SURFACTANT ASSISTED EXTRACTION OF POLYHYDROXYALKANOATE SYNTHESIZED BY *Cupriavidus necator*

Thesis

Submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

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DECLARATION

by the Ph.D. Research Scholar

I hereby *declare* that the Research Thesis entitled **SURFACTANT ASSISTED EXTRACTION OF POLYHYDROXYALKANOATE SYNTHESIZED BY *Cupriavidus necator*** *w*hich is being submitted to the **National Institute of Technology Karnataka, Surathkal** in partial fulfillment of the requirements for the award of the Degree of **Doctor of Philosophy** in **Chemical Engineering** is a *bonafide report of the research work carried out by me*. The material contained in this Research Thesis has not been submitted to any University or Institution for the award of any degree.

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CERTIFICATE

This is to certify that the Research Thesis entitled **SURFACTANT ASSISTED EXTRACTION OF POLYHYDROXYALKANOATE SYNTHESIZED BY *Cupriavidus necator*** submitted by **SIVANANTH M**, (Register Number: **121162CH12F03**) as the record of the research work carried out by him, i*s accepted as the Research Thesis submission* in partial fulfillment of the requirements for the award of degree of **Doctor of Philosophy**.

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ABSTRACT

Polyhydroxyalkanoates or Bioplastics, are synthesized as reserve food source by microbes under nutrient depleted condition and in the presence of surplus carbon source. *Cupriavidus necator*, a gram-negative soil microbe, was used to valorize crude glycerol derived from biodiesel plant into PHA in an anaerobic and unsterile mode of batch fermentation. Medium optimization studies were performed by one variable at a time approach and it was found that maximum PHA production of 11.96 g/L, was achieved at optimized medium composition of 9 wt% crude glycerol, 0.75 g/L ammonium sulphate, 2 g/L sodium bicarbonate, 7.5 mM copper sulphate & 4 mM magnesium sulphate, pH adjusted to 6.8 and incubated at 30ºC, 150 rpm for 72 hours. NMR & FTIR based structural characterization revealed that the PHA synthesized is a novel long chain length terpolymer and is named as Poly [3-Hydroxybutyrate-co-3-Hydroxyvalerate-co-3-hydroxy 4-methoxyphenyl valerate] (P3HB-co-HV-co-MeOPhHV). Novel PHA synthesized has glass transition temperature of -14.34ºC, melting temperature of 104.85ºC while the onset of degradation was found to be at 250.64ºC. Average molecular weight of the polymer was found to be 994 KDa while the average molecular number is 615 KDa and polydispersity index is 1.616. Different micellar extraction based purification techniques were developed to separate and purify synthesized PHA from biomass present within the crude fermentation broth. The maximum purity of 92.49% was obtained by performing nonionic surfactant based cloud point extraction during batch studies, continuous cloud point extraction in RDC, revealed that variation in operational variables such as flow rate and rotor speed reduced the overall purity to 88%. low frequency sonic waves assisted cloud point extraction increased the PHA purity to 94.28%. Gum arabic – mixed surfactant coacervate complex assisted microfiltration of PHA resulted in a purity of 97.08 % Maximum separation factor of 3.045 was achieved when crude broth was run in reverse phase C18 column with TX100+methanol based hybrid micellar mobile phase.

Keywords: Polyhydroxyalkanoate, *Cupriavidus necator*, Cloud point extraction, Ultrasonication, Membrane extraction, Chromatography

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ABBREVIATIONS

^{13}C	Carbon NMR
^{1}H	Proton NMR
AOT	Di-octyl sodium sulfosuccinate
CAC	Critical aggregation concentration
CDW	Cell dry weight
CMC	Critical micelle concentration
CTAB	Cetyl trimethyl ammonium bromide
CPE	Coud point extraction
CP	Coud point
DDAB	Didodecyl dimethyl ammonium bromide
EDTA	Ethylenediaminetetraaceticacid
EO	Ethylene oxide
ESI-MS	Electron spray Ionization- Mass spectrometry
FTIR	Fourier transform Infra red
GMO	Genetically modified organisms
GPC	Gel permeation Chromatography
HB	Hydroxy butyrate
HHx	Hydroxy hexanote

HLB	Hydrophile Lipophile Balance
HV	Hydroxy valerate
LC-MS	Liquid Chromatography Coupled Mass Spectrometry
lcl-PHA	Long Chain Length PHA
LDPE	Low density polyethylene
LLE	Liquid Liquid Extraction
MALDI-TOF-MS	Matrix assisted laser desorption/ionization coupled mass spectrometry
mcl-PHA	Medium Chain Length PHA
MLC	Micellar liquid chromatography
MWCO	Molecular weight cutoff
NMR	Nuclear Magnetic resonance
OE	Oxyethylene
PE	Polyethylene
PEG	Polyethylene glycol
PET	Polyethylene terepthalate
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PHB-co-HD	Polyhydroxybutyrate-co-Hexadeconate
PHB-co-HV	Polyhydroxybutyrate-co-valerate
PHB-co-HV-co-4HB	Polyhydroxybutyrate-co-hydrxyvalerate-co-4hydroxybutyrate
PHB-co-HV-HHX	Polyhydrobutyrate-co-hydroxyvalerate-co-hydroxyhexanoate
PHHx-co-HD	Polyhydroxyhexanoate-co-Hexadeconate
PI	Poly dispersity
PLA	Poly lactic acid
PONPE	Polyoxyethylene nonyl phenyl ether
PP	Polypropylene
ppm	parts per million
RP	Reverse phase
RP-HPLC	Reverse Phase High Pressure Liquid Chromatography

RPM	rotation per minute
scl-PHA	Short Chain Length PHA
TBABr	Tetra butyl ammonium bromide
TBPFO	Tetrabutyl ammonium perfluorooctanoate
TLL	Tie line length
TMN6	Tergitol 6
TX100	Triton X 100
TX114	Triton X 114
TX45	Triton X 45
UACPE	Ultrasonication Assisted Cloud Point Extraction
UV/Vis	Ultraviolet/Visible

CHEMICAL ABBREVIATIONS

$Al(NO_3)_3$	Aluminium nitrate
$Ca(NO_3)_2$	Calcium nitrate
$CaCl_2$	Calcium chloride
$CDCl_3$	Deuterated chloroform
CG	Crude glycerol
CO_2	Carbon dioxide
CsCl	Caseium chloride
$CuSO_4$	Copper sulphate
GA	Gum arabic
K_2HPO_4	Dipotassium hydrogen phosphate
KBr	Potassium bromide
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
$Mg(NO_3)_2$	Magnesium nitrate
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
Na_2HPO_4	Disodium hydrogen phosphate

Na_3PO_4	Sodium phosphate
NaBr	Sodium bromide
NaCl	Sodium chloride
NaH_2PO_4	Sodium dihydrogen phosphate
NaI	Sodium Iodide
NaSCN	Sodium cyanide

SYMBOLS, NOTATIONS & UNITS

°C	degree celsius
C/N	carbon to nitrogen ratio
Da	dalton
g	gram
g/L	gram/Litre
g/L/h	gram/Litre/hour
g/mol	gram/mol
Gpa	giga pascal
kDa	kilo dalton
kg	kilogram
kHz	kilo hertz
kPa	kilo pascal
L	litre
m	meter
m/m %	mole/mole %
m/v %	mole/volume %
m/v %	mass/volume %
m/z	mass to charge ratio
MDa	Mega dalton
MHz	Mega hertz
mL	millilitre
mm	millimeter

M_n	Average molecular number
mol %	mole %
MPa	Mega pascal
mV	milli volt
M_w	Average molecular weight
N_{ag}	Aggregation number
N_{ag}	Aggregation number
nm	nano meter
v/v %	volume / volume %
vol%	volume %
w/v	weight/volume
w/w %	weight/ weight %
W_o	Water content
wt%	weight %
ΔH_f	Heat of fusion
µg	Microgram
µL	microlitre
τ	Shear bond strength
Φ	Holdup

CHAPTER 1

INTRODUCTION

In the current scenario, sustainability has become a keyword in the field of process and product design, owing to the adverse effects of modernization with increasing human population and for which earthly resources are utilised in an uncontrollable level (Ananstas and Zimmerman 2003, Dale 2003). Replenishing fossil fuel and other chemical sources have made industries across the globe take a paradigm shift towards bio-based products and its production, for more than a decade now. Though microbes have been extensively employed, it was not handled and used effectively until 18th century from several B.C. and the invention of the microscope and the physiology of microbes pave the path for several microbial applications in different fields. Novel developments in the field of genetic engineering by 20th century have led to commercialization of biotechnology in the global market. Bio based products have extensively used in various fields such as food, medical and biopharmaceutical, environmental, paper and pulp, leather, paint, fine chemicals etc. (Soetaert and Vandamme 2006, Hatti-kaul 2007, Wenda et al. 2011, Gartland et al. 2013). Apart from developing nations, underdeveloped and developing countries are investing in biotechnology as a result of abundant resources available from agricultural waste and there is a large scope of valorising agricultural, industrial and domestic waste into useful products via biosynthesis. 170 billion tonnes of biomass has been reported to be produced across the globe, out of which only 3.5 % has been utilised by human mankind, which is a major concern across the globe (Soetaert and Vandamme 2006).

1.1 Microbial Polyhydroxyalkanoate

Among several bio-products that have been identified and commercialised, microbial polyhydroxyalkanoates or bio plastics as they are commonly referred to are one such microbe-derived product. PHA is bio-polyester, with materialistic properties similar to that of commercial synthetic plastic which is of petrochemical origin, over the recent few years there has been a huge rise in its demand and market across the globe as a replacement towards synthetic plastics (Philp et al. 2013). The amount of

used non-degradable synthetic plastics that are disposed of in the landfills and water sources including oceanic waves accounts for several tonnes and are considered to be a threat to the world. Due to the adverse effect of synthetic plastics, that owe environmental threat, it has been banned for its usage in several countries. Microbial PHAs offer several distinct advantages such as production from renewable raw materials ranging from simple sugars to complex waste sources, helps in large scale valorisation of waste, higher production, easily biodegradable and no environmental threat, recycling and reuse etc. PHA is synthesised by microbes as reserved food source (Dawes and Senior 1973); microbial metabolic pathway stimulates the production of PHA under limited nutrient condition (oxygen, nitrogen, phosphorous, sulphur, Iron etc.) in the presence of excess carbon source (Koller et al. 2010).

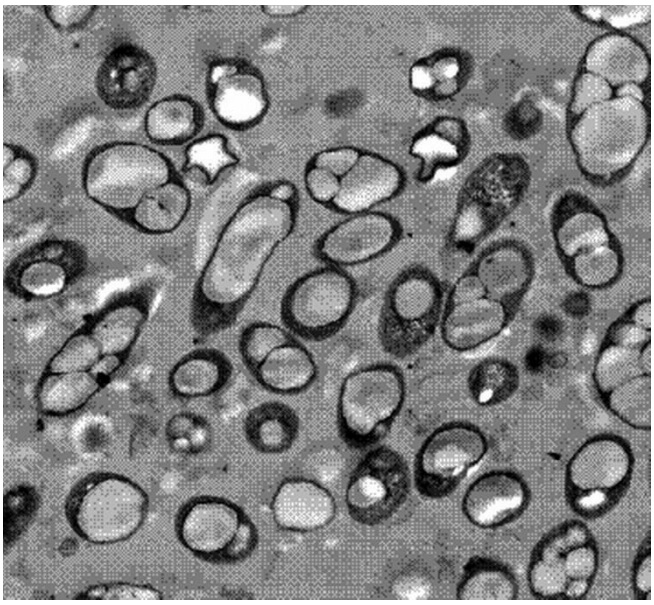


Figure 1.1 TEM image of PHA within *Cupriavidus necator*, magnification – 1/30000 (adopted from Koller et al. 2012).

Several microbes including common to novel isolated gram positive, gram negative bacteria and cyanobacteria have been explored for their potentiality towards PHA production from various carbon sources (Chen 2009, Keshavarz and Roy 2010). As a result of advanced techniques in the field of biology such as genetic engineering and molecular biology has resulted in research and development of Recombinant strains anchoring PHA synthesising genes from wild strains. This enhances the testing

and utilisation of complex carbon sources, increase in the gene copy number and development of cost effective process such as unsterile fermentative synthesis of PHA. Since the market value of PHA depends upon the raw materials and operational cost, extensive research in developing an economic yet highly profitable process towards PHA production is of current interest among researchers.

PHA molecules are amorphous and are lipid in nature and pertain as water insoluble inclusion bodies with a size range of 0.2-0.5 μm within the microbial cells. The number of PHA granules present within the cells has been reported to remain constant between 8 and 12 (Sudesh et al. 2010, Braunegg et al. 1998). Since PHA is amorphous in nature, exerting stress on the microbe results in metabolic activity of intracellular depolymerisation enzymes on PHA and PHA loss within the microbe. The molecular size of PHA molecule varies from a few kDa to MDa and usually depends upon the type of carbon source available and fermentation conditions maintained during its production.

1.1.1 Classification and properties of PHA

About 150 different PHA molecules have been reported till date and their major classification is based on several factors such as nature of carbon source - bio-based carbon source or a mixture of bio-based carbon source mixed with the synthetic carbon source. Depends on the number of carbon atoms present in a monomeric unit of PHA, it will be classified as short chain length PHA (scl –PHA) (3 to 5 carbon atoms), medium chain length PHA (mcl –PHA) (6 to 14 carbon atoms), long chain length PHA (lcl-PHA) (> 14 carbon atoms) (Fig 1.2). Scl-PHA and mcl-PHA are most commonly synthesised by microbes using simple to meagrely complex substrates, while lcl-PHA are synthesised by microbes when complex carbon sources with long chain carbon atoms are present in the fermentation medium. Further, different types of polymer are synthesised based on the combination of monomers such as homopolymer (made up of same monomeric units) and copolymer (consisting of different monomeric units interlinked by covalent bonding) (Fig 1.3). Based on the chemical composition of PHA, it will be classified as PHA with aliphatic units or aromatic units or those that comprise both aliphatic and aromatic units (Tan et al. 2014).

PHB is the most common PHA to be synthesised and to be utilised as replacement of LDPE. Though Young's modulus and tensile strength are good enough than PP, elongation to break (%) is very less compared to that of PP (Table 1.1). PHB in its native form is stiff and brittle, however, after extraction and processing, they are observed to acquire good mechanical strength. The presence of different monomers in PHA declines the mechanical strength (Young's modulus) and copolymer becomes more flexible, unlike stiff homopolymer. Depending upon the position of the copolymer and mol% of the monomeric composition of the copolymer, the mechanical strength varies. The presence of 4HB decreases crystallinity of the PHA increases the elongation to break (%) which imparts elasticity to the biopolymer. Similar results have been reported for the presence of increasing mol. % of HV in PHBV copolymer. The addition of longer chain monomeric units such as HHx in a copolymer makes the PHA molecule more amorphous, elastomeric and sticky in nature and drastically reduces the melting point while increasing the elongation % of the PHA molecule (Sudesh et al. 2010, Akaronye et al. 2010 and Tan et al. 2014).

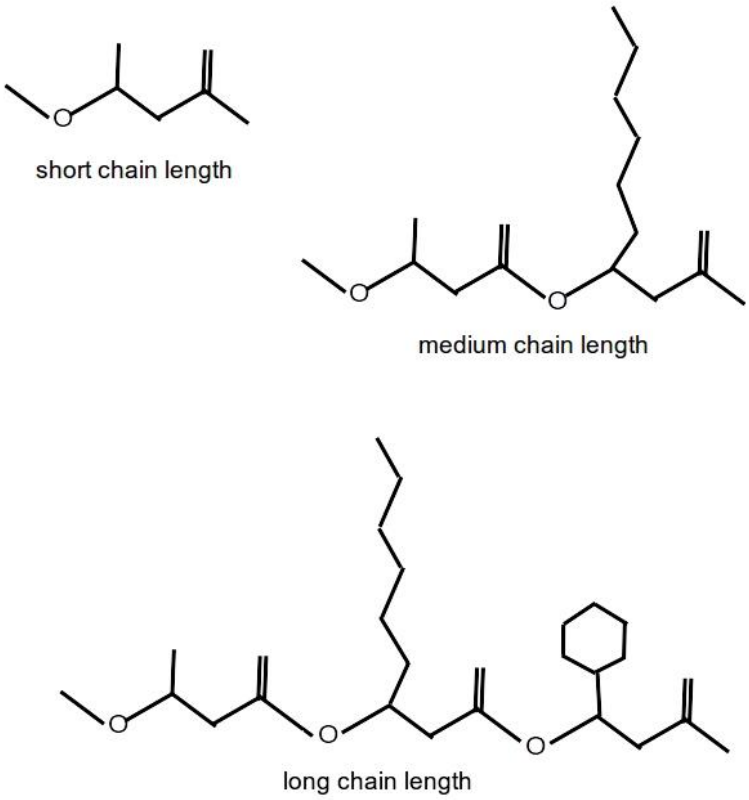


Figure 1.2 Classification based on chain length of PHA.

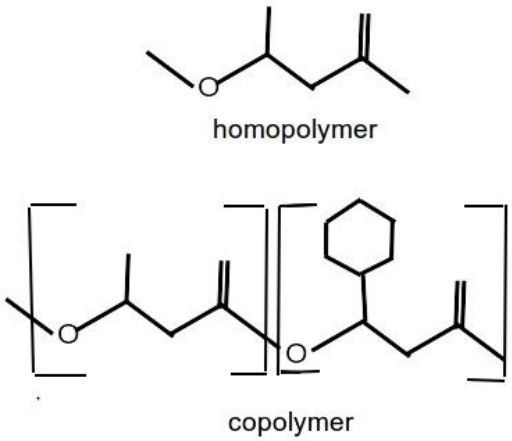


Figure 1.3 Classification of PHA based on the composition of monomeric unit.

Table 1.1 Physical properties of natural bio plastics and synthetic plastic (adopted from Akaronye et al. 2010)

Plastic		Polymer	Melting Temperature (°C)	Glass Transition Temperature (°C)	Elongation to Break (%)	Tensile strength (MPa)
Natural Bio plastic	scl-PHA	P(3HB)	180	4	5	40
		P(4HB)	53	-48	1000	104
	mcl-PHA	P(3HB-co-20% 3HV)	145	-1	50	20
		P(3HB-co-16% 4HB)	150	-7	444	26
		P(3HB-co-6% 3HD)	130	-8	680	17
Synthetic Plastic		Polypropylene	176	-10	400	34.5
		Polystyrene	240	100	-	50

1.1.2 Applications of PHA

Based on the physiochemical properties, especially the mechanical strength, PHA has been utilised in different fields for varied applications. PHB and its variance are chiefly employed in the medical and pharmaceutical field owing to its

biodegradable nature, increased biocompatibility, ability to reshape and resize according to the application. Apart, they are also used in the packaging industry, as a result of comparatively stronger polymeric properties such as those of PP and PE (Bucci et al. 2005). Copolymers are often used in the packaging industry as a result of low melting temperature and high flexibility and have also been found to degrade at a faster rate when exposed to the environment compared to homopolymers (Bugnicourt et al. 2014). As a result of flexibility, different PHA copolymers and blend of PHA and other polymers have been tested for their efficacy as shopping bags, disposable cups, utensils, diapers, in the manufacture of female hygienic products, razors etc. (Reis et al. 2008). PHA based packaging materials offer low oxygen and moisture permeability, and UV resistance compared to LDPE while exhibiting comparatively good mechanical strength like PE or PP or PET. However, production and processing cost of PHA based packaging materials is considered to be a barrier towards its commercialization (Petersen et al. 1999, Khosravi-Darani and Bucci 2015).

Biological origin of PHA offers increased biocompatibility, which has enabled PHA to be tested *in vivo* and *in vitro* for several medical and pharmaceutical applications to replace the use of several synthetic polymers (Wu et al. 2009). PHB is widely found in most of the organisms, and their enhanced interaction with bio-macromolecules allows it to be found in the cell cytoplasm, membranes and intracellular fluids (Reusch 1995). They have been observed to solubilise and transfer salts across membrane barriers (Reusch 1992), which is an important aspect for any foreign macromolecule to be utilised for medical applications.

Different PHA molecules have been used in the production of medical devices that can be utilised in the treatment of cardiovascular diseases. Cardiovascular diseases are considered to be one of the major reasons for increased mortality in this modern age, as a result of unusual food habits and physical psychological pressure among humans. Medical devices that are to use for cardiovascular treatments need to express low immunogenicity, non-toxic, durability and resistance to degradation during enhanced usage etc. (Valappil et al. 2006). Trileaflet valve made up of mcl-PHA were tested by implanting the same in the pulmonary position of lamb model and it was found that the implant resulted in no thrombosis formation even after 120 days (Sodian et al. 2000). Blend of PGA with P(3HHx-co-3HO), resulted in no

thrombosis formation while a mild valvular regurgitation was seen after 6 months of implanting the device (Stock et al. 2000). In another study, trileaflet valve made up of PGA coated with P4HB was found to grow in size with the lamb's growth which inferred that such implants are helpful in the long term treatment such as those in the treatment of children (Hoerstrup et al. 2000).

PHA has also been tested for its efficacy as vascular grafts, porous P4HB patch seeded with vascular cells from ovine peripheral vein was implanted into lamb peripheral pulmonary artery, after 169 days, reformation of tissue was observed with no signs of thrombosis or stenosis and reduced the risk or re-operation(Stock et al. 2000). In another study by Optiz et al. (2004), P4HB patch seeded with vascular smooth muscle cells was incubated in pulsed flow bioreactor; the authors after a certain period of incubation time were able to observe tissue formation with mechanical strength equal to that of the native aorta. PHO was found to enhance enzyme activity and tissue regeneration when tested in rat models and the PHA material was found to undergo slow *in vivo* degradation, over a certain period of time (Marois et al. 1999).

Low platelet adhesion, erythrocyte contact, and haemolysis reactivity were observed. Surface modification of PHBHHx, by ammonia plasma treatment or fibronectin coating, was found to enhance the growth of human umbilical vein endothelial cells (Qu et al. 2005). High haemocompatibility and cytocompatibility have been observed when PHBHHx was tested as blood-contacting material (Qu et al. 2006). However, such medical studies are still in research level and have not been extended to clinical trials as a result of compliance mismatch (Salacinski et al. 2002).

PHB conduits have been successfully tested and used for nerve regeneration in rat models (Hazari et al. 1999), PHB conduit seeded with nerve cells were able to regenerate in the case of peripheral nerve injury (Terenghi et al. 2002), peroneal nerve injury (Mohanna et al. 2003) and long gap nerve injury repairs (Mohanna et al. 2005). Biodegradability is an important factor when nerve conduits are tested and the degradability should match to that of nerve regeneration (Mohanna et al. 2005). However, PHB and PHBHHx show slower degradation rate compared to faster nerve regeneration, development of novel PHA based nerve graft implants are extensively studied to increase the degradation rate.

In a study conducted by Galego et al. (2000), a combination of P(HB-co-8%HV)/ HA (30% w/w) showed a mechanical strength of 62MPa which is on par with that of human bones. PHBV with incorporated HA was found to show low inflammatory response and higher mineralisation rate (Cool et al. 2007). PHBHHx as a single polymer had been reported to enhance osteoblast attachment, proliferation and differentiation compared to that of HA incorporated PHBHHx (Li et al. 2005, Wang et al. 2005). PHB reinforced HA has been found to favour bone tissue adaptation response, while the newly organised bone structure was seen to form close to the implant and thickness of the newly formed bone was found to increase with time (Luklinska and Schluckwerder 2003, Kose et al. 2003). PHBV scaffolds with preseeded bone marrow stromal cells were found to enhance *in vivo* tissue repair and have been compared to that of calcium-phosphate loaded collagen (Kose et al. 2003). Natural coral (Shamsuria et al. 2004), tricalcium phosphate (Liu et al. 2007, Zheng et al. 2007) and other biomaterials have been used to blend with PHA to identify novel hybrid bone scaffolds that can be used in bone replacement.

Identification of novel scaffolds in cartilage tissue engineering is of worldwide interest as cartilage once damaged, has very limited self-healing feature and often leads to medical conditions such as osteoarthritis and functional loss of joints, while surgery cannot replace the damage (Schulz and Bader 20007). A scaffold made up of individual PHA and PHA blends have been tested towards the proliferation of chondrocytes, the authors were able to infer that PHA blends enhanced cell proliferation compared to individual PHA (Deng et al. 2002, Sun et al. 2005). Medical imaging techniques proved that incorporation of PHBHHx in PHB was found to enhance the surface property that facilitated anchoring of type II collagen filaments and their penetration into the scaffolds (Kose et al. 2005). PHBV matrices were tested for tissue repair by Wang et al. (2008); the authors found that the implanted matrices stimulated early cartilage formation compared to that of collagen containing calcium phosphate. PHBV and PHBHHX have been identified as effective cartilage tissue regeneration materials, with high surface integrity and accumulation of tissue.

As PHA molecules are hydrophobic and express encapsulation capacity, several mcl-PHA molecules have been tested for its efficacy as drug delivery system owing to its low melting temperature and lower crystallinity %. Ueda and Tabata

(2003) found that PEGylation of PHA nano spheres resulted in increased blood circulation time. PHA based drug delivery systems showed increased release of antibiotics at the required site. As a result of their nano size, PHA nano spheres can penetrate tissues and are readily taken up by the cells and a high loading and release efficiency was observed in PHB and PHBHHx compared to that of PLA nano spheres (Xiong et al. 2010).

3HB has been found a novel application as a nutrient supplement as 3HB exists as ketone bodies in blood and are used as an alternative energy source in the brain when glucose is depleted (Gasior et al. 2006). Oral administration of 3HB was found to increase serum alkaline phosphate activity with calcium deposition and prevented reduction of bone mineral density. In another study, oral administration of 3HB ketone bodies has been found to control seizure, metabolic diseases, protein catabolism, appetite and parenteral nutrition while it can be used to treat diabetes, neurodegenerative disorders, and epilepsy (Martin et al. 2002).

PHA films have been tested as oil blotting material. PHA and PHB copolymers were found to contain oil absorption to about 80 % (Sudesh et al. 2007). PHA films have been tested for their absorption characteristics and they have been researched upon for their efficiency on dye removal. It was found that when PHB films were used, they absorbed Batik dye up to 38 % while it increased to about 80 % when the PHB material was electro spun to nanofibers due to the increase in surface area. Titanium oxide incorporated PHB nanofibres have been employed in waste water treatment that combines both absorption and photo-oxidation process (Sridewi et al. 2011). Electro spun nanofibers of PHB have been effectively tested for decontamination of water, it has been reported that the nanofiber was effectively able to separate gram positive, gram negative bacteria and yeast from water, the fibre material with microbe can be left to decompose in a safe and effective manner. PHA has also been tested for its efficient release of herbicides for long term usage in a slower rate.

PHA has been effectively tested towards its potentiality as fuel, acid hydrolysis of mcl-PHA molecules results in methyl ester derivatives. It has been found that the blends of hydroxyalkanoate methyl esters with gasoline or diesel lowered heat of combustion while it increased the heat of combustion when mixed

with ethanol (Chen 2009, Andreeßen et al. 2014).The presence of hydroxyl and carboxyl groups enhances its usage as precursor molecule toward the production of aromatic compounds, vitamins, antibiotics etc. (Chen and Wu 2005, Orts et al. 2008). Compared to chemical synthesis, catalytic conversion of PHA is considered safe and economical. PHA is utilised in the production of latex based paints.

1.1.3 Commercialization of PHA

The global market for PHA production and its usage was estimated to be around 70 million USD in 2015 and is expected to grow in the coming years. Among the different continents, Asia has been identified to be a potent market towards commercialization of PHA owing to the presence of rich and varied resources that can be utilised for PHA production, apart from South American countries. Owing to their varied applications, several companies across the globe have invested in RandD, Production, and separation of PHA for commercial use. Numerous unit operations are involved starting from production till separation and purification; there is always a constant search towards identification and development of an economic process design. As a result of the high cost involved in PHA production, companies are looking for a simple yet sustainable approach that could lower the cost of operation while yielding high market value product (Chen 2009).

Several types of PHA have been researched upon by industries for their production and application, PHB, PHBV, P3HB4HB, PHBHHx are few commercially available polymers. A few industrially employed common microbial strains are *Ralstonia eutropha*, *Alcaligenes latus*, *Pseudomonas putida*, *Pseudomonas oleovorans*, *and Aeromonas hydrophila*, *Escherichia coli* (Sudesh et al. 2010, Akaronye et al. 2010, and Tan et al. 2014). Following problems are foreseen by the companies in achieving high productivity of PHA such as, achieving high PHA content in shorter time, increased PHA content and larger granules within the microbes, development and utilisation of novel medium consisting of varied waste complex carbon sources and that enables growth of novel isolated microbes, scale up of fed-batch to continuous mode of fermentative synthesis of PHA, novel separation methods that are cheap yet profitable. Though large scale production of PHA has been well documented, solvent extraction has been employed to separate PHA from the

biomass, owing to their high solubility in chlorinated solvents. Search for novel separation techniques that could enhance the purification while treating large volumes of feed, to retain PHA nativity and to gain higher yield is still pursued by researchers and they are trying to bring up a solution via dedicated research in the field of downstream processing of PHA.

Table 1.2 Companies involved in the production of PHA (adopted from Chen 2009).

Polymer	Company	Country	Application
PHB	Chemie Linz; BTF	Austria	Packaging and drug delivery
	Biomers; BASF	Germany	Packaging and Drug delivery
	Monsanto	USA	Raw materials
	Mitsubishi	Japan	Packaging
	Biocycles	Brazil	Raw materials
	Tianjin Northern Food; Jiangsu Nan Tian	China	Raw materials
PHBV	ICI	UK	Packaging
	BASF	Germany	Packaging and Raw materials
	Monsanto	USA	Raw materials
	Zhejiang Tian An	China	Raw materials
Varied PHA	Metabolix; Tepha; ADM; PandG; Meredian	USA	Packaging, Raw materials, medical applications
	Kaneka	Japan	Packaging
	Bio-On	Italy	Raw materials
PHBHHx	Jiangmen Biotech Ctr	China	Raw materials
P3HB4HB	Tianjin Bio-Science	China	Packaging and Raw materials

1.2 Liquid - Liquid Extraction

Choice and selection of an appropriate separation technique in the bio industry is highly influenced by several factors such as nature of the source from which bio-product needs to be separated – microbial, plant or animal cells and location of the product – intracellular or extracellular product, required yield and purity of the product, handling and treatment of bulk feed and the overall operating cost. Fundamentals of any bio separation process are same as that of any other chemical separation process and are broadly classified based on the phase in which the product is available (Singh and Singh 1996, Keller et al. 2001). Considering the fact that all living cells are solid in nature, the primary step of any bio separation is Solid-liquid separation such as filtration or clarification to separate the cells from the medium, followed by other appropriate techniques that lead to purification of the desired bio product. When the desired product is excreted into the surrounding medium by the cells, most often LLE techniques is adopted industrially to enrich the product in one particular solvent based on the product's selective solubility. Salt precipitation, chromatography and membrane separation are few other separation techniques that are industrially employed towards product separation. However, researchers and engineers are looking ahead for alternative separation techniques that can handle a large volume of feed in lesser time, and that offers enhanced selectivity and productivity during separation and are of low operational cost which also offers sustainable operation (Keller et al. 2001, Przybycien et al. 2004).

Liquid-liquid extraction, commonly known as solvent extraction, is a common technique that is widely used from lab scale to industrial level by employing immiscible liquid phases as extractants. LLE has been practised since 19th century wherein chemists have utilised organic solvents to extract chemicals from water. The efficiency of the process is denoted by partition coefficient which is defined as the ratio of the concentration of solute in the organic phase to that of the aqueous phase. The partition coefficient of a solute depends on the temperature of the separation system, pH, concentration of Ion species and other parameters involved and are related to the Gibbs energy difference of the system.

$$Partition\ coefficient\ (k) = \frac{solute\ Concentration\ in\ organic\ phase}{solute\ concentration\ in\ aqueous\ phase} \qquad (1.1)$$

To carry out an efficient LLE, the solvents used should be partially miscible or immiscible on maintaining an isobaric (constant pressure) or isothermal (constant temperature) condition. Solvents used in LLE are required to possess characteristic features like miscibility - solvents miscibility with other solvents and water should be taken into consideration while designing the system, density- density difference between the solvent used and the aqueous phase should have a notable difference and solubility – solvents have solubility limit within aqueous phase and attain their mutual saturation level and the solute also possess a solubility factor based on which it gets solubilised in the organic phase or the aqueous phase or in both.

Although, solvent extraction offers higher extraction efficiencies, considering the volume of solvent required to treat and extract bio molecules of interest from the source, pertains to be cost consuming. The recycling of solvent requires and separation of impurities from the used solvent again adds up to the overall cost of the process. As most of the solvents are apolar in nature, they exhibit high hydrophobicity and as a result impose denaturing effect on bio molecules. Polar solvents are mild but are of low efficiency. The choice of solvent based on the nature and source of the bio molecule to be separated, the volume of feed and extent of purity determines the efficiency of a solvent extraction process. Scale up of the solvent extraction process is a difficult task as it involves handling of several gallons of volatile solvent and imposes safety issue and risk during large scale operations. Most of the solvents are non-renewable and are of petrochemical origin and as a result, the concept of extraction has taken a paradigm shift from conventional solvent based extraction techniques to modern extraction techniques involving surfactants, ionic liquids, supercritical fluid etc., which are known to be green solvents.

1.2.1 Surfactant assisted extraction

Surface active agents or surfactants, they are commonly referred to be organic compounds, are either amphiphilic or amphipathic in nature. A surfactant has two distinct parts with one of the part expressing hydrophilicity while the other part expresses hydrophobicity. Hydrophilic part of the surfactants are referred to as surfactant head and are observed to interact with polar solutes and solvents via dipole-dipole or ion-dipole interaction, while the hydrophobic part is referred to as surfactant

tail that interacts with apolar solutes and solvents via hydrophobic interactions (Myers 2005). With the addition of surfactant to a polar solvent such as water, surfactant monomers align on the surface of the water, as shown in figure 1.4 in such a way that the hydrophilic head interacts with water, while surfactant tails are exposed away from the aqueous environment. Surfactant monomers present on the surface replace water molecules and as a result of lesser interaction between surfactant head groups and water molecules compared to that of water - water interaction. A similar phenomenon is found to occur with the addition of surfactant monomer to two immiscible liquids. The surfactant head groups are aligned towards the comparatively polar liquid, while hydrophobic tails are exposed towards the apolar liquid and thereby reducing the interfacial tension between the two liquids as shown in figure 1.4.

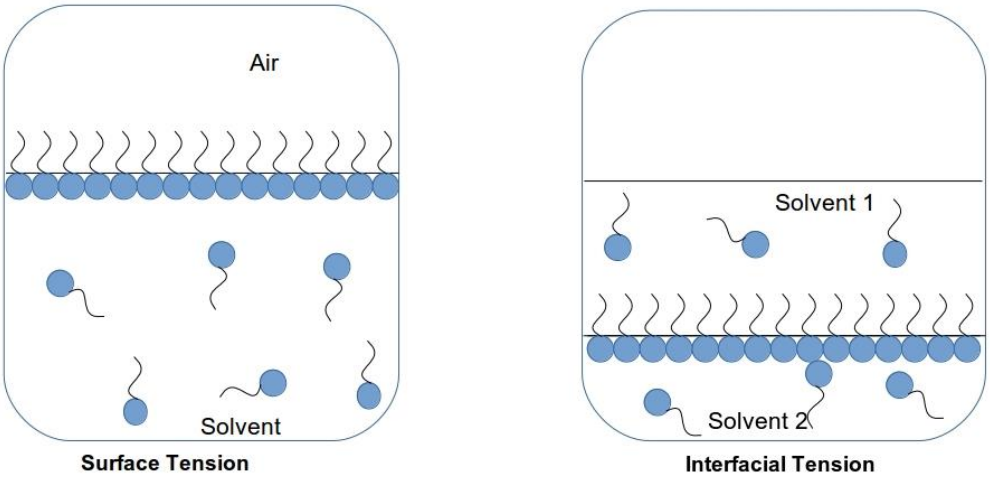


Figure 1.4 Surfactant's alignment in air-liquid and liquid-liquid phases.

Surfactant based LLE is a biphasic system made up of an aqueous phase and an organic phase, where solutes are partitioned from one phase to another based on the solute solubility in either aqueous or organic phase. (Harrison 1993, Rodrigues et.al. 1999A and 1999b). Organic phase consists of micelles that are considered to be a thermodynamically stable isotropic dispersion of two immiscible liquids stabilised by surfactant interfacial film. Micelles are often known as oil – in - water (O / W) micro emulsions while reverse micelles are known as water - in - oil (W / O) micro emulsions. The addition of surfactant to water results in the formation of micelles and addition of such micelle solution to an immiscible solvent, usually a nonpolar organic

solvent, such as hexane, toluene, isooctane, chloroform etc. results in the formation of reverse micelles. Micelles compose a hydrophobic core made up of hydrophobic tails of surfactants, while the surfactants hydrophilic head groups are in contact with water, the addition of hydrophobic solute such as oil, partitions the oil to the hydrophobic core forming an O/W micro emulsion. In the case of reverse micelles, hydrophilic water core was formed through the restructuring of surfactants, which is the water core is enclosed by surfactant head groups, while the surfactant tails protrude into the surrounding bulk nonpolar solvent. The hydrophilic solutes get solubilised often in the hydrophilic core of reverse micelles. The reverse micelles are often formed using charged surfactant head groups and as a result of which hydrophilic charged solutes electrostatically interact with the ionic surfactant head groups and remains solubilised within the water core (Rabie and Vera 1996, Pessoa Jr and Vitolo 1997).

1.2.2 Classification of surfactants

Surfactants are classified based on their dissociation in water. On addition of surfactant monomers to water, surfactant head groups have been observed to undergo two different types of interaction. Physical adsorption of surfactant head groups via weak van der Waal interactions, chemical adsorption between solute and surfactant head groups via electrostatic interactions. A surfactant that undergoes physical adsorption and possesses uncharged or neutral head groups are referred as nonionic-surfactants. Surfactants that impart electrostatic interaction possess charged surfactant head groups which are either negatively charged (anionic) or positively charged head groups (cationic). Increasing the hydrocarbon chain of a surfactant tail leads to increasing surface tension, whereas increasing ethylene oxide units in surfactant tail such as those in nonionic surfactants lead to decrease in surface tension.

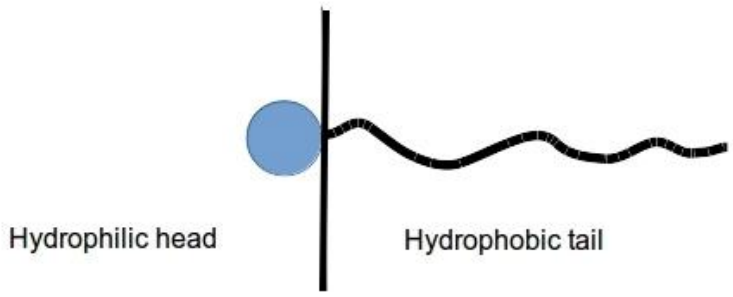


Figure 1.5 Representation of surfactant monomer.

Anionic surfactants are dissociated into a polar binding anion and a cation, the head group is usually made up of an alkaline metal such as Na+, K+ or a quaternary ammonium. These are the commonly used surfactants and involve 50% of the world production. Alkyl sulphates are the most commonly used anionic surfactants that are employed as foaming and wetting agents, sodium, ammonium or ethanolamine salts of dodecyl or lauryl sulphate are used in the formation of the micellar system and lauryl sulphate is an extremely hydrophilic surfactant used in the purification of hydrophilic molecules. Alkyl ether sulphates have 0 to 5 Ethylene oxide groups which enhance the packing of polar heads in a compact manner between the air water interphase and give a better tolerance to divalent cations giving them an extreme foaming capability. Apart, a series of compounds such as sulphated alkanolamines, glyceride sulphates, and different classes of sulfonates also form the anionic surfactant series.

On dissociation, cationic surfactants produce hydrophilic cation and an anion and the head group is usually a halogen compound. Since their production involves high-pressure hydrogenation reaction, it is more expensive than the anionic surfactants. Due to which it is often used as a bactericide and as corrosion inhibitors. They are considered to be weak foaming agents and detergents and cannot be mixed with anionic surfactants unless catanionic synergy is required. Fatty amines, salts, and their quaternary derivatives form the cationic surfactant group. Fatty amines are classified under cationic surfactants due to their cationic properties at acidic pH, even though they are anionic in nature.

Nonionic surfactants have their head group made of amides, esters, alcohols, ether or phenol which do not dissociate in a hydrophilic environment and most of the nonionic surfactants have a polyethylene glycol chain which is formed by polymerization of ethylene oxide that results in a hydrophilic chain, hence are called polyethoxylated nonionic. Polycondensation of polypropylene oxide results in polyether, which is hydrophobic in nature. Nonionic detergents are good wetting agents, emulsifiers, and exhibit a very low toxicity level. Polyethylene oxide chains are globally hydrophilic, but then the methylene group imparts hydrophobicity to the whole molecule; with increasing EO chain, the hydrophobicity also increases.

Ethoxylated alcohols and alkyl phenols, ethoxylated thiols, fatty acid esters and nitrogenated ethoxy chains form the major part of the nonionic surfactants.

Amphoteric or zwitterionic surfactants as they are called dissociate into anionic and cationic groups, they are pH sensitive and are anionic at low pH and cationic at high pH conditions and amphoteric at intermediate pH conditions. Even though their production cost is high, they are less toxic and are highly biocompatible and so are chiefly employed for biological applications. Amino and imido-propionic acids are the commonly used amphoteric surfactants. Polymeric surfactants are a new class of surfactant group that dissociates into the hydrophilic and lipophilic group and are employed in formulations. Gemini surfactants coined by Menger is another important class of surfactants that possess two hydrophilic, charged head groups linked together by a spacer and free floating tails, they are found to exhibit high surface tension at lower surfactant concentration, high wetting ratio etc. over the conventional surfactants.

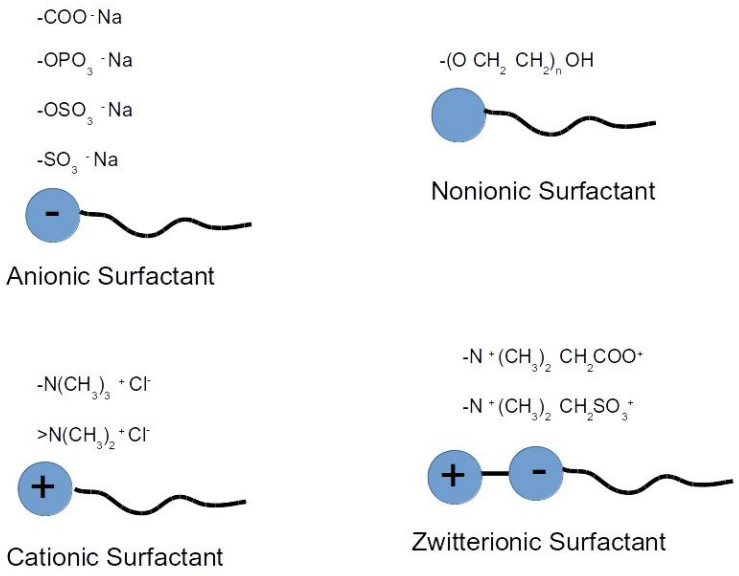


Figure 1.6 Classification of surfactants based on the head group.

Surfactant properties depend upon the balance between the surfactant head group and its tail which is expressed as Hydrophile Lipophile Balance (HLB) number (Shinoda 1983). As HLB increases above 5, surfactant attains hydrophilicity and is readily soluble in polar solvents, HLB less than 5 represents lipophilicity and so the surfactants undergo active solubilisation in most of the apolar organic solvents. Although most of the surfactants with low HLB are readily soluble in water, their

solubility limit ranges from µM to a few mM. The higher concentration of such surfactants increases the viscosity of the solution and sometimes forms viscous gels.

1.2.3 Micellar and reverse micellar extraction

Micelle and reverse micellar extraction of a solute consist of two steps namely, forward extraction where in the solute of interest is partitioned into the micelle/reverse micelle from its feed and back extraction which involves the back extraction of the partitioned solute into fresh stripping phase. Micelles and reverse micelles are dynamic in nature and often aggregate or collide with each other resulting in the solute transfer or in the transfer of surfactant monomers from the surrounding environment into the surfactant aggregates (Rabie and Vera 1996). Since the work on the reverse micellar extraction of proteins from the feed solution by Luisi et al. (1988), considerable interest has been laid by different researchers across the globe in employing surfactant based extraction system towards separation and purification of a wide range of solutes.

The extraction efficiency of a surfactant based two phase separation system depends upon the effect of different process variables such as pH, additive effects on the critical micelle concentration of a surfactant system. Variation in the process variables affect the water content of the reverse micelle and bring about a change in the number of surfactant units within a micelle, which in turn influence the solute partitioning into micelle / reverse micelle.

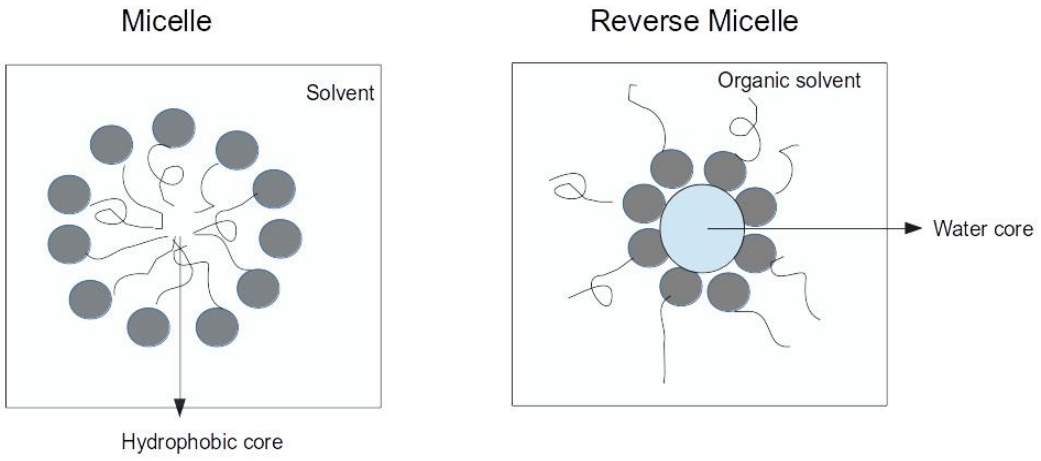


Figure 1.7 Diagrammatic representation of micelle and reverse micelle-micelle.

Aggregation number of a micelle is defined as the ratio of an average molecular weight of reverse micelle to that of the molecular weight of the surfactant.

$$N_{ag} = \frac{Average\ molecular\ weight\ of\ reverse\ micelle}{Molecular\ weight\ of\ surfactant} \qquad (1.2)$$

Water content is defined as the ratio of moles of water to moles of surfactant in a reverse micelle.

$$W_o = \frac{moles\ of\ Water}{moles\ of\ Surfactant} \qquad (1.3)$$

Having known the surfactant concentration, aggregation number can be used to find the number of micelle/reverse micelle units within a system and water content is used to determine the size of a reverse micelle. Ionic surfactant based reverse micellar extraction is more often used for the separation of bio molecules from the feed mixture. Considering the complexity and high density of the fermentation broth, reverse micelles are employed to enhance the mass transfer of the desired solute from the broth to water core of reverse micelle which is hydrophilic in nature and offers high specificity due to the presence of charged head groups protruding into the water core. Change in process variables such as pH, the addition of salt, co-surfactant, and co-solvent enhances or declines the solute transfer into reverse micelles. A few examples of surfactant based extraction of solutes are listed in Table 1.3.

Table 1.3 Different solutes separated using surfactant based extraction

Surfactant employed	Solute extracted	Reference
TX114	Cadmium, copper zinc and lead in water	Ghaedi et al. 2009
TX114	Cobalt and nickel	Giokas et al. 2001
TX114/CTAB/TX100	Allura red	Pourreza et al. 2011
TX114	Lead	Tavallali et al. 2010
TX114	Pediocin like bateriocin	Boyaval et al. 2000

TX114	Estrogen	Wang et al. 2006
PONPE 5	Mercury	de Wuilloud et al. 2002
PONPE-7.5	Gold (III)	Akita et al. 1998
TX114	Arsenic	Shemirani et al. 2005
TX100	Eosin Dye	Purkait et al. 2005
TX100	Sudan dye	Liu et al. 2007
TX100	Congo red	Purkait et al. 2004
TX100	Rhodamine B	Pourreza 2008
CTAB/TX100	Orange II	Pourreza and Zareian 2009
TX100	Carmoisine and Brilliant blue	Pourreza and Ghomi 2011
TX114	Phthalate esters	Ling et al. 2007
PONPE 10	Phenol	Akita and Takeuchi 1995
TX114	Bisphenol and β-napthol	Zhong et al. 2011
TX114	Penicillin residues	Kukusamude et al. 2010
CTAB- affinity reverse micelles (Concanavalin A ligands)	Bromelain	Hebbar et al. 2008
AOT	Lipase	Aires-Barros and Cabral 1991
AOT/Brij 30	Monoclonal antibody (IgG1)	Günther and Stuckey 2010
CTAB	Organic dye	Pandit and Basu 2002
AOT	Amino acids	Dövyap et al. 2006
AOT	Alkaline phosphatase	Gonnelli and Strambini, 1988
AOT	Penicillin acylase	Bansal-Mutalik and Gaikar 2003
CTAB	Soy hull peroxidase	Lakshmi and Raghavarao 2010

Aliquat 336	α - chymotrypsin	Joivalt et al. 1990
AOT	Lectin	He et al. 2015
SDS	Basic Yellow 28, Basic Blue 41, and Basic Red 46	Ueda et al. 2011

Compared to any other liquid-liquid extraction, surfactant based extraction techniques offer several advantages such as enhanced specificity and selectivity towards the solute, ease in handling, and alteration in system properties for the better extraction efficiency, as a single step extraction of both hydrophilic and hydrophobic solutes, ease in design of experiments and scale-up, the use of bio-based surfactants and its biodegradability after use, reuse and recycling of surfactants, lower time for extraction and two phase formation.

The major drawback of surfactant based technique is the disposal of used surfactant stream and the reusability of surfactants. The surfactant systems have been extensively researched upon for their recycling and their restored extraction efficiency. The two phase formation in surfactant based aqueous two phase extraction systems of require an external field such as heat or sound or pressure. However, the invention of novel intensified processes which incorporate the surfactant micellar systems is widely studied for the reduction of overall operational cost with higher selectivity.

1.3 Scope and Objectives of the research work

With the increasing demand towards the production of different types of PHA and its application in various fields, the global market of PHA is expected to reach 3.45 million metric tonnes in 2020 (Shen et al. 2010). Companies that are involved in the production of PHA have shifted their focus towards enhanced production and purification of PHA through extensive research and development to significantly cut short the production cost and to identify an appropriate process design that results in high profit yet is economically feasible. Although sustainability has paved the way towards valorisation of waste from agriculture, industry and domestic waste towards PHA production, classification of separation technique with respect to the PHA synthesised by the microbe and on the PHA physiochemical properties are very

limited and still remains to be a question. Global industries have widely accepted solvent extraction followed by other purification steps such as centrifugation and drying to polish the PHA which meets the market quality (Chen 2009). However, utilising toxic and replenishable petrochemical solvents have found to pose a serious environmental threat and lead to the extinction of fossil fuels. Though PHA expresses a higher solubility in chlorinated solvents, earlier reports suggest the deterioration of its physical properties and native form due to the harsh effect of solvent, which becomes a constraint towards the application in medical and health care industries (Verbeek and Bier 2011). Alternatively, micelle based aqueous two phase extraction technique may be exploited for the PHA extraction and purification, as it was well developed and has been extensively used for the separation of hydrophobic solute from the feed. Unlike any other LLE technique, micellar extraction offers high selectivity towards the hydrophobic solutes.

The current topic of research intends to design an effective and efficient surfactant based separation technique to separate PHA from the feed mixture in its native form. Since PHA exists in the form of amorphous granules associated with proteins and lipids, micelle based extraction techniques have to be designed to separate PHA from the feed at maximum possible purity and recovery. The research topic is put forth in such a way that the design and operation of the developed separation process enhance the extraction efficiency of PHA and also is a sustainable design that could be utilised to separate similar hydrophobic solute of interest from its feed sources. Accordingly, the following objectives were formulated for the present work.

- To synthesise polyhydroxyalkanoate (PHA) using biodiesel derived crude glycerol as the carbon source and *Cupriavidus necator* DSM 428 as the microbial strain and optimise the medium components and physical parameters for the maximum production of PHA.

- To characterise the structural, physical and mechanical properties of the synthesised PHA.

- To develop and design suitable surfactant based extraction/purification techniques for the simultaneous extraction and purification of PHA.

- To study the effect of process variables in each of the developed surfactant based extraction/purification techniques.

1.4 Organization of Thesis

Chapter 1: Brief information on Polyhydroxyalkanoate including classification and PHA characteristic features (Physical and mechanical properties) were discussed along with the importance of bio-separation while highlighting the significance of surfactant based LLE process.

Chapter 2: Elaborative report on the production of polyhydroxyalkanoate (PHA) using biodiesel derived crude glycerol as the carbon source and *Cupriavidus necator* DSM 428 as the microbial strain. A comprehensive literature survey on the synthesis of PHA, experimental protocol, the effect of medium components and process condition on the production of PHA, and physiochemical characterization of synthesised PHA are also explained in this chapter.

Chapter 3: Nonionic surfactant induced cloud point extraction of synthesised PHA from crude fermentation broth was presented. A brief literature survey on the cloud point extraction and the types and characteristics of surfactant, method development and optimisation of process parameters and their effect on PHA extraction by considering individual and mixed surfactants and chromatographic analysis of purified sample are explained in this chapter. Continuous cloud point extraction of PHA, which includes the hydrodynamics and mass transfer characteristics of PHA on maintaining different operating conditions of a continuous contactor, is also reported.

Chapter 4: Ultrasonication assisted cloud point extraction of PHA from crude broth is highlighted in the chapter. A comprehensive literature survey, design and development of ultrasonication assisted cloud point extraction method, the effect of process parameters on extraction efficiencies and chromatographic analysis of the purified PHA are presented.

Chapter 5: Design and development of gum Arabic – mixed micelles based coacervate induced encapsulation of PHA and its release during microfiltration is elaborated. Effect of process parameters on membrane extraction efficiencies and flux of the membrane process at different operating conditions has been elaborately discussed.

Chapter 6: Development and validation of nonionic surfactant TX100 based micellar liquid chromatographic separation of PHA from the crude fermentation broth method has been reported. Effect of process variables on the peak efficiency is explained in detail.

Chapter 7: Highlights a consolidated summary and conclusion of all the developed separation process and comparison of respective extraction efficiencies, pros and cons of the processes. The overall conclusion enlisting both upstream and downstream processes developed towards the synthesis of PHA by *Cupriavidus necator* and its separation and purification from the broth.

Chapter 8: Bibliography

CHAPTER 2

PRODUCTION OF PHA

2.1 PHA granules

Primarily, PHA is synthesised by the microbes as reserved food source which also acts as stress regulator under UV irradiation, heat, and stress shock. Cell metabolic pathways such as glycolysis, Krebs cycle that is involved in respiration, β-oxidation, de novo fatty acids synthesis which are responsible for the fatty acid conversion, amino acid catabolism, Calvin Cycle, and serine pathway are all interlinked with PHA synthesis. Substrates are converted into Acetyl-CoA, the most common metabolic intermediate, which is directed towards PHA synthesis and accumulation. PHA granules exist in the form of inclusion bodies as shown in figure 2.1, and are made up of 97.7% PHA, 1.87% protein and 0.46% lipid contents (McCool and Cannon 1999). These inclusion bodies have an inner core made up of hydrophobic amorphous PHA enclosed by phospholipid monolayer consisting of catabolic – PHA synthase and PHA depolymerase and non-catabolic PHA synthases, the key enzymes that are involved in PHA synthesis which takes place within the cytoplasm of the microbial cell. Carbon source with up to 6 carbon atoms is converted into soluble 3-Hydroxyacyl-CoA by the catabolic enzymes that are structurally transformed as explained above to form insoluble PHA granules. Four different classes of PHA synthases have been classified till date based on their substrate specificity. PHA synthase, the key enzyme of PHA production, is classified under α/β hydrolase family of enzymes, and are involved in functions similar to that performed by cellular lipases whose presence is noted in the interface of the aqueous phase and a hydrophobic membrane layer. Hydrophobic C terminal of PhaC class I and II are attached to the granule, while in class III and IV, PhaE and PhaR have a hydrophobic terminal attached to the granule (Tan et al. 2014, Mezzina and Pettinari 2016). NMR study conducted by Doi et al. (1995) on copolymer synthesis in *Ralstonia eutropha* revealed that random copolymerization takes place, where the addition of monomer to growing PHA chain is independent of the monomeric unit present at the growing chain end. Enzymatic action on the control of PHA chain length remains unclear. Similarly, a single PHA synthase enzyme unit synthesising multiple polymer chains

and PHA chain initiation and termination also remain unclear. In vitro study on PhaC revealed that molecular weight of the polymer or more specifically, the growth of PHA molecule within the microbial cell, is controlled by the enzyme concentration and is independent of substrate concentration in the feed.

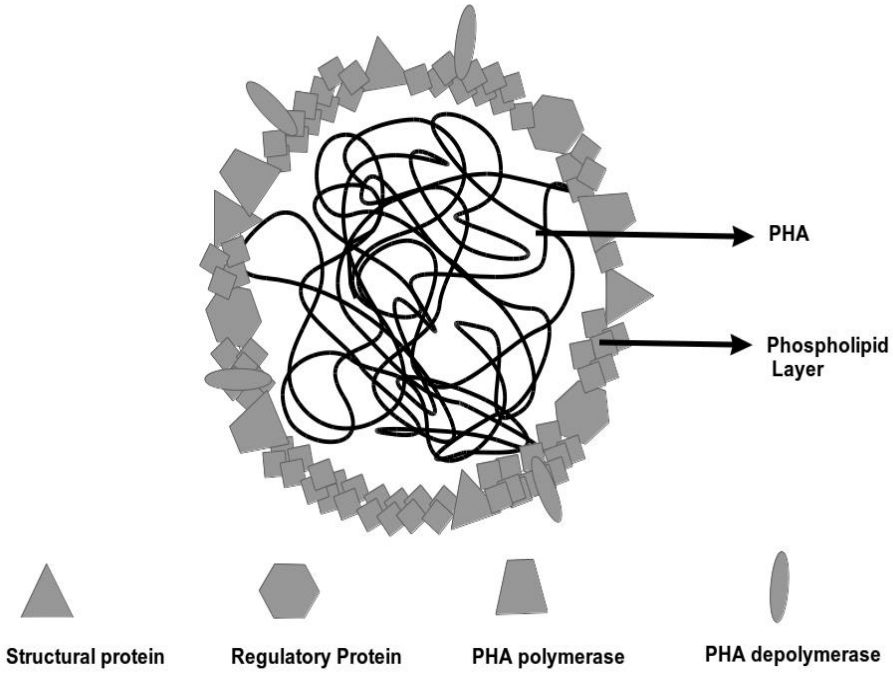


Figure 2.1 Schematic representation of PHA granule.

Earlier studies on NMR and DSC analysis infer that PHA exists as amorphous granules rather than in its crystalline state (Inoue et al. 1989) and TEM analysis further proved that PHA within the cell exists as multiple granules (Cai et al. 2009). Horowitz and Sanders (1995) studied the growth of artificial PHB granules and its purification using surfactants. It was observed that the PHB molecules are in the amorphous state rather than their crystalline structure as a result of nucleation of PHB. The authors believe that the PHA's amorphous state is as a result of the size of the granules and is not a biological effect.

Structural studies on PHA granules have reported that the enzymes associated with PHA synthesis are found on the lipid bilayer covering the PHA granule. The PHA depolymerase is responsible for the metabolism of synthesised PHA and the synthesised PHA is also reported to be embedded within the lipid bilayer. Other proteins associated with the lipid bilayer, commonly referred to as Phasins is PhaP,

which is the most abundant protein reported to be found on the lipid bilayer of *Ralstonia eutropha*. These PhaP proteins have a similar function to oleosins found in plants that are involved in stabilising the PHA granules and preventing their coalescence. Hydrophobic C terminal of Phasins have been found to act as granule binding domain and have also been found to involve in granule localisation within the cell. In a study involving deletion of PhaP gene resulted in the formation of larger granule while over expression of the gene resulted in a large number of smaller granules (Steinbüchel1995, Bernd 2003, Tan et al. 2014, Mezzina and Pettinari 2016).

2.2 Valorisation of Biodiesel derived crude glycerol

Increased dumping of waste from different sources and problems related to its disposal and environmental issues has paved a way towards identification and utilisation of simple to complex waste as an effective carbon source, which has become worldwide interest among researchers in the recent past. Complex waste streams are often made up of a mixture of various carbon and other nutrients and are considered to be very helpful in synthesising PHA with varied yet effective materialistic properties that can be utilised for high-end applications. Agricultural waste, biodiesel waste, waste from the oil industry, dairy industry waste are a few examples of complex waste streams that have been well utilised for PHA production. Crude glycerol has been used as an active raw material towards the production of 1.3-propanediol, citric acid; hydrogen based fuels, docosahexaenoic acid, other lipids, dihydroxyacetone, succinic acid and PHA via fermentation. Crude glycerol is also extensively employed for the production of other fine chemicals via chemical catalysed reactions. A variety of microbes have been documented to utilise glycerol as carbon source towards the production of PHA, *Pseudomonas oleovorans* NRRL B-14682, *Pseudomonas corrugata* 388, *Paracoccus denitrificans* and *Cupriavidus necator* DSM 545, *Zobellella denitrificans* MW1, Mixed microbial consortia (MMC). However, it is important to consider that the waste streams are spent feed or processed water stream that contains a lot of heavy metals, organic compounds, non-fermentable materials that might hinder the microbial growth and PHA accumulation. The dedicated research is always required in this regard before utilising the waste streams for PHA production.

2.3 Literature Survey

2.3.1 Metabolic pathways of PHA production

PHA accumulation occurs in the presence of surplus carbon source and limited macro nutrients or micro nutrient condition prevails in the medium. Microbes have been categorised into two classes based on the uptake of nutrients, the first group includes microbes that synthesise PHA during limited nutrient conditions while the second group accumulates PHA during exponential growth phase and doesn't require nutrient limiting condition. PHA accumulation while the growth of microbe takes place is common in microbes such as *Alcaligenes latus*, *Methylobacterium* sp. (Nath et al. 2008) and recombinant *E. coli*. Other microbes such as *Cupriavidus necator*, *Azotobacter vinelandii*, *Haloferax mediterranei* and *Pseudomonas hydrogenovora*, synthesise and accumulate PHA, devoid of its link to cell growth. In *Pseudomonas* 2F (Braunegg et al. 1999, 2002), PHA production was found to reach a maximum after carbon source depletion, which proves that the key enzymes involved in PHA production, their activity and resulting PHA accumulation varies from microbe to microbe and depends on the carbon source and other nutrient conditions maintained.

Depending upon the nature and composition of the carbon source and the microorganism employed, appropriate metabolic pathways are activated within the microbe that metabolises carbon and stores PHA in the cytoplasm. The carbon source is converted into Acetyl-CoA by the action of pyruvate dehydrogenase during glycolysis, on further metabolic reaction carried out by a series of enzymes, PHAs are formed. In the presence of increased carbon source, Acetyl-CoA from Krebs cycle blocks 3-Ketothiolase (PhaA) responsible for PHA production and excess Acetyl-CoA is diverted for cell respiration and growth. Though production of R-hydroxyalkyl-CoA from the usual metabolic pathway has been reported, conversion of R-hydroxyalkyl-CoA to PHA remains unclear and needs to be explored. Till date, 37 enzymes have been reported that are involved in PHA synthesis (Tan et al. 2014).

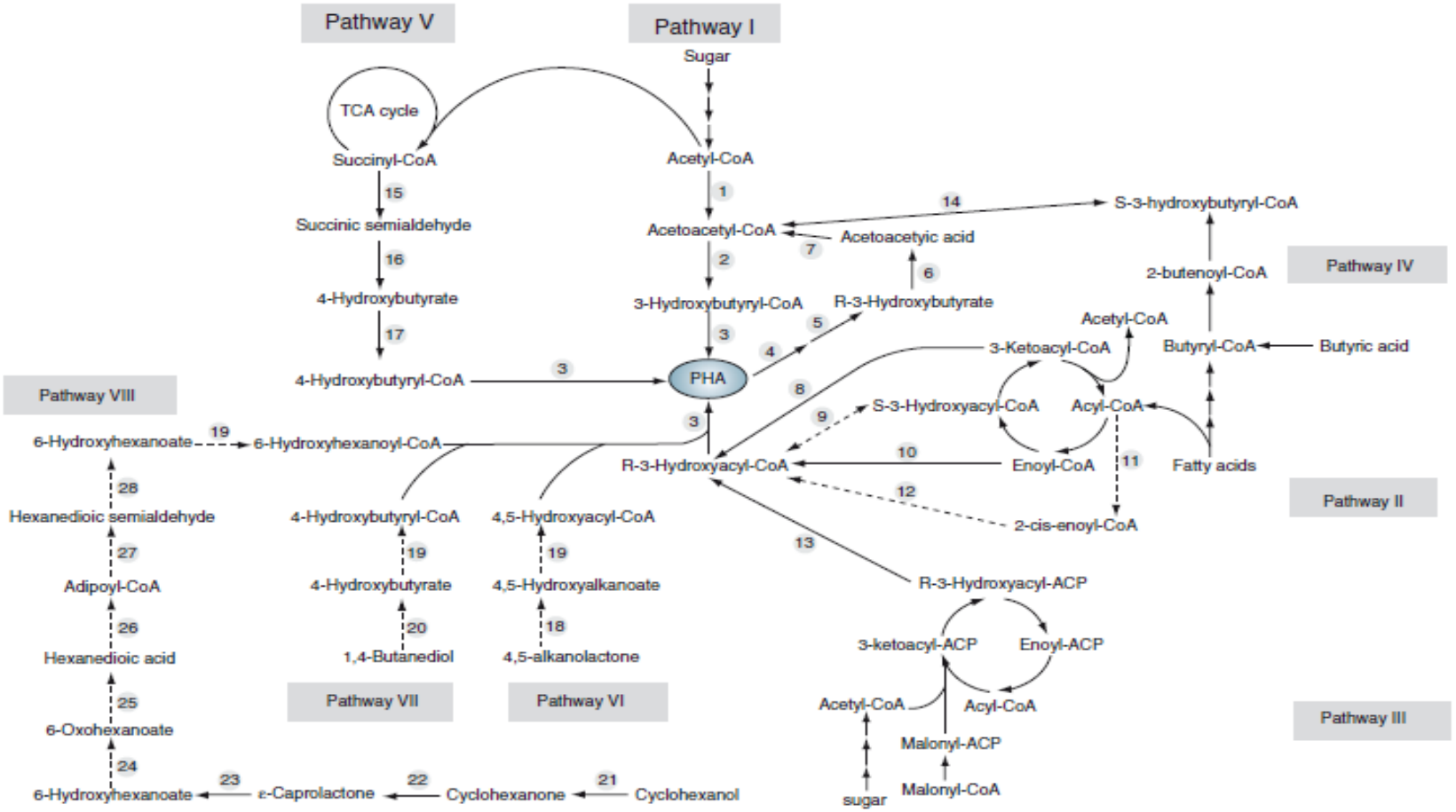


Figure 2.2 Metabolic pathways involved in the production of PHA from various carbon sources (adopted from Sudesh et al. 2000).

Table 2.1 Enzymes involved in the metabolic pathway towards PHA production (adopted from Sudesh et al. 2000 and Tan et al. 2014).

Pathway	Enzyme	Enzyme name	Reference
Pathway I	PhaA	β-ketothiolase	Sudesh et al. 2000
	PhaB	NADPH dependent acetoacetyl-CoA reductase	
	PhaC	PHA synthase	
Associated pathway	PhaZ	PHA depolymerase	
		Dimer hydrolase, (R) -3-Hydroxybutyrate dehydrogenase, Acetoacetyl-CoA-synthetase	
Pathway II	FabG	3-Ketoacyl-CoA reductase	
		Epimerase	
	PhaJ	(R)–Enoyl-CoA hydratase/enoyl-CoA hydratase I	
		Acyl-CoA oxidase, putative Enoyl-CoA hydratase I	
Pathway III	PhaG	Putative 3-Hydroxyacyl-ACP-CoA transferase	Sudesh et al.2000, Zheng et al. 2005, Taguchi et al. 1999
	FabD	Malonyl-CoA-ACP-transacylase	
Pathway IV		NADH-dependent Acetoacetyl-CoA reductase	Chohan and Copeland 1998
	SucD	Succinic semialdehyde dehydrogenase	Valentin and Dennis 1997
Pathway V	4hbD	4-Hydroxybutyrate dehydrogenase	
	OrfZ	4-Hydroxybutyrate-CoA:CoA transferase	
Pathway VI		Lactonase	Valentin and steinbuchel 1995

Pathways VII		putative hydroxyacyl-CoA synthase, putative Alcohol dehydrogenase	Xie and Chen 2008
Pathway VIII	ChnA	Cyclohexanol dehydrogenase	Brzostowicz et al.2003
	ChnB	Cyclohexanone monooxygenases	
	ChnC	Caprolactone hydrolase	
	ChnD	6-Hydrohexanone dehydrogenase	
	ChnE	6-Oxohexanoate dehydrogenase, semialdehyde dehydrogenase, putative 6-Hydroxylhexanoate dehydrogenase, putative Hydroxyacyl-CoA synthase	

2.3.2 Carbon sources and microbes employed for PHA production

Saccharides or carbohydrates are the most abundant and readily consumed carbon source by the microbes. The oligosaccharides and polysaccharides are generally converted into a simpler form (monosaccharide) which can then be utilised by the microbe for PHA accumulation. During metabolism, sucrose, a simple sugar obtained from sugar bearing plant sources is converted into fructose and glucose, which is then up taken by microbes such as *Azotobacter vinelandii*, *Alcaligenes lactus*. Recombinant strains of *Ralstonia eutropha* harbouring genes from *Mannheimia succiniciproducens* was found to utilise sucrose directly but then the productivity was very less about 0.0046 g/L/h (Park et al. 2015). Molasses obtained from sugar industries are waste by-products that are utilised as cheapest sugar source towards PHA production. However, availability of molasses is subjected to seasonal production of sugar bearing crops. Lactose, milk sugar obtained in the form of whey from the dairy industry, is a discharged waste after cheese manufacturing. Whey contains about 35-50 g/L of lactose in it and a number of bacteria have been screened to utilise lactose for the production of PHA. Starch, cellulose, and hemicelluloses are a few examples of polysaccharides. Starch is obtained from rice, sorghum, potato, it is said to be the complex form of glucose interlinked by α – 1, 4 - glycosidic bonds. Lignocellulose contains about 40 % – 50 % cellulose, 20 % – 50 % hemicelluloses and 20 % – 30 % lignin. Lignin is an unfermentable aromatic compound while

hemicellulose is made up of polysaccharides such as xylose, mannose, arabinose, galactose, and rhamnose. Cellulose is an interlinked β – 1, 4 - a glycosidic form of D-glucose, which cannot be metabolised and so, is excreted by the human body. Lignocellulose needs to be treated extensively for delignification to excrete out cellulose and hemicellulose. With microbes that can feed upon such complex sugar sources, liquefied wood has also been researched upon as potential carbon source towards PHA production.

Apart from sugars, lipids are readily accepted as the carbon source by a variety of microbe. The lipid content in the medium is hydrolyzed to short chain length fatty acids by lipase from the microbe. After transport of the fatty acids across the cell membrane, β-oxidation of fatty acids takes place to synthesise PHA. Complex fat content such as tallow which is a mixture of waste animal fat obtained from the animal slaughtering has been tested for their efficacy to be used as carbon source. It was found that their low melting point has been a problem in hindering the fermentation process, however, when used with methanol mcl-PHA were reported to be synthesised by the microbe. *Ralstonia eutropha* has been found to utilise tallow to synthesise PHA where in 80 % cell accumulation was achieved. When gum arabic was used as an emulsifier in the presence of tallow, Riedel et al. (2015), was able to achieve PHA production of 26 g/L. Various plant oils and waste from plant oil industry such as olive oil factories have been tested for PHA production and it has been reported that they have been readily consumed by fatty acid consuming bacteria, owing to liquid form (Riedel et al. 2012). Glycerol from biodiesel industry prevails to be the current topic of interest among researchers, owing to its abundant availability and uptake by a number of microbes to synthesise a variety of PHA molecules with varied materialistic properties. Hydrocarbons have also been tested for its utilisation as complex carbon source towards PHA production, most of the *Pseudomonas* spp., have been found to synthesise PHA from hydrocarbons (Kanaly and Harayama 2000, Tobin and O'Connor 2005). A list of different carbon sources and microbes that were involved in PHA production are enlisted in table (2.2)

Table 2.2 List of carbon sources and microbes employed towards PHA production.

	Microbial strain	PHA productivity (g/L or % CDW)	CDW (g/L)	Reference
Sugar Beet molasses	*Azotobacter Vinelandii* UWD	PHB:19-22	N/A	Page 1992
	Azotobacter Vinelandii UWD	PHB: 36	N/A	Chen and Page 1997
Sugar cane molasses	*Bacillus sp.* Jma5	PHB: 25-35% dry weight	30 (batch), 70 (fed batch)	Wu et al. 2001
	Bacillus megaterium ATCC 6748	PHB: 2.2 (43% Cell dry weight)	5	Chaijamrus et al. 2008
	Bacillus megaterium BA-019	PHB :30.5 (42% Cell dry weight)	72.6	Kulpreecha et al. 2009
Soy molasses	*Pseudomonas corrugata*	mcl-PHA: 5-17%	1.5-3.6	Solaiman et al. 2006
	Bacillus sp. CL1	PHA : 90%	3.42	Full et al. 2006
Hydrolyzed soy and malt	*Bacillus sp.* HF-1, HF-2	PHA: 18.42	32	Law et al. 2001
Hydrolyzed whey	*Ralstonia eutropha* DSM545	PHBV : 2.25	4.5	Marangoni et al. 2002
Whey and Whey Hydrolysates	*Methylobacterium sp.* ZP24	PHB: 1.1	5.25	Yellore and Desai 1998
	Methylobacterium sp. ZP24	PHA : 2.6-5.9	5.1-9.9	Nath et al. 2008
	Bacillus megaterium CCM 2037	PHB: 1.48	2.87	Obruca et al. 2011
	Recombinant *E. coli* GCSC 6576	PHB: 109	87	Wong et al. 1998

	Microbial strain	PHA productivity (g/L or % CDW)	CDW (g/L)	Reference
	E. coli GCSC 4401	PHB: 96.2	119.5(fed batch)	Ahn et al. 2000
Hemicellulosic fraction of poplar wood	*Pseudomonas pseudoava*	PHB: 6.57	1.5	Bertrand et al. 1990
Xylose with levulinic acid	*Burkholderia cepacia* ATCC 17759	PHBV: 1.3-4.2	9.5	Keenan et al. 2006
Hemicellulosic hydrolysates	*Burkholderia cepacia* ATCC 17759	PHB: 2.0	5.1	Keenan et al. 2006 a
xylose, glucose from sugar cane bagasse	*Burkholderia cepacia* IPT 048 and *B. sacchari* IPT 101	PHB: 34.8	60	Silva et al. 2004
Wheat bran hydrolysate	*Halomonas bolivensis* LC1	PHB: 4	9	Van-Thuoc et al. 2008
cellulose in tequila bagasse	*Saccharophagus degradans* ATCC 43961	PHA: 1.5	2.55	Munoz et al. 2008
Formic acid, acetic acid, furfural and acid soluble lignin	*Ralstonia eutropha*	PHB: 6.1-6.8	10.7-11.1	Yu et al. 2008
Lard, butter oil, olive oil, coconut oil, soybean oil	*Pseudomonas aeruginosa* and *Pseudomonas resinovorans*	mcl-PHA: 2.1	1.6-2.8	Ashby et al. 1998

	Microbial strain	PHA productivity (g/L or % CDW)	CDW (g/L)	Reference
Castor seed oil, coconut oil, mustard oil, cottonseed oil, groundnut oil, olive oil, and sesame oil	*Comamonas testosteroni*	mcl-PHA : 87.5% cell dry weight	N/A	Thakor et al. 2005
Lard and coconut oil	*Pseudomonas putida*	PHA: 0.9-1.6	2 to 4	Solaiman et al. 2001
Palm kernel oil, palm olein, crude palm oil and palm acid oil	*Wautersia eutropha* mutant	PHA:3.3	8.3	Loo et al. 2005
Glycerol	*Bacillus megaterium*	PHB: 62%		Naranjo et al. 2013
	Cupriavidus necator DSM 545	PHB: 62%		Cavalheiro et al. 2009
	Cupriavidus necator	PHB: 0.92	Specific growth rate - 0.11 h-1	Tanadchangsaeng and Yu 2012
	Methylobacterium rhodesianum and *Cupriavidus necator* DSM 11348	PHB: 0.254,g/l/h		Bormann and Roth 1999
	Z. denitrificans	PHB:74%	1.25 g/l/h	Ibrahim and Steinbüchel 2009
	Haloferax mediterranei	PHBHV4HB:75.4 %		Hermann-Krauss et al. 2013

	Microbial strain	PHA productivity (g/L or % CDW)	CDW (g/L)	Reference
Jatropha based biodiesel derived glycerol	Marine bacteria SM-P-3M	PHA: 0.306	0.404	Shrivastav et al. 2010
Methane	*Methylocystis spp.*	PHBV: 39 %		Myung et al. 2015
Methane	type II methylotrophs	PHB: 0.54		Khosravi-Darani et al. 2013

2.3.3 Role of *Cupriavidus necator* and crude glycerol in the production of PHA

Cupriavidus necator is a hydrogen oxidizing bacteria, known as Knallgas bacteria is capable of switching to aerobic and anaerobic growth conditions and can adapt to heterotrophic or autotrophic growth. The bacterium was first reported in the year 1987 by Makkar and Casida, it was found that the bacteria behave as a non-obligatory predator feeding on other gram-negative and gram-positive soil bacteria and fungi (Casida 1988). The microbe is strong resistance to copper and presence of copper in the medium stimulates microbial growth. *Cupriavidus necator* genome has been well sequenced and their metabolic pathways have been studied. Hydrogen oxidizing enzyme, Hydrogenase is the main product of interest for which the microbe has been well researched upon. As a result of their hydrogen fixing ability, the microbe has been extensively researched upon for their ability to support life in space. The organism has undergone a lot of change in its name from its isolation to till the past decade (Vandamme and Coenye 2004).

As a promising alternative fuel, biodiesel has gained worldwide importance over the last decade, research on novel sources for biodiesel production, method development, and optimization, scale up to have been the topic of research among scientist looking ahead for a sustainable fuel that could replace fossil fuels. A major by-product of the biodiesel industry, glycerol is as well increasing at an alarming rate, 10 % (w/w) of raw material is converted into glycerol during biodiesel production. 30 million gallon plant is expected to produce 11,500 tons of glycerol every year, it is estimated that the biodiesel industry has resulted in 4 billion gallons of crude glycerol across the globe (Ayoub and Abdullah 2012). Valorisation of crude glycerol into useful products has been researched upon to make a profit out of such large abundant waste source, while its purification to pure glycerin and its usage in the pharmaceutical and medical field as an active pharmaceutical ingredient or in any other field is a cost consuming process. The catalyst employed, impurities present in the feed determine the purity of crude glycerol and its composition, crude glycerol contains glycerol, ash, and methanol apart from soap and other impurities such as salt in most cases.

Glycerol is transported across the membrane barrier via passive diffusion. Glycerol uptake is facilitated by an integral membrane protein, Glycerol facilitator

(GlpF), glycerol in the cytoplasm is converted into Glycerol-3-Phosphate by Glycerol kinase (Glpk) and the catabolic product remains in the cytoplasm until further metabolic pathways are stimulated. GlpF is a highly selective protein facilitating non-ionic compounds and facilitates 100 - 1000 times transport of glycerol to cytoplasm from the surrounding medium and is not saturated even at concentrations > 200 mM. A large number of microbes can grow anaerobically in the presence of glycerol; glycerol can be readily converted into Acetyl-CoA which is the main product for the synthesis of PHA (Jensen et al. 2002, Stroud et al. 2003, Henin et al. 2008). PHB obtained from glycerol fermentation has been reported to be of low molecular weight, as a result of the termination of chain propagation caused by covalent esterification of glycerol to PHB.

Glycerol utilisation happens via fatty acid metabolism, apart from PHB, mcl-PHA is synthesised via this metabolic pathway as long chain fatty acids are converted into PHA. The number of carbon atoms in the fatty acid determines the monomer formed and the different types of fatty acids present in the medium lead to the formation of copolymers. Fatty acids present in the medium are converted into Acyl-CoA by the action of thiokinase and CoA-transferase, Acyl-CoA is further oxidized via β-oxidation pathway to form Acetyl-CoA which is further converted to 3-Hydroxy-acyl-CoA. PHA_{mCL} synthases are the key enzymes responsible for the conversion of 3-Hydroxy-acyl-CoA into PHA. The presence of saturated aliphatic fatty acids in the medium leads to conversion of the same into Acetyl-CoA and Propionyl-CoA, the presence of side chained fatty acids and those with functional groups are converted into other CoA esterases that are then taken down to form 3-Hydroxy-acyl-CoA (Mothes et al. 2007, Chen 2009).

PHA molecules are synthesised as homopolymers or copolymers. In a study performed by Gao X et al. (2012), genes related to β-oxidation were related which enhanced the direct conversion of PHA similar to the chain length of fatty acid added to the fermentation medium. Genetic engineering of *Pseudomonas putida* KT2442 leads to a novel genetically engineered strain that can utilise carbon source with up to 10 carbon atoms, forming homopolymers. Most of the wild strains have been reported to synthesise PHA copolymers containing PHB and another PHA molecule interlinked together to form the biopolymer. The cost of producing copolymer is high

as to synthesise HV, HHx, propionate and lauric acid respectively should be added to the medium. Most of these raw materials are toxic, genetically modified strains have been generated to overcome this problem. Block copolymers are synthesised by covalent bonding of two or more unique monomers. Random block copolymer production results in novel PHA materials with varied materialistic properties. It is inferred that presence of PHB as a monomer unit in random block copolymer offers mechanical strength and its materialistic properties; however, depending upon the mol % of a certain monomer, the overall materialistic properties vary.

High cell density was achieved by maintaining a linear growth phase of *C. necator* in the fed-batch mode when glucose and fructose were used as carbon source. In another study conducted by Fereidouni et al. (2011), maximum PHB content was achieved when fructose was fed during exponential phase, adding sodium acetate to the medium in the presence of fructose increased the yield of PHB. 81 % PHA accumulation was achieved when a pulse of soy bean oil (40 g/L) was fed to a bench scale bioreactor, growth limitation was observed on exhaustion of minerals in the medium. Tanadchangsaeng and Yu (2012) studied the production of PHB by *C. necator*, the authors found that the productivity was less than that observed in the presence of glucose and the specific growth rate attained a maximum of about 0.11 h-1. The microbe was found to follow gluconeogenesis to synthesise glucose from glycerol that can be utilised for cellular activity by the microbe, glycerol as carbon source was found to affect the biomass growth rate, PHB productivity and its polymer characteristics. Two stage batch fermentation in the presence of rice straw hydrolysate revealed that higher degrees of nitrogen deficiency lead to maximum PHA accumulation and maximum PHA accumulation was found to occur in the first 12 hours from PHA accumulation initiation stage. The presence of aromatic compounds in the medium was found to restrict PHA accumulation while fed batch mode enhanced the production of PHA. Aromatic compounds are metabolized by *C. necator* via 2, 3-dioxygenase pathway. In a study on dual feeding of levulinic acid and sodium propionate, it was found that PHBV was synthesised with 80 mol % HV content. Levulinic acid was utilised for cell growth and proliferation while sodium propionate was metabolized to 3-HV (Berezina 2012). *C. necator* is able to utilise complex carbon source and in a study conducted by Koller et al. (2015) liquefied

wood was converted into PHBV. High concentrations of liquefied wood inhibited the cell growth. Mazur et al. (2009) have utilised the biodiesel of rice bran oil as a carbon source and obtained PHB accumulation of 61 %.

2.3.4 Fermentative modes of PHA production

Although all three modes of fermentation - Batch, Fed batch and continuous cultivation of PHA has been well studied and documented. Industrially fed batch cultivation is employed for the production of PHA, as a result of higher productivity of PHA and biomass. Hartmann et al. (2006) employed octanoic and undecenoic acids as carbon source and cultivated *Pseudomonas putida* in a batch mode to check out the production of PHA and found that the molar fraction of each monomer in the mcl-PHA produced varied significantly in the exponential phase than during stationary phase and inferred that the organism utilises different substrate kinetics and was found to accumulate more PHA during initial stages of nitrogen limitation. The viability of *Pseudomonas oleovorans* was found to decrease with limiting nitrogen concentration and PHA accumulation and was concluded that batch cultivation is not a suitable method for PHA production.

Fed Batch fermentation is considered to be the suitable method for production of PHA to achieve cell mass and PHA productivity. Operating parameters are altered and maintained in such a way that both biomass production and PHA production are well maintained and doesn't affect each other, to do so dissolved oxygen concentration and nitrogen concentration are limited. During fed batch production of PHA from *Alcalingenes latus,* dissolved oxygen supply was controlled without limited nitrogen condition which resulted in increased biomass during exponential phase and later nitrogen limited conditions were introduced which resulted in PHA accumulation. Single stage fed batch fermentations during nitrogen limited conditions resulted in a poor accumulation of PHA. Sun et al. (2007), discovered that optimal strategy for PHA production in fed batch to feed growth limiting substrate at the same rate at which substrate utilisation takes place ensures that only PHA metabolic pathway is activated and no by-products are formed.

During the single stage continuous fermentation increased biomass growth and decreased PHA production was reported at increasing dilution rate. Early induction of nutrient limitation resulted in maximum PHA accumulation and less biomass growth

and vice versa condition was observed for late induction of nutrient limitation. Hartmann and co-workers (2006) found that varying the dilution rate had an effect on the monomeric composition of PHA synthesised. Zinn et al. (2003) limited both carbon and nitrogen and found that PHAs were produced with tailor made properties which are employed in the production of PHA from carboxylic acids, where in limited nutrients doesn't affect the growth of the microbe and also enhance the productivity. (Akaronye et al. 2010)

The dissolved oxygen concentration has also had an effect on the production of PHA, dissolved oxygen % of 1 - 4 % air saturation resulted in a decrease in production rates of 3HB due to lower glucose uptake rates yet 3HV production increased in the system containing *Cupriavidus necator* that utilised glucose and propionic acid. Under oxygen limited condition, oxidative loss of CO2 from propionly - CoA is avoided resulting in increased production of 3HV from propionate at the expense of 3HB (Lefebvre et al. 1997). During PHA production in aerobic conditions, 50 % of the carbon source is utilised for respiration while only a lesser amount of carbon source is accumulated as PHA.

2.3.5 Separation of Polyhydroxyalkanoate

Biomass containing PHA inclusions are to be treated appropriately for the release and separation of PHA from other cellular impurities. Cellular proteins and the lipopolysaccharide layer surrounding PHA can act as pyrogen causing severe medical issues and sometimes lethal, if not separated from PHA before it is processed for any direct consumption/utilisation. Selection of any downstream processing technique to separate PHA depends upon the number or amount of PHA present in a cell, the type of PHA present, microbial strain (presence of cell envelope), and percentage purity required (Kunasundari and Sudesh 2011, Koller et al. 2013). However, the impact of the downstream technique on PHA materialistic properties is hardly considered. In recent times owing to its varied applications and the necessity to retain its materialistic properties, selection of an appropriate separation technique is considered to be a crucial step. A few cellular impurities that are considered to be pyrogen are listed in table 2.3.

Table 2.3 List of cellular content associated with PHA.

Cellular content	Separation method	Reference
Lipids	Degreasing	Braunegg 1998
Proteins	Enzymatic treatment – Protease	Holmes et al. 1982
DNA	Enzymatic treatment – Nuclease	Boynton et al. 1999
Endotoxins (Lipopolysaccharide layer-Gram negative bacteria)	Non-polar solvent extraction	Volova et al. 2003

The solubility of PHA in any given solvent or solvent mixture is important to determine the type of process design to be developed and adopted. The economy of the separation process depends upon the equipment design, raw materials used, PHA yield and purity obtained and the feasibility to use or process the separated PHA material. The water content in the feed might hinder PHA extraction from the cells and needs to be removed appropriately by separation of cells from medium and drying or lyophilisation of the biomass, which could be treated for PHA removal. Such treated cells are dissolved in polar to intermediate nonpolar solvents to remove lipids that could give odour and colour to the PHA material. Several purification techniques have been employed and have been studied by various researchers, which are listed in table 2.4. Even though advanced techniques have been identified, industrially solvent extraction is employed as it is robust, scalable and comparatively of cheaper operation cost and the process is well established. Chlorinated solvents are added to solubilise PHA material from the pre-treated biomass, followed by precipitation of PHA by adding “PHA anti-solvent” such as low chain alcohols/water/acetone. The addition of PHA anti-solvent precipitates PHA from the solution while solubilising lipids and other non-PHA cellular materials. Non-halogenated solvents such as tetrahydrofuran, acetic acid have also been tested to extract PHA from the biomass. Mcl-PHA is readily soluble in both halogenated and non-halogenated solvents than scl-PHA.

Table 2.4 Different downstream techniques developed to separate and purify PHA from biomass.

Separation Technique employed	System employed	Strain Employed	Yield and Purity	Reference
Solvent Extraction	Chloroform	*Bacillus cereus* SPV	Yield 31% Purity 92%%	Valappil et al. 2007
	Chloroform	*Cupriavidus necator* DSM 545	Yield 96% Purity 95%	Fiorese et al. 2005
	1,2-Propylene carbonate	*Cupriavidus necator* DSM 545	Yield 95% Purity 84%	Fiorese et al. 2005
	Acetone-water Process		Yield 80-85%	Narasimhan et al. 2008
	Methyl tert-butyl ether	*Pseudomonas putida* KT2440	Yield 15-7.5 wt%	Wampfler et al. 2010
	Methylene chloride	*Cupriavidus necator*	Purity 98%	Zinn et al. 2003
	Non halogenated solvents- isoamy propionate, propyl butyrate, isoamyl valerate etc.	*Cupriavidus necator*	~Purity 90%	Mantelatto and Durao 2008
	Acetone, room temperature	*Pseudomonas putida* GPo1	Yield 94%	Elbahloul and Steinbuchel 2009
Digestion Method				
Surfactant	SDS	Recombinant *E. coli*	Purity 99% Yield 89%	Choi and Lee 1999
	Palmitoyl carnitine	*Cupriavidus necator, Alcaligenes latus*	Degree of lysis : 56-78%	Lee et al. 1993
Sodium Hypochlorite		*Cupriavidus necator*, Recombinant *E. coli*	Purity 86% Purity 93%	Hahn et al. 1995
		Cupriavidus	Purity 98%	Berger et al.

Separation Technique employed	System employed	Strain Employed	Yield and Purity	Reference
		necator DSM 545		1989
Self-flotation of cell debris	Chloroform	*Zobellella denitrificans* MW1	Yield 85% Purity 98%	Ibrahim and steinbuchel 2009
Dissolved air flotation	Enzymatic hydrolysis, sonificatin , flotation	*Pseudomonas putida*	Purity 86%	Van Hee et al. 2006
Aqueous two phase system	Microbispora sp culture-ATPS	*Bacillus flexus*	Yield 50% Purity 95%	Divyashree et al. 2009
Gamma irradiation	Radiation-chloroform	*Bacillus flexus*	Yield 45-54%	Divyashree and shamala 2009
Air classifcation		*Cupriavidus necator, E. coli*	Yield 90% Purity 97% , Yield 85% Purity 95%	Noda 1998
Spontaneous liberation		*E. coli*	Autolysis of 80%	Jung et al. 2005
Cell fragility	Chloroform	*Bacillus flexus*	Yield 43%	Divyashree and shamala 2010
	Sodium hypochlorite	*Bacillus flexus*	Yield 50%	
	Alkaline hydrolysis	*Bacillus flexus*	Yield 50%	

Kunasundari and Sudesh (2011) have listed out the pros and cons of different separation techniques developed to separate and purify PHA from biomass. Although, solvent extraction technique offers several advantages such as removal of lipids and proteins from the PHA inclusion bodies (Endotoxin removal), ease in design, scale up and operation of the technique, very low extraction time and high recovery of PHA; several disadvantages such as large scale use of volatile, toxic and non-renewable petrochemical based solvents to dissolve a very limited amount of PHA, high cost

insisted by large volume of solvent used during the extraction technique, environmental threat imposed by usage of hazardous solvents, difficulty in extraction of mcl-PHA and lcl-PHA as a result of increasing viscosity and elasticity exerted on PHA solubility affects the extraction efficiency, loss of polymer nativity and separation of valuable by-products not possible during conventional solvent extraction technique. While, chemical digestion offers advantages such as ease in scale up, solubilisation of non-PHA cellular content and higher recovery of product, it imposes disadvantages such as loss of PHA nativity and digestion of non-PHA content results in low PHA purity. Enzyme based separation technique offers advantages such as mild, non-denaturing extraction of PHA from biomass while the separation of by-products is also possible. Enzyme based extraction impose huge operational cost as a result of the use of enzymes, difficulty in design and scale up of enzyme based extraction technique, lower efficiency during reuse of enzyme, further separation steps required to extract PHA from the complex feed containing cytoplasmic content. Mechanical disruption techniques offer pros such as ease in design, operation and scale up, very low operational cost, low generation of waste, however, high degree of loss in PHA nativity as a result of heat generated during mechanical operation, mechanical disruption offers very low purity and it is considered to be a pre-purification technique towards release of intracellular PHA from the biomass into the surrounding medium, further purification techniques are required to reach the desired purity of PHA. Super critical fluid extraction impose huge operation cost owing to maintenance of high temperature and pressure to generate super critical CO_2 however it offers low specificity towards PHA extraction and lesser purity since the super critical fluids possess the ability to solubilise most of the lipid content. Though Polymer based ATPS is a well-established separation technique, use of polymer and its interaction with PHA remains unclear and as a result design, operation of large scale operation remains to be a question. Novel extraction techniques such as gamma irradiation are of high cost and difficulty to scale up to large scale operations due to the hazardous effects of continuous use of gamma rays on health issues.

2.3.6 Instrumental methods of PHA analysis

Lemoigne was the first to analyze PHB synthesised in *Bacillus megaterium* in the year 1926, by solubilising PHB in chloroform and diethyl-ether and saponification. Later, Wilkinson developed estimation protocol using the spectrophotometer; the insoluble PHA inclusions were a problem to read the solution. Law and Slepecky (1961) introduced crotonic acid assay to determine the amount of PHB synthesised by converting the PHA into crotonic acid in the presence of sulphuric acid. α, β - unsaturated bonds of crotonic acid make it easier to read at 235 nm in a spectrophotometer. PHA quantities up to 5µg can be measured using this technique. Wallen, in the year 1974 developed NMR technique to analyze PHB synthesised from activated sludge and was extracted using hot ethanol (95%). Braunegg et al. (1978) converted PHA into methyl ester by performing acid-hydrolysis, as only one methyl ester can be obtained for a PHA studied under acid hydrolysis. The method was more appropriate and the obtained methyl ester was quantified in GC by comparing it to an internal standard. Karr et al. (1983) developed an HPLC based method to detect crotonic acid obtained after methanolysis of PHA. GC analysis of PHA can be performed only after the PHA is completely converted into a volatile compound. PHA is hydrolyzed and acylated before running in GC. GC coupled to atomic emission detector can detect the presence of halogen in PHA and monomeric unit of PHA.

Since moisture content is not a problem in operating HPLC, unlike GC, PHA can be readily analyzed by HPLC. The dehydrated fatty acids can be analyzed without derivatizing the samples. *Cis* and *trans*-configuration of crotonic acid formed after acid-hydrolysis can be easily detected using HPLC, which is not possible with any other methods. It has been reported that GC analysis underestimates the PHA content up to 40 % and HPLC has been found to be a better alternative in quantifying and qualitatively analyzing PHA, when coupled to an appropriate detector such as MS. MALDI-TOF-MS have been currently explored for their potentiality to analyse PHA, MS can detect the exact mass of PHA. The results obtained are highly accurate, automated and offer reproducibility.

2D NMR can be used to analyze the *cis/trans* bonds, double bonds, epoxide, halogen or acetylated groups of PHA. NMR assists in the detection of different

monomeric units of PHA such as in the case of copolymers or block copolymers, unlike HPLC or GC, coupled to a detector. ^{13}C NMR has been used to detect the type of polymer – homopolymer or copolymer (Dai et al. 2008). NMR has also been used to determine crystallinity, chain dynamics, PHA content in the biomass and its molecular weight. A major disadvantage of PHA detection by NMR is its lower resolution to detect longer alkyl chains, overlapping of proton and carbon signals can cause misinterpretation of data. FTIR has been applied to detect the type of polymer within the biomass or in its purified form. Characteristic wavelengths observed can be analyzed to represent the structure of bio polyester present within the biomass. FTIR analysis is chemical free and requires only a limited time of analysis. However, they cannot predict the nature of the polymer (homopolymer or copolymer) and the change in monomeric compositions.

Gel permeation chromatography is employed to analyze the M_w and M_n of the biopolymer. Polystyrene standards of varying molecular weight are used to construct the standard calibration curve. Polymer molecular weights of 5 to 10000 kDa are predicted by using Styragel HMW 6E column, while those ranging from 0.2 to 2000 kDa are predicted using PLgel Mixed-C column. Often two or more columns are connected in series to obtain the absolute M_w and M_n. The ratio of M_w and M_n is referred to as polydispersity index. Thermal properties are often studied to reveal the appropriate thermal degradation temperature, melting point so that it can be used in making blends and during processing of PHA for various applications. DSC offers quantitative and qualitative information of PHA, TGA offers the study of loss of mass with respect to thermal degradation of PHA sample tested. A simultaneous thermal analysis combining both TGA and DSC enables varied thermal analysis during a single operation reducing the time and cost of analysis.

Table 2.5 Analytical techniques employed to study PHA.

Technique	Analysis	Reference
UV	Quantitative determination of PHB	Law and Slepeckey 1961
LC	Monomer composition	Hesselmann et al. 1999, Grubelnik et al. 2008

GC	Monomer composition	Lee and Choi 1995, Furrer et al. 2007
NMR	Polymeric composition and Functional groups present in the polymer	Dai et al. 2008
MALDI-TOF-MS	Structure – polymeric composition and Functional groups present in the polymer	Saeed et al. 1999
GPC	Molecular size and polydispersity	Li et al. 2009
DSC	Glass transition and melting temperature, enthalpy and crystallinity	Galia 2010
TGA	Degradation temperature, PHA content	Katime and Cadenato 1995
FTIR	Structure, functional group analysis, crystallinity, PHA content	Hong et al. 1999, Chen et al. 2009
Mechanical testing machines	Tensile strength, Young's modulus, % Elongation to break etc.	Wu and Liao 2014

2.4 Aim and scope of the work

Earlier reports prove that *Cupriavidus necator* has been successfully and effectively employed towards the production of PHA from various carbon sources. Although production process has been well established by considering crude glycerol as carbon source, the design of a cost effective yet profitable fermentation process remains to be a question. Aeration and maintaining sterility during fermentation are cost increasing steps and design of a fermentation process to synthesise a range of PHA molecules from different carbon sources and microbial strain will not only increase the PHA productivity but also reduces the overall production cost. In this context, the present chapter aims to develop a batch fermentation process that

effectively utilises crude glycerol as a carbon source by employing *Cupriavidus necator* to synthesise PHA in an unaerated and unsterile fermentation mode.

2.5 Materials and Methods

2.5.1 Microorganism

Cupriavidus necator DSM 428 was procured from IMTECH, Chandigarh, India. The obtained lyophilized sample was activated by suspending a little amount of it in sterilized nutrient broth and the inoculated medium was incubated at 30ºC maintained at 150 RPM in an incubator shaker for 24 hours. Post-incubation, fermentation broth was used as seed culture for further experiments.

2.5.2 Materials

Standard Poly 3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) (12 mol %) was purchased from Sigma Aldrich, India. Magnesium Sulphate and sodium hydroxide were purchased from CDH, India. Ammonium sulphate, disodium hydrogen phosphate, dilutes hydrochloric acid, concentrated sulphuric acid and sodium bicarbonate were purchased from Rankem, India. Copper sulphate and dipotassium hydrogen phosphate were purchased from Merck, India.

Deionised water was used throughout the experiments and room temperature was maintained unless and otherwise stated.

2.5.3 Biodiesel derived glycerol

Biodiesel was prepared from karanja oil as described in the literature (Patil and Deng 2009). As a result of esterification of oil, two immiscible phases were obtained containing upper biodiesel and lower crude glycerol. The phases were left to separate based on gravity in a separating funnel. Once a clear interface was achieved, the top phase and the bottom phase were separated carefully. Bottom crude glycerol phase was washed twice with warm water to remove soap and residual impurities and washed crude glycerol was stored at room temperature until further use.

2.5.4 PHA production and quantification

The Minimal salt medium used for microbial growth was prepared by dissolving the following salts in deionised water, $(NH_4)_2S0_4$ - 1 g/L, K_2HPO_4 - 1.5 g/L, Na_2HP0_4 .$12H_20$ - 9 g/L, $MgSO_4.7H_20$ – 0.2 g/L. Volume of the medium in the

flasks was maintained at 150 mL and crude glycerol was added to the prepared medium and the contents were transferred to 250mL conical flask. A known volume of seed culture as described in section 2.5.1 was transferred to the conical flask containing fermentation medium in an open environment. The flasks were sealed with cotton plugs and the mouth of the flask and the sealed cotton were wrapped together with several layers of parafilm to maintain anaerobic condition. Parafilm wrapped conical flasks were incubated at 30ºC and 100 RPM in an incubator shaker for 72 hours. After incubation, the fermentation broth was subjected to crotonic acid assay (Law and Slepckey 1961) to estimate the amount of PHA synthesised.

A modified version of the conventional crotonic acid assay (Law and Slepckey 1961) was developed to estimate the amount of PHA present in the fermentation broth. A known weight of PHBV was dissolved in 5 mL of chloroform in a test tube, 5 mL of deionised water was added to the tube and the tube was let in a boiling water bath for 10 minutes. After 30 minutes, the tubes were withdrawn and were let to cool down to room temperature. From the bottom phase, 20µl of the sample was withdrawn and was added to a fresh tube containing concentrated H_2SO_4, the total volume was made up to 5 mL using conc. H_2SO_4. The tube was subjected to heating in a boiling water bath for 10 minutes and after which the tube was cooled down to room temperature. The absorbance of the solution was read at 235 nm, which corresponds to the crotonic acid. Different concentrations of standard PHBV were subjected to crotonic acid assay and the resulting absorbance at 235 nm was used to construct a standard graph, the slope obtained was used to calculate unknown concentration of crotonic acid.

To quantify the amount of PHA in the fermentation broth, aliquots of fermentation medium after incubation were taken in a beaker and the content was homogenized for 10 minutes at low speed. From the homogenized sample, a known volume was transferred to a pre-weighed centrifuge tube and the tube was subjected to centrifugation at 5000 RPM for 10 minutes. The supernatant obtained was stored separately, while the tube containing the pellet was subjected to drying in a hot air oven maintained at 100ºC for one hour. After incubation, the tube was cooled down to room temperature and the weight of the tube was noted down. Weight obtained is

considered as post weight of the tube that equals the cell dry weight of the biomass present in the volume of fermentation broth considered for PHA analysis.

The pellet obtained was resuspended in 1 mL of chloroform and 1 mL of water was added to the tube, the tube was subjected to heating at 100ºC for 10 minutes. After heating, the tube was cooled down to room temperature and 20 µl of the chloroform phase was withdrawn and was added to a fresh tube containing conc. H2SO4, the total volume was made up to 5 mL using conc. H_2SO_4. The tube was subjected to heating in a boiling water bath for 10 minutes and after which, the tube was cooled down to room temperature. The absorbance of the solution was read at 235 nm, which corresponds to crotonic acid. Obtained absorbance value was compared to the standard graph, slope obtained from the standard graph was used in the back calculation of the concentration of PHA. The difference between pre weight and post the weight of the tube gives the cell dry weight and crotonic acid assay estimates the amount of PHA present in the obtained cell dry weight.

2.5.5 Optimization of medium components

Effect of medium components on PHA productivity was studied by following one variable at a time approach. The study was performed to obtain the respective variable effect on the PHA production by varying one of the medium components while keeping the other components fixed. Effect of carbon source was studied by varying the concentration of biodiesel derived crude glycerol added to the medium between 1 and 12 wt%. The flasks containing varying concentrations of crude glycerol was incubated and after incubation, PHA extraction and quantification as explained in section 2.2.4 was followed. Crude glycerol concentration which gave maximum PHA production was fixed to study the effect of nitrogen source; ammonium sulphate concentration was varied from 0.5 g/L to 1.5 g/L. After incubation, the fermentation broth was subjected to extraction and crotonic acid assay. The amount of crude glycerol and nitrogen source concentration which gave maximum production of PHA was fixed to study the effect of sodium bicarbonate. Sodium bicarbonate concentration was varied between 1 and 2 g/L. After fixing the sodium bicarbonate concentration which gave maximum PHA production, the effect of pH (6 to 7.5), RPM (50 to 250), copper sulphate concentration (1 to 10 mM).

Cu_2SO_4 was studied since it is known to stimulate the growth of *Cupriavidus necator* in the medium. The system variables which gave maximum production of PHA were fixed to study the effect of enzyme cofactor, Mg2SO4 and. Mg2SO4 concentration was varied from 1 to 5 mM.

2.5.6 PHA characterization

The PHA obtained in the present fermentation process was purified through conventional method (solvent extraction) for the physiochemical and mechanical characterization of PHA. Fermentation broth with maximum PHA production was subjected to homogenization for 10 minutes at low to medium speed. The homogenized broth was transferred to a separating funnel, to which chloroform: methanol in a ratio of 2:1 (V/V) was added, separating funnel was left undisturbed for the formation of immiscible phases. Bottom phase containing organic solvent was transferred to a dry glass petri dish and the solvent mixture was allowed to evaporate at room temperature. After solvent evaporation, the crude sample obtained was dried by passing nitrogen at a very low flow rate until maximum moisture content was removed. Resulted crude PHA was subjected to physicochemical and mechanical characterization.

NMR studies were conducted by performing ^{1}H (400 MHz) and ^{13}C (100 MHz) in Bruker 500 MHz instrument, Germany. $CDCl_3$ was used as the internal reference. A known weight of the PHA sample was dissolved in 1 mL of $CDCl_3$; Chemical shifts were reported down field from 0.00 ppm. The spectrum obtained was compared and analyzed for relative peaks at different ppm, and the chemical structure of PHA was predicted.

FTIR analysis was performed by dissolving a known weight of solvent extracted PHA to 1 mL of chloroform and the solution mixture was added to KBr pellets. Once the solvent got evaporated, FTIR spectra were recorded using Shimadzu FTIR IR-Prestige 21 Spectrophotometer, Japan. Spectrum scans were recorded by performing 20 scans at a resolution of 2 cm^{-1}, between a wavelength range of 4000 cm^{-1} and 400 cm^{-1}.

Thermogravimetric analysis was performed in TA Instruments SDT-Q600 instrument. A known weight of the sample was considered for the study and the

sample analysis was performed at a rate of 10°C min^{-1} from room temperature to 600°C under nitrogen flow. Empty aluminium pan was used as the reference weight. DSC analysis was performed in Mettler Toledo DSC 822e, sample was taken in an aluminium pan provided with a closing lid. Sample within the pan was subjected to a temperature change of – 50 ºC to 400ºC at a heating rate of 10ºC per minute. Raw data obtained was analyzed using inbuilt software for T_G and T_M. Crystallinity was calculated from the enthalpy value obtained at T_M,

$$Crystallinity\ \% = \frac{\Delta H_m at\ melting\ point}{\Delta H_m of PHB} \qquad (2.1)$$

Where ΔH_m of PHB (Enthalpy at melting point of PHB) – 146 J/g.

Gel permeation chromatography was performed in Turbo Matrix-40, Perkin Elmer unit coupled with Plgel 5µm Mixed D column (300mm X 7.5 mm X 5µm) equipped with mixed bead column bed. 100 % THF was used as mobile phase and maintained at a flow rate of 1 mL/min. A known weight of the sample was dissolved in THF and was injected into the column. The peaks obtained were compared to the standard graph made up of polystyrene beads with varying molecular weight ranging from 2590-275300. Average Molecular weight (M_w), Average molecular number (M_n) and Polydispersity were determined using the software.

Shear bond strength of PHA was determined via the method discussed by Kulkarni et al. (2010). Triplets of glass, wood and acrylic sheets (Height X Width – 100 X 25 mm) were placed in a desiccator with silica gel for 24 hours to remove moisture content. The polymer was homogeneously spread to the ends of strips with a superficial area of 5 ± 0.5 cm^2 and the ends with polymer were placed over each other and were hand pressed for about one minute. The thickness of the polymer layer was detected using vernier caliper. Binder clips were used to hold glued pieces together until curing time. Each of the different set of samples prepared was maintained in 10ºC, 40ºC and 100ºC for 24 hours. After curing, the samples were cooled down to room temperature and the binder clips were removed. The samples were subjected to tensile strength testing in ZWICK ROELL, Z020 with 20 KN load cell. Pre-load capacity of 0.1 N and the test speed was maintained at 1 mm/min.

Stress to strain graph was noted and from the data obtained, the type of failure and the adhesive strength were determined. Shear bond strength was calculated using the equation (2.2)

$$\tau = \frac{F}{A} \qquad (2.2)$$

τ – Shear Bond Strength

F – Force applied (N/m^2)

A – Superficial area covered with adhesive (m^2)

2.6 Results and Discussion

2.6.1 Effect of carbon source

Experiments were conducted to study the effect of carbon source; biodiesel derived crude glycerol (1 to 12 wt%) in the fermentation medium. As shown in figure 2.3, it can be observed as biodiesel derived crude glycerol concentration was varied, the biomass production was found to increase till 3 wt% of crude glycerol, and biomass production was found to decline and remained almost constant with further increase in carbon source concentration. Biomass content was found to increase significantly beyond 9 wt% of crude glycerol concentration in the fermentation medium. It is inferred that with increasing concentration of crude glycerol, the amount of PHA synthesized was found to increase and reached a maximum at 9 wt% of crude glycerol while with further increase in carbon source, PHA production was found to decline.

At concentrations ranging between 4 and 8 wt%, carbon source is metabolized to form PHA while a minimum amount of crude glycerol is utilised for energy synthesis and other metabolic activities. As the concentration of crude glycerol increases to 9 wt%, the excess carbon available in the medium may be converted to energy utilized for the biomass production and consecutively towards PHA synthesis and storage within the biomass. The excess carbon source in the medium beyond 10 wt% of glycerol inhibits the PHA synthesis and the carbon uptake is re-routed to energy metabolism and cell growth and division. Results obtained in this study are in accordance to those reported by Zhu et al. (2010) that increasing crude glycerol concentration in the fermentation

medium resulted in lower biomass and PHA productivity, while; Sindhu et al. (2011) have mentioned that maintaining higher concentrations of glycerol in the medium lead to lower PHA accumulation as a result of substrate inhibition.

During fed batch cultivation which resulted in higher PHA accumulation (Garcia et al. 2013). At lower concentrations of crude glycerol, the fermentation takes place in a batch mode while at increased concentrations the fermentation takes place in a fed batch mode owing to the competitive uptake of glycerol molecules metabolized and stored as PHA granules. Similar results were observed by de Paula et al. (2017), on utilizing higher concentrations of crude glycerol (10 to 50 g/L) towards PHA synthesis by novel species of Pandoraea, 63 % accumulation in the biomass has been recorded by the authors.

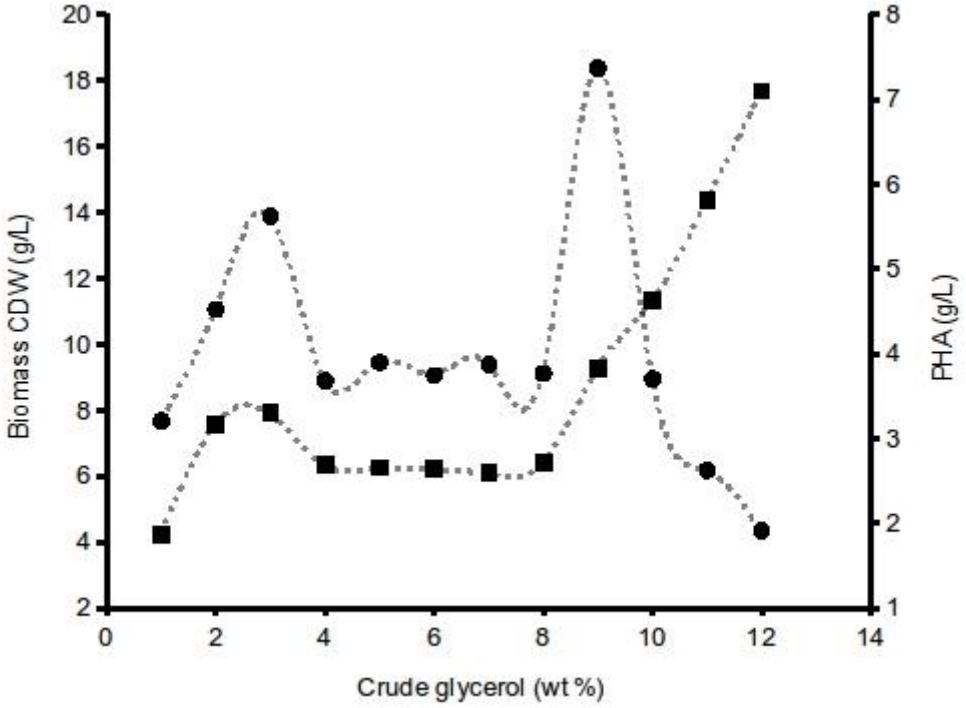


Figure 2.3 Effect of carbon source on production of biomass (■) and PHA (●).

2.6.2 Effect of Nitrogen source

Nitrogen is one of the macro-nutrients that influence the PHA production and accumulation in a microbe. The effect of ammonium sulphate concentration (0.5 - 1.5 g/L) as nitrogen source added to the medium and its effect on biomass production and PHA accumulation were studied. It is observed from figure 2.4, that the lower

concentration of ammonium sulphate lead to nutrient depleted condition and stimulates metabolic conversion of crude glycerol to PHA. However, maximum PHA accumulation was obtained on maintaining nitrogen source concentration as low as 0.75 g/L. Presence of nitrogen based impurities in the crude glycerol might be utilized by the microbe which supports cell maintenance while enhancing PHA accumulation at ammonium sulphate concentration of 0.75 g/L. On increasing the concentration of ammonium sulphate beyond 0.75 g/L, it emanated a drastic decrease in PHA production but stimulated cell growth and maintenance. Ammonium sulphate supplied to the medium is primarily uptaken by the microbe and metabolized towards cell maintenance, via production of essential enzymes and proteins. Thus, presence of excess nitrogen in the fermentation broth enhances protein production and as a result biomass accumulation is observed on maintaining ammonium sulphate concentrations above 0.75 g/L. Lower concentrations (0.5 g/L) of ammonium sulphate are not sufficient enough to enhance cell growth, as a result there is minimum biomass production and PHA accumulation.

The results obtained in this study are in accordance to those reported in a study conducted by Beaulieu et al. (1995), it was found that ammonium sulphate is a potent nitrogen source compared to nitrate, phosphate and chloride salts of ammonium, and lower concentrations of ammonium sulphate has been reported to enhance PHA accumulation. Similar results have been reported by Daneshi et al. (2010), PHA production by *C. necator* was found to increase from 0.08 to 0.17 g/L/h with decreasing $(NH_4)_2SO_4$ concentration from 3 to 0.75 g/L at a fixed carbon concentration of 80 g/L corn syrup. Effect of Carbon/Nitrogen ratio at fixed carbon concentration was studied by Ahn et al. (2015) and the authors found that the microbe is said to take time to enter in to nitrogen depleted condition, when both nitrogen and carbon sources are present in the medium and also it highly depends on the incubation time. Hence the result obtained in the present work on maintaining higher concentrations of ammonium sulphate that resulted in low PHA accumulation is justified. The authors also referred that high nitrogen deficient condition needs to be maintained to attain maximum PHA accumulation. Increasing carbon/nitrogen ratio also has effect on PHA accumulation and reported to increase on increasing the ratio

from 3.6:1 to 360:1 due to the generation of microbial cells and in turn leads to higher PHA accumulation.

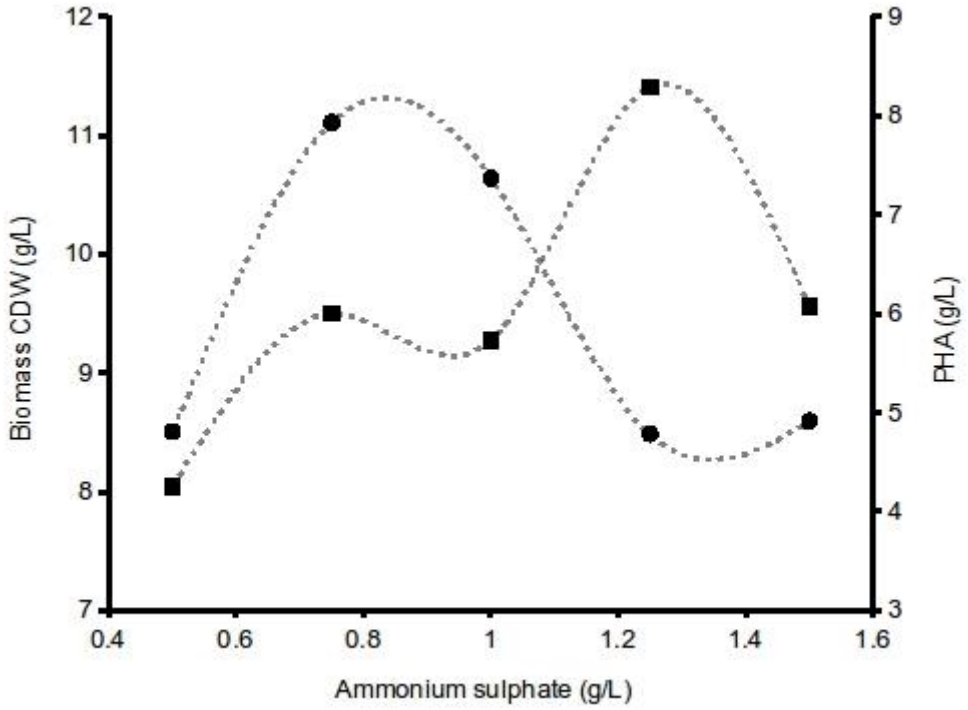


Figure 2.4 Effect of nitrogen source on production of biomass (■) and PHA (●).

2.6.3 Effect of sodium bicarbonate

Berezina (2013), reported that when low concentrations of oxygen was maintained in the medium towards PHA production by *Cupriavidus necator*, it resulted in lower biomass but increased PHB content. In the current research work, sodium bicarbonate was added to the medium to lower the oxygen content and present an oxygen free environment to the microbe. Earlier reports suggest that sodium bicarbonate has been used in maintaining and in the inorganic supply of CO_2 for cell growth (Lee et al. 2003). Hence the experiments were conducted by varying sodium bicarbonate concentration between 1 - 2 g/L, to study their effect on the production of PHA at a fixed carbon and nitrogen source concentrations of 9 wt% crude glycerol and 0.75 g/L ammonium sulphate, respectively. As shown in figure 2.5, sodium bicarbonate at lower concentrations had a very little effect on the PHA accumulation. At lower concentrations, the amount of CO_2 in the environment is very less, as a result oxygen trapped in the head space of the flasks disrupts the PHA formation

within the cells and glycerol is primarily routed towards cell maintenance rather than PHA accumulation. Subsequent increase in the concentration of sodium bicarbonate results in an effective CO_2 environment and in the accumulation of PHA. However, the addition of sodium bicarbonate was found to decrease the biomass and PHA production compared to that of the variation in ammonium sulphate concentration as seen in figure 2.4. Presence of excess CO_2 in the environment disrupts the energy metabolism and thereby results in low biomass production.

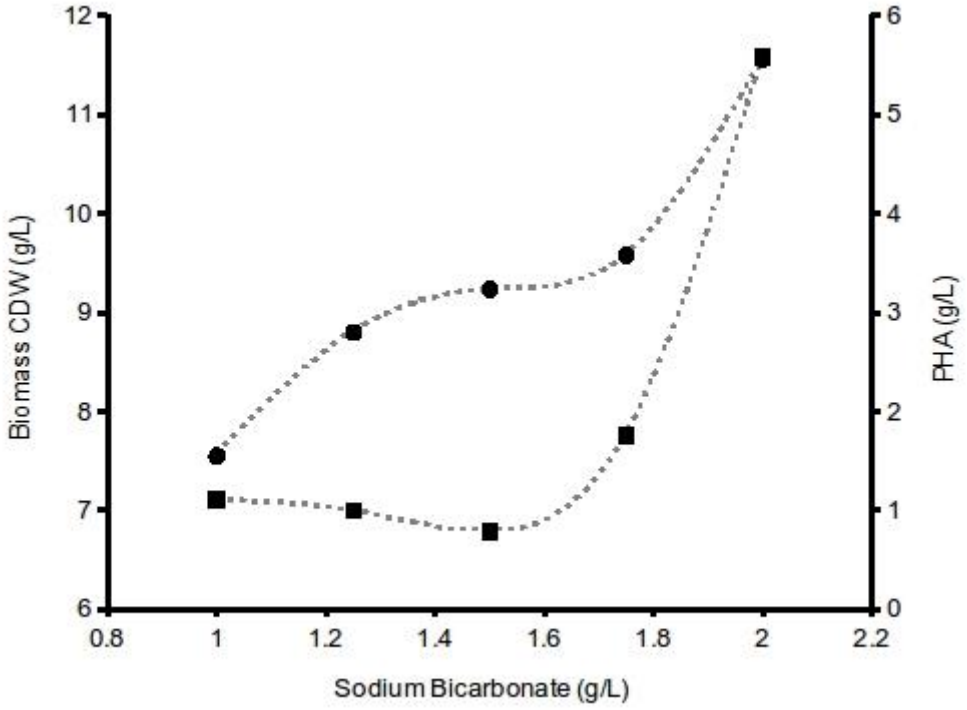


Figure 2.5 Effect of sodium bicarbonate on the production of biomass (■) and PHA (●).

Results obtained by studying the effect of sodium bicarbonate are in accordance with the study conducted by Tanaka and Ishizaki (1994), who have reported the effect of carbon dioxide and hydrogen on PHA production by *Alcaligenes eutrophus*. At oxygen depleted conditions, biomass production was found to remain constant, whereas PHA accumulation was found to increase. By maintaining dissolved oxygen concentration of 1 to 4 % air saturation valerate production was induced compared to butyrate, even though similar metabolic pathway is involved in the production of both the monomeric units. Prevailing low oxygen condition in the medium prevents oxidative loss of CO_2 from Propionyl-CoA that in turns lead to the

formation of monomers other than PHB (Lefebvre et al. 1997). Thus, increasing sodium bicarbonate concentration in the fermentation medium should have induced oxygen depleted condition, thereby increasing the production and accumulation of PHA within the biomass.

2.6.4 Effect of copper salts

Effect of copper as microbial growth initiator and on the PHA accumulation was studied by varying copper sulphate concentration from 1 to 10 mM, in the medium containing 9 wt. % crude glycerol, 0.75 g/L ammonium sulphate and 2 g/L sodium bicarbonate. From figure 2.6, it is inferred that with increasing concentrations of copper sulphate PHA accumulation was found to increase and reached maximum at copper sulphate concentration of 7.5mM. Lower concentrations of copper sulphate have been inferred to initiate microbial growth and PHA accumulation while increasing concentrations of copper ions in the medium resulted in complex formation with other macro and micro nutrients and their precipitation, which inhibits microbial growth and PHA accumulation (Casida L.E. 1988, Helm et al.2008).

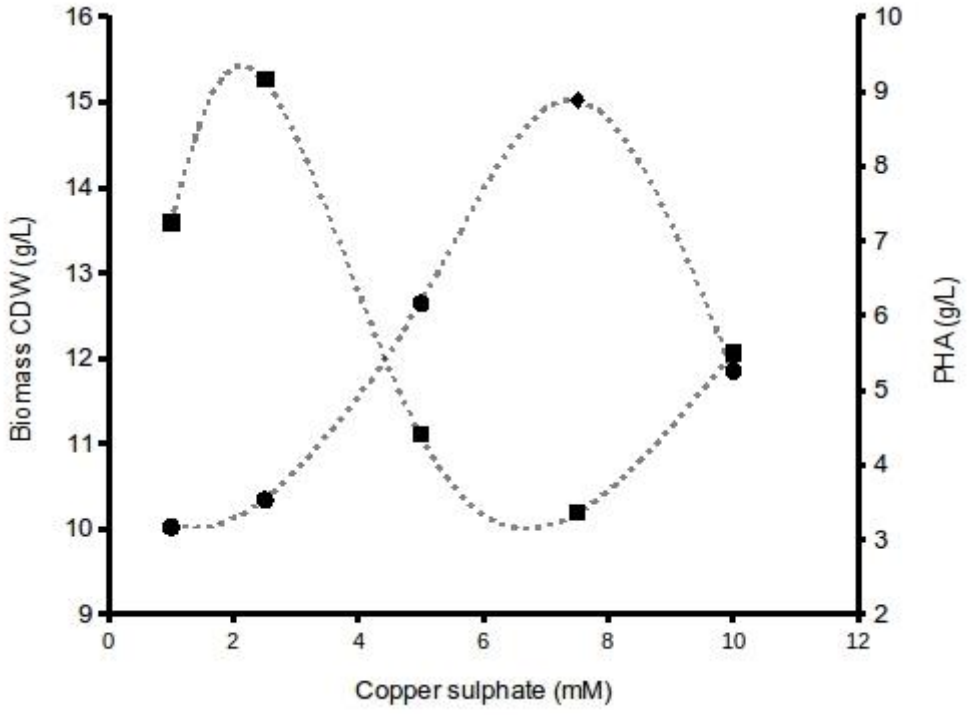


Figure 2.6 Effect of copper sulphate on the production of biomass (■) and PHA (●).

2.6.5 Effect of broth pH

The effect of pH on PHA accumulation was studied by varying the fermentation broth pH between 6.0 to 7.5 as shown in figure 2.7. It is observed from figure that the maximum production of PHA occurred on maintaining broth pH of 6.8. Increasing broth pH from 6 to 6.8 resulted in a higher productivity of biomass and PHA and reached their maximum values at pH 6.8. With further increase in broth pH and on reaching neutral pH of 7.0, biomass production was found to decline while a significant decrease in PHA production was also observed. As most of the enzymes involved in PHA production show their maximum enzymatic activity at the pH of 6.8, PHA accumulation reached a maximum of 8.88 g/L. Travenier et al. (1997) reported that PHA production and biomass production were low on maintaining acidic pH, as a result of the influence of pH on trace elements such as copper sulphate, that hinders the enzymatic activity and metabolic pathway. A study conducted by Filipe et al. (2001) showed that maintaining system pH below neutral pH induces the carbon uptake and its metabolism that leads to PHA accumulation with the microbes.

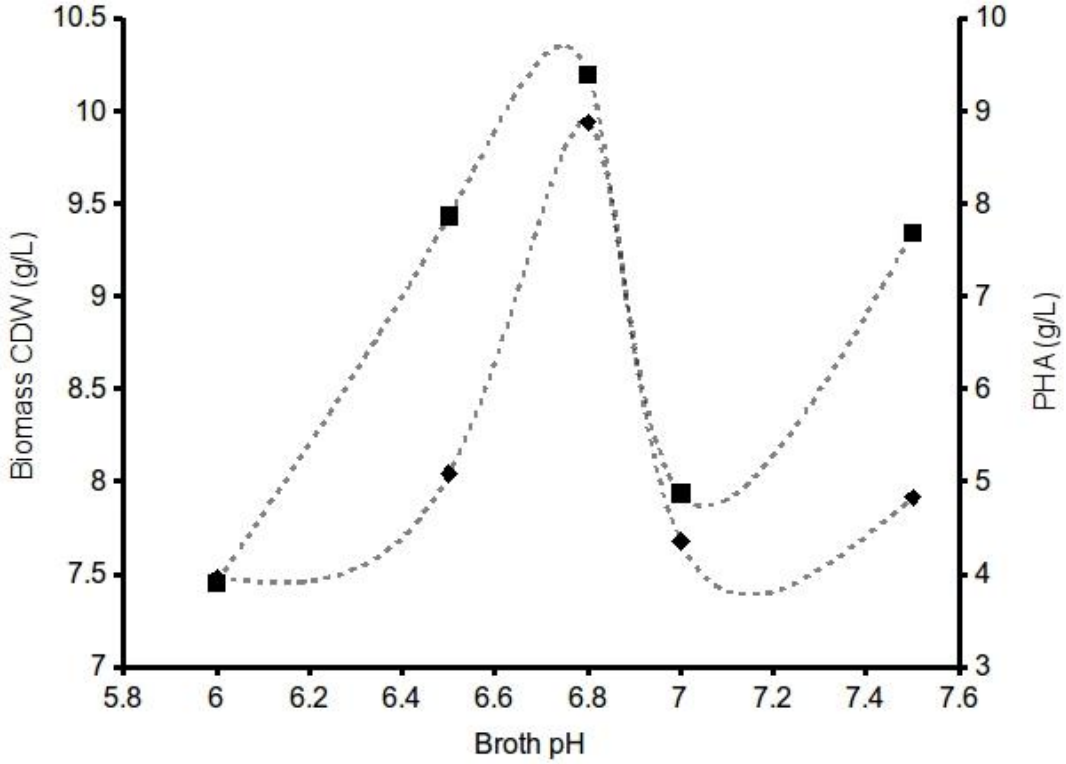


Figure 2.7 Effect of fermentation broth pH on the production of biomass (■) and PHA (●).

2.6.6 Effect of Agitation rate

Effect of speed of rotation on the biomass production and PHA accumulation was studied by varying rotational speed between 50 and 250 RPM. From figure 2.8, it is inferred that maximum PHA production of 10.82 g/L occurred at 150 RPM, while 50 RPM and 250 RPM resulted in very low PHA accumulation. This decreasing effect might be attributed to the fact that increased agitation aids in shear stress experienced by the biomass that in turn affects the metabolic activity of the biomass, thereby reducing biomass production and PHA accumulation (Wei et al. 2011), while 50 RPM might not be sufficient enough to enhance the mass transfer of glycerol from the medium to the microbial cells.

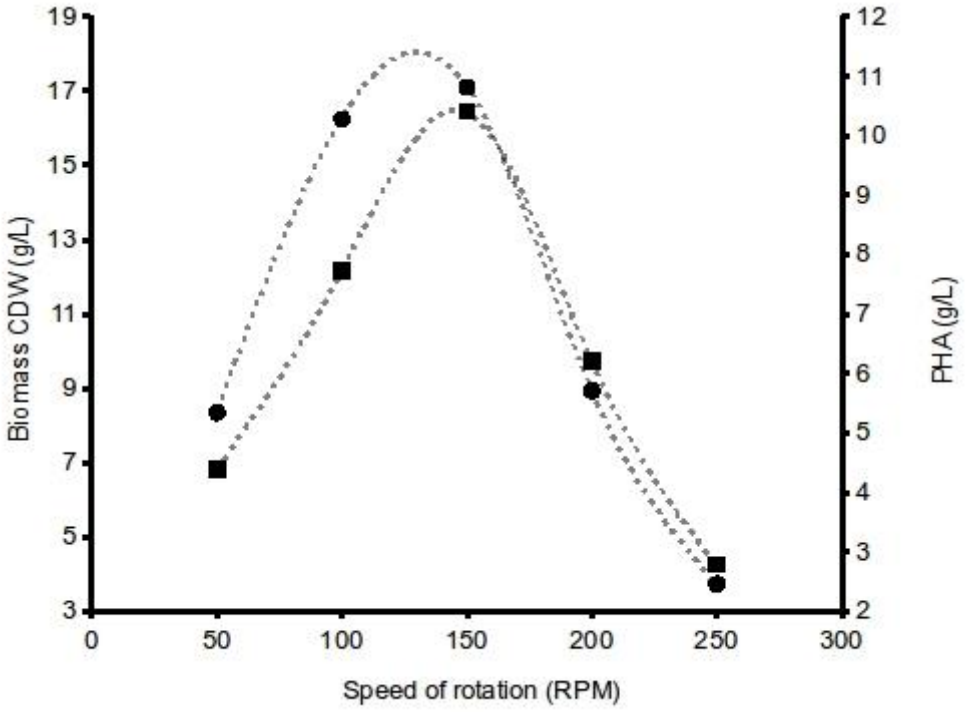


Figure 2.8 Effect of the speed of rotation on the production of biomass (■) and PHA (●).

2.6.7 Effect of magnesium sulphate

Magnesium is an enzyme cofactor and is predominantly involved in the metabolic pathways for proper functioning of enzymes inside a cell. Thus, variation in the micronutrient concentration is expected to influence the biomass growth and PHA accumulation within the microbes. Effect of addition of magnesium sulphate was

studied by varying its concentration from 1 to 5 mM and the results obtained are represented in figure 2.9. It was found that with increasing concentration of magnesium sulphate, biomass concentration and PHA production was found to increase and reached a maximum at magnesium sulphate concentration of 4mM. With further increase in the cofactor concentration, both biomass and PHA production was found to decline. Magnesium is a vital cofactor involved in the glycolysis and Krebs cycle (Saris et al. 2000), which are the pre-steps in the production of PHA. Most of the glycolytic enzymes have been reported to be sensitive to magnesium ions in the medium. Results obtained in this study are in accordance with the available literature, with increasing magnesium concentration in the medium, metabolic enzymes and PHA accumulation are enhanced. Higher concentrations of magnesium result in precipitation with other metal ions present in the medium that in turn affects the biomass growth and PHA accumulation.

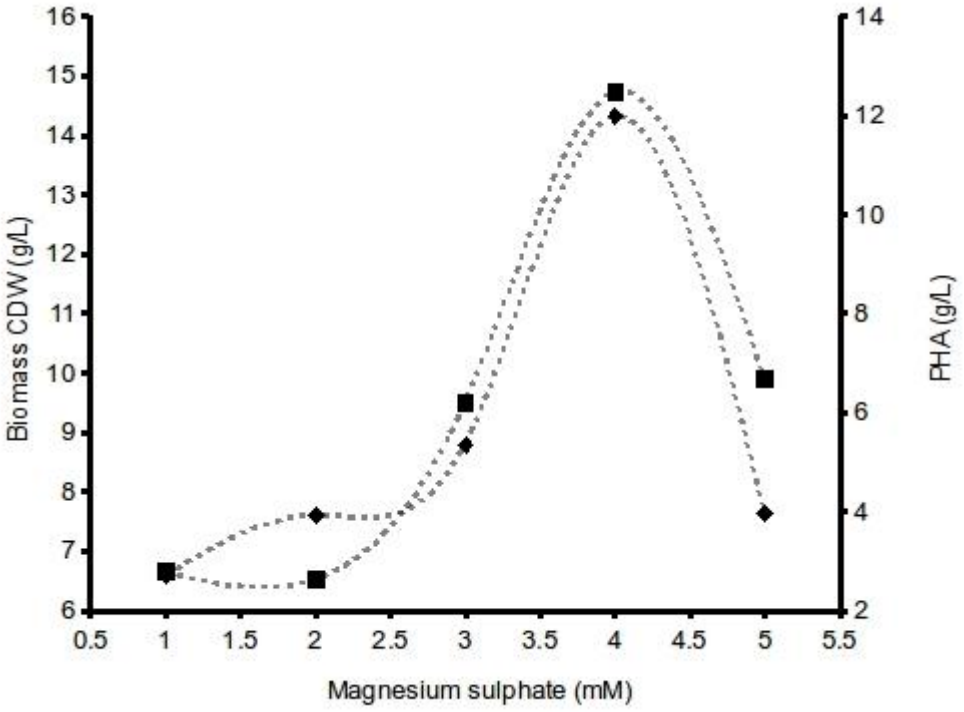


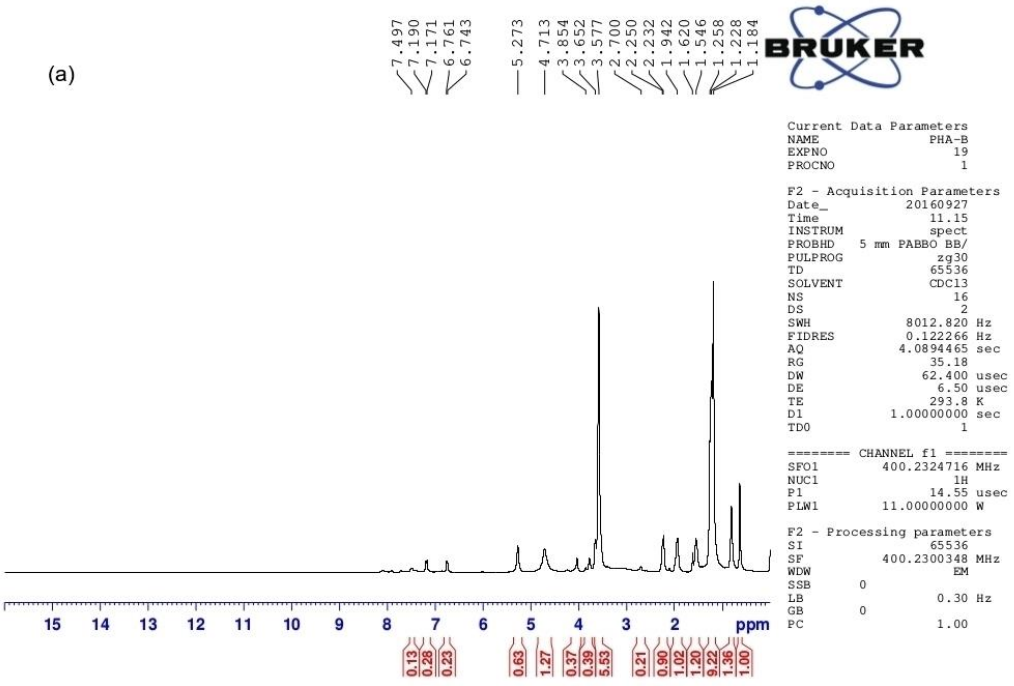
Figure 2.9 Effect of magnesium sulphate on the production of biomass (■) and PHA (●).

2.6.8 PHA characterization

Physiochemical and mechanical strength studies as described in section 2.5.6 were conducted and the results obtained were analyzed to derive the chemical structure of PHA synthesised by *Cupriavidus necator* and physical properties of PHA were inferred.

2.6.8.1 NMR analysis

Chemical structure of the biopolymer synthesized by *Cupriavids necator* was studied using proton and carbon NMR. Based on the chemical shift assignment for each proton (figure 2.10a) and for carbon resonance (figure 2.10b), the polymer structure was confirmed to be that of a random terpolymer.



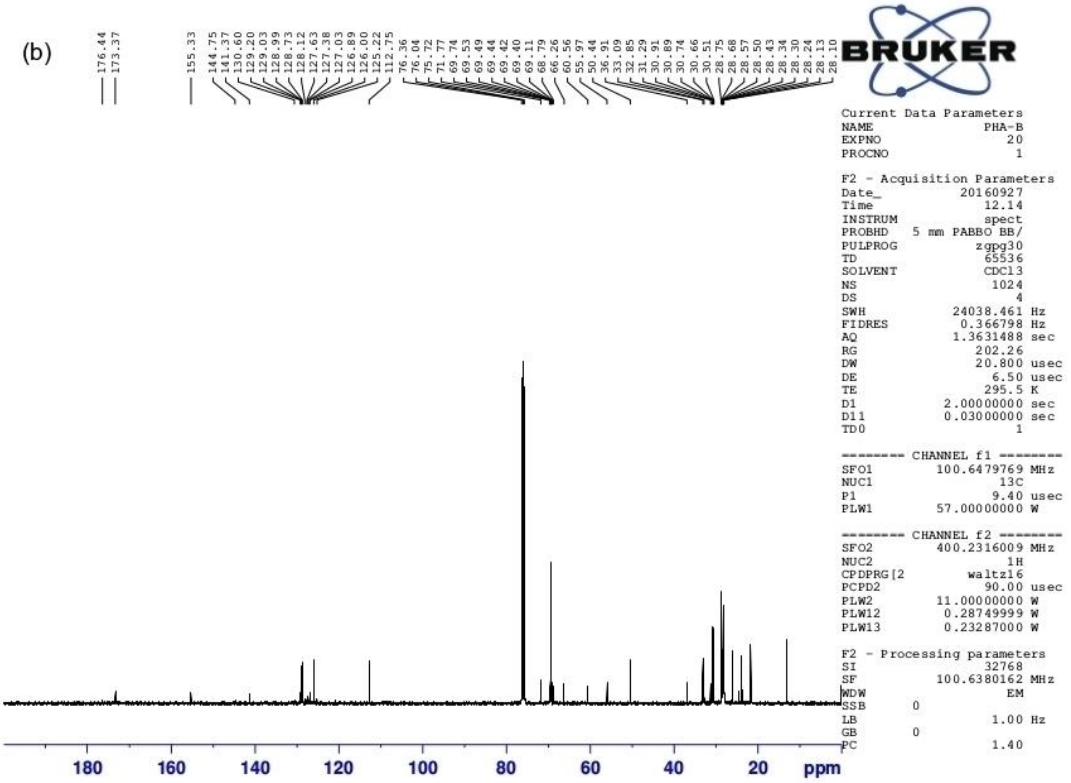


Figure 2.10 (a) ^{1}H NMR spectra of PHA, (b) ^{13}C NMR spectra of PHA

The polymer contains two types of monomers; one of them bearing aromatic ring and another with aliphatic carbon unit. Respect to aromatic monomer, the benzene ring should be a p-disubstituted ring with a methoxy group (-OCH_3), which is confirmed by a ^{13}C-NMR peak at 57 ppm and represents 6-7%, while the aliphatic content is about 94-93%. With respect to aliphatic monomers, the ratio between the signals 5.5-4.5 and 2.3-0.9 ppm is 1 to 7 as seen in figure 2.10 (a), which means that the content between 3-hydroxybutyric acid and 3-hydroxyvaleric acid is nearly 1:1. The ratio of both signals is 1 to 8, but the spectrum of methanol appears at 3.5 ppm (CH_3) and 1.1 ppm (OH). From the chemical shifts it can be concluded that the synthesized biopolymer is a terpolymer comprising 45-46% hydroxybutyrate, 45- 46 % hydroxyvalerate and 7 - 8% Methoxy phenyl valerate. PHA synthesized has been estimated to contain 21 carbon atoms forming an lcl-PHA.

2.6.8.2 FTIR analysis

FTIR spectrum obtained for lcl-PHA synthesised by *Cupriavidus necator* is represented as figure 2.11. The peaks between 1500-800 cm^{-1}represent crystallinity and as the % T increases, the graph represents that the polymer is more amorphous than crystalline. Peaks around 2900 cm^{-1} represent C=O group, 1460 represents C-H bond in CH_2 and the peak at 1460 represents C-H bond in CH_3. Series of bonds from 1000 to 1300 represents stretching of C-O in ester group. Bands around 3300 cm^{-1} should represent water molecules attached to PHA, signal at 2900 cm^{-1} represent the stretching vibration of C-H (which includes CH, CH_2, and CH_3).

The methylene carbons (-CH_2-) are represented by distinct two peaks at 2924 and 2854 cm^{-1} respectively. This is the main type of carbon that is comparable to bands in polyethylene obtained at 1737 cm^{-1}.The band at 1637 cm^{-1} corresponds from hydroxyl groups from the end-chains of the polymer. C=O stretching from esters (-CO_2R-) is present as a sharp signal at around 1100 cm^{-1}, while the other band from ester bonds (stretching vibration of C-O) obtained as a strongest band at 997.50.

Thus, the obtained chemical structure was correlated with those obtained from NMR structure and the novel PHA is named as Poly [3-Hydroxybutyrate-co-3-Hydroxyvalerate-co-3-hydroxy 4-methoxyphenyl valerate] (P3HB-co-HV-co-MeOPhHV) and the mol % of different monomeric units within PHA is estimated to be about, 45 – 46 % hydroxybutyrate, 45 - 46 % hydroxyvalerate and 7 – 8 % Methoxy phenyl valerate. The chemical structure of novel lcl-PHA characterized is represented as figure 2.12.

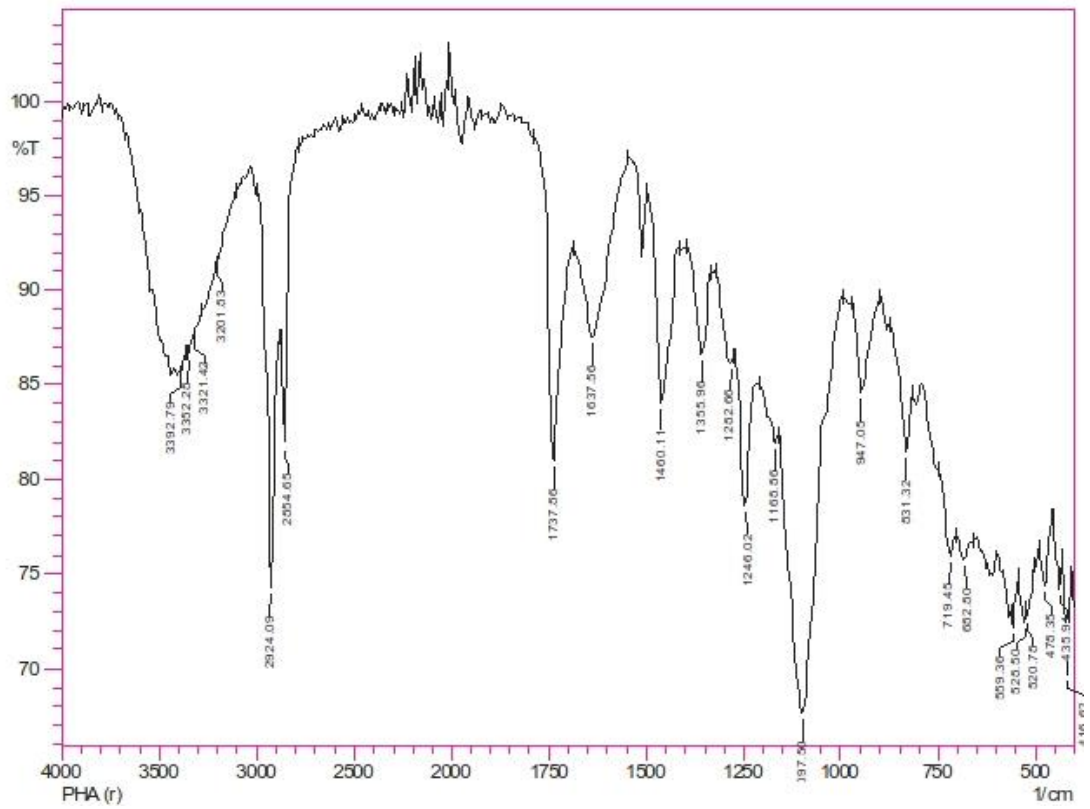


Figure 2.11 FTIR spectra of PHA.

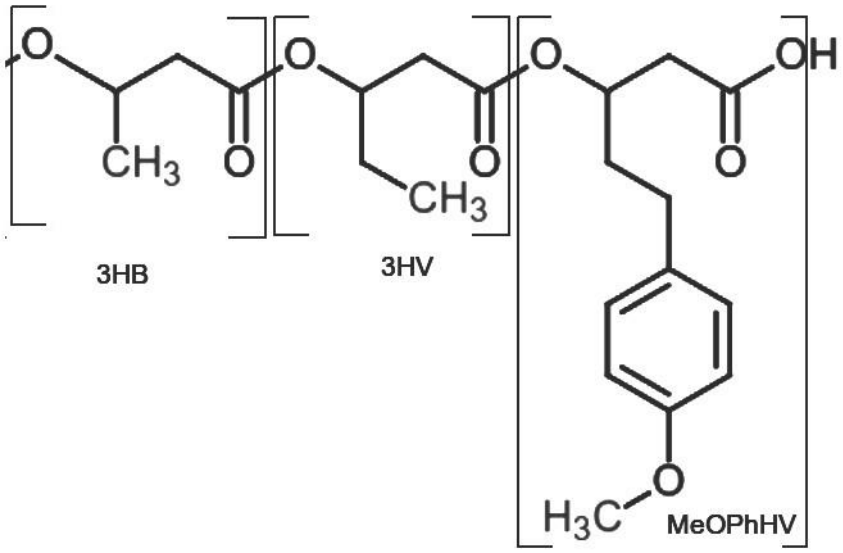
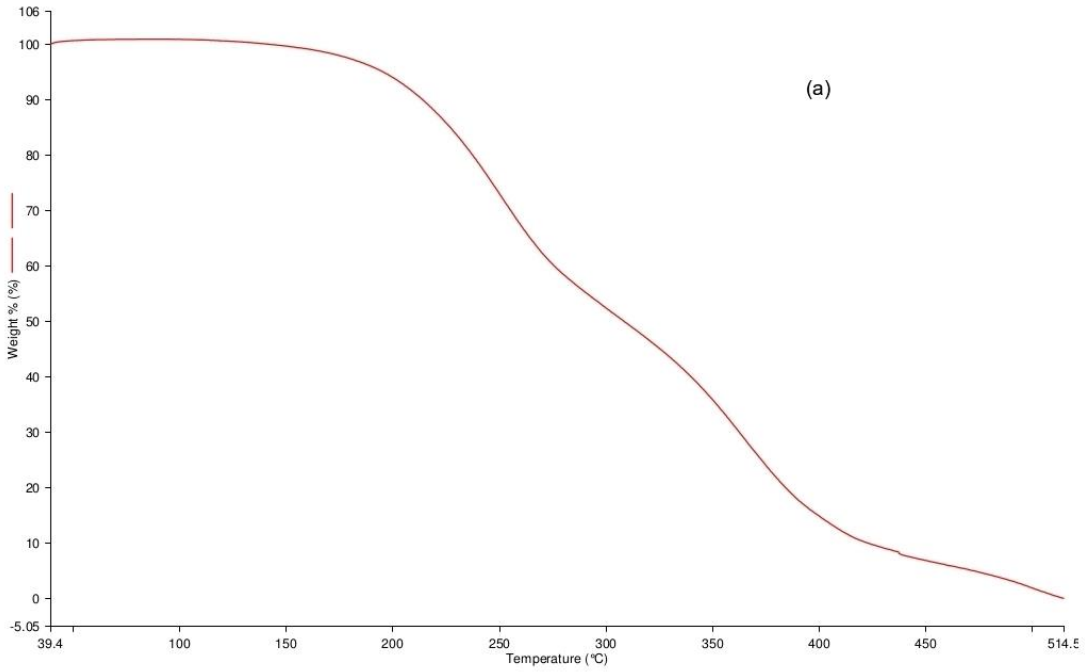


Figure 2.12 Predicted structure of PHA - Poly [3-Hydroxybutyrate-co-3-Hydroxyvalerate-co-3-hydroxy 4-methoxyphenyl valerate] (P3HB-co-HV-co-MeOPhHV).

2.6.8.3 Thermal stability studies

Biopolymer synthesised by *Cupriavidus necator* at optimized conditions in the present study was subjected to Thermogravimetric studies. Thermogravimetric analysis was performed between 38ºC to 550ºC, with an increment rate of 10ºC/minute. From figure 2.13a, it can be observed that the polymer underwent two step degradation, the first degradation takes place at 250.64ºC and the second degradation step was found at 364.38ºC, while the sample completely charred out at 514.5ºC. On heating, interlinked bonds in PHA are broken down to form individual monomeric units and about 50-60 % of the total polymer is degraded during the first step while the remaining polymer is degraded during the second step. Around 400ºC, complete degradation of the polymer takes place and the total weight percentage becomes 0 % at 514.5ºC.



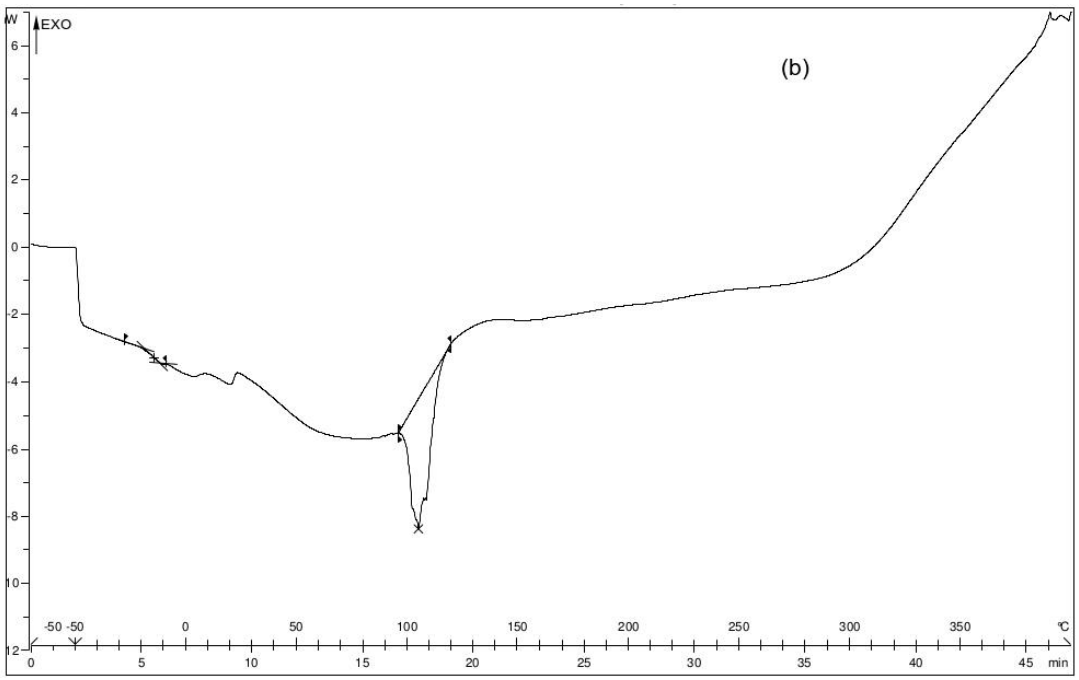


Figure 2.13 (a) TGA curve of PHA, 18 (b) DSC curve of PHA.

Differential scanning calorimetry was performed to analyze the glass transition temperature and the melting temperature of the PHA synthesised by *Cupriavidus necator*. DSC studies were performed between -50ºC and 400ºC. From the graph, as shown in figure 2.13b, it os observed that the glass transition temperature of the PHA produced in the present study was -14.34ºC while melting of the biopolymer took place at 104.85ºC. From the obtained enthalpy value, the crystallinity of the PHA material was calculated as 17 % crystalline. The PHA produced in this study is amorphous when compared to a crystalline polymer obtained after solvent extraction as reported in the literatures (Muhammadi et al. 2015).

2.6.8.4 Gel permeation chromatography

The Gel permeation chromatography (GPC) studies for the PHA synthesized, revealed that the average Molecular weight (M_w) of 994 and the average molecular number (M_n) of 615 leads to the Polydispersity index of 1.616. In general, if the ratio of M_w/M_n value falls within the range of 1.1-2.0, the synthesised PHA molecule is a moderately dispersed polymer. Similarly, the Polydispersity index between 1.5 and 2.0 represents that the polymer is obtained via chain reactions. The obtained value of 1.616 for the present PHA justifies that the PHA is synthesized via metabolic cycle

within the microbe. The Higher average molecular weight of PHA is about 1579, which is usually difficult to measure with precision and is associated with the diffusion of PHA across the column. Taidi et al. (1994) have reported that the presence of carbon source such as glycerol resulted in low molecular weight PHA compared to that of scl-PHA obtained in the presence of simple carbon sources such as methanol and sodium succinate. Similar results have been reported on the utilisation of biodiesel derived glycerol towards the production of PHA by employing *Burkholderia cepacia* ATCC 17759 (Zhu et al. 2010).

2.6.8.5 Shear bond strength analysis

On physical observation of synthesised PHA at room temperature, polymer existed as sticky viscous liquid and there was no change in the physical state of the polymer as observed in the case of PHB or PHBV. As it is difficult to analyze the polymer mechanical strength in the form of strips, PHA synthesised in this work was used as adhesive. As seen in figure 2.14, shear bond strength was found to be high in the case of acrylic, while considerably low values were obtained for glass. No significant effect was seen in the case of wood strips and was concluded that novel PHA cannot be used in the case of wood. It is inferred from the graph, with an increase in curing temperature from 20ºC to 100ºC, the bond strength was found to increase in the case of acrylic. The maximum bond strength of 12.66 MPa was obtained on maintaining a curing temperature of 100ºC, while it was maximum of about 1.31 MPa in the case of glass at 40ºC. Synthesised novel PHA is a complex terpolymer with three different monomeric unit and presence of ring carbon deteriorates the molecular strength of the polymer. However, the promising result obtained from the bond strength of acrylic justify that the polymer can be used as bio-adhesive which is less hazardous in bio-based applications.

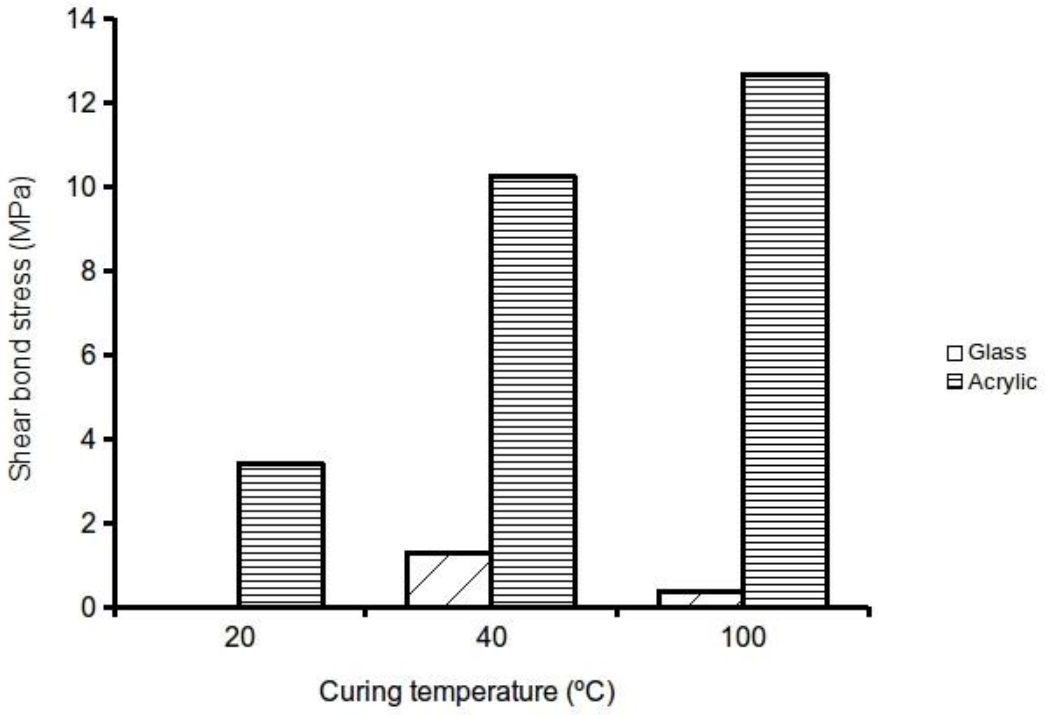


Figure 2.14 Shear bond strength analysis of PHA.

2.7 Comparison of physical properties of novel lcl-PHA with other PHA synthesised from complex carbon source

Pseudomonas oleovorans was used to synthesise mcl-PHA from aliphatic hydrocarbons as carbon source, glass transition temperature of synthesised PHA was found to vary between -25.8 and -43.1ºC while melting temperature was found to be between 38.9 and 47.6 ºC, the effect of carbon source was found to be reflected in the variations in the physical properties of PHA synthesised. Higher molecular weight PHA with average molecular weight varying between 178 kDa and 330 kDa was synthesised as a result of aliphatic monomeric units. Ashby and Foglia (1998) were able to synthesise mcl-PHA from different sources of triglycerides and found that with increasing unsaturation in the side chains of the polymer, thermal stability decreased and molecular weight of the polymer was found to be in the range of 10^5 g/mol.

Poly(3-hydroxydodecanoate-co-3- hydroxy-5-phenylvaleric acid) [P(3HDD-co-3HPhV)] and poly(3-hydroxy-5-phenylvaleric acid) [P(3HPhV)], were synthesised from fatty acids by mutant strain of *P. entomophila* L48, *P. entomophila* LAC23. The

authors found that with increasing mol % of 3HPhV from 0 to 100 mol %, average molecular weight of the polymer was found to decrease from 10.4×10^4 Da to 4.41×10^4 Da. Melting temperature was found to decrease from 82.4 to 50.4ºC, the homopolymer has been reported to be viscous sticky liquid at room temperature.

Mizuno et al. (2014) successfully synthesised PHA with phenyl groups, poly(3HB-co-3-hydroxy-3- phenylpropionate) [P(3HB-co-3H3PhP)] by employing *Ralstonia eutropha* harbouring PhaC1 Ps gene from *Pseudomonas sp*. 61-3. *Pseudomonas putida* KT2440 was used in the cultivation of poly(3-hydroxy- ω -phenyl alkanoate)s [P(3HPhA)s], poly(3-hydroxy-5-phenylvalerate) [P(3H5PhV)] and poly(3-hydroxy-4-phenylbutyrate-co-3-hydroxy-6-phenylhexanoate) [P(3H4PhB-co-3H6PhHx)] from different acids such as racemic 3-hydroxy-3-phenylpropionic acid, 3-phenylpropionic acid, cinnamic acid, 5-phenylvaleric acid, and 6-phenylhexanoic acid. Average molecular number of P(3HB-co-3H3PhP) has been reported to be about $6.5\text{-}9.7 \times 10^4$ while melting temperatures were about 135-158ºC and glass transition temperature was 14.6ºC. Average molecular number of P(3H5PhV) was reported to be 7.9×10^4, while that of P(3H4PhB-co-3H6PhHx) was 11.3×10^4. Glass transition temperature of P(3H5PhV) and P(3H4PhB-co-3H6PhHx) was lower compared to that of P(3HB-co-3H3PhP).

Cruz et al. (2015) studied the synthesis of mcl-PHA from olive oil deodorizer distillate by employing *Pseudomonas resinovorans* in fed batch fermentation mode. PHA synthesised was found to be a terpolymer composed of varying mol % of 3-hydroxyocatonate, 3-hydroxydecanoate, 3-hydroxyhexanoate, 3-hydroxydodeconate, and 3-hydroxytetradeconate. The molecular weight of the PHA was about 0.3×10^5 g/mol and polydispersity index of 1.5. The melting point of PHA synthesised was 36ºC and glass transition temperature of −16ºC, PHA was in amorphous form with the crystallinity of 6 %. Shear bond strength results reveal that mcl-PHA has a good bonding strength towards wood and glass. Muangwong et al. (2016) have studied the synthesis of novel mcl-PHA from crude glycerol derived from biodiesel refinery with waste cooking oil as its source. Authors were able to synthesise homopolymeric 3-hydroxy-5- *cis*-dodeconate, with a molecular weight of 3.6×10^4 Da.

Considering the above references, PHA synthesised in this research work has comparatively higher thermal stability and crystallinity but lower molecular weight

and shear bond strength, attributed to the chemical composition of the carbon source used and the conditions maintained during growth. Earlier reports suggest that carbon source feeding strategy highly influenced the molecular weight of the polymer. Cross linking of the monomeric units of the polymer gives a compact structure to the polymer backbone thereby decreasing the molecular mobility of the units within the polymer, which leads to increase in glass transition temperature. It has also been found that with increasing monomeric composition, crystallinity declines. Low molecular weight can be attributed to the effect of crude glycerol present in the medium. Crude glycerol has been reported to involve in termination of PHA chain termination via covalent esterification of glycerol to end units of PHA growing chain that in turn reduces the molecular weight of PHA and influences thermal and mechanical properties (Ashby et al. 2005, Zhu et al. 2010). Lower crystallinity is caused as a result of comparatively lower mol % of PHB and as a result of the presence of aromatic ring within the MeOPhHV unit of lcl-PHA. As a result of low crystallinity and the amorphous nature, currently synthesised PHA was able to be tested as an adhesive by performing shear bond strength unlike the mechanical testing protocol carried out in the case of PHB and PHBV sheets. Pappalrado et al. (2014) have reported preparation of PHA sheets for a polymer with low crystallinity; the technique involves the dissolution of PHA in toluene and casting it over water layer. The protocol did not result in the formation of sheets but instead resulted in precipitated oil layer that once removed from water layer resulted in the formation of viscous liquid at room temperature. The retransformation in structure might be as a result of incorporation of water molecules to P3HB-co-HV-co-MeOPhHV synthesised in this work. Comparatively higher melting and glass transition temperatures were obtained for the novel lcl-PHA synthesised by *Cupriavidus necator* in this work to that of other PHA from complex carbon sources as discussed above, as a result of higher mol % of PHA with lower mol % of MeOPhH.

2.8 Summary

Based on PHA productivity microbes are classified into two types (i) Microbes that synthesise PHA devoid of nutrient depleted conditions (ii) Microbes that synthesise PHA in nutrient depleted conditions. In the presence of an excess of crude

glycerol, *Cupriavidus necator* falls under the second category where in depletion of nitrogen, sulphur, phosphorous, potassium or oxygen stimulates PHA production. It is inferred that on maintaining C/N ratio of 120 with crude glycerol concentration of 9 wt%, ammonium sulphate concentration of 0.75 g/L, sodium bicarbonate concentration of 2 g/L, copper sulphate – 7.5 mM and magnesium sulphate concentration of 4 mM, and broth pH adjusted to 6.8 and speed of rotation - 150 RPM, resulted in maximum PHA production of 11.96 g/L. The presence of copper sulphate stimulated biomass growth as a result of which PHA productivity increased while magnesium sulphate, enzyme cofactor significantly influenced the PHA production. During nutrient depletion conditions, the energy required to maintain the cell growth and maintenance decreases thereby the carbon substrate is utilised to generate energy rather than to store or accumulate PHA (Lee 1996, Choi et al.1998). Structural characterization revealed that the PHA synthesised from crude glycerol by *Cupriavidus necator* is a novel lcl-PHA terpolymer consisting 21 carbon atoms and comprised of 45-46% hydroxybutyrate, 45- 46 % hydroxyvalerate and 7 - 8% methoxy phenyl valerate. The novel PHA synthesized is named as Poly [3-Hydroxybutyrate-co-3-Hydroxyvalerate-co-3-hydroxy 4-methoxyphenyl valerate] (P3HB-co-HV-co-MeOPhHV). The presence of ring substitute in the terpolymer influenced the physical properties of PHA. Phenyl group increased the thermal stability of PHA but had a declining effect on the mechanical strength. As a brown, viscous crude liquid, synthesised PHA was tested for its adhesive property and was found to have a positive effect on adhesion of acrylic sheets and a less significant effect on adhesion of glass.

CHAPTER 3

CLOUD POINT EXTRACTION OF PHA

Development of separation process to extract hydrophobic compounds from the complex mixtures like fermentation broth has been of wide research interest, as large scale operations involve higher operation and maintenance cost and most of the process are difficult to scale up to industrial level. With invent of surfactants and their varied types, aqueous two phase micellar separation is found to be an effective extraction process for hydrophobic compounds. Cloud point extraction is one such micelle based extraction, which has been widely researched upon for the extraction of highly concentrated solutes such as heavy metals and dyes into a very low volume of the surfactant rich micelle phase. Cloud point extraction is based on the ability of the surfactant micelles to form surfactant rich micelle phase when introduced to temperature change. With increasing temperature, surfactant solution attains turbidity, during which maximum solute transfer occurs from the feed to the surfactant micelles as a result of the solute-micelle interaction. CPE has attributed increased interest among researchers, considering the fact that it is an aqueous based separation technique and the solutes nativity is retained during the extraction process. Solute partition coefficients can be influenced and enhanced by varying several systems and operational parameters such as pH, temperature, surfactant type and concentration, solute concentration etc. The advantage that CPE offers compared to any other solid-liquid extraction or liquid liquid extraction is that even very small volumes of feed can be treated to obtain higher solute preconcentration factor.

3.1 Formation of Micelles

The addition of surfactants to a solvent increases the monomeric surfactant concentration and the micelles are formed beyond a certain surfactant concentration termed as critical micellar concentration (CMC). CMC is the lowest surfactant concentration, required to form micelles in the solution. As surfactant aggregates are formed there is a decrease in entropy that arises from the breaking up water's hydrogen bonded structure and formation of differently structured water. The hydrophobic part of the surfactant orders the water molecule within the vicinity of the

hydrocarbon chain and so to minimize the entropy for the formation of surfactants cluster around and form aggregates resulting in reduced number of water molecules attached to the hydrocarbon chain. The process is enthalpically favourable and entropically unfavourable that causes exposure of hydrophobic part of the monomers which involve hydrophobic interaction among the surfactant tails to form a cluster even at low concentrations. This phenomenon leads to absorption and desorption caused by thermal motions within the system which makes the surfactant aggregate in dynamic nature. The number of micelles that is involved in forming a surfactant micelle is known as aggregation number, about 60 to 100 surfactant monomers are usually accommodated within a micelle.

Surfactant micelles compose a strong hydrophobic core made up of surfactant tails, charged surfactant head groups of micelle form stern layer which are covered by counter ion binding Gouy Chapman electrical double layer that neutralizes the charge of the surfactant head groups. Micelles formed by nonionic surfactant head groups are larger than those formed by ionic surfactants, owing to the bigger sized head groups of the nonionic surfactant monomers; as a result of nonionic surfactant head groups often undergo steric repulsion from one another exposing the surfactant tails and the palisade region, which is strongly hydrated compared to that of hydrophobic core (figure 3.1).

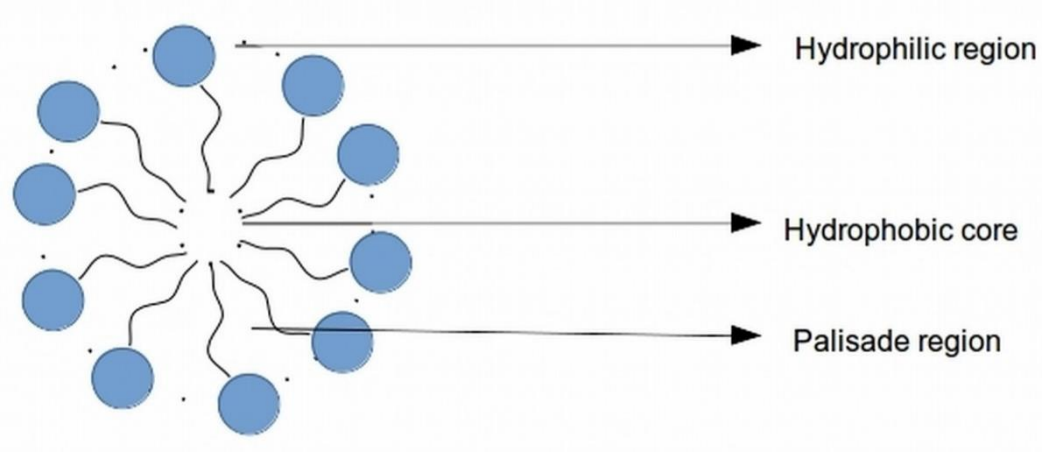


Figure 3.1. Solute binding sites in micelle.

CMC of a micellar solution is altered with the addition of co-surfactant, solvent or additives such as electrolytes. With the increase in temperature, the solubility of the micelles decreases; thereby single aqueous surfactant solution separates into two

coexisting isotropic phases, namely micelle rich lower phase and an upper aqueous phase. Most of the hydrophobic solutes get partitioned in the surfactant rich bottom phase while the hydrophilic solutes partition into the aqueous phase. Most of the nonionic, zwitterionic surfactants possess cloud point < 100ºC, while ionic surfactants cloud point temperature exceeds 100ºC (Mukherjee et al. 2011). However, the cloud point temperature found to decrease or increase the presence of co-surfactant and additives such as salts or polymers or organic compounds. At cloud point temperature, homogeneous surfactant solution undergoes two phase formation and exhibiting turbidity as a result of the formation of the micellar network. With further increase in temperature beyond cloud point temperature, the micelles or coacervates settle down as micellar rich bottom phase coexisting with a top aqueous rich phase. A few surfactants are known to possess two cloud points, where the micelles reshape from rod like micelles to worm like micelles or continuous bilayer. Cloud point observations are done visually; however, light scattering techniques such as laser beam scattering, dynamic light scattering, and refractometry are used to confirm the presence of micelles (Mukherjee et al. 2011).

With increasing temperature, solubility of surfactant increases in solution forming a homogeneous surfactant solution, the temperature at which highest solubility is obtained is termed as krafft point while with increasing temperature, micelles settle down as separate phase, the onset of separation is denoted by turbidity of the solution micelles expressing Tyndall effect and the temperature at which two phase formation begins is termed as cloud point temperature. Krafft point is predominantly discussed in systems made up of ionic surfactants while cloud point is related to non-ionic surfactants. Above the krafft point, a lot of surfactant monomers are solubilised and a maximum decrease of surface or interfacial tension is observed. Recent studies revealed the fact that cloud point of ionic surfactant is related to the surface charge of the aggregates and increasing electrostatic repulsion might bring down the cloud point of the system.

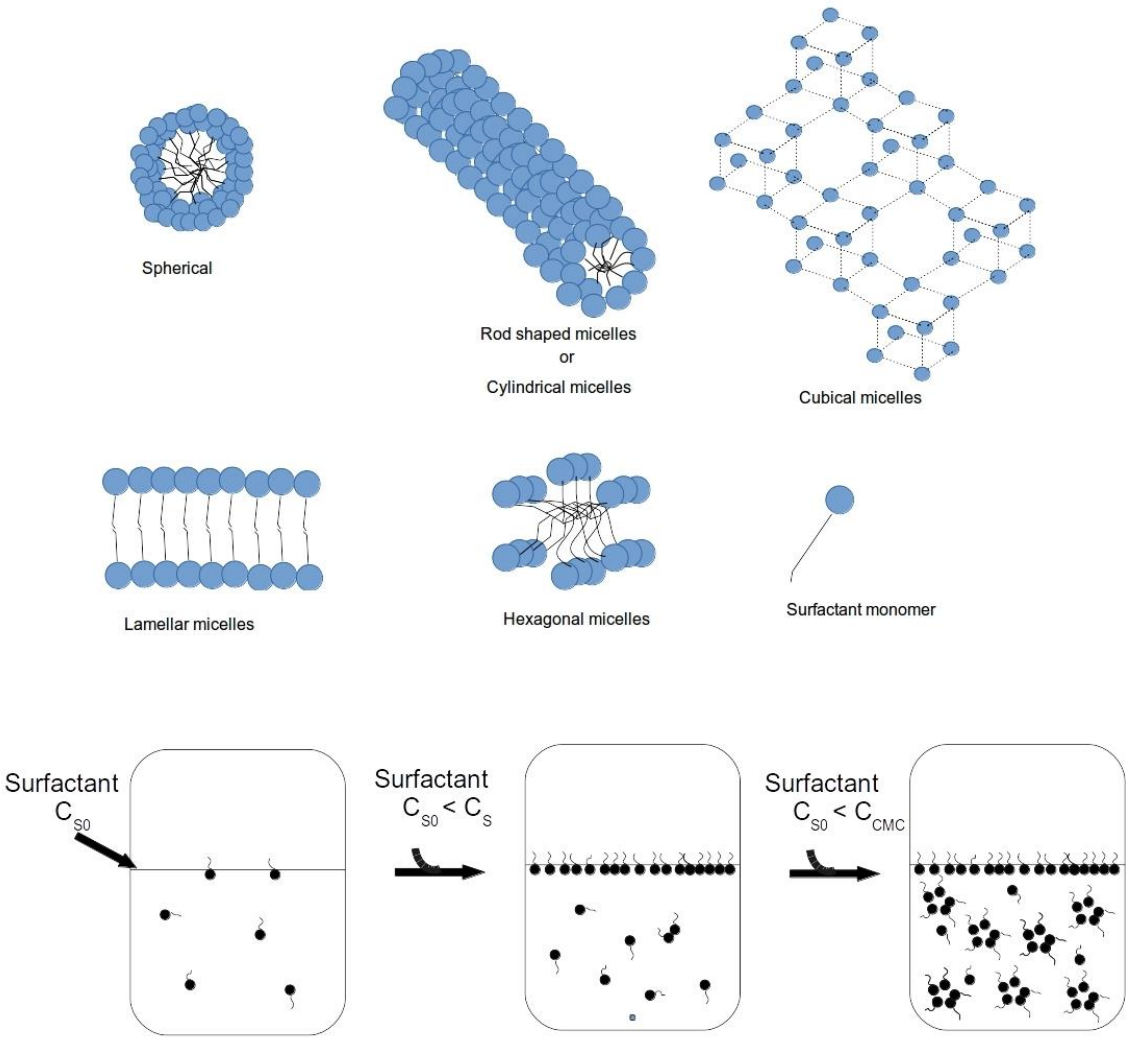


Figure 3.2. Micellization and different shapes of micelles (C_{s0} – Initial surfactant concentration, C_s – increasing surfactant concentration, C_{CMC} – surfactant concentration at CMC.

3.2 Application of Cloud point extraction

During CPE, random and dynamic mass transfer of solutes occurs between the fed solution and the surfactant micelles. A system containing surfactant, different solutes when subjected to temperature change, surfactant micelles have been observed to undergo structural rearrangement. Such rearrangement of surfactant tails within the micelle leads to exposure of hydrophobic sites. Strongly hydrophobic solutes present in the feed undergo hydrophobic interaction with the hydrophobic micellar core. Solutes that are comparatively less hydrophobic in nature are found to interact with

the palisade region of the micelle as shown in figure 3.1; such solutes are equally hydrophobic and hydrophilic in nature and can interact with both, surfactant head and the tail. Hydrophilic solutes collectively interact with the surfactant head groups and water molecules present in between the surfactant head groups via hydrogen bonding. With the increase in temperature, replacement of water molecules present in between the surfactant head groups takes place; as a result, hydrophilic solutes are rejected into the water rich top phase. Though solute transfer is influenced by system pH, cosurfactant, solvent, the presence of an additive, solutes present in the palisade region are highly influenced by such parameters compared to solutes in the water phase or those present within the surfactant micellar core. As a result solutes present in the palisade region are either repelled into the water phase or they remain interacting with the surfactant tails. With the exposure of surfactant tails, hydrophobic solutes are encapsulated and are retained within the micelles that settle down as single micelle rich bottom phase as shown in figure 3.3.

Cloud point extraction (CPE) has been extensively researched upon towards extraction of metal agents like zinc, silver, cobalt, mercury, manganese, copper, chromium and lanthanides; organic pollutants like humic acid, fulvic acid, phenols and phenylamines; dyes like yellow dye, malachite green, crystal violet and proteins including bacteriorhodpsin, cytochrome c and other hydrophobic membrane proteins from their respective feed mixtures. Most of the heavy metals are hydrophobic in nature and so undergo hydrophobic interaction with the surfactant micelles and are partitioned into the surfactant rich bottom phase. Bio molecules as a result of their cellular origin are mostly hydrophilic in nature, except membrane proteins and lipids and other hydrophobic solutes. As a result of hydrophilicity, most of the cellular proteins are partitioned into the top aqueous phase or remain interacting with the micelle over the palisade region. However, membrane proteins are encapsulated by the surfactant micelles, while lipid molecules are involved in the formation of micelles by interaction with surfactants.

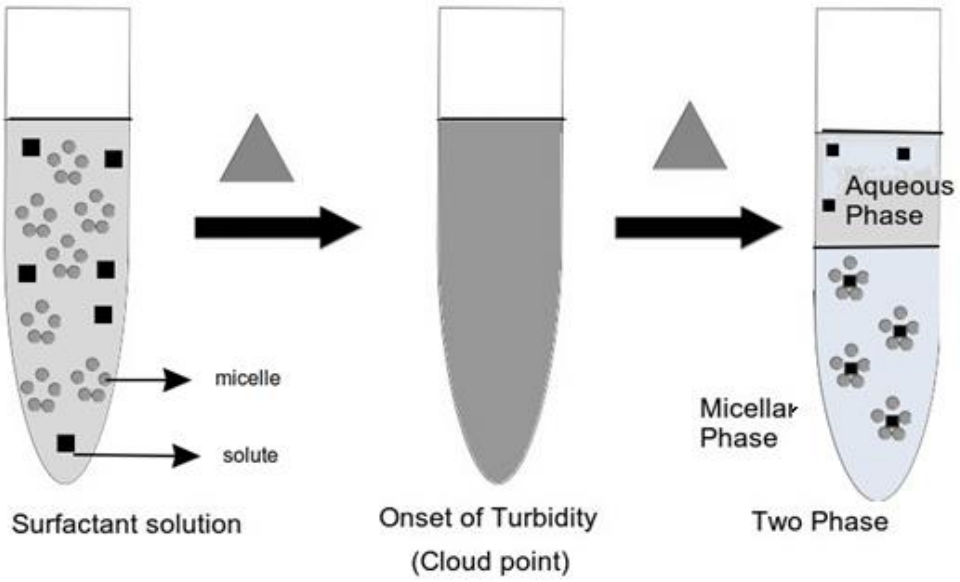


Figure 3.3 schematic representation of cloud point extraction of solute.

3.3 Continuous Micellar Extraction

Over the recent years, development of new extractants with superior selectivity and efficiency of solute separation has led to an increasing interest in the research and development and usage of agitated columns towards the treatment of different bimolecular systems. Currently, more than 25 different extractors have been designed and employed for industrial LLE operations. Conventional LLE units such as spray column, PRDC, mixer-settler units and packed columns have been studied by several researchers for their extraction efficiency during continuous operation. However, column extractors are widely preferred for continuous operation for the following reasons, less capital and operational cost, ease in design and scale up. The presence of more than one theoretical stage offers higher purification factor compared to any other continuous extraction unit.

Considering the fact that micelle based CPE of solutes remains to be in the batch mode and lab scale, not many reports are available on the continuous cloud point extraction of solutes in column extractors. Trakultamupatam and Scamehorn (2010) studied the continuous extraction of aromatic compounds toluene and ethyl benzene from wastewater using t-octylphenolpolyethoxylate. The authors found that the volumetric mass transfer of solutes was highly influenced by operational parameters of RDC such as rotor speed, phase flowrate and presence of additives.

From the results, the authors were able to infer that solute partitioning was found to double during continuous operation compared to CPE at batch mode. Similar results were obtained on the study of extraction of phenol using TX114 (Ingram et al. 2012)

3.3.1 Rotating disc contactor

Among the various column extractors used for the extraction purpose, rotary agitated extraction column such as the rotating disc contactor (RDC) possess better operational flexibility than conventional sieve plate, packed and spray columns. Designed and developed by Reman in 1951(Gross and Skelton 1951), RDC possesses high efficiency per unit height, high throughput, low driving power, low cost and provide increased yields over other conventional extractors (Maria et al. 1997). RDC is a cylindrical column with a central rotating shaft carrying equally spaced discs, positioned at the centre of each mixing compartment generated by stator rings, which provides a larger interfacial area and better contact between two phases. Stator rings act to convert the long cylindrical column into multi staged LLE unit. During LLE operation, the lighter phase is introduced from the bottom which is transported to the top passing via descending heavier phase sent from the top inlet. Compartments within the RDC or simply, the mixing zones are within which mass transfer between light and heavy phase takes place. Rotor ring present within each mixing zone efficiently involves in creating a vortex that in turn leads to breaking of lighter dispersed phase into smaller droplets, that increases the mass transfer coefficient.

These advantages have led to the wide use of RDC in the petroleum industry for furfural and sulphur dioxide extraction, propane deasphalting, sulfolane extraction and caprolactum purification. RDC has also been extensively used in the extraction of bio molecules – recombinant cutinase (Carneiro-da-Cunha et al., 1994), α-toxin (Cavalcanti et al., 2008), ascorbic oxidoreductase (Porto et al., 2004), glucose-6-dehydrognase (Hasmann et al. 2007), α-lactalbumin (Kalaivani and Regupathi 2016) and also to carry out enzyme catalysed esterification reaction (Oliveria et al., 2000).

Mass transfer within RDC depends upon the hydrodynamic conditions of the phases employed and differ from system to system based on the physical properties of the phases and the structural characteristics of RDC and flow parameters employed during LLE. Several modification have been proposed to a conventional RDC

structure to improve its performance that depends upon LLE system used; asymmetric rotating disc contactor, perforated disc contactor, modified rotating disc contactor and open turbine rotating disc contactor are a few modified RDCs employed to study mass transfer and hydrodynamic studies.

3.4 Literature review

3.4.1 Cloud point temperature and Critical micelle concentration (CMC)

According to pseudo phase separation model, micelles are treated as a separate phase. CMC of a micellar solution is altered with the addition of co-surfactant, solvent or additives such as electrolytes. Most of the nonionic, zwitterionic surfactants possess cloud point < 100ºC while ionic surfactants cloud point temperature exceeds 100ºC. A few ionic surfactants have also expressed more than one cloud point on varying the temperature, for example, TBPFO possess two cloud points, structural studies indicate that in the first cloud point region the micelles were rod shaped and behaved as Newtonian fluid and in the second cloud point system the micelles were worm like and possess pseudoplastic properties. Viscometry, Refractometry, laser scattering techniques are the commonly employed methods to study the formation and confirmation of cloud point in an aqueous surfactant solution. Cloud point temperature of a certain surfactant depends upon the number of OE units present in the surfactant, with increasing OE unit, cloud point temperature is said to increase. However, with increasing carbon chain length CP was found to decrease, as a result of increasing hydrophobicity exerted by the surfactant. For longer alkyl chain surfactants the CMC value is between the range of 10^{-4} – 10^{-2} M, a lower CMC value is achieved by increasing the molecular mass of the hydrophobic part of the surfactant or by lowering the temperature or with the addition of additives such as electrolytes, polymers of uncharged organic species. The physiochemical properties of micelle vary above and below its CMC value and are often used to determine the CMC of a certain surfactant. CMC of a surfactant solution is influenced by several factors such as surfactant tail – increasing hydrophobic hydrocarbon tail of a surfactant results in decrease in CMC, surfactant head group – nonionic surfactants possess low CMC values and high aggregation number, with increasing EO unit, hydrophilicity increases thus increasing the CMC value, For cationic surfactants, micelle size

increases with the presence of counter ion in the order $Cl^- < Br^- < I^-$ while for anionic surfactants it increases in the order $Na^+ < K^+ < Cs^+$; ionic surfactants with organic counterpart show low CMC values and higher aggregation number.

The presence of salt reduces electrostatic repulsion between surfactant head groups of ionic micelle and results in the growth of micellar size while higher surfactant concentration results in a change in micellar shape and often leads to breakage and formation of smaller secondary micelles. For nonionic surfactants, increasing temperature results in turbidity of micelles known as cloud point, at which CMC decreases while micellar size increases, however, temperature change is more significant for ionic micelles. Similar parameters influence the micellar shape, increasing surfactant concentration has a predominant effect on the shape of micelles. Micelles formed at CMC are usually spherical in nature and with the further addition of surfactant concentration form cylindrical rod like micelles and with further addition, micelle shape shifts to hexagonal arrays as a result of close packing of micelles. Further increase in surfactant concentration causes the formation of secondary micelle phase which is lamellar in nature while in a few surfactants cubic phase is seen with the transition of surfactant micelles from heaxagonal arrays into cubic micelles.

3.4.1.1 Effect of Temperature on CMC

CMC value varies with the charge of the polar head group and hardly depends on the temperature and pressure yet, a few surfactants have been observed to show a drastic change in CMC above the temperature of 100ºC. CMC decreases as the hydrophobic part of the surfactant increases and is more rapid for nonionic than ionic surfactants (Lindman 1984). The CMC of ionic surfactants were found to decrease half with the addition of one methylene group to the hydrocarbon chain (Attwood 1970).For chains containing more than 16 carbon atoms the decrease in CMC was not significant and the further appreciable effect was seen due to coiling of the chain (Mukerjee 1967) and in branched hydrocarbon chain, the effect was less compared to that of the straight chain. NMR (Florence and Parfitt 1971) and ultrasonic (Craber and Zana 1970) measurements have reported that the rate of dissociation of monomer from micelle is between the range of $10^{-9} – 10^{-2}$ per second. A Life time of a micelle

depends on the hydrocarbon chain length, dissociation degree, aggregation number and additive effect (Hoffman et al. 1985). At low salt concentration, ionic micelles change their aggregation number in a particular fashion whereas at high salt concentration they coalesce and break into monomers (Lebner et al. 1981).

3.4.1.2 Effect of additives on CMC

The addition of salts induce hydration or dehydrate the OE unit of surfactant tail in a micelle, causing increase or decrease in the cross sectional area of the hydrophobic core of a micelle, that in turn increases or decreases the CP (Collins and Washabaugh 1985). Effect of additives on cloud point temperature of a surfactant system is a very commonly and mostly studied topic of research as most of the nonionic surfactants possess cloud point at above 60ºC and as nonionic surfactants are chiefly employed for various applications in food, cosmetics and pharmaceutical industries, reducing the cloud point of such systems is a necessity to make the process much more efficient and cost effective to be operated. The position of the solubilized additive in the micelle determines the cloud point temperature and most importantly the variation in CMC. An additive, when added to the surfactant solution can localize itself in between the surfactant head groups reducing inter-micellar repulsion and steric repulsion between surfactant head groups or in the micellar core made up of hydrophobic tails dehydrating the OE units (Yu and Xu 1989). Nature of the additive added and its concentration, presence of one or more additives, surfactant and its type, temperature, pressure has an overall effect on micellar CMC.

Akbas and Batugoc (2009) found that anions imposed an effect on attenuated water structure present among the micelles and their replacement, thereby declining cloud point temperature of the TX405 surfactant system. Effect of halogens on cloud point was found to decrease in the order $F^- > Cl^- > Br^-$, while other addition of other anions showed the following order $PO_4^{3-} > SO_4^{2-} > NO^{3-} > Br^-$.Studies on the effect of addition of salts such as NaI, Na_2SO_4, NaSCN, NaCl, NaBr, LiCl, KCl, $CaCl_2$, $Ca(NO_3)_2$, $MgCl_2$, $Mg(NO_3)_2$, $A1(NO_3)_3$ to SDS and TX100 surfactants resulted in decrease in the Cloud point temperature of the surfactant systems as a result of charge distribution and inter-micellar repulsion. The author has also reported that anion with high lyotropic number declines counter ion binding of cation to micelle and resulted

in a decrease in cloud point temperature. The addition of SDS to TX100 resulted in the inclination of cloud point temperature of nonionic surfactant system as a result of inter-micellar repulsion (Marszall 1998). The addition of thiocyanate to PONPE-7.5 surfactant solution resulted in an increase in cloud point temperature as a result of Free energy transfer (dehydration and solvation energy) of an ion from aqueous to micellar phase and repulsion of hydrophobic chains of the surfactant (Rathman and Scamehorn 1984). In studies conducted by Ajith and Rakshit (1993) and Ajith et al. (1994) on the addition of NaCl to brij-35:1-propanol (80:20) resulted in decline in cloud point temperature as a result of a reduction in inter-micellar repulsion. In different studies conducted by Shinoda and Takeda (1970), Tokiwa and Matsumoto (1975) and Collins and Washabaugh (1985), salting in effect of salts such as NaSCN, $Ca(SCN)_2$ lead to increase in cloud point temperature, while salts such as NaCl, Na_2SO_4 resulted in salting out of the surfactant system there by declining the cloud point temperature. Goutev et al. (1998) reported that addition of KCl to TX100 surfactant solution resulted in declining cloud point temperature as a result of dehydration of OE unit and structural disorder of surfactant tails within the micelle. Similar results were obtained by Molina-Bolivar (2002), with the addition of CsCl, LiCl and NaCl to TX100 surfactant system. The salts were found to decline cloud point temperature in the following order, LiCl<CsCl<NaCl.

Gonzalez and Travalloni-Louvisse (1989), found that addition of ethanol to TX100 surfactant system increased the cloud point temperature as a result of enhanced solubility of the surfactant in water/ethanol system. Kabi-Ud-Din et al. (1996) found that addition of aliphatic alcohols to SDS/TBABR surfactant system resulted in penetration of alkyl chain of alcohol into the micellar core. The bulk head groups of aliphatic alcohols are reported to penetrate in between surfactant head group replacing water from the micellar head group and decreased the cloud point temperature. In a study conducted by Li et al. (2009) on the addition of water soluble alcohols to Tergitol 15-S-7, Tergitol 15-S-9 and Neodol 25-7 surfactants observed that cloud point temperature was found to decrease with increasing chain length of alcohols studied. Increasing alcohol chain length offers increased hydrophobicity and replacement of water molecule thereby causing inter-a micellar interaction that causes a decline in cloud point temperature. Similar results were obtained by Alauddin et al.

(2009), the authors observed that alcohols reduced cloud point temperature of TX100 in the following order decanol > octanol > heptanol > hexanol > butanol.

Iwanaga et al. (1998), studied the effect of addition of glycerin, 1,3-butanediol, ethylene glycol, and PEG 400 to heptaethylene glycol dodecyl ether (C12EO7), the authors found that increasing glycerin concentration, decreased the cloud point temperature and vice versa effect was observed with the addition of 1,3-Butanediol, ethylene glycol and PEG 400. The addition of Glycerin to the surfactant solution is said to induce hydrophobicity, while the addition of other polyols induced hydrophilicity to surfactant. Studies on the effect of the addition of water soluble polymers to Tergitol 15-S-7, Tergitol 15-S-9 and Neodol 25-7, resulted in a decrease in the cloud point temperature as a result of increasing inter-micellar repulsion among the micelles. Alauddin et al. (2009), found that addition of alkane to TX100, resulted in a decrease in cloud point temperature in the following order decane > octane > heptane > hexane. Ruiz et al. (2001) have reported that addition of ethylene glycol to TX100 increased the cloud point temperature of the surfactant system as a result of steric repulsion of micelles caused by a decrease in dielectric constant declining Van der waal ineraction among micelles. Mahajan et al. (2004) reported the effect of the addition of glycol oligomers and triblock polymers (TBP) to Tween 20 and Tween 80. The authors found that increase in polymer concentration decreased the cloud point temperature as a result of hydrophobicity imposed by polymer that facilitates dehydration of micelles and reduction in cloud point temperature. Zhao and Chen (2006) found that excluded volume effect is the reason for the reduction in cloud point temperature of TX100 and TX114 surfactant systems, with the addition of polymers like 2-hydroxyethyl cellulose and hexadecyl modified 2-hydroxyethyl cellulose. Jan et al.(2007), found that addition of carboxylic acids -ethanoic acid , propanoic acid , butanoic acid, and hexanoic acid to TX100 resulted in a decrease in cloud point temperature due to the dehydration effect imposed by carboxylic acids.

Mata (2006), found that addition of SDS to TX100 resulted in an increase in cloud point temperature as a result of inter-micellar repulsion caused by surfactant head groups present within the micelles. Similar results were observed by Panchal et al. (2006), with the addition of sodium alkyl sulphate and alkytrimethyl ammonium bromides to TX114.

The addition of non-electrolytes such as urea and dioxane to SDS/TBABr mixed surfactant system, resulted in inclination in cloud point temperature as a result of hydration of micelles imposed by water-structure making additives (Kumar et al. 2000). Similar results were observed by Asakawa et al. (1995), with the addition of urea and thiourea, which inclined the cloud point temperature and increasing concentration of the additives, resulted in declining cloud point temperature. This effect is attributed to the combined effect of counter-ion dissociation degree (β) of micelles and solubility of micelles. Lakshmi and Nandi (1976), found that addition of glucose to SDS/TBABr mixed surfactant system resulted in a decrease in cloud point temperature as a result of dehydrating effect caused by sugar moieties (water structure breaking additive). Similar results were observed with the addition of sugars- xylose, arabinose and dextrose. On studying the addition of amino acids-glycine, alanine, leucine and phenylalanine to SDS/TBABr, it was found that Alanine and glycine interact with surfactant head group replacing water and cause micelle coalescence leucine and phenylalanine interacts with the surfactant tail .

3.4.2 Report on cloud point extraction

Several authors have designed and studied the extraction of solutes via CPE and a few recent kinds of literature involving cloud point extraction of diverse solutes are enlisted in table 3.1. A brief discussion on the parameters studied and the effect of operational parameters on the extraction efficiency is given below. A few operational parameters that were found to be commonly studied by the authors are system pH, the addition of salt, volume ratio, incubation temperature etc.

Table 3.1 Consolidated table on literature review of solutes extracted by CPE.

Solute and source	Surfactant employed	Reference
polychlorinated dibenzo-*p*-dioxins from water sample	polyoxyethylene 10 lauryl ether (POLE)	Sanz et al. 2002
Cyanobacterial Toxins (Microcystins)	Aliquat-336	Kwok-Wai Man et al. 2002
Hexahistidine-tagged EGFP from Recombinant *E. coli*	Nickel chelated TX114	Wang et al. 2004

Polycyclic aromatic hydrocarbon (PAH) from water stream	Tergitol 15-S-5, Tergitol 15-S-9, Neodol 25- 7	Hung et al. 2007
Anthraquinones from *Morinda citrifolia*	TX-100 and Genapol X-080	Klathevest et al. 2009
Copper, zinc, iron and Nickel from biological and environmental samples	TX114	Ghadei et al. 2009
arprocarb (AC), carbofuran (CF), isoprocarb (IC), and fenobucarb (FC) from corn	TX100	Zhou et al. 2009
Codeine from aqueous solution	TX114	Mashhadizadeh and Jafari 2010
Sulphanomides in milk	Tween 20, TX100 and TX114	Zhang et al. 2011
Iron in beer samples	TX114	Filik and Giray 2012
Clavulanic acid (CA) from Fermentation broth (*Streptomyces* sp.)	C10E4, AOT and CTAB	Hang et al. 2013
Bergenin from *Ardisia japonica*	TX114	Xing and Chen 2013
humic and fulvic acid from natural water	TX114 , CTAB	de Wuilloud et al. 2013
single-chain antibody fragment (scFv) from Supernatant of yeast (*Pichia pastoris*) fermentation broth	TX114	Malpiedi et al. 2014
Fatty acids from microalgae cultures (*Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Scenedesmus obliquus*))	Triton X-114, Tergitol TMN 6, Tergitol 15-s-7	Glembin et al. 2014
Standard Cutinase	Dehypon LS54 (surfactant) and Dextrin (polymer)	Mutalib et al. 2014

Fatty acids from microalgae *Scenedesmus obliquus*	TX114	Glembin et al. 2014
Reactive Blue 19	Nonylphenol polyethoxylate with-9.5 EO units	Melo et al. 2014
palladium (II), silver (I) and gold (III) from aqueous stream	TX114	Mortada et al. 2014
Nickel (II) from saline sulfate medium	TX100	Youcef et al. 2015
hiamine (vitamin B1), niacinamide (vitamin B3), pyridoxine (vitamin B6), and riboflavin (vitamin B2) from plasma and urine samples	TX100	Heydari and Elyasi 2014
Antiretroviral drugs - Abacavir (ABC), Efavirenz (EFV), Lamivudine (3TC) and Nelfinavir (NFV) from human plasma	TX114	Hunzicker et al. 2015

Sanz et al. (2002) studied the extraction of polychlorinated dibenzo-*p*-dioxins from water sample using polyoxyethylene 10 lauryl ether (POLE). The authors found that with increasing concentration of NaCl, recovery of solute was found to increase and similar results were obtained with increasing surfactant concentration. However, recovery was found to remain constant at higher surfactant concentrations. Maximum recovery of above 90 % has been recorded by the authors. Kwok -Wai Man et al. (2002) studied the cloud point extraction of Cyanobacterial Toxins (Microcystins) using Aliquat-336. Cloud point extraction conditions were optimized to 2.5 mM Aliquat-336 and 75 mM Na_2SO_4 at 25°C, to attain maximum recovery of Microcystins.

Wang et al. (2004) studied the extraction of Hexahistidine-tagged EGFP from Recombinant *E. coli* using Nickel chelated TX114. The authors have reported that with increasing the TX-Ni mole ratio, partitioning of EGFP to micelle phase was found to increase from 0.34 to 2.5. Recovery of 83% and Purity of 70% were obtained at an optimized condition of 2 wt% TX114, and TX-Ni mole ratio of 0.1, the addition of 500mM NaCl in the presence of 20mM Tris-HCl buffer whose pH was adjusted to 8.0.

Tergitol 15-S-5, Tergitol 15-S-9, Neodol 25-7 had been used to cloud point extract Polycyclic aromatic hydrocarbon (PAH) from the water stream by Hung et al. (2007). It had been reported that on studying the effect of salts (NaCl, NaI, Na_2SO_4, Na_3PO_4 and $CaCl_{2)}$, NaI was found to increase the cloud point temperature of the surfactant system, while the addition of other salts studied decreased the cloud point temperature of all the surfactant systems. Increasing concentrations of Tergitol 15-S-9 in the presence of 0.45 M Na_3PO_4 was found to increase recovery of PAH. Klathevest et al. (2009) studied the cloud point extraction of anthraquinones from *Morinda citrifolia* using TX-100 and Genapol X-080. The authors found that with increasing concentrations of TX100 and Genapol X-080, recovery of anthraquinone from its source was found to increase. The incubation temperature of 75ºC for 30 minutes, at TX100 concentration of 1 (v/v %) improved the recovery as high as 100 %. Copper, zinc, iron and Nickel were extracted from biological and environmental samples using TX114. The authors found that the heavy metal extraction was found to increase until pH - 8.5 and with further increase in pH the extraction efficiency was found to decrease. Maintaining temperature range of 45 – 50ºC has been reported to enhance metal complex formation and its interaction with the surfactant micelles. The higher temperatures lead to de complexation and reduce the recovery. Recovery of about 100 % has been reported on maintaining the following conditions, TX114 concentration of 0.13 (w/v) %, pH – 8.5, NaCl – 0.2 M (Ghaedi et al. 2009). In another study arprocarb (AC), carbofuran (CF), isoprocarb (IC), and fenobucarb (FC) were cloud point extracted from corn using TX100 (Zhou et al. 2009). The authors found that 4 volume % TX100 gave maximum recovery of pesticide from the feed, while the increase in surfactant concentration lowered the recovery. With increasing concentration of Na_2SO_4 (12 - 22 w/v %), recovery was found to increase until 18 %

sodium sulphate concentration. Maintaining incubation temperatures above 30ºC has been found to result in instability of the pesticides that in turn lead to a decline in pesticide recovery.

Codeine was cloud point extracted from aqueous solution using TX114. The authors have reported that maximum recovery of codeine was recorded on maintaining system pH of 4.5. Maximum recovery of codeine was observed at 2.5 mM buffer concentration and when 5 µM of bromothymol blue was used as ligand, that resulted in the maximum recovery and higher dye concentrations has been reported to have had no significant change in the recovery of codeine (Mashhadizadeh and Jafari 2010).

Zhang et al. (2011) studied the extraction of sulphanomides from milk using Tween 20, TX100 and TX114. TX100 and TX114 were found to enhance the recovery of sulphanomides, however, TX114 was observed to increase the viscosity of the extraction system. Maximum recovery was observed at ammonium hydroxide concentration of 95 µL, with further increase in salt concentration recovery was found to decline. Maintaining a concentration of 0.4 mL of n-butyl alcohol enhanced the phase separation and recovery of solutes into micellar phase and with increasing temperature (30-70ºC), recovery was found to increase until 50ºC and with further increase in temperature. An optimized condition of TX100 concentration of 60 g/L, the volume of ammonium hydroxide - 65 µL, volume of n-butyl alcohol - 0.4 mL, and incubation temperature of 50ºC, resulted in recovery almost equal to 100 %.

Filik and Giray (2012) studied the extraction of Iron from beer samples using TX114. On varying the pH, the authors found that maintaining system pH at 5, enhanced the separation of Fe (II) and Fe (III) by forming metal-ligand species that interacts with micelles. 5-Br-PADAP was used as complexation agent and its concentrations had been varied (2-20 µM), it is reported that 10 µM of 5-Br-PADAP was sufficient to enhance the extraction of Iron from the feed. An optimized extraction condition of 0.25 w/v % TX114, concentration of 5-Br-PADAP at 10 µM, pH adjusted to 5, and cloud point temperature of 50ºC, 0.54 mM EDTA as chelating agent lead to recoveries of 97 – 101 % in different beer samples.

Organic acids - humic and fulvic acid was extracted from natural water using TX114 and CTAB. It has been reported that with the addition of alcohol, lowest

ethanol concentration studied was found to enhance phase separation and recovery of solutes, maintaining alcohol concentration above 0.5 v/v % resulted in low recovery. Buffer concentration of 0.05 M enhanced phase separation and recovery of organic acids, while the presence of 0.012 % w/w CTAB in the feed enhanced the recovery to about 100 %, the absence of CTAB and varying pH and on maintaining pH above neutral pH, recovery was found to be less than 20 %. Recovery of 99.9 % was observed when pH (8) adjusted TRIS buffer solution of concentration 0.05 M, TX114 concentration of 0.2 % w/w and CTAB concentration of 0.012 % w/w was maintained (de Wuilloud et al. 2013).

TX114 was used to extract Bergenin from *Ardisia japonica* (Xing and Chen 2013). Optimized extraction condition of TX114 concentration of 7 v/v %, pH adjusted to 7, liquid/solid ratio of 100:1 (mL/g), 25 % m/v sodium chloride, maintaining 70ºC for 10 minutes resulted in recovery of 87.2 %. Haga et al. (2013) had studied the extraction of clavulanic acid (CA) from fermentation broth containing *Streptomyces* sp., using C10E4, AOT and CTAB as surfactants. On employing C10E4/ CTAB mixed surfactant system, partitioning of CA to the micelle rich phase increased with increasing concentration of CTAB within the mixed surfactant system, as a result of enhanced electrostatic interaction between CA and cationic micelles. In the case of C10E4 / AOT mixed surfactant system, the presence of AOT was found to influence and interrupt CA estimation. At 12 % (w/w) of CTAB, increasing temperature of the system, enhanced partitioning of CA to micelle rich phase, while with increasing concentration of CA at a given cloud point temperature, solute partitioning is reported to decline as a result of excess CA in the system and its interaction with the micelles. Recovery % in the presence of AOT/C10E4 micellar system was about 43.0 %, K - 0.67, on maintaining cloud point temperature of 29ºC, while recovery % in the presence of CTAB/C10E4 micellar system was about 21.5, K-1.44 at cloud point temperature of 40.7ºC.

Standard Cutinase was cloud point extracted using Dehypon LS54 (surfactant) and Dextrin (polymer) by Mutalib et al. (2014). The authors have reported that on varying the pH between 6.0, 6.5, 7.0, 7.5, and 8.0, cutinase attains a net negative charge above its pI-4.3 and as a result, the enzyme was found to partition into the micellar phase. On maintaining 22 % w/w Dehypon LS54 and 12.5 % w/w Dextrin,

pH adjusted to 8.0, and cloud point temperature maintained at 25ºC, the recovery of 65 % with a purification factor of 6.92 was obtained.

Glembin et al. (2014) studied the effect of process variables on cloud point extraction of fatty acids from different microalgae cultures (*Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Scenedesmus obliquus*) by employing different surfactant such as Triton X-114, Tergitol TMN 6, Tergitol 15-s-7. The authors found that algal cultures showed biocompitability in the decreasing order Tergitol 15 s 7 > Triton X-114 > Tergitol TMN 6. Haddou et al. (2006), studied the extraction of Phenol and benzyl alcohol from an aqueous stream using Oxo-$C_{10}E_3$, Oxo-$C_{13}E_9$. The authors found that with varying concentrations of NaCl, the addition of 10 wt% of NaCl lowered the cloud point temperature drastically from 69ºC to 38ºC. The presence of 25 wt% NaCl in the extraction system, lead to the recovery of phenol – 96 %, as the introduction of salt lowered the solubility of the micelles and phenol and enhanced its micelle - solute interaction. On studying the effect of anions, the authors have inferred that presence of sulphate ions induce a decline in cloud point temperature less than 10ºC at 10 wt% of Na_2SO_4, compared to 10 wt% of NaCl. Recovery of 95 % for phenol and 90 % of benzyl alcohol had been obtained at an optimized extraction condition of 0.15 wt% of phenol and on maintaining cloud point temperature of 50ºC.

Malpiedi et al. (2014) studied cloud point extraction of single-chain antibody fragment (scFv) from Supernatant of yeast (*Pichia pastoris*) fermentation broth using TX114. The authors studied different process variables and found that with the addition of supernatant, cloud point temperature of surfactant solution was found to decrease to -14ºC. Effect of broth loading (30-90 w/w %), the concentration of salts NaCl and $MgSO_4$ (0-10 w/w %) were studied and, the authors found that the addition of salts resulted in salting out effect of the micellar phase, by replacing the water molecules present between the micelles which enhanced the coalescence of micelles and phase separation. Effect of affinity ligand has been studied by considering two differeny dyes, Cibacron Blue F3GA (CB) and Fabsorbent™ F1P HF (HF). The addition of dyes was observed to enhance partitioning of antibody fragments and other proteins to the surfactant rich phase. Antibody fragment partitioning was higher with the addition of HF compared to CB. An optimized

condition of 4 (w/w %) TX114, 60 (w/w %) of yeast fermentation supernatant, Fabsorbent F1P HF – 0.008 (w/w %), pH -7 and at cloud point temperature of 24ºC, resulted in Purity of 32 %, Recovery of 88 % with a maximum Purification factor of 2.

Reactive Blue 19 was extracted from aqueous solution using Nonylphenol polyethoxylate with 9.5 EO units as surfactant by Melo et al. (2014). The authors report that with increasing incubation temperature of 65 - 75ºC, extraction efficiency was found to decrease. Increasing surfactant concentration 3 - 6 (m/m %), resulted in increasing dye extraction efficiency as a result of increasing number of micelles and their interaction with the dye. As mole ratio of dye to surfactant was increased, extraction efficiency was found to increase until 0.5, after efficiency declined due to excess dye in the system. Recovery of 87 % was achieved at the optimized condition of NP9.5 EO concentration of 7 wt%, Reactive Blue 19 concentration of 100 ppm and on maintaining the cloud point temperature of 65ºC.

TX114 was used to cloud point extract fatty acids from microalgae *Scenedesmus obliquus*. On varying the surfactant concentration, it has been reported that with increasing TX114 concentration from 3 to 5 wt%, fatty acid accumulation was found to increase in the micelle phase as result of the availability of a large number of micelles and their enhanced interaction with the fatty acids. Optimum condition of

3 wt% TX114, cloud point temperature of 37ºC has been employed during continuous pilot plant operation to attain maximum recovery of fatty acids (Glembin et al. 2014).

Heydari and Elyasi (2014) studied the extraction of Vitamins, Thiamine (vitamin B1), niacinamide (vitamin B3), pyridoxine (vitamin B6), and riboflavin (vitamin B2) from plasma and urine samples using TX100. It has been observed that the presence of ion pair agent enhanced the partitioning of vitamins into surfactant micelles and a maximum recovery was obtained at TX100 concentration of 0.2 (w/v) %. Maximum extraction was obtained at system pH of 3 while increasing pH leads to lower recovery.

Metals such as palladium (II), silver (I) and gold (III) were extracted from an aqueous stream using TX114 by Mortada et al. (2014). Maximum recovery of 99 -102 % has been recorded at TX114 concentration of 0.05 (w/v) %, adjusted pH of 6.0 ,

HCPTS - 0.05 mM, 25 °C. The authors have reported that maintaining system pH between 6 – 8, maximum recovery of metals were observed as their ability to form complex with 4-(*p*-chlorophenyl)-1-(pyridin-2-yl)thiosemicarbazide (HCPTS) was found to increase. Recovery was found to increase with increasing TX114 concentration until 0.05 w/v %, with further increase in surfactant concentration extraction efficiency was found to remain unaltered.

TX100 was used to cloud point extract Nickel (II) from saline sulfate medium by Youcef et al. (2015). Increasing pH lead to increasing extraction of nickel until pH-8.74 and with further in pH, recovery was found to decrease in the presence of 5 wt% TX100 and at cloud point temperature of 65ºC. Maximum recovery of nickel (II) has been recorded in the presence of TX100 concentration of - 5 wt%, with further increasing surfactant concentration, recovery has been observed to decrease. Similarly, Maintaining 60ºC resulted in the maximum recovery of nickel while further increase in incubation temperature leads to lower recovery. Optimized condition of Nickel (II) – 1.7mM, Schiff base ligand – 5.1 mM, TX100 - 5 wt.%, Na_2SO_4 - 7 wt.%, pH = 8.8, resulted in maximum nickel recovery of 90 %

Hunzicker et al. (2015) studied the extraction of antiretroviral drugs - Abacavir (ABC), Efavirenz (EFV), Lamivudine (3TC) and Nelfinavir (NFV) from human plasma using TX114. With increasing surfactant concentration, recovery of ABC and 3TC were found to increase, while recovery of EFV and NFV were found to decrease. On studying the effect of pH (2.5 – 11.5), the authors found that with increasing system pH until 5, recovery of the drug was found to increase and with further increase in pH, recovery remained unchanged. Phase separation and recovery were found to increase with increasing incubation temperature from 45 until 65ºC and with further increase in temperature, recovery was found to decline. Maximum recovery of 81 -107 % have been reported at optimized extraction conditions.

3.4.3 Hydrodynamic and mass transfer studies in RDC

Knowledge of the phase holdup is one of fundamental importance in the design, scale up and operation of RDC as it is needed to calculate the interfacial area per unit volume (Kalaichelvi et al. 1998). The performance of an RDC or any extraction unit depends on the amount of solvent present in the extractor; when a

amount of solvents exceeds the feed, a larger quantity of the solute is transferred/extracted and the phenomenon depends on the equilibrium characteristics of the system. From an operational point of view knowledge of the dispersed phase holdup is essential for inventory purposes such as scale up of the unit. Earlier literature suggest that several authors have published empirical correlations for several types of columns (Kumar and Hartland 1984, Dalingaros et al. 1986, Kumar and Hartland 1988, Kumar and Hartland 1995, Stockfleth and Brunner 2001) that have been further extensively employed to study modified columns and their hydrodynamic properties. Correlations available in the open literature elaborate the estimation of hold up that are restricted to a limited range of applications, in terms of column geometry, physical properties of the system as well as the rotor speed (Kalaichelvi et al. 1998).

Carneiro-da-Cunhal et al. (1994) studied the extraction of a recombinant cutinase from an aqueous solution to a reversed micellar phase of AOT in Isooctane. By using perforated rotating disc contactor (32 mm diameter and 160 mm height). Studies were carried out at constant flowrates for different time periods. It was found that with an increase in the time period the yield was increased to 78% at 70 minutes.

Kumar and Hartland (1995) proposed a correlation for the determination of holdup in RDC for both with mass transfer and without mass transfer conditions. They considered dispersed phase hold up to be a function of power dissipation per unit mass, continuous and dispersed phase velocities, physical properties, compartment height and gravitational constant. They also stated that the rotor diameter also possessess a significant role in the case of dispersed phase hold up.

Moris et al. (1997) studied the hydrodynamic behaviour of an RDC (72mm in diameter, operating height of 1.1 m, 22 mixing compartments) using the two phase system water-kerosene. The Total hold up was found to increase with rotor speed, total throughput and organic/aqueous phase flow ratio increases. The local holdup was measured at different column heights and was observed that it was maximum at the centre of the column and minimum at the upper and lower parts of the column. Experimental data of the hydrodynamic characteristics of pilot-plant size RDC obtained for a wide range of operation conditions were compared with results

predicted by published correlations. All the correlations considered predicted high holdup values.

Kalaichelvi and Murugesan (1998) developed a new correlation for the direct estimation of dispersed phase hold up from known operating system variables. Experiments were carried out in a cylindrical glass RDC (0.0762 and 0.1m dia. and 0.9m height). It was found that initially, the holdup increases gradually with dispersed phase flowrates and with further increase in the flowrates (both continuous and dispersed) as well as rotor speed, the holdup increases sharply. It was observed that the variation in the dispersed phase holdup is due to the effect of more fundamental variables viz., rotor speed, column geometry, phase flowrates and the physical properties of the systems used. Correlation involving Froude number, phase flowrates, Morton number and geometrical factors was proposed for both no solute transfer as well as mass transfer conditions. A separate correlation for no agitation condition was also proposed. Two regions of operations were also noted including region 1, where the dispersed phase holdup is nearly independent of rotor speed, covers the operation at low rotor speeds. Region 2 covers higher rotor speeds and higher dependency of dispersed phase hold up on rotor speed was observed.

Sarubbo et al. (2003) studied the effect of dispersed phase velocity, system composition and disc rotation speed on column holdup and it was found that the dispersed phase holdup increased with increasing the dispersed phase velocity and disc rotation speed. The experiments were carried out on a perforated rotating disc contactor (160mm height, 32mm internal diameter, 3 and 4 stages) using the system comprising of cashew nut tree-gum and PEG 4000. Studies were carried out at two tie line length. The variation of holdup for various tie line lengths was attributed to the phase viscosities. It was found out that the holdup decreased on increasing the tie line length and the number of discs had no considerable influence on the holder. The holdup was found to increase with the dispersed phase flowrate and the rotor speed.

Regupathi and Murugesan (2004) have reported their studies on prediction of continuous phase axial mixing in rotating disc contactor and about the advantages of rotating disc contactors when compared to the other types of liquid-liquids extraction equipment like packed, sieve tray, spray towers and pulsed columns. These advantages include simplicity in construction, high through with relatively low power

consumption, free from plugging and higher efficiency due to the circular motion of rotor disc which enhances the turbulence in the contacting liquid phases.

Soltanali et al. (2009) investigated the extraction of protein by reverse micelle extraction in rotating disc contactor with and without stators. They noted that the dispersed phase hold up increased with increase in rotor speed. In the absence of stator rings, it was observed that the holdup data increased considerably. They explained that it might be due to the size of the discs because without stator rings the discs were bigger and closer to the column wall causing greater obstacles to phase flow and diminished the velocity of the droplets. The experimental data were fitted with Kumar and Hartland equation and good fitting was observed for low rotor speeds whereas in the case of higher rotor speeds the experimental holdup data were found to be much higher than the calculated ones.

Kadam et al. (2009) studied the hydrodynamic and mass transfer characteristics of asymmetric rotating disc contactors using two contactors of 5 litres and 60 litre volumes. Studies were carried out on different aqueous organic systems with toluene forming the organic phase and water and aqueous PEG solutions forming the aqueous phases. It was found that the dispersed phase holdup increased with the increasing impeller speed, linearly with dispersed phase velocity, increased nominally with continuous phase velocity and was found proportional to $(P/V)^{0.4}$, where P is the power input per unit mass and V is the volume of the column. A correlation for the determination of dispersed phase holdup was proposed. RDC has been successfully used for reverse micellar extraction and not many studies have been carried out on the hydrodynamics and mass transfer studies of RDC using surfactant based LLE systems. Physical property studies of the system are inevitable in the hydrodynamic study of RDC and there lies a necessity to study the physical property studies of the surfactant based LLE systems beforehand to study the hydrodynamic of the system in RDC.

3.5 Aim and scope of the work

In general, nonionic surfactants such as TX100 and TX114 have been widely used for the separation of compounds owing to their mild nature towards the solute molecules. Nonionic surfactant based cloud point extraction of solute from the

feed involves stronger hydrophobic interaction between solute and the micellar hydrophobic core. Such interactions can be altered by varying the surfactant concentration, addition of cosurfactant, Ionic strength and pH which in turn influences the mass transfer of solute during forward and backward extraction. Biodegradability of most of the nonionic surfactants, minimum amount of surfactant which is required to form micelles, hydrophobicity based extraction, higher specificity and efficiency and ease in scale up and the possibility of recycling surfactant solution during continuous extraction process are considered to be advantageous over any other separation process. Hence, this present work aims to study the effect of process variables on extraction efficiency of PHA using nonionic surfactant based micelles via cloud point extraction. Though fixed temperature surfactant based extraction of PHA using individual non-ionic surfactants has been previously reported by Yang et al. (2011), this chapter aims to study the cloud point extraction based separation and purification of PHA from the biomass present within crude fermentation broth.

3.6 Materials and Methods

3.6.1 Materials

Surfactants -Triton X 100 (TX100), Triton X 114 (TX114) and Tergitol 6 (TMN6), Polymers- Polyethylene glycol (PEG) 4000, 6000 and 8000, Standard Poly(3-Hydroxybutyrate–co-3 hydroxyvalerate) PHBV (12 %) were purchased from Sigma Aldrich, India. Sodium sulphate, sodium chloride, ammonium sulphate and ammonium chloride were purchased from CDH, India. HPLC grade acetonitrile, HPLC grade trifluoroacetic acid (TFA) and concentrated sulphuric acid (H_2SO_4) (98%) were purchased from Merck, India. Deionised water was used during the protocols and the experiments were conducted at room temperature unless and otherwise stated.

LABINDIA analytical UV 3000 + UV/Vis spectrophotometer and Shimadzu HPLC LC 20 A series were used for the UV spectral analysis and Shimadzu HPLC LC-MS 2020 was used for fixed UV spectrum and Mass spectrometry analysis. *Cupriavidus necator* DSM 428 from MTCC, IMTECH Chandigarh, India was used in the synthesis of PHA in submerged batch fermentation process under limited ammonium sulphate as nitrogen source and abundant crude glycerol as a carbon source in the medium.

About 85 % of PHA accumulation was calculated by performing crotonic acid assay as described in section 2.2.5. The fermentation broth after incubation was used as such for the cloud point extraction protocol.

3.6.2 Cloud point extraction protocol

The effect of individual surfactant concentration on the extraction of PHA from the fermentation broth was studied by considering nonionic surfactants TX100, TX114 and TMN6 at varying concentrations of 1 to 10 wt%. Equal volumes of broth and surfactant solution of varying concentrations studied, were added to dry pre-weighed tubes and the tubes were subjected to temperature change (from room temperature to 80ºC) in a temperature controlled water bath to induce cloud point formation and separation of two phases. The phase formation was examined visually for the appearance of turbidity which is the onset of cloud point and the clear separation of two distinct phases. Later, the tubes were withdrawn and were allowed to cool down to room temperature and were further subjected to centrifugation at 5000 RPM for 10 minutes. After discarding the supernatant the tubes were dried in a hot air oven for 1 hour at 100ºC. The tubes were allowed to cool down to room temperature and the post-weight of the tubes was recorded. The difference of post-weight and pre-weight of the tube denotes the cell dry weight (CDW) of biomass present in the volume of broth considered for the study. The pellet obtained was subjected to chloroform treatment and a required amount of the chloroform dissolved sample was used to analyze the PHA content by performing crotonic acid assay as described in section.2.5.4. Absorbance noted at 235 nm in the UV/Vis spectrophotometer was used to calculate the amount of PHA present and the same was used to calculate the extraction efficiency using the following formulas

$$Purity\ \% = \frac{PHA\ \ extracted}{Biomass(CDW)} \times 100 \qquad (3.1)$$

$$Recovery\ \% = \frac{PHA\ extracted}{initial\ crude\ PHA} \times 100 \qquad (3.2)$$

The effect of mixed surfactants-TX100+TX114, TX114+TMN6, TX100+TMN6 on the cloud point extraction of PHA from the biomass was studied for surfactant mixtures forming total weight % of 1, 5, and 7. Recovery % and purity % in the presence of mixed surfactant systems were calculated. The Mixed surfactant system which gave maximum purity % was considered to study the effect of fermentation broth pH (2 to 9) and the combination of the pH and surfactant mixture composition which gave maximum purity of PHA was considered for further purification steps. Effect of additives was studied by considering polymer- PEG 4000, 6000 and 8000 with the concentrations of 0.1, 0.5 and 1 wt%. Similar study on additive effect was performed by adding electrolytes. Salts from the Hofmeister series, ammonium sulphate, ammonium chloride, sodium sulphate, sodium chloride were considered at a concentration range of 0.1 to 1M was used for the extraction study.

3.6.3 Chromatographic analysis of purified PHA

HPLC analysis was performed for the systems which gave maximum purity of PHA during individual extraction steps. Rezex ROA organic acid H+ (8%) column from Phenomenex, USA was used for the analysis and 0.014N H_2SO_4 was used as the mobile phase. Column oven temperature of 30º C and PDA temperature of 40ºC was maintained. Chromatograms obtained at 235 nm were compared to analyze the peak intensity and the retention time of the peaks.

Further in a similar chromatographic study, HPLC analysis was performed for the samples of standard PHBV in RP-column capcell pak C18 MG II type maintained at 30ºC with the mobile phase of HPLC grade acetonitrile and water at 50:50 (vol:vol) and a flowrate of 1mL/min. Similarly, the micelle phase of the system that gave the highest purity of PHA during the cloud point extraction as such and the chloroform derived micelle phase of the same system were also analysed at the same column condition. 20µl of the samples were injected into the column separately and the UV spectrum at 235 nm was recorded along with the mass scan obtained from ESI-MS. Nitrogen was used as nebulizing gas and drying gas maintained at a flowrate of 1.5 L/min and 15 L/min respectively. MS unit heat block was maintained at 200ºC while ion interphase temperature was maintained at 350ºC. The raw data obtained was

processed using HPLC software and was analyzed for the negative ion peaks and the base peak obtained corresponding to the retention time of PHA.

3.6.4 Continuous cloud point extraction

3.6.4.1 Design of RDC

A modified RDC was designed as shown in figure 3.4, to study continuous cloud point extraction of PHA from fermentation broth using nonionic surfactant based micelles. During the study, following points were taken into consideration to achieve successful design and operation of the extractor, fermentation broth containing PHA accumulated microbial cells, was to be used as continuous phase; nonionic surfactant solution containing micelles was to be employed as dispersed phase. Cloud point temperature was maintained by preheating the broth and the surfactant solution before passing into the column to enhance interphase formation within RDC.

Rotating Disc Contactor used for the study was made up of Perspex provided with a central rotating shaft carrying equally spaced discs, positioned at the centre of each compartment made up of stator rings. The stator ring coupled with the rotor ring partitions the column into multiple stages. The central shaft, stator and rotor rings were made up of mild steel while the inlet and outlet ports were made up of brass. The light (dispersed) and heavy (continuous) phases are to be introduced at the middle and at the bottom of the column, respectively; the counter current flow of the phases was maintained during the study. The speed of rotation was regulated by a digital rotation speed meter supplied with DC voltage and the rotation per minute was measured by a laser source attached with the digital speedometer. Silicone tubes were used for the supply of continuous and dispersed phase. Cloud point extracted micellar phase outlet at the bottom was fitted with an adjustable limb to facilitate control over the interphase formed between the continuous and the dispersed phase.

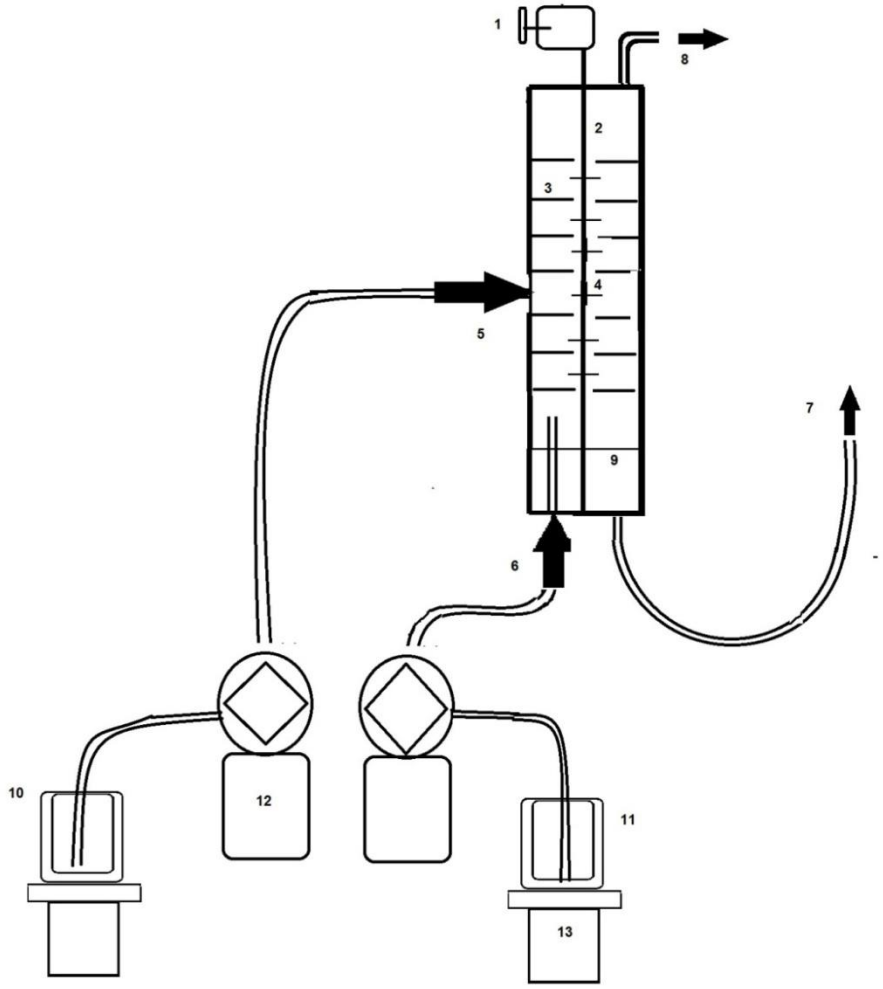


Figure 3.4 Schematic representation of Modified Rotating Disc Contactor used in continuous CPE of PHA. 1 – AC motor, 2-rotating shaft, 3-stator ring, 4- rotor ring,5- dispersed phase (micellar solution) inlet, 6-continuous phase (fermentation broth) inlet, 7-dispersed phase (micellar phase) outlet, 8-continuous phase (spent fermentation broth) outlet, 9-interphase, 10- micellar solution in jacketed vessel, 11-fermentation broth in jacketed vessel, 12-peristaltic pump, 13-magnetic stirrer.

Active volume of the column is defined as the volume occupied by the liquid inside the column. This volume will be less than the total volume of the column due to the presence of column internals. The active volume of the RDC was measured by initially filling the column completely with water and then draining the entire water into a measuring cylinder and the volume drained was measured. Dimensions of the modified RDC are tabulated in table 3.2.

Table 3.2 Dimensions of RDC used for continuous cloud point extraction of PHA.

Inner diameter of the column, D_c (m)	0.0044
Height of the column, H (m)	0.43
Rotor diameter, D (m)	0.0025
Stator ring diameter, D_s (m)	0.003
Height of the compartment, Z_c (m)	0.002
Number of compartments	9
Total volume of the column (mL)	650
Active volume of the column (mL)	560

3.6.4.2 Continuous micellar extraction of PHA

Micellar system that gave maximum purity of PHA during batch cloud point extraction studies was chosen as the dispersed phase; while 20 times diluted crude fermentation broth was used as the continuous phase. The fermentation broth was diluted to decrease the thickness of interphase formed as a result of protein and cellular impurities aggregation which is commonly encountered during continuous LLE. Dispersed phase and continuous phase were preheated in a jacketed vessel by circulating hot water from the water bath. The temperature of the dispersed phase and continuous phase were maintained more than the cloud point temperature of the micellar system (36ºC). Jacketed vessels were stirred with a magnetic stirrer and both the phases were stirred separately to avoid precipitation of surfactant micelles and cellular proteins respectively. The phases were let to flow into RDC by employing variable speed peristaltic pumps. The dispersed phase entering the column comes in contact with the cellular impurities and PHA and other media components and settles down as a micelle rich bottom phase; as the temperature of the column is well beyond the cloud point temperature of the surfactant system. At the maintained condition, the interphase formation was observed and the adjustable limb connected to the micellar outlet was adjusted to maintain the level of interphase at the bottom of the column, i.e. below the lowest stator ring. Once, steady state was achieved which is visually confirmed by a steady interphase maintained within RDC. Dispersed phase hold up was measured by the displacement method. Once the steady state of interphase is

achieved, the contactor is left undisturbed for a while to allow tiny droplets of dispersed phase distributed among the continuous phase to settle down over the bottom micellar phase. Displacement of interphase can be visually observed with the rising up in the dispersed phase volume as a result of settling of dispersed phase trapped within the continuous phase that was distributed amidst the column internals. Volume occupied by the dispersed phase after displacement of the interphase was measured and dispersed phase hold up is calculated using equation 3.3,

$$\text{Dispersed phase holdup},\phi = \frac{\text{Volume of the dispersed phase}}{\text{Total contacting volume}} \qquad (3.3)$$

The variation of dispersed hold up, mass transfer coefficient, purity and recovery % of PHA were studied by varying dispersed phase and continuous phase flowrates and speed of rotation. The rotor speed was varied from 50 RPM to 150 RPM while the dispersed flowrate was varied from 35 to 45 ml/min and by also varying continuous phase flowrate from 15 to 45 ml/min. One variable at a time approach was maintained, by keeping one of the parameters as a variable and the other two parameters constant. Dispersed phase hold up was calculated as described above, purity and recovery % were calculated as elaborated in section 3.4.2. Mass transfer coefficient was calculated by measuring the solute PHA concentration at inlet and out let during the steady state operation (Equation 3.4) (Porto et al. 2000)

$$K_d a = \frac{L\left[\frac{(lnC_i) - KC}{C_o - (KC)}\right]}{V} \qquad (3.4)$$

Where, $K_d a$ – Mass transfer coefficient (min^{-1})

L – Dispersed Phase flowrate (mL/min)

C_i - Concentration of solute in let (mg/mL)

C_o – Concentration of solute in outlet (mg/mL)

V -Volume of dispersed phase at steady state (mL)

K- Partition coefficient

C -Amount of solute in micellar phase (mg/mL)

3.7 Results and Discussion

3.7.1 Effect of surfactant

Extraction studies were performed by considering nonionic surfactants TX100, TX114 and TMN6 due to their mild and non-denaturing characteristics on bio molecules mostly involving proteins (Tani et al. 1998). Cloud point shift on the addition of fermentation broth to the surfactant solutions were studied and it was observed that the cloud point temperature was found to decrease with increasing concentrations of TX100 and TMN6 while it increased with increasing concentrations of TX114 as shown in figure 3.5.

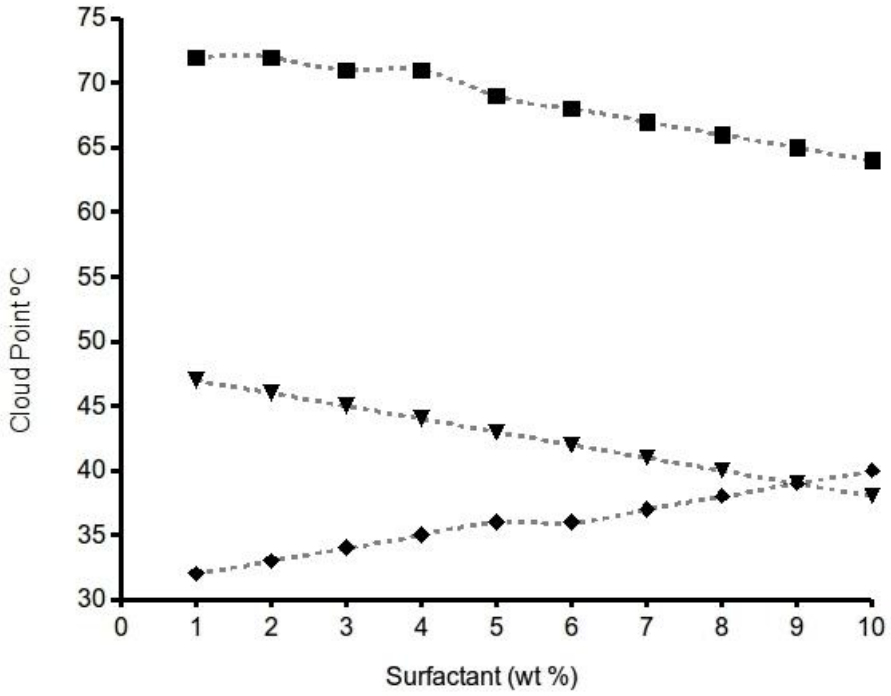
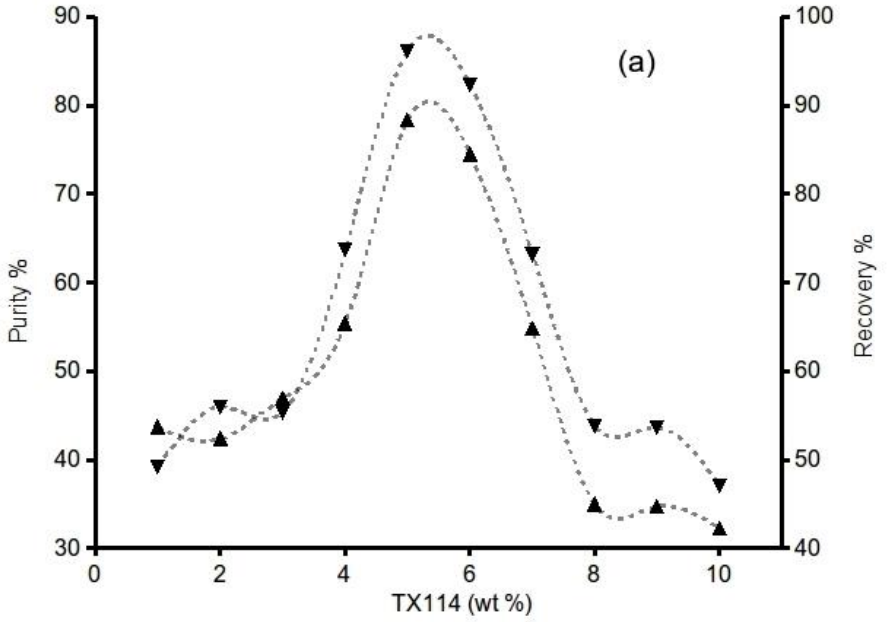
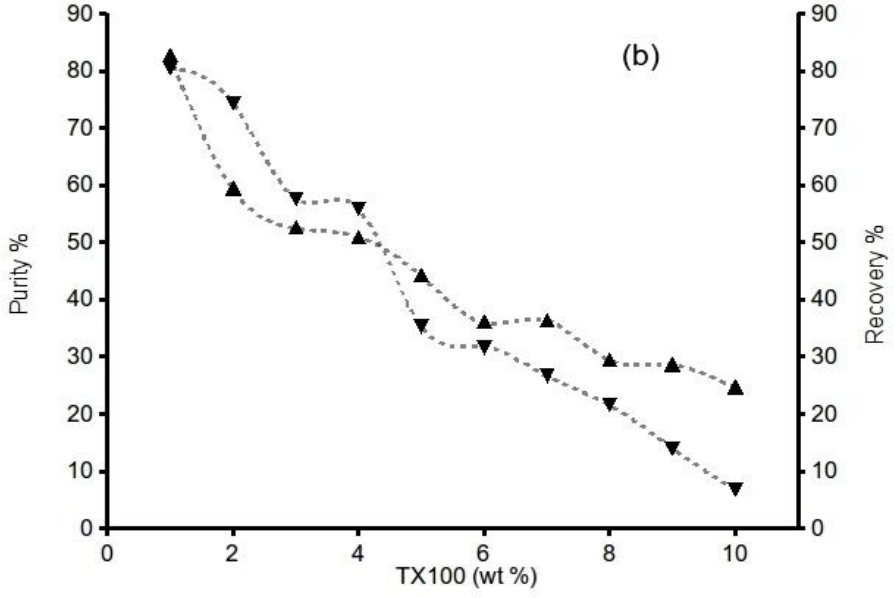


Figure 3.5 Cloud point temperature variations of different concentrations (wt%) of surfactants in the presence of fermentation broth. ■ - TX100, ▼ - TMN 6 , ◆ - TX114.

On addition of surfactant to the fermentation broth, surfactant monomers solubilise the lipid bilayer of the cell wall and forms micelles thereby leading to cell disruption and cell leakage (Helenius and Simons 1975). Hydrophobic tail of the surfactants involves them in masking the hydrophobic domains of the protein molecules and also interacts with hydrophobic solutes, PHA and lipid molecules while the hydrophilic head groups are solubilised in the surrounding water. As a result of stronger hydrophobic interaction between micelle and hydrophobic PHA molecules

in the medium, a stable micelle-PHA complex is formed as explained by necklace bead model (Hansson and Lindman 1996) and settles down as a micelle rich bottom phase (coacervate phase). Purity and recovery % of PHA were found to increase in the order of TX100 < TMN6 < TX114 as shown in figure 3.6 (a,b,c).





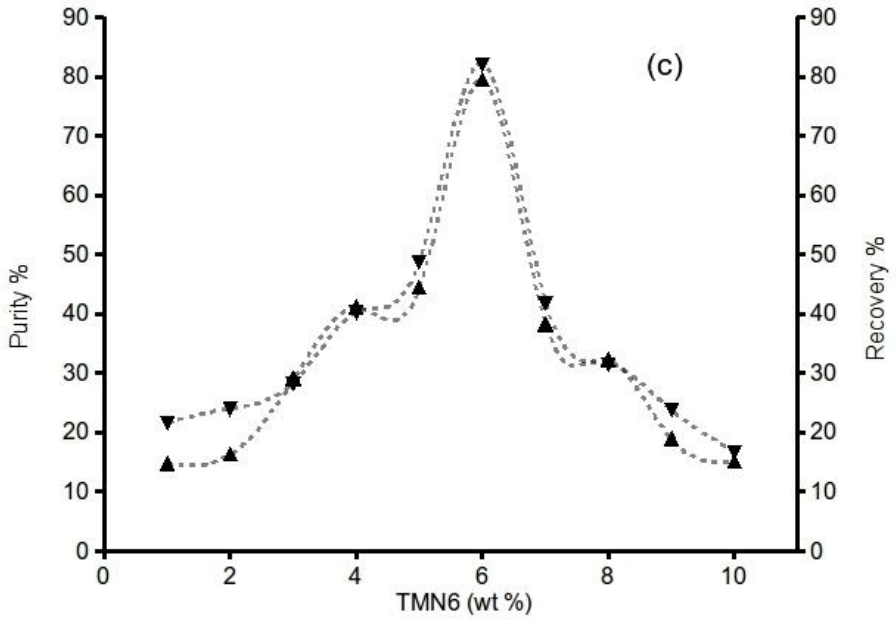


Figure 3.6 Purity % - ▼ and recovery % - ▲of PHA in the presence of varying concentrations of individual surfactants (a) TX114 (b) TX100 (c) TMN6.

As Hydrophile Lipophile Balance (HLB) value of the surfactant (HLB value of TX114 - 12.4, TMN6 - 13.1 and TX100 - 13.5) increases with increasing surfactant concentrations, purity and recovery of PHA were found to decrease as a result of a decrease in hydrophobicity of the surfactant system (Egan 1976). At increased surfactant concentrations, the excess monomeric surfactants present in the solution encapsulate the proteins and other bio molecules and settle down along with the micelle-PHA complex, which leads to decrease in the purity and recovery of PHA. Similar results have been obtained by several authors on employing TX114 in cloud point extraction of solutes; increasing surfactant concentration was found to incline cloud point temperature of the feed and also increased the extraction of bergenin from *Ardisia japonica* (Xing and Chen 2013).

3.7.2 Effect of mixed surfactant

Mixed surfactant systems were studied to decrease the cloud point temperature and increase the extraction efficiency of the micelle system due to the variation in HLB value of the system at different surfactants and their varying concentrations. As a result of mixing the surfactants at different concentrations, individual surfactant mixture's HLB value differs from one another and as a result, their extraction

efficiency and their respective cloud point temperature vary (Egan 1976). It was observed that the cloud point temperature of mixtures containing TX114; TX114+TMN6 and TX114+TX100 had lower cloud points than TX100+TMN6. The extraction efficiency was found to decline with their respective increase in the HLB value in the following order TMN6+TX114 > TX100+TX114 > TMN6+TX100. Higher concentrations of TMN6 and TX100 present in the mixture leads to increase in the HLB value, in turn, increasing the the extraction of hydrophilic solutes and reducing the purity and recovery of PHA extracted from the fermentation broth.

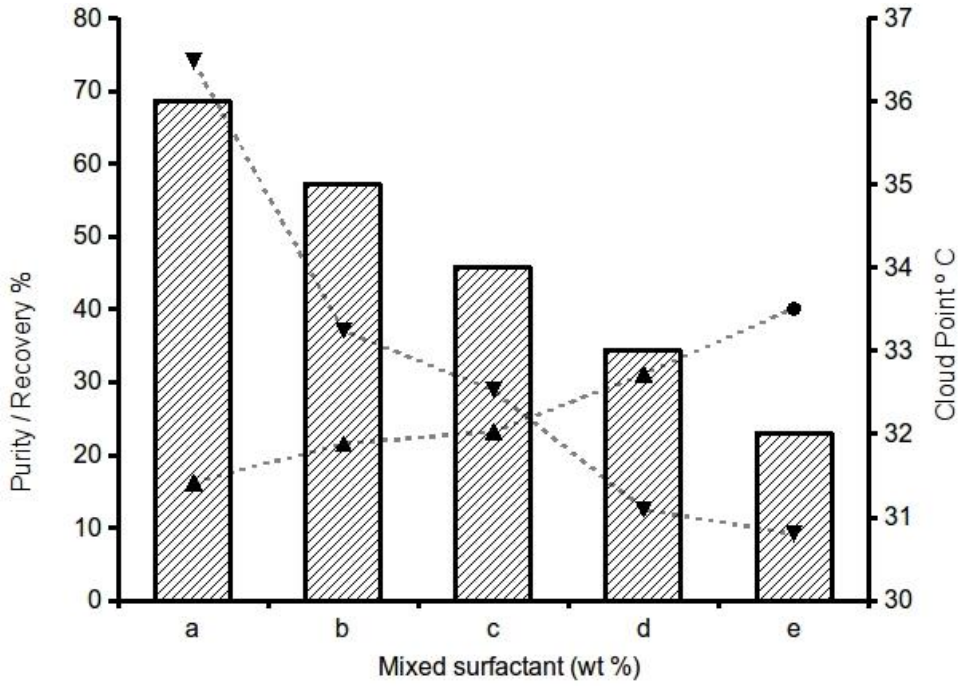


Figure 3.7 Purity % - ▼ and recovery % - ▲ and cloud point temperatures (Diagonal line bars) of surfactant mixture – TX114+TMN6 at 5 wt%: a – 4.5% + 0.5%, b - 3.75%+1.25%, c - 2.5%+2.5%, d - 1.25%+3.75%, e - 0.5%+4.5%.

As seen in figure 3.7, the purity % was found to increase with increasing concentrations of TX114 in the surfactant mixture TX114+TMN6 while the recovery % decreased owing to increase in hydrophilicity with the increase in TMN6 concentration to the mixture. TX114 and TMN6 combinations that make up to total weight % of 5: TX114-4.5%+TMN6-0.5% was observed to give maximum purity % of 74.13 while the cloud point was found to be at 36ºC and the recovery % was about

16.18. Similar effects have been reported in the literature by Paleologos et al. 2005, substantiating that the increasing concentration of TX114 in the surfactant mixture of TX114 and SDS had a positive influence on the extraction efficiency of chromium. Extraction efficiency of other mixed surfactant systems studied is represented in appendix I.

3.7.3 Effect of pH

Effect of variation in broth pH was studied to increase the PHA recovery % with higher purity, as pH has a direct effect on structural reconfirmation of proteins and its interaction with the micelles. The fermentation broth pH was varied between 2 to 9 during the extraction and the obtained results are shown in figure 3.8. The cloud point of the systems remained same at 36ºC as that of mixed surfactant solution of 5 wt% of TX114+TMN6, even on varying the broth pH. As observed in the figure, the purity and recovery % was found to increase until pH 3 and decreased as the pH moves from acidity to basicity. At an acidic pH of 3, most of the proteins attain a net positive charge and are attracted towards the aqueous phase thus resulting in a maximum purity % of 79.38. Further increase in broth pH leads to charge variation of the proteins present in the solution with respect to their corresponding isoelectric point. The presence of both positive and negatively charged proteins results in dipole interaction and aggregate formation that settles down in the micelle rich phase thereby decreasing the extraction efficiency. Similar effects were observed by Yang et al. (2013), and maximum purification was obtained at a pH of 3.77 on the recovery of PHA using anionic surfactants.

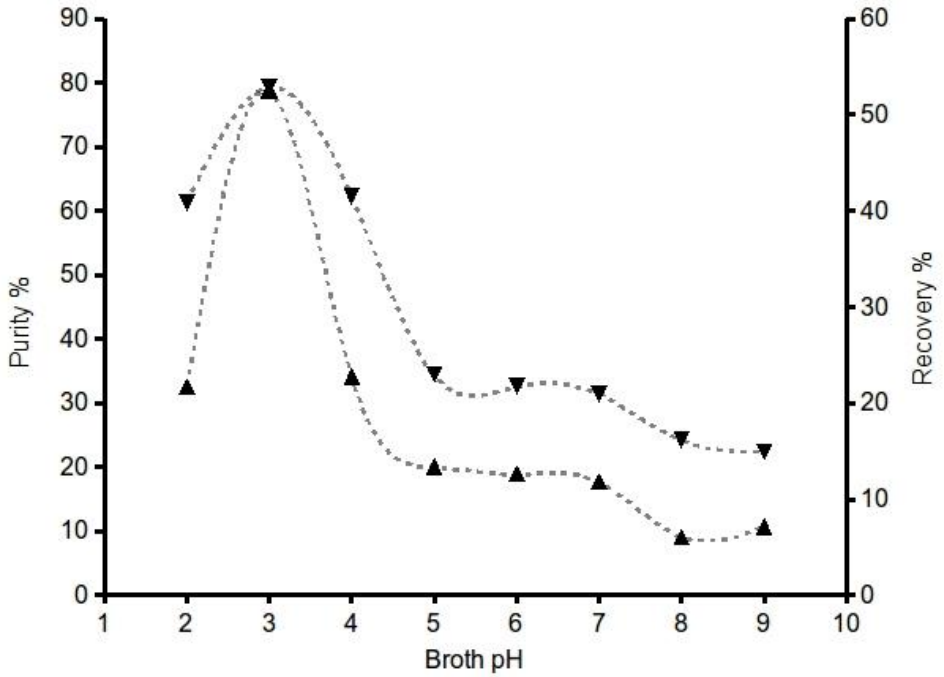


Figure 3.8 Effect of broth pH on purity % - ▼ and recovery % - ▲.

3.7.4 Effect of additives

The presence of additives has a significant role in the reduction or increment of cloud point of the micellar system and also has an effect on the extraction efficiency by modulating the intermolecular forces between the solute and the micelles (Mukherjee et al. 2011). In order to further increase the extraction efficiency further and to reduce the cloud point temperature of the mixed micelle systems, additives including polymer – PEG of varying molecular weight – 4000, 6000, 8000 and electrolytes like from the hofmesiter series like- sodium sulphate, sodium chloride, ammonium sulphate, ammonium chloride were considered.

3.7.4.1 Effect of PEG

Figure 3.9 depicts the effect of the addition of varying concentrations of different PEG molecules as an additive on cloud point temperature. It is inferred from the figure that with increasing concentration of a given PEG, cloud point temperature was found to decrease drastically. It was also found that the increasing molecular weight of PEG at a given concentration decreases the cloud point temperature significantly.

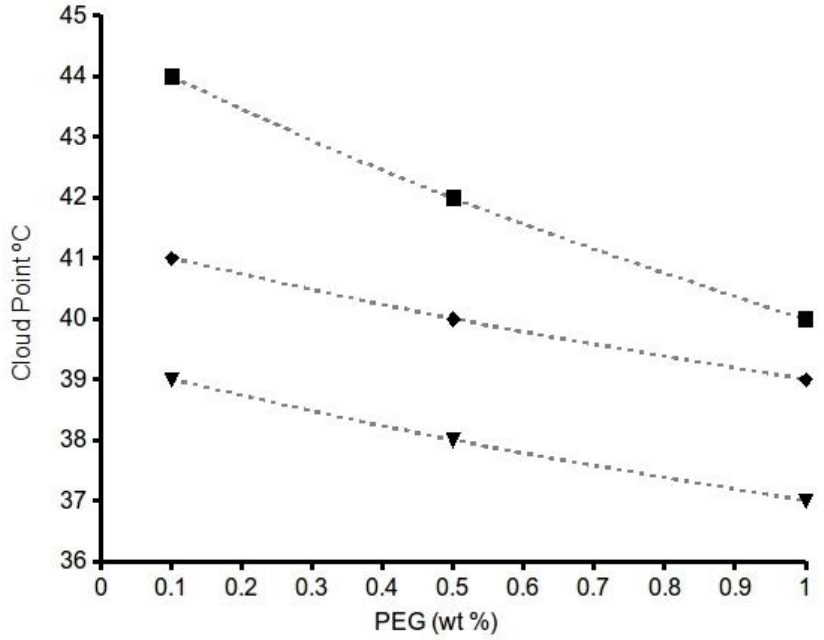
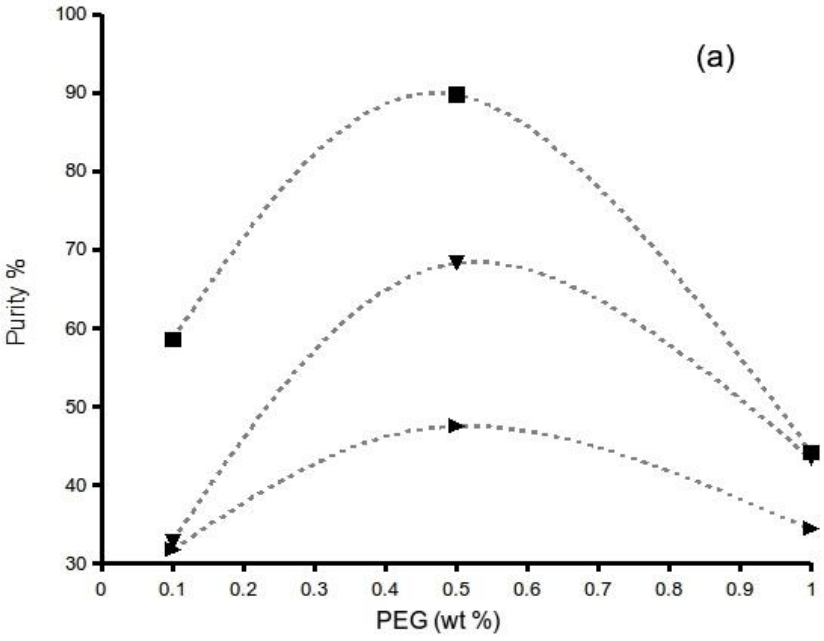


Figure 3.9 Effect of varying molecular weight and concentration (wt%) of PEG on cloud point temperature in the presence of TX114+TMN6 mixed micelle system. ■ - PEG 4000, ◆- PEG 6000 and ▼ - PEG 8000.



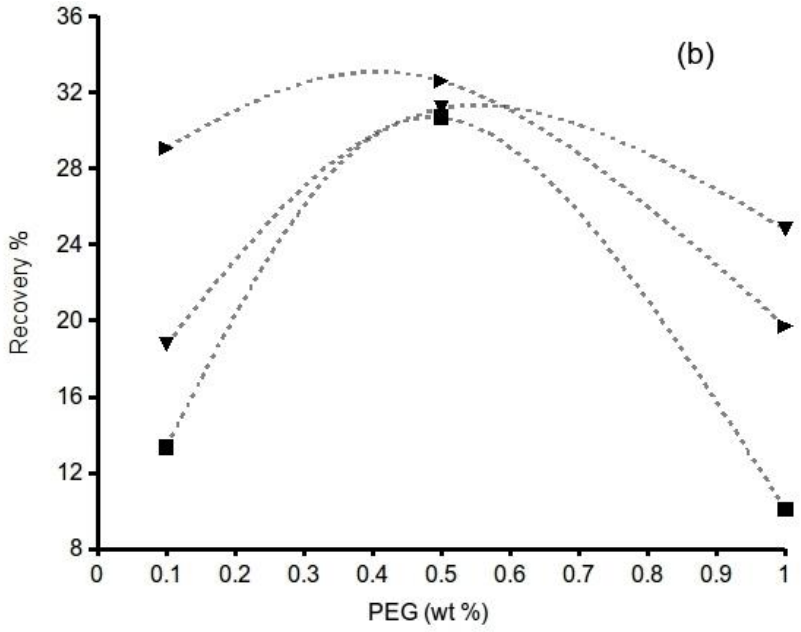


Figure 3.10 Effect of varying molecular weight and concentration (wt%) of PEG on purity % (a) and recovery % (b) in the presence of TX114+TMN6 mixed micelle system. ■ - PEG 4000, ◆ - PEG 6000 and ▼ - PEG 8000.

PEG molecules, when added to surfactant solution forms polymer-surfactant aggregates with surfactant micelles as explained by necklace bead model and they lead to a reduction in the surfactant concentration required to form micelles which are usually lower than that of CMC and is known as critical aggregation concentration (CAC) (Hansson and Lindman 1996). As a result of the PEG-surfactant interaction, cloud point was found to decrease with increasing molecular weight and their varying concentrations (Naqvi and Khatoon 2011). The purity of PHA extracted into the micelle rich phase was found to increase while the recovery % decreased with increasing molecular weight and concentrations of PEG. As PEG molecules are strongly hydrophilic compared to surfactants, they solubilise themselves in the aqueous layer and aid in the separation of proteins, while surfactants solubilise most of the hydrophobic solutes in the micellar phase (Sivars and Tjerneld 2000). As observed in figure 3.10 (a,b), purity and recovery % was found to increase with the addition of PEG molecules until 0.5 wt% and with further increase in molecular weight and concentration purity and recovery of PHA decreased, as a result of

increase in hydrophobicity of the PEG-Surfactant system (Saitoh et al. 1994, Tani et al. 1997).

3.7.4.2 Effect of electrolytes

Four different electrolytes in the concentration range of 0.1 to 1 M were also used as additives along with PEG. As shown in figure 3.11, with increasing concentration of ammonium sulphate, sodium sulphate and sodium chloride, the cloud point temperature was found to decrease; while with increasing concentration of ammonium chloride, cloud point temperature was found to increase in the presence of mixed surfactant system of TX114+TMN6. At low concentrations such as 0.1 M, cloud point temperature was found to decrease in the order ammonium sulphate > sodium sulphate > sodium chloride, which indicates that the cationic group has a major impact on the cloud point temperature of the mixed surfactant system. The ethylene oxide unit of the surfactant tails gets hydrated or dehydrated depending on the anions present in the salt, which in turn leads to surfactant's structural deformation and thereby increasing or decreasing the cloud point of the system.

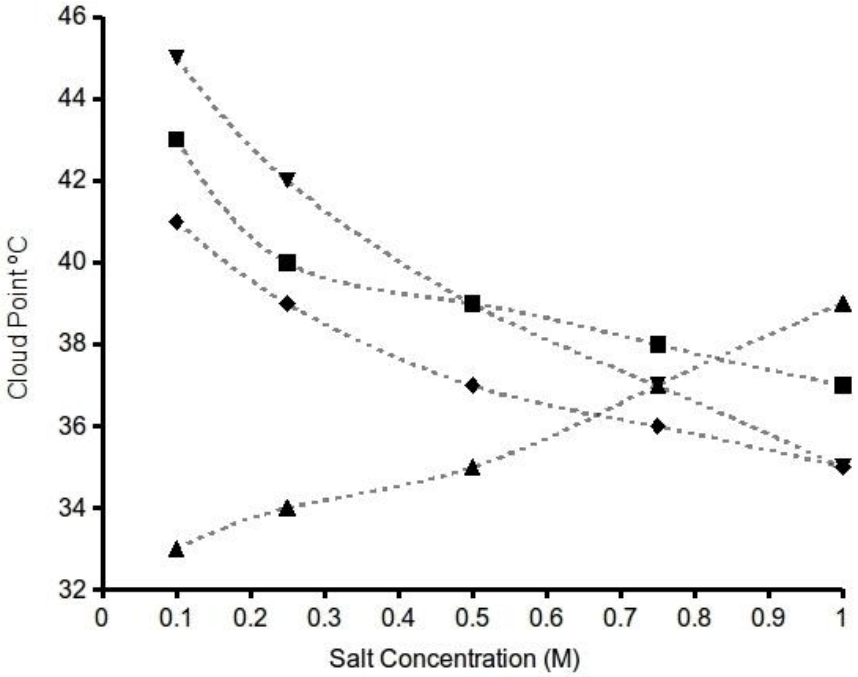


Figure 3.11 Effect of different salts and its varying concentrations (M) on cloud point temperature in the presence of TX114+TMN6 mixed micelle system. ▼ - ammonium sulphate, ■ - sodium sulphate, ◆ - sodium chloride and ▲ - ammonium chloride.

From figure 3.11, it is concluded that the addition of sulphate salts increases the cloud point temperature, but chloride salts decrease the cloud point temperature of the system. However, the increase in the concentration of sulphate and chloride salts resulted in a decrease and increases the cloud points, respectively as a result of ionic charge and hydrophobicity imparted on the micelles. Similar effects are observed for cations on hydration and dehydration of the surfactant micelles. The larger ionic radius of ammonium compared to that of sodium has resulted in the reduction of cloud point temperature due to the screening of hydrogen bonding between water and the adjacent micelles and expose the hydrophobic bonds, which in turn leads to micelle-micelle interaction and micelle coalescence and forms a micellar rich lower phase (Israelachvili 2011, Parikh et al. 2013).

The presence of electrolytes in the medium imparts electrostatic interaction or repulsion with proteins while the interaction between nonionic surfactant micelles and PHA is purely hydrophobic. At low concentrations (0.1 M), purity and recovery of PHA were high in the presence of ammonium sulphate, ammonium chloride, sodium sulphate, while ammonium chloride gave the lowest purity among the salts studied. However, increasing salt concentration results in replacement of water molecules between micelles thereby enhancing the formation of micellar phase. During this phenomenon, as a result of the salt-surfactant interaction, hydrophobic core of the surfactant micelles are well exposed that interacts with hydrophobic cellular impurities apart from PHA, thereby reducing the purity and recovery of PHA. Presences of salts also induce protein aggregation and precipitation as a result of exposure of hydrophobic domains of the proteins that affect the extraction efficiency of PHA. From the experiments, it was observed that the addition of 0.1 M ammonium chloride provides a maximum purity of 92.49 % and with a recovery of 84.4%. Since ammonium chloride has a mild effect on the proteins, most of the proteins were solubilised and retained in the aqueous phase and thereby increase the purity and recovery % of PHA in the micelle phase which is evident from the results as observed in figure 3.12 (a,b).

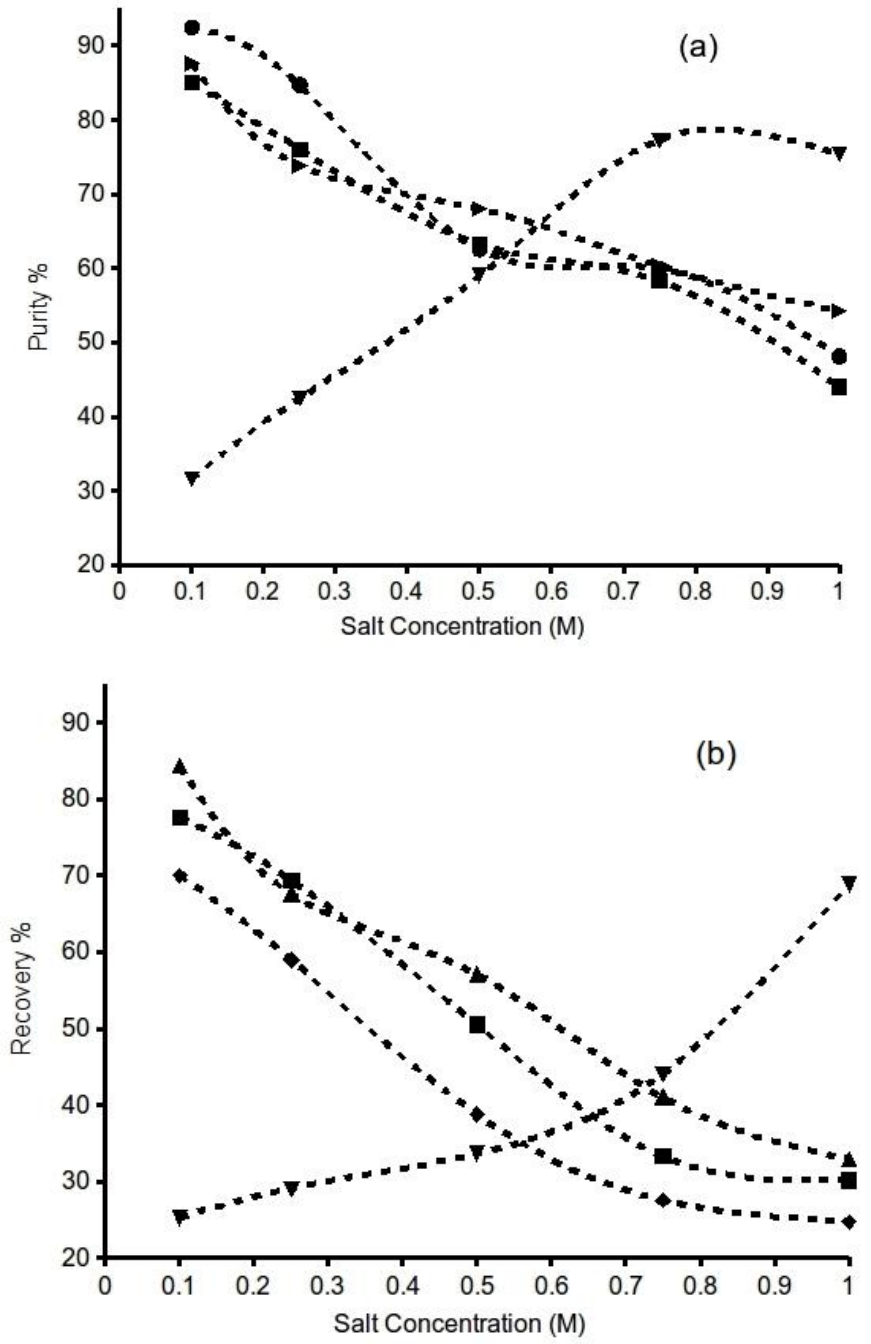


Figure 3.12 Effect of different salts and its varying concentrations (M) on purity % (a) and recovery % (b) in the presence of TX114+TMN6 mixed micelle system. ▼ - ammonium sulphate, ■ - sodium sulphate, ◆ - sodium chloride and ▲ - ammonium chloride.

Similar results have been reported in several cloud point extraction studies involving the addition of salts, CPE of polychlorinated dibenzo-*p*-dioxins from water sample using POLE was found to increase with increasing concentration of NaCl; however, at high salt concentrations recovery declined (Sanz et al. 2002). In another study involving extraction of palladium (II), silver (I) and gold (III) from an aqueous stream using TX114, the presence of Na_2SO_4 enhanced recovery compared to $NaNO_3$ and NaCl. The presence of Sulphate ions has been reported to induce CP less than the addition of chloride ions (Mortada et al. 2014). However, in our study, the presence of mixed surfactant system imposes significant effect in the presence of ions, compared to the effect of ions in the presence of individual surfactants (figure 3.6 a and b).

3.7.5 Chromatographic analysis of PHA

Results obtained from crotonic acid assay were verified by performing HPLC analysis in Rezex ROA organic acid H+ (8%) column. PHA samples with maximum purity from individual cloud point extraction steps were converted to crotonic acid assay and the same was injected to the column maintained at the given column conditions. It is deduced from figure 3.13 (a, b) that the peak intensity of PHA samples obtained during cloud point extraction by individual surfactants, was increasing in the order TX100 < TMN6 < TX114, which is in agreement with the purity % of PHA obtained via crotonic acid assay. Figure 3.13b, depicts the effect of all the process variables like mixed surfactants and their concentration, broth pH and additives (PEG and salt) and their concentration on the PHA purity. It was found that the peak intensity was increasing for samples in the order TX114+TMN6 (4.5%:0.5% - wt%:wt%) < pH 3 < PEG 4000 (0.5 wt%) < ammonium chloride (0.1 M). The maximum peak intensity corresponding to maximum purity and recovery % of PHA was obtained for the mixed surfactant system with a total surfactant concentration of 5 wt% (4.5 wt% TX114 and 0.5 % TMN6) at pH 3 and with the addition of 0.1 M ammonium chloride as additive at a cloud point temperature of 45ºC.

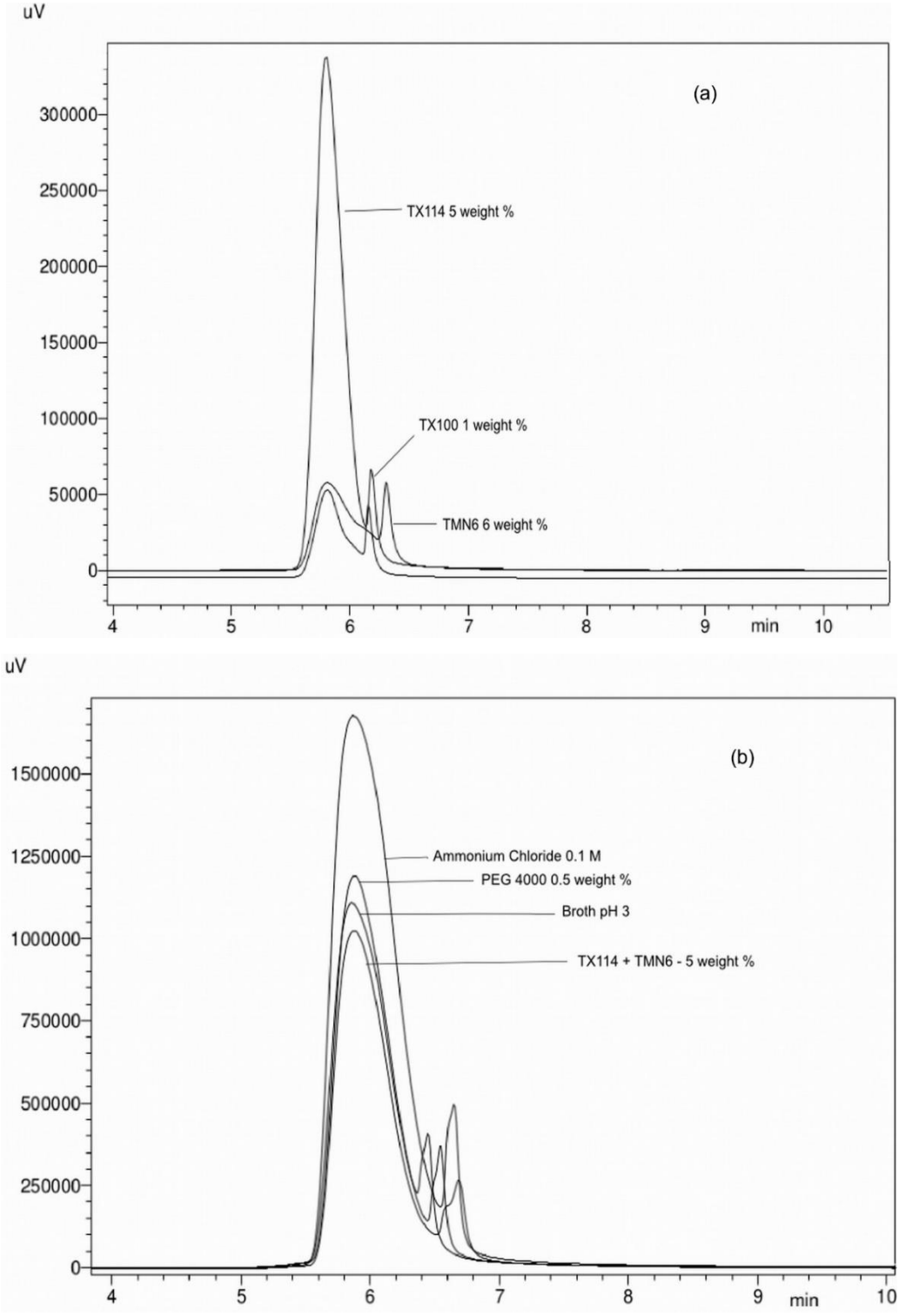


Figure 3.13 HPLC peaks obtained at 235 nm for (a) individual surfactants - TX114, TMN6, and TX100. (b) mixed surfactants, broth pH and additives – PEG and Electrolyte.

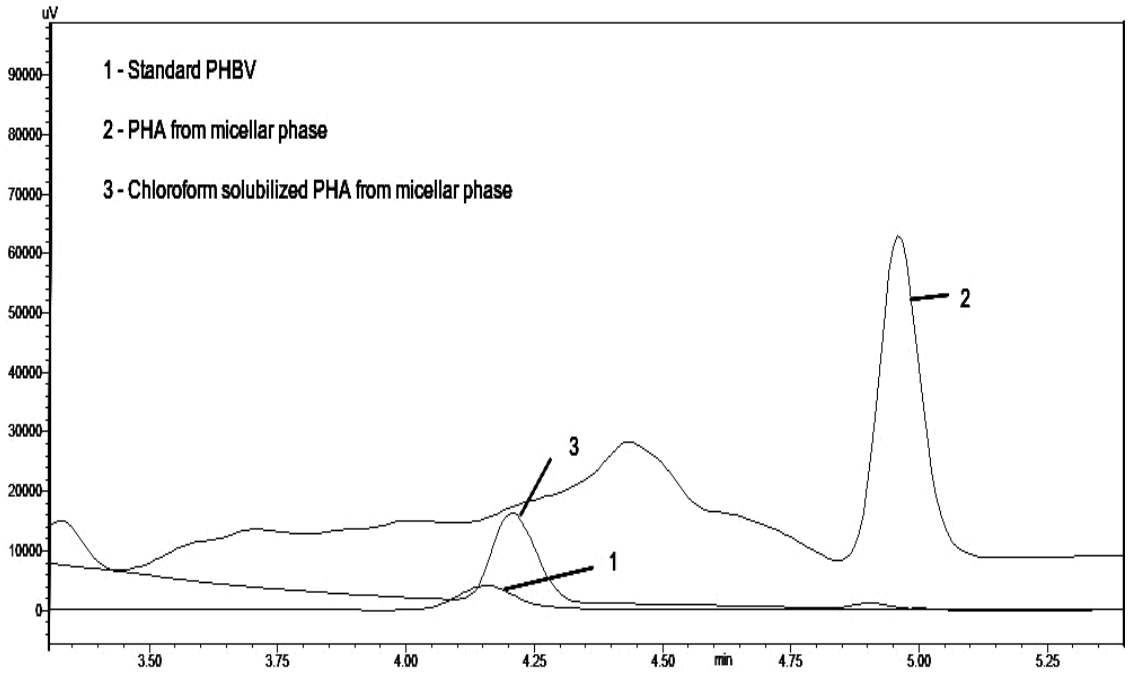


Figure 3.14 HPLC analysis of standard PHBV, PHA from micellar phase and chloroform solubilised PHA from micellar phase at UV-235 nm.

Figure 3.14 shows the comparison of retention time of standard PHBV peak with that of PHA from micellar phase of CPE and chloroform solubilised PHA from micellar phase of CPE. It was observed that the standard PHBV had a retention time of ~ 4.1 minute while PHA obtained through cloud point extraction had a retention time of about ~ 4.9 minutes, while chloroform derivatized PHA from cloud point extracted micellar phase had a retention time of ~ 4.2 minutes. Comparison of negative ion peaks obtained from mass spectrometry of PHA from micellar phase of CPE and chloroform solubilised PHA from CPE as observed in the figure, indicated that the base peak of the PHA extracted via cloud point extraction was 327 while that of Chloroform derived PHA from cloud point extraction method resulted in a m/z of 277. This effect might be as a result of screening effect of chloroform on PHA, which results in disruption of PHA nativity.

It was observed that conventional chloroform extraction of PHA resulted in a purity of 88.76% while sodium hypochlorite treatment resulted in purity of 90.47 % and ultrasonication of crude broth at 4 KHz for 5 minutes resulted in a purity of 78.37 %. Cloud point extraction of PHA molecule derived purity % of 92.49 and a recovery of 84.4 %.

3.7.6 Continuous micellar extraction of PHA

Continuous cloud point extraction was studied in modified RDC as discussed in section 3.4.4.2. Dispersed phase made up of TX114 (4.5 wt%) + TMN6 (0.5 wt%) and continuous phase comprising 20 times diluted fermentation broth with the addition of 0.1 M ammonium chloride, whose pH was adjusted to 3.0 was used to study the hydrodynamic and mass transfer characteristics. Cloud point temperature of the batch system that gave maximum extraction efficiency was 36ºC and hence continuous phase and dispersed phase were heated to about 70ºC in separate jacketed vessels attached with a circulating water bath before fed to RDC.

During continuous micellar extraction of PHA, micelles (dispersed phase) are fed through the middle dispersed phase inlet as shown in figure 3.4. Micelles that enter the RDC are diluted and dispersed randomly by the diluted fermentation broth (continuous phase) entering from the continuous phase inlet at the bottom of RDC. As an effect of dilution of micelles, surfactant monomers and micelles are dragged by the upward flow of continuous phase towards the continuous phase outlet at the top of the column. However, maintaining temperature higher than the cloud point temperature results in displacement of water droplets around the micelle and causes micelle-micelle interaction.

Inter-micellar interactions result in the coalescence of smaller micelles to form larger micelles that on entering the mixing zone are again disrupted to form secondary micelles by the shear force exerted by rotor and stator rings. This process of micelle formation, breakage and reformation of secondary micelles occurs as a continuous cycle of events, as a result of which interaction of solutes and micelles are enhanced that in turn leads to higher mass transfer efficiencies. Micelle disruption and formation of secondary micelles are highly influenced by the operating variables such as rotor speed and phase flowrates. Hence, speed of rotation (50, 100 and 150 RPM), dispersed phase flowrate (35, 40 and 55 ml/min) and continuous phase flowrate (15, 30 and 45 ml/min) were varied to study their effect on dispersed phase holdup, mass transfer coefficient, % purity of PHA and % yield of PHA.

3.7.6.1 Effect of rotor speed

Rotor speed is an important operating condition that controls the dispersed phase droplet breakage and droplet coalescence which are the key factors that affect the dispersed phase holdup and mass transfer characteristics during continuous LLE. The effect of rotor speed on dispersed phase holdup was studied at varying continuous and dispersed phase flowrates. Results obtained are shown in figure 3.15, it can be observed that irrespective of the phase flowrates, holdup was found to increase with increasing rotor speed. At a given rotor speed, holdup values were observed to increase with the dispersed phase flowrate which further increased with increasing continuous phase flowrate. Dispersed phase within the continuous phase in each compartment is scattered by the shearing action of centrally placed rotor discs. This causes turbulence in the liquid which is proportional to the disc rotation speed. With the increase in rotor speed, larger micelles are broken down to fine droplets, which spend more time within the mixing zones, thus increasing the dispersed phase holdup.

As shown in figure 3.16, PHA mass transfer coefficient was found to increase with increasing rotor speed, which indicates that the higher turbulence created by the movement of rotor disc in the mixing zone facilitated the mass transfer of PHA from continuous phase to the dispersed phase droplets. The increasing rotor speed resulted in more number of smaller sized micelles in the column owing to the breakdown of larger micelles that comes into contact with the moving disc. This decreases the terminal velocity of the smaller droplets, while it increasing its retention time within the column and increases the interfacial area for mass transfer of PHA from bulk continuous phase to the micellar droplets (Zhang et al. 1981, Tong and Furusaki 1995). The repeated coalescence and re-dispersion of drops in the column enhances the rate of mass transfer through the surface renewal model. Similar results have been reported by Trakultamupatam et al. (2005a) during the continuous cloud point extraction of toluene using OP (EO) 10 nonionic surfactant system

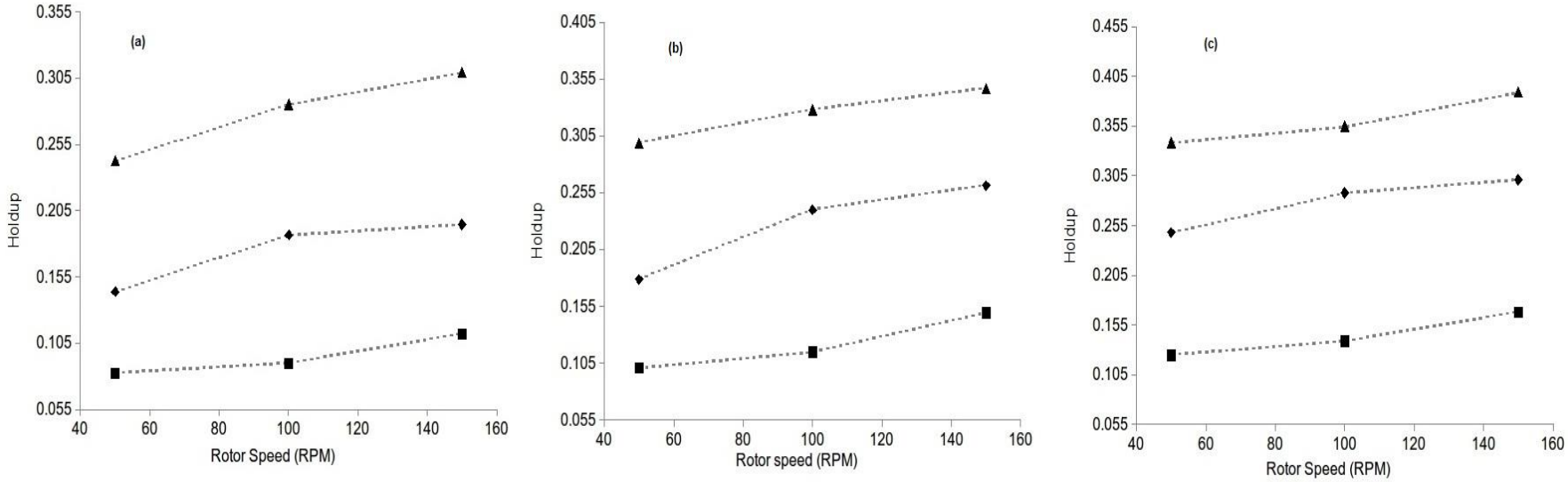


Figure 3.15 Effect of rotor speed on holdup, on maintaining continuous phase flowrate (a) 15 mL/min, (b) 30 mL/min and (c) 45 mL/min, with variations in dispersed phase flowrate ■ - 35 mL/min, ◆ - 45 mL/min and ▲ - 55 mL/min.

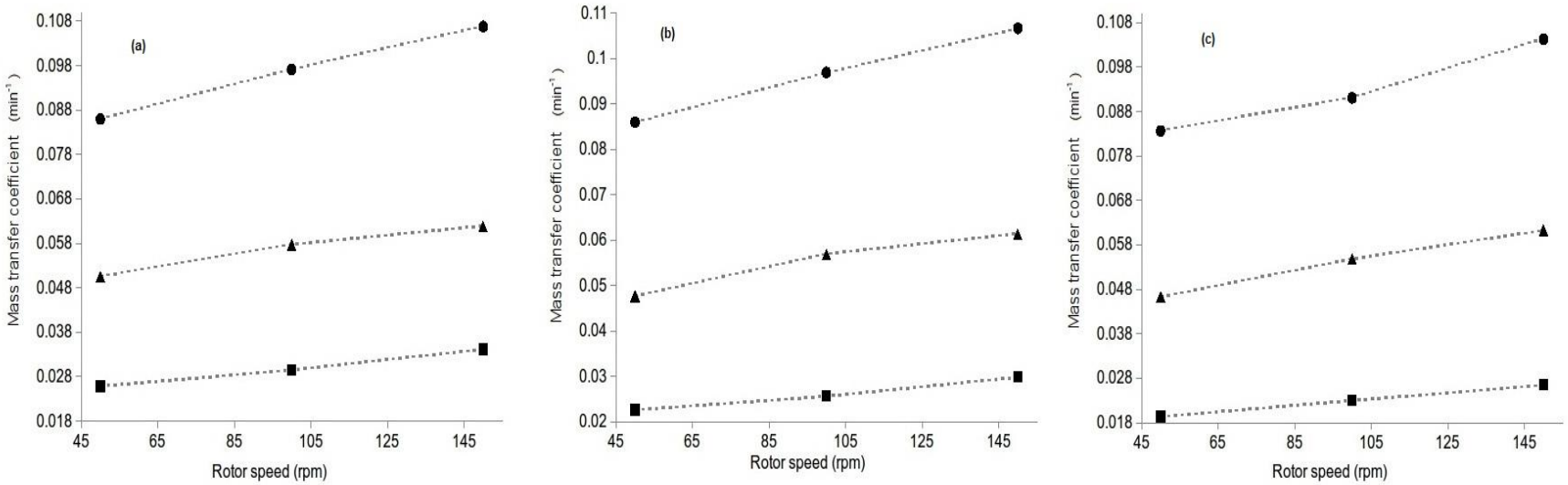


Figure 3.16 Effect of rotor speed on mass transfer coefficient, on maintaining continuous phase flowrate (a) 15 mL/min, (b) 30 mL/min and (c) 45 mL/min, with variations in dispersed phase flowrate ■ - 35 mL/min, ▼ - 45 mL/min and ● - 55 mL/min.

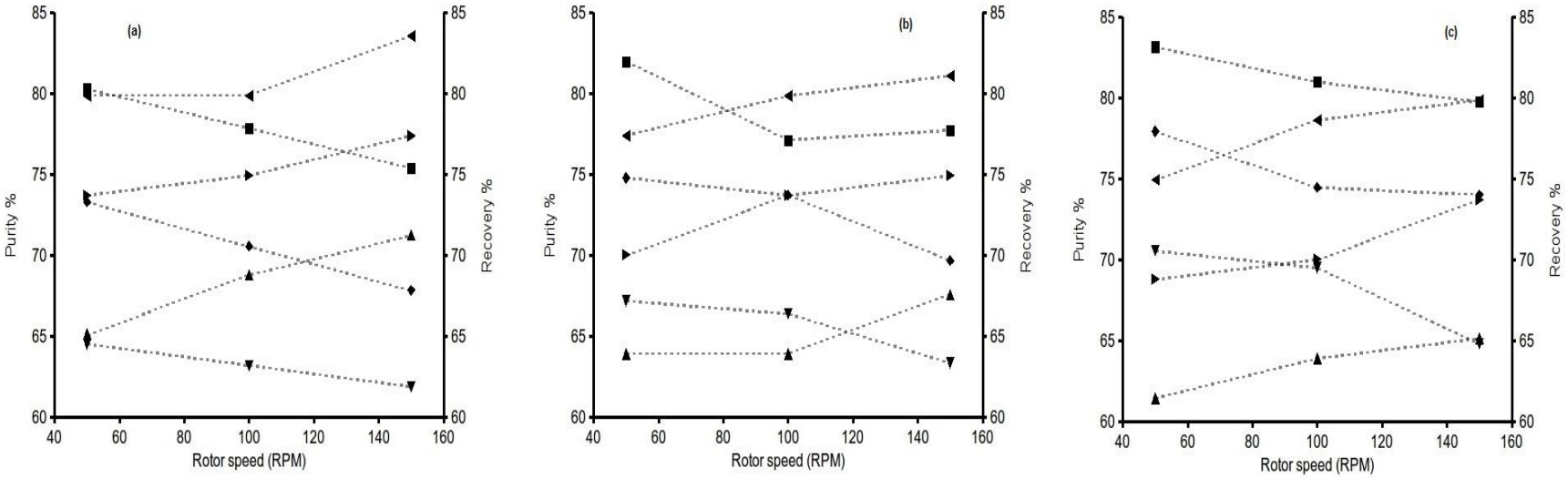


Figure 3.17 Effect of rotor speed on extraction efficiency, on maintaining continuous phase flowrate (a) 15 mL/min, (b) 30 mL/min and (c) 45 mL/min, with variations in dispersed phase flowrate Purity %: ▼ - 35 mL/min, ◆ - 45 mL/min and ■ - 55 mL/min; Recovery %: ▲ - 35 mL/min, ▶ - 45 mL/min and ◀ - 55 mL/min.

3.7.6.2 Effect of continuous phase flowrate

As shown in figure 3.18, it can be seen that with increasing continuous phase flowrate, holdup was found to increase due to the exertion of drag force by the uprising continuous phase liquid on the counter balancing dispersed phase droplets from settling down. Dispersed phase droplets are enforced with combined forces such as buoyancy, drag and friction of droplets. As continuous phase flowrate is increased, pressure exerted by the excess continuous phase on the micelles within the mixing zone increases which in turn increases the residence time of the micelles (Zhang et al. 1981, Tong and Furusaki 1995). At the steady state operation, the number of smaller micelles present in the system interacts with each other and leads to its coalesce and settle down over bottom micellar phase thus increasing the dispersed phase holdup.

At constant dispersed phase velocity and rotor speed, mass transfer coefficient of PHA was found to decrease with increase in continuous phase flowrate as shown in figure 3.19. As discussed earlier, the observed decrease in mass transfer efficiency could be as a result of decrease in mean residence time of the continuous phase within the column with increasing continuous phase flowrate. In addition excess biomass with respect to micelles causes limited interaction of micelle and PHA while other hydrophobic cellular impurities also interact with the micelles. This competitive binding leads to reduction in PHA mass transfer from continuous to dispersed phase

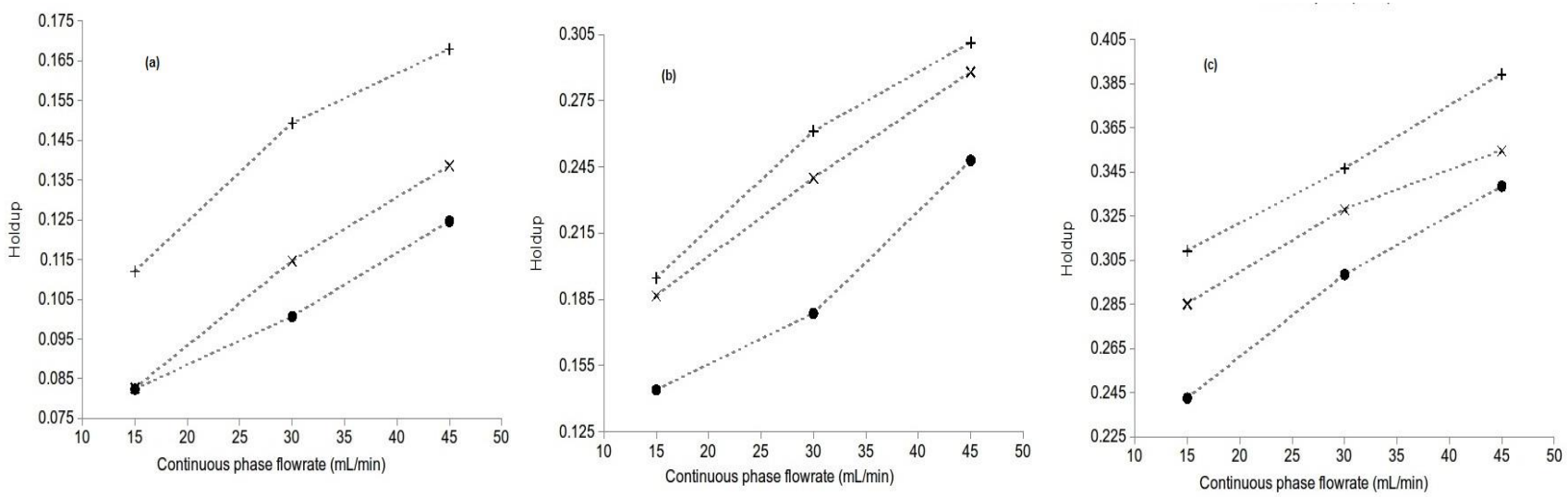


Figure 3.18 Effect of continuous phase flowrate on holdup on maintaining dispersed phase flowrate (a) 15 mL/min, (b) 30 mL/min and (c) 45 mL/min, with variations in rotor speed ● - 50 RPM, × - 100 RPM and + - 150 RPM.

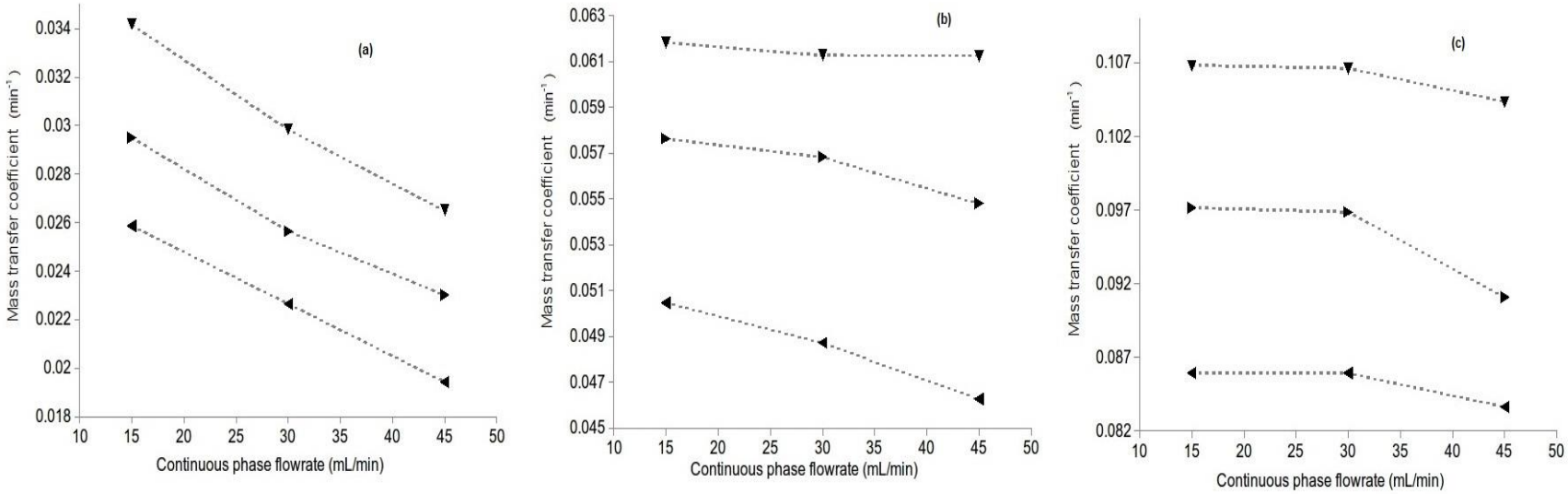


Figure 3.19 Effect of continuous phase flowrate on mass transfer coefficient on maintaining dispersed phase flowrate (a) 15 mL/min, (b) 30 mL/min and (c) 45 mL/min, with variations in rotor speed ◀ - 50 RPM, ▶ - 100 RPM and ▼ - 150 RPM.

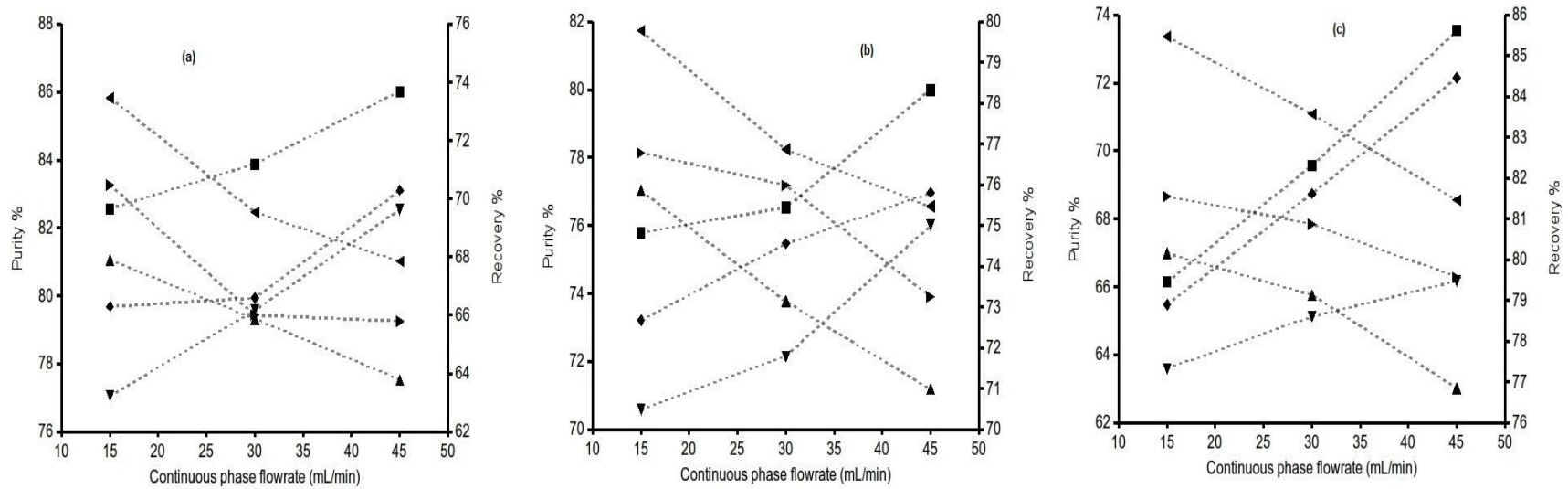


Figure 3.20 Effect of continuous phase flowrate on extraction efficiency of PHA dispersed phase flowrate (a) 15 mL/min, (b) 30 mL/min and (c) 45 mL/min, with variations in rotor speed, Purity % : ▲ - 50 RPM, ▶ - 100 RPM and ◀ - 150 RPM; Recovery % : ▼ - 50 RPM, ◆ - 100 RPM and ■ - 150 RPM.

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3.7.6.3 Effect of dispersed phase flowrate

As flowrates of dispersed phase were increased, dispersed phase holdup was found to incline increase as shown in figure 3.21. The effect is as a result of increasing number of micelles within the column with increasing dispersed phase flowrate. With The increased in micelle concentration, kinetic and buoyancy forces acting on the micellar droplets increase and eventually results in the formation of large number of smaller micelles. Downward velocity of such smaller micelles is very less and is retained within the mixing zone by the uprising continuous phase which increases the residence time of the micelles. Thus increasing residence time results in increase in dispersed phase holdup. Increase in dispersed phase holdup with dispersed phase flow indicates increase in interfacial area available for mass transfer (Zhang et al. 1981, Tong and Furusaki 1995). Thus, PHA mass transfer coefficient was found to increase with the dispersed phase flowrate.

When the number of micelle within the RDC increases with respect to a particular continuous phase flowrate, it leads to enhanced interaction of the hydrophobic cellular impurities with the micelle; this causes competitive binding of PHA with micelle and reduction in mass transfer coefficient.

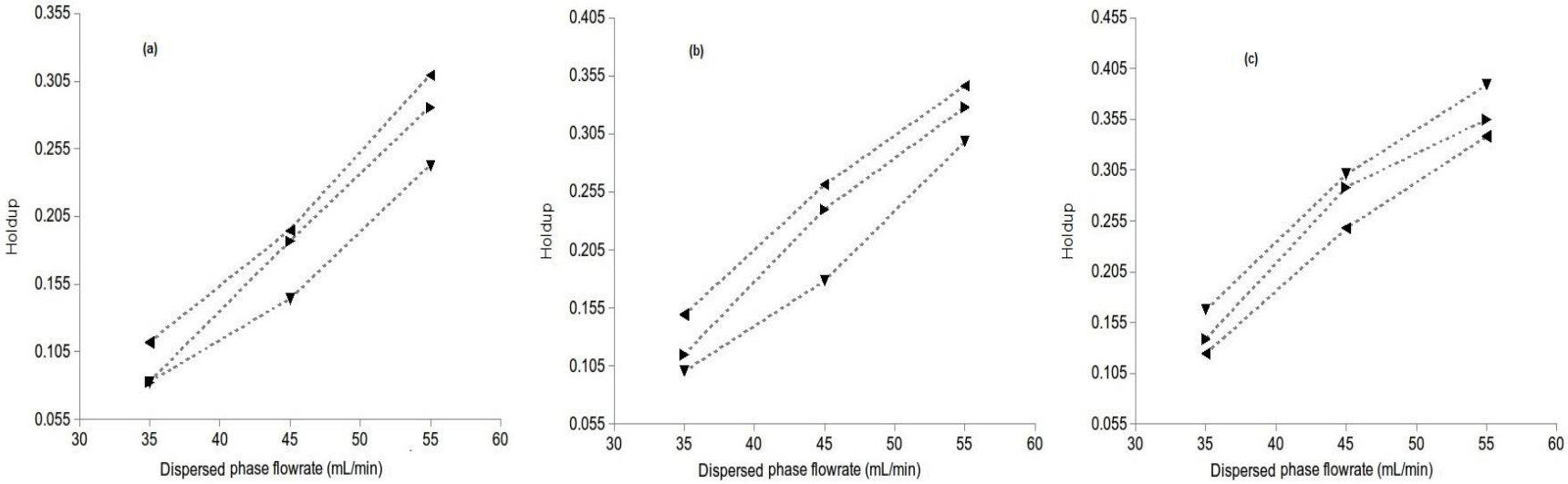

Figure 3.21 Effect of dispersed phase flowrate on holdup of PHA on maintaining rotor speed (a) 50 RPM, (b) 100 RPM and (c) 150 RPM, with variations in continuous phase flowrate ◀ - 15 mL/min, ▶ - 30 mL/min and ▼- 45 mL/min.

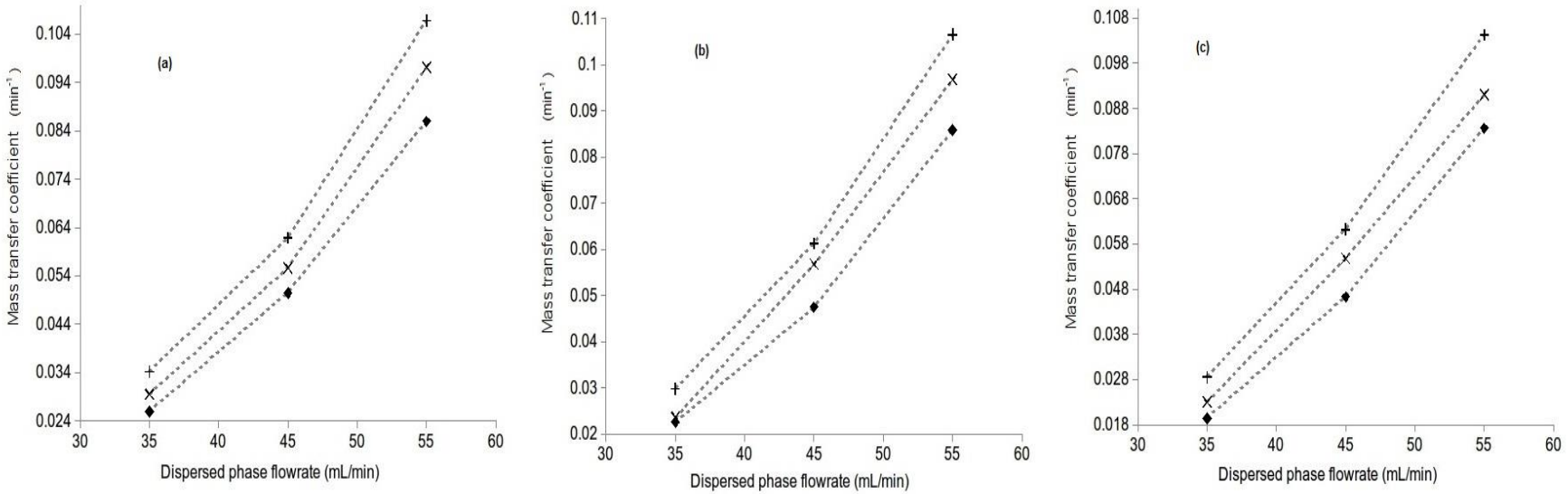

Figure 3.22 Effect of dispersed phase flowrate on mass transfer coefficient on maintaining rotor speed (a) 50 RPM, (b) 100 RPM and (c) 150 RPM, with variations in continuous phase flowrate ◆ - 15 mL/min, ×- 30 mL/min and + - 45 mL/min.

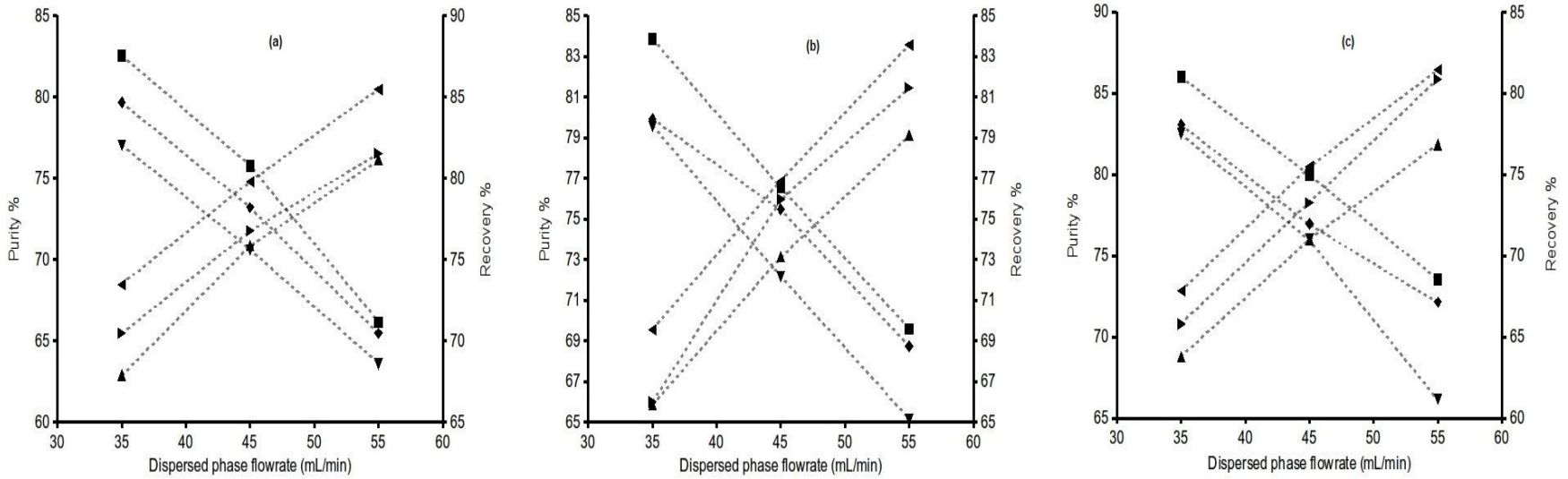

Figure 3.23 Effect of dispersed phase flowrate on extraction efficiency of PHA dispersed phase flowrate (a) 15 mL/min, (b) 30 mL/min and (c) 45 mL/min, with variations in rotor speed, Purity % : ▼ - 50 RPM, ◆ - 100 RPM and ■ - 150 RPM; Recovery % : ▲ - 50 RPM, ▶ - 100 RPM and ◀ - 150 RPM.

3.7.6.4 Effect of operating conditions on % purity and yield

As shown in figure 3.17, it was observed with increasing rotor speed for the dispersed phase and continuous phase flowrates studied, recovery of PHA was found to increase while PHA purity % was found to decrease drastically. As rotor speed increases, micelles entering the column are broken down into smaller secondary micelles. During breaking and reformation of secondary micelles, surfactant tails interact with PHA, thereby enhancing the PHA recovery %. However, exposure of hydrophobic tails will also enhance the interaction of micelles with other hydrophobic cellular impurities. As a result of which PHA purity was found to decrease. As continuous phase flowrate was increased, the number of micelles available to disrupt cell membrane and to interact with the hydrophobic solutes is very limited. As a result surfactant micelles interact with strongly hydrophobic solutes such as PHA and encapsulate them, there by leading to an increase in PHA purity while PHA recovery % remained to be low (figure 3.20). However, with increasing dispersed phase flowrate for a given continuous phase flowrate and rotor speed, purity % was found to decrease while PHA recovery increased (figure 3.23). As dispersed phase flowrate is increased, the number of micelles entering the column increases. Large volume of micelles present inside the column can readily interact with the microbial cells and disrupt them, thus micelles can encapsulate PHA and other hydrophobic solutes present within the column.

It was also found that the overall purity obtained by performing continuous cloud point extraction of PHA was about 89 %, while purity of PHA obtained from batch process was 92.49 %. The decrease in PHA purity on scale up from batch to continuous operation is attributed to the fact that during continuous operation, micelles are repeatedly disrupted and interact with the continuously flowing diluted fermentation broth. While the level of impurity is fixed during the batch operation, impurity level keeps changing during continuous operation and as a result of which large number of impurities are entrapped by surfactant micelles which in turn declines the overall purity obtained.

3.8 Summary of the research work

Considering the biodegradability and ease in handling of micellar solution, PHA was successfully extracted and purified from biomass in its native form. By optimizing the process variables, currently the developed nonionic surfactant based cloud point extraction of PHA resulted in purity of 92.49 %. It was found that the presence of individual surfactants resulted in higher purity compared to those in the presence of mixed surfactant systems. Presence of TX114 in the surfactant mixture, lead to achieve the cloud point temperatures below 40ºC, which are easy to maintain without much variations and ease in scale up. Addition of high molecular weight PEG was found to impart hydrophobicity, as a result and hence the cloud point temperature found to drastically reduce in the presence of PEG 8000 compared to the addition of PEG 4000 to the mixed surfactant system. Similarly, PHA purity was found to be high in the presence of PEG 8000 compared to that of lower molecular weight PEG molecules. Addition of ammonium chloride to the mixed surfactant system decreased the cloud point temperature while it increased the PHA purity and recovery compared to other salts studied. RP-HPLC analysis confirmed that the polymer's nativity was retained to a larger extent, as nonionic surfactant based micelles are mild in extracting biomolecules. Continuous cloud point extraction of PHA in modified RDC, maximum purity % of 86.01 and recovery % of 85.48 was obtained. Decrease in purity % compared to the batch studies is a result of influence of the operational parameters such as flowrate and rotor speed. Mass transfer coefficient and holdup were also found to be influenced by operational parameters. It is inferred from previous literature, that maintaining temperature beyond cloud point temperature of the surfactant system results in increase in micellar size (Komaromy-Hiller et al. 1996, Glatter et al. 2000). Hence, the mass transfer of solute from continuous to dispersed phase found to increase, it can be seen that by maintaining operating temperature above the cloud point temperature (> 36ºC), resulted in increase in PHA mass transfer coefficient as different operational variables were studied. The results obtained are in accordance with the results obtained for the continuous cloud point extraction of toluene using surfactant micelles by Trakultamupatam et al. (2005b).

CHAPTER 4

ULTRASONICATION ASSISTED CLOUD-POINT EXTRACTION OF PHA

4.1 Basics of cavitation

Cloud point extraction, though has attained major importance among researchers and separation scientist towards extraction and purification of hydrophobic solutes by influencing and increasing the specificity offered by surfactant micelles, maintaining cloud point temperature during large scale operation is cost consuming and difficult. For surfactants or surfactant mixtures, even when the cloud point temperature is of operational range, maintaining isothermal condition during continuous operation is tedious and is influenced by the environmental conditions that in turn affect the extraction efficiency. Cloud point of a surfactant system can be induced by exerting physical force by changing pH or introducing sonic waves, mixing, magnetic properties, microwave etc. Among the parameters listed, sonication is widely used in biotechnology for cell disruption, especially when the desired solute of interest is intracellular in nature. Ultrasonication has been employed as a major extraction technique to extract solutes from various sources such as plant materials, mammalian and microbial cells. Since, ultrasonication ensures maximum cell breakage and leakage of cellular material; it is often employed as a primary downstream processing technique, before performing major separation technique such as extraction or adsorption.

Sound waves when introduced into a physical medium travel across the medium compressing and stretching the molecular space of the medium. The average distances between the molecules of the medium vary as they oscillate from their mean position. When high negative pressure is exerted by the sound waves, the distance between the molecules exceed the distance required to maintain the physical state of the medium and as a result voids are created across the physical medium which are generally known as cavitation bubbles. Cavitation bubbles undergo continuous compression and rarefaction and with the repetitive cycle they implode releasing high energy within the medium. When Low frequency ultrasonic waves are introduced,

equal sized bubbles as a result of definite interval between compression and rarefaction that results in continuous smooth growth of bubbles resulting in stable cavitation. High frequency sonic waves leads to formation of large sized bubbles as a result of uneven and frequent formation and growth of bubbles and subsequently, the bubble implosion may release high pressure and temperature. Further it leads to liquid agitation and solute degradation, while lower intensity of the waves increase the amplitude of vibration and hence enhance cavitation. Cavitation is achieved at lower temperatures compared to high temperatures, as formation of solvent vapour and it's filling up within the void bubbles is prevented at lower temperature and so the expected effect of cavitation can be well achieved. Stable cavitation as a result of even and equal sized bubbles are often employed in the bio industry, while transient cavitation leads to denaturation of bio molecules owing to release of high temperatures and pressure in the range of 5000ºC and 2000 atmospheric pressures and are often employed for high viscous liquid medium.

Bubble size distribution and free energy generated during cavitation has been a major research of study in the field of sonication. Macrobubbles, microbubbles and nanobubbles are created during cavitation; bubble size > 50µm are known as macrobubbles, 200 nm- 50µm are microbubbles and those less than 200nm constitute nanobubbles. As a result of larger size and the amount of gas present, macrobubbles overcome the buoyant force and so escape to the surface and burst, microbubbles undergo constant size shrinkage and collide while nanobubbles have been observed to even stay for months that are stabilized by hydrogen bonds. Cavitation is influenced by the physiochemical parameters related to the sonication process like, nature and composition of the medium, medium pH, presence of additives such as salts, frequency, sonication time, type of sonicator used etc.

4.1.1 Applications of ultrasonication in (Bio) chemical industries

Ultrasonication was first used by Harrison and Pandit for cell disruption by cavitation generated through a throttling valve. Earlier reports on cavitation on release of intracellular proteins suggest that low pressure homogenization are not effective unlike high pressure homogenizers, since cavitation is experienced apart from

mechanical shear stress exerted over the cells. Save et al. published that even though cavitation releases high temperature and pressure and generation of free radicals, activity of the enzymes released from the cells were retained, as a result of exposure of intense conditions for a shorter time which cannot affect the enzymes. On adjusting the process parameters like the type of sonicator used and other process variables, the cell disruption can be complete or it can lead to cell leakage. Ultrasound has been successfully employed towards the emulsification of two immiscible liquids require mechanical mixing. Introduction of sonic waves enhances the dispersion of one liquid into the other immiscible liquid by causing a shift in the liquid interfaces. Ultrasonication enriches the formation of emulsion even in the absence of surfactants and the process is easy to operate and scale –up at of low cost. Ultrasonication is applied in various fields ranging from petrochemical, mining and metallurgical fields towards extraction of suspended solids from the feed. Apart, ultrasonication is predominantly used in the field of food, pharmaceutical and cosmetic industries towards varied applications, specifically targeting the stabilization of emulsions to increase the shelf life of the product. In biotechnology/ biochemical Engineering, sonicating waves are used for cell disruption, especially focusing on selective release of intracellular proteins, sludge treatment, enhancing transesterification reactions for the production of biofuels, enzyme extraction, enzyme catalysed waste treatment process, Reduction of moisture in fruit extracts, crystallization, biosensors, etc.,.

4.1.2 Ultrasonication assisted cloud point extraction

Wood and Loomis were the first to perform ultrasonication based emulsification in the year 1927, however the process was patented in the year 1944. Ultrasonication emulsification is said to be a two stage process; introduction of sonic waves leads to dispersion of oil phase into water as droplets, while in the second stage droplets formed are broken down to secondary droplets of nanosize as a result of turbulence and shear force caused by cavitation. Introduction of surfactant deform the droplets and form the nanosized emulsions. The amount and type of surfactant and its HLB value decides the formation of nanosized emulsions. Formation of micelles is enhanced by surfactants whose HLB value varies between 8 and 16. Low frequency

sonic waves lead to the formation of even and nano sized droplets varying from 100 – 400 nm droplet size. However, the droplet size greatly depends upon the ultrasonication time, surfactant used, amount of hydrophobic solute and volume ratio of oil (when creating oil in water droplets). It has been found that the transparency and stability of emulsion droplets formed via ultrasonication were high compared to other emulsification methods. Presence of sonic waves in a surfactant systems cause a structural rearrangement of surfactants within a micelle. As the micelles were reshaped, their corresponding extraction efficiency also varies. Repetition with the introduction of sonic waves, releases high temperature and pressure. The abundant energy release during implode of microbubbles entrapped between the micelles leads to the structural transformation of micelles. Addition of surfactant lowers the surface tension of the solution, there by cavitation is enhanced at low frequencies.

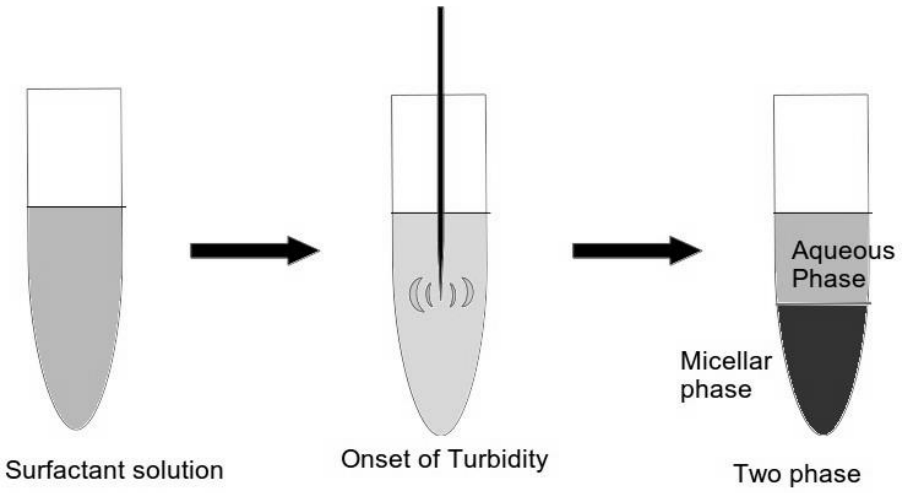

Figure 4.1 Schematic representation of UACPE.

Mixed surfactant offers higher stability to bubbles as a result of screening of intra-micellar repulsions caused by similar sized head groups. Repulsive forces between micelles enhance the formation of bulk micellar phase, while presence of surfactants in bubble prevents bubble coalescence. At lower surfactant concentrations, the charge of surfactant head group had no effective influence on the bubble growth, however at higher surfactant concentration the surfactant type and charge influences on the bubble growth according to Li et al. (2010). Increased chain length of

surfactant offers lower mass transfer resistance thereby enhancing diffusion of gas into the bubbles. Increased gas flux across the surfactant layer during rarefaction, leads to growth in bubble size as a result the imploding effect has a larger effect on the water molecules present between the micelles. With continuous sonic cycle and implosion, inter-micellar network is formed as the water molecules are replaced and there by exposing the micelles to form the single bulk micellar phase.

4.2 Literature review

The ultrasonication assisted liquid-liquid extraction of bio-molecules from the complex sources are well established, however it was less studied for the surfactant assisted extraction processes. Literatures discussed in the following are comprehended as table 4.1. The following section briefly discuss the effect of various operational parameters such as system pH, presence of additives, sonication time, incubation time and temperature on the extraction efficiency.

Table 4.1 Literature survey on solutes extracted using UACPE.

Source and solute	Surfactant	Reference
Calcium, Arsenic, Iron from tea leaf samples	TX100, CTAB, DDAB	Borkowska-Burnecka et al. 2004
Polyaromatic hydrocarbons (pyrene, anthracene, penanthrene)	TMN6	Yao and Yang 2007
Polybrominated diphenyl ethers (PBE) from water and soil sample	TX 100, TX 114 and PONPE-7.5	Fontana et al. 2009
Estrogens in urine	TMN6	Zou et al. 2012
Benzimidazole anthelmintics from water and milk samples	TX114	Santaladchaiyakit and Srijaranai 2012
Adrenaline from different	TMN 6	Qin et al. 2012.

milk samples		
Chromium (III) and (VI) from environmental water samples	CTAB	Hashemi and Daryanavard 2012
Selenium from water	TX114	Wen et al. 2014
Sulfite in beverages	TX114	Altunay et al. 2015
Copper from water	TX114	Yang et al. 2015

Hashemi and Daryanavard (2012) studied UACPE of chromium (III) and (VI) from environmental water samples using CTAB. The recovery of chromium was found to decrease with increasing pH as acidic condition enhances the oxidation of chromium and its interaction with surfactant micelles. The lower sonication time of 2 minutes lead to better interaction of chromium with surfactant micelles. The recovery of chromium was found to increase until 50mM CTAB concentration and decreases with further increase in CTAB concentration. At low iodide concentration, reduction of chromium is less and as a result complexation between CTAB micelles and ions are low, while at high iodide concentrations competitive binding of ions takes place that reduces the overall efficiency of the process. Efficiency was found to increase with increasing iodide concentration until a concentration of 0.8mM, with further increase efficiency was found to decrease. At the optimized condition the authors reported a recovery of 99 - 100 %.

Sulfite present in beverages was extracted using TX114 by Altunay et al. (2015). The authors reported a maximum recovery at system pH of 10. At pH below 10, formation of imine bisulfite complex that interacts with TX114 micelles are less; while at pH above 10, deprotonation of complex occurs and so the extraction efficiency was less. Nile Blue A, a redox sensitive ion-pairing agent that interacts with imine bisulfite was added to the system and it was found that with increasing Nile Blue A concentration, the efficiency was found to increase until 0.6 mL volume of Nile Blue A. Further the effect of additives Pyrogallol and Bromide ions were

studied and the obtained results were reported. The efficiency was found to increase with increasing surfactant concentration in the feed and reached a maximum at TX114 volume of 0.2 mL. The temperature was varied before subjecting the feed to UACPE and the solution became turbid at incubation temperature above 45°C as cloud point temperature of TX114 is less than the temperature range studied. However, change in temperature had no significant change in efficiency. Ultrasonication time of 20 minutes gave maximum recovery of 97 – 103 % of imine bisulfite complex (Altunay et al. 2015).

Borkowska-Burnecka et al. (2004) studied the sonication assisted extraction of calcium, Arsenic, Iron from tea leaf samples using TX100, CTAB, and DDAB. Addition of TX100 resulted in increased leaching efficiency of Barium, Manganese, Magnesium, Nickel and Phosphor from black tea leaves, while recovery of barium and nickel were more than 100 % and recovery of phosphor was ~ 100 %. DDAB lead to decrease in recovery while CTAB had no significant effect on recovery of heavy metals from tea leaves. Presence of CTAB and DDAB improvised the leaching efficiency of lead, manganese, magnesium and phosphor from tobacco leaves and the leaching efficiencies were similar for both surfactants. Addition of TX100 remained insignificant with respect to recovery of heavy metals and maximum recovery of above 100 % has been reported.

Yang et al. (2015) studied sonication assisted CPE of copper from water by employing TX114. The authors found that as ultrasonication time was increased (5 - 30 minutes) the recovery of copper was found to increase and reach maximum at 20 minute. As the equilibration temperature was raised, recovery has been reported to increase and reach its maximum at 55ºC. Effect of chelating agent, Diethyldithiocarbamate (DDTC) (0.01-0.1 m/v %) has been studied and the authors found that addition of 0.03 % (m/v) of DDTC lead to increase in recovery while with further increase in chelating agent concentration, co-extraction of chelating agent by micelles were observed that lead to decrease in recovery of copper. Maximum recovery of 94 – 109 % has been recorded as the system pH was further varied from 6 to 11.

Polybrominated diphenyl ethers (PBE) were extracted from water and soil sample using TX 100, TX 114 and PONPE-7.5 (Fontana et al. 2009). No significant change was observed in extraction efficiency with change in pH (2–12), while presence of citrate buffer was found to enhance the structural change of surfactant micelles and its interaction with PBE. The extraction efficiency was found to increase and reach a maximum at 4mM buffer concentration, when study the effect of different buffers and their varying buffer concentrations (citrate (pH 5.6), acetate (pH 4.0), phosphate (pH 7.0) and tetra borate (pH 9.22)). TX1000 and TX14 were found to enhance extraction of PBE while effect of PONPE-7.5 on recovery was insignificant. 0.4 g/L of TX114 lead to maximum recovery of PBE. Addition of salt, NaCl (0-3.4 M), lead to increase the micelle size and interaction with PBE via, hydrophobic interaction. 0.4 M NaCl resulted in maximum recovery of PBE. Increasing incubation temperature has been reported to lead to lower solubility of micelles and their interaction with PBE, while maximum recovery has been achieved at 70ºC. The authors noted that the increasing sonication time has significant improvement in recovery until 4 minutes and further increase in sonication time is said to have no significant effect on recovery. Maximum recovery of 97-108 % has been achieved by Fontana et al. (2009).

Wen et al. (2014) studied the extraction of selenium from water using TX114. The authors found a maximum recovery of heavy metal at 20 minutes when they study the effect of ultrasonication time (2-30 minutes).TX114 concentration higher than 0.06 % (v/v) lead to decrease in extraction efficiency, at higher concentrations the chelating agent was co-extracted into micelle rich phase, while maximum recovery of selenium occurred at dithizone concentration of 12.5 µM at the pH of 1. Maximum selenium recovery of 92 – 107 % has been obtained by the authors at optimized extraction condition.

Yao and Yang (2007) studied ultrasonication assisted cloud point extraction of Polyaromatic hydrocarbons (pyrene, anthracene, penanthrene) (PAH) using TMN6. During continuous extraction process, distribution of PAH in micelles were found to increase till 1 hour and then remained constant with increasing time. Maximum

recovery of PAH were obtained at an ultrasonication power of 300 W and maintaining low power less than 300 W had no significant effect on recovery. The authors found that there was no much significant change in recovery on varying the system volume from 100 to 500 mL and temperature. Low flow rates enhanced the effect of ultrasonication on CPE of PAH, however increasing PAH concentration resulted in lower extraction efficiency as a result of competitive binding of excess PAH with surfactant micelles.

TMN6 was used to extract Estrogens in urine sample via UACPE by Zou et al. (2012). The authors found that estrogens recovery was found to increase with increasing Tergitol TMN-6 concentration from 0.2 to 0.5 % v/v and extraction efficiency decreased steeply with further increase in TMN6 concentration. Extraction efficiency reached a maximum at 60 minutes. The recovery was enhanced by maintaining the incubation temperature at 45ºC, while low temperatures had no significant effect on recovery. Addition of NaCl, enhanced phase separation by displacing free water present between the micelles and resulting in coalescence of micelles. 2.0% (w/v) gave maximum extraction and with further increase in NaCl concentration, recovery of estrogens was found to decrease. The recovery increased and reached maximum at neutral pH and increase until 45 minutes of sonication time. Optimized conditions lead to maximum Estrogens recovery of 85 – 104 %.

TX114 was used to study the extraction of benzimidazole anthelmintics from water and milk samples by Santaladchaiyakit and Srijaranai (2012). The authors found that the addition of various salts containing sodium increases the extraction of benzimidazole in the order of CH_3COONa > Na_2SO_4 > Na_2CO_3 > NaCl. Benzimidazole remains neutral in the studied pH range (6-12), and so it has been concluded that presence of salt influences the extraction process and not the system pH. The salt CH_3COONa at 7 % (w/v) gave maximum recovery. Extraction was found to increase with increasing surfactant concentration until 0.75 % (w/v) and with incubation at room temperature. Maximum recovery of the solute was found to be around 68 – 112 %, being extracted from different water and milk samples.

Adrenaline was extracted from different milk samples using TMN 6 by Qin et al. (2012). On studying different process variables and their effect on ultrasonication assisted cloud point extraction efficiency, the authors found that the increasing TMN 6 concentration (0.5–6 % (v/v)) resulted in recovery increased recovery till 3 % and then the recovery was found to decline. Addition of sodium chloride at 3 % enhanced the recovery of adrenaline compared to sodium sulphate. The recovery was found to decline with increasing temperature (35 to 65ºC) as a result of decline in complex formation with surfactant micelles. Increasing equilibration time (20 to 50 minutes) enhanced adrenalines recovery by micelles, however after 40 minutes extraction of adrenaline was found to decrease. Maximum adrenaline recovery of 95 – 98 % was achieved by the authors.

4.3 Aim and scope of the work

Although, nonionic surfactants have been actively employed towards separation of solutes from feed mixture, maintenance and handling of cloud point systems formed by ionic surfactants are rather difficult. Most of the ionic surfactants have cloud point temperature greater than 100ºC, to maintain such higher temperatures and to study the effect of process variables to attain maximum purity and recovery of PHA is a difficult task. Considering the advantages of external force induced cloud point formation apart from heating, this chapter aims to study the effect of low frequency sonic waves to induce the formation of cloud point systems. This chapter aims to study the effect of sonic waves on cloud point systems formed by ionic surfactants and nonionic surfactants and their encapsulation capacity of PHA. Different process variables including sonication factors such as sonication time, sonication frequency and micelle related parameters such as individual surfactant concentration, mixed surfactants, broth pH, and effect of additives are to be tested for their effective influence on extraction of PHA from the biomass.

4.4 MATERIALS AND METHODS

4.4.1 Materials

Surfactants -Triton X 100 (TX100), Triton X 114 (TX114), Dioctyl sodium sulfosuccinate (AOT), cetyltrimethyl ammonium bromide (CTAB), Polymers-Polyethylene glycol (PEG) 4000, 6000 and 8000, and standard Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) (12 %) were purchased from Sigma Aldrich, India. Sodium sulphate (Na_2SO_4), sodium chloride (NaCl), ammonium sulphate ($(NH_4)_2SO_4$) and ammonium chloride (NH_4Cl) were purchased from CDH, India. HPLC grade acetonitrile and concentrated sulphuric acid (H_2SO_4) (98%) were purchased from Merck, India. Deionised water was used during the protocols and the experiments were conducted at room temperature unless and otherwise stated.

Sonics vibra-cell VCX 130, USA was used for ultrasonication studies, the unit contains a 6 × 113 mm (diameter × height) probe tip made up of autoclavable titanium alloy with a net power output of 130 watts, and frequency of 20 kHz and is maintained within a sound proof box to prevent noise emission and to maintain isothermal condition. LABINDIA analytical UV 3000 + UV/Vis spectrophotometer, India was used for the UV spectral analysis and Shimadzu HPLC LCMS 2020, Japan was used for chromatographic analysis. Fermentation broth with PHA production of 11.96 g/L as described in chapter 2 was used in the development of extraction protocol.

4.4.2 Extraction Protocol

Ultrasonic probe tip is chiefly employed for cell rupture owing to its increased shear force and lower radical formation. Sonication was performed by inducing sonic waves by altering the input frequency value to the controller while the sample mixture was taken in graduated, preweighed centrifuge tubes. The tubes were placed in a beaker filled with ice to avoid denaturation effect caused as a result of heat released during ultrasonication. Effect of pure TX100 surfactant concentration on low frequency sonic wave assisted CPE of PHA from fermentation broth was studied by varying the concentration between 1 to 10 wt.%. Surfactant solutions and

fermentation broth were added to preweighed centrifuge tubes and the tube was subjected to ultrasonication at an initial frequency of 8 kHz for 3 minutes with a pulse interval of 2 seconds. After sonication, the tubes were observed for formation of two phase and the tubes were let to cool down to room temperature before PHA estimation was performed. Amount of PHA encapsulated by the micelles in the bottom surfactant rich phase was estimated by performing crotonic acid assay as described in 2.5.4, and the purity and recovery of PHA obtained were calculated using the equations 3.1 and 3.2 respectively.

TX100 concentration, which gave maximum purity % was fixed for the further extraction studies; effect of sonication frequency on purity % and recovery % was studied by varying the frequency between 2 to 10 kHz for 3 minutes and with a pulse interval of 2 seconds. TX100 concentration and frequency which gave maximum purity of PHA were fixed to study the effect of sonication time between 2 to 10 minutes. Mixture of surfactants comprising nonionic surfactant-TX100 (concentration as taken above) was added to other surfactants, nonionic – TX114/ anionic – AOT/ cationic – CTAB and the effect of its varying concentrations (1 to 5 wt. %) was studied. Effect of additives were studied by considering different electrolytes from Hofmeister series (sodium chloride, sodium sulphate, ammonium chloride and ammonium sulphate) at varied concentrations of 0.1 to 1 M and Polymer – PEG 4000, 6000 and 8000 (0.1 to 1 wt. %) in the presence of different surfactant mixtures. Overall extraction efficiencies were compared and the system with maximum purity was subjected to chromatographic analysis.

4.4.3 Chromatographic analysis

LCMS analysis was performed for the system with highest purity % of PHA obtained via low frequency sonic wave assisted CPE. 20 µl of the same was injected to a reverse phase column, capcell pak C18 MG II type maintained at 40ºC. Mobile phase comprising acetonitrile: water at a ratio of 70:30 (vol:vol) was passed through the column at a flow rate of 1 mL/min. Chromatographic peaks were obtained at 235 nm and the fractionated sample corresponding to the retention time of PHA was passed on to ESI-MS, as programmed. Nitrogen with a flow rate of 1.5 L/min and 15

L/min was used as nebulizing gas and drying gas respectively. MS unit heat block was maintained at 200ºC, while ion interface temperature was maintained at 350ºC. The raw data obtained was processed using LCMS software and was analysed for chromatogram and mass peaks. Similar run was also performed for standard PHBV dissolved in chloroform. Chromatograms of standard PHBV and PHA obtained via the current process were compared and studied for their retention time.

4.5 Results and Discussion

Crude fermentation broth was utilised to determine the amount of PHA present in the broth by performing modified crotonic acid assay protocol and about 85 % of PHA accumulation was found in the microbial cells. PHA extracted was observed to be amorphous and viscous in nature even on exposure to open environment, viscous nature of the PHA material perpetuated when dissolved in water. Cavitation as a result of introducing sonic waves, leads to increased permeability of membranes and its thinning; parameters such as physical parameters of the microbe, growth status and presence of outer cell membrane (lipopolysaccharide and protein layer in gram negative bacteria) determines the efficiency of sonication. Low frequency ultrasound offers increased sonochemical destruction of living cells and increased extraction of cellular content (Sinisterra 1992, Gogate and Kabadi 2009, Majid et al. 2015). Presence of surfactant in the feed mixture and introduction of sonic waves causes enhanced penetration of surfactant monomers across the lipopolysaccharide layer of gram negative bacteria and its dissolution, on further presence of sonic waves, cell permeability or cell wall disruption is possible and improvised mass transfer of cellular contents into the surrounding medium.

4.5.1 Effect of Individual Surfactant

Initially, sonication assisted CPE was performed at 8 kHz of sonication frequency operated for 3 minutes with 2 seconds pulse interval. Effect of individual surfactant was studied by considering TX100 and its varying concentrations between 1 to 10 wt. %. Figure 4.2 (a, b) represents the purity % and recovery % of PHA obtained respectively. It is inferred from the graph that purity % and recovery % were found to increase with increasing TX100 concentration and at higher concentrations

of TX100 both purity and recovery of PHA declined. Maximum purity % of 84.7 with a PHA recovery % of 26.1 was obtained in the presence of 3 wt. % of TX100.

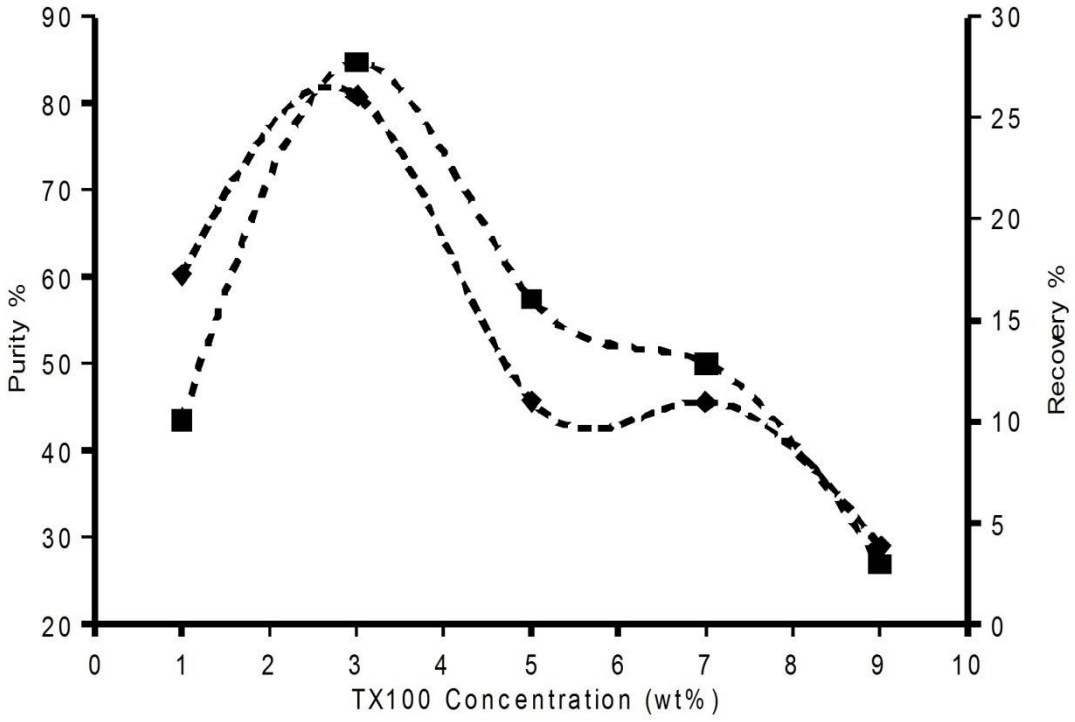

Figure 4.2 Effect of Triton X 100 concentration on PHA purity % - ■ and Recovery % - ◆.

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Earlier reports suggest that with the addition of surfactant, they assemble to form a thin film along the bubble surface creating a no-slip boundary condition that increases micro streaming of the bubble until bubble implosion. Increase in micro streaming capacity, enhances the mass transfer across the bubble surface. During continuous compression and rarefaction of bubbles, surfactant density in a bubble increases during compression which inclines the mass transfer resistance, expansion of bubble leads to decrease in surfactant density and mass transfer resistance (Leong et al. 2011). Higher concentrations of surfactants have been found to increase the growth rate of a bubble resulting in early onset of surface oscillations that creates micro streaming in its vicinity. At low surfactant concentrations, large sized bubbles are formed as not much surfactant is available to cover the whole interfacial area.

Drop size is reduced with increasing surfactant concentration and the number of bubbles originating at maximum surfactant concentration is less than those obtained at lower surfactant concentrations.

During cavitation, surfactant are adsorbed on the bubble surface on the vapour-water interface and diminishes the bubble coalescence rate which results in the formation of uniform sized bubbles stabilized by micelles (Cho et al. 2005, Zhang and Maeda 2006, Leong et al. 2011). Surfactant type (charge and chain length) determines the bubble size; with increasing concentration of surfactants bubble size was found to increase by rectified diffusion and also lead to decrease in surface tension as stated by Leong et al. (2011). Hydrophobic interaction between PHA and the surfactant micelles increases with increasing surfactant concentration, which is strong enough to form micelle-PHA complexes and settle down in the bottom coacervate phase and most of the hydrophilic cellular impurities are present in the top aqueous phase (Hansson and Lindman 1996). However, high surfactant concentrations results in increase in HLB value of the system which enhances micelle-protein interactions that leads to the reduction in purity % and recovery % (Kunieda and Shinoda 1985). Reports on calcium, arsenic and iron extraction from tea leaf samples with TX100 as surfactant suggest that addition of TX100 resulted in increased leaching efficiency of barium, manganese, magnesium, nickel and phosphor from black tea leaves (Borkowska-Burnecka et al. 2004). Similar effect of surfactant concentration was reported for the extraction of estrogen from human urine samples by employing Tergitol 6 in UACPE (Zou et al. 2012) and the extraction of polybrominated diphenyl ethers using TX114 (Fontana et al. 2009).

4.5.2 Effect of sonication frequency

Effect of sonication frequency on the purity % and recovery % was studied in the presence of TX100 at a fixed concentration of 3 wt% and a sonication time of 3 minutes with 2 seconds interval, the results obtained are represented as figure 4.3 (a,b). From figure, it is inferred that as the frequency is increased from 2 to 6 kHz, PHA purity was found to increase and reach a maximum of 88.23 % at 6 kHz. However with further increase in sonication frequency, purity % was found to decline.

Frequencies >6 kHz leads to generation of large number of bubbles in a shorter span around the sonic wave source that are not good enough to form void on collision.

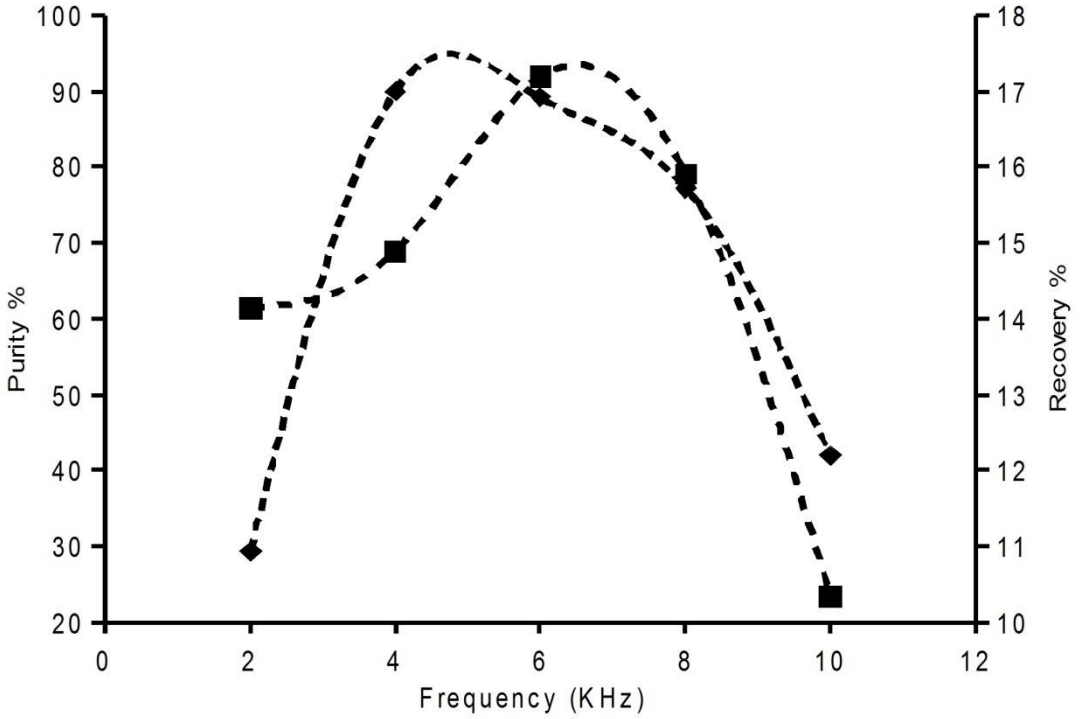


Figure 4.3 Effect of Frequency (kHz) on PHA purity % - ■ and Recovery % - ◆.

Earlier reports suggest that the viscosity of micelle phase increases with increasing frequency. Longer wavelengths are propagated at low frequencies creating large sized microbubbles while smaller microbubbles are created at high frequencies as a result of shorter wavelength. Presence of large sized microbubbles result in higher shear stress compared to that of smaller sized microbubbles created at high frequencies (Yusof and Ashokkumar 2015). Thus low frequencies result in adiabatic implode which can disrupt the microbial cell and aid in the release of cellular components into the surrounding medium. Presence of TX100 in the solution reduces the surface tension and intense the effect of cavitation on the microbial cell surface for ruptures and cell leakage (Cameron et al. 2009). From figure 4.3b, it is to be noted that increased frequency of sonic waves lowered the recovery of PHA from the medium. It was also observed that the volume of coacervate phase decreases at

frequencies > 6 kHz as a result of repulsion of scattered water molecules among the micelles that result in micelle-micelle interaction. These stronger micellar interactions attract cellular impurities thereby declining the PHA purity and recovery.

4.5.3 Effect of sonication time

TX100 concentration of 3 wt% and sonication frequency of 6 kHz was fixed to study the effect of sonication time which was varied between 2 to 10 minutes. From figure 4.4a, it is inferred that the purity % was maximum at shorter sonication time (6 min) and with further increase in sonication time, purity of PHA obtained was found to decrease. Similar results were observed for the effect of sonication time on recovery of PHA from the fermentation broth. Exposure of sonic waves for longer durations ensures increased cell rupture and leakage (Guerrero et al. 2001).

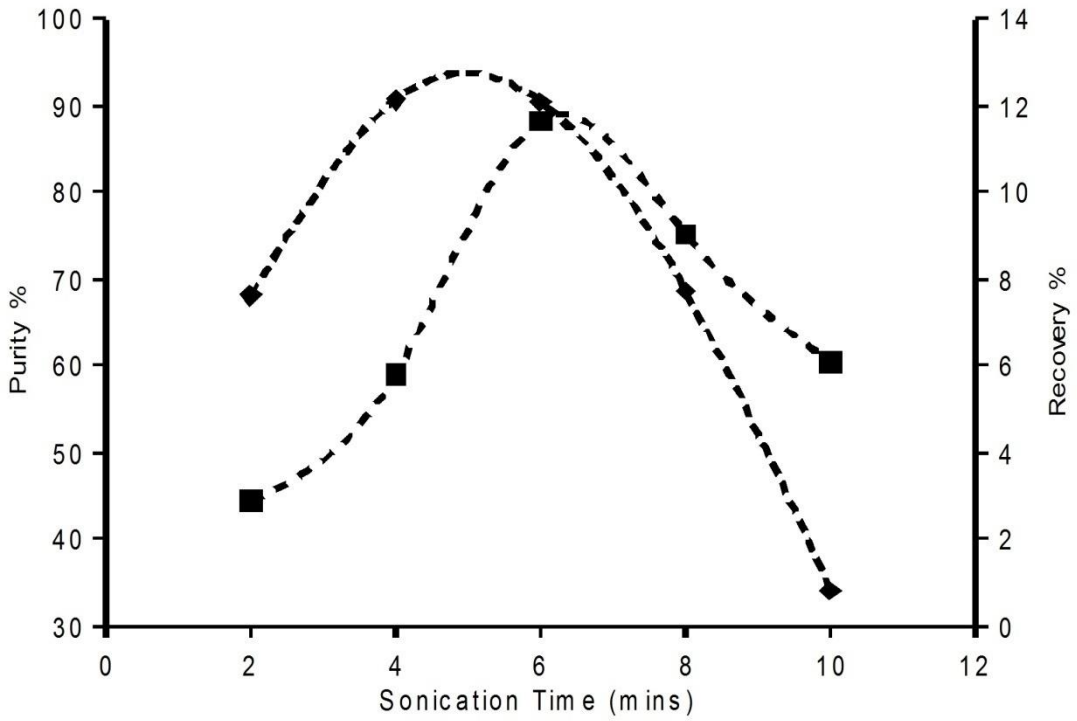


Figure 4.4 Effect of sonication time (minutes) on PHA purity % - ■ and Recovery % - ◆.

However the heat generated results in denaturation of protein and breakage of PHA chain length. These denatured proteins further precipitate out in to the micellar phase which lowers the purity and yield of PHA. Reports on the effect of sonication

time on UACPE of Polybrominated diphenyl ethers from water and soil sample using nonionic surfactants TX100, TX114 and PONPE-7.5 that optimum sonication time of 4 minutes increased the extraction efficiency and with further increase in sonication time the recovery was found to decline (Fontana et al. 2009).

4.5.4 Effect of fermentation broth pH

When the broth pH was altered from acidity to basicity, purity % was found to increase initially and with further inclination towards basic pH, purity and recovery of PHA was found to decline as seen in figure 4.5. Acidic pH causes protonation of amino acids thereby increasing the hydrophilicity of the proteins,that in turn leads to repulsion of the same into the top aqueous phase.

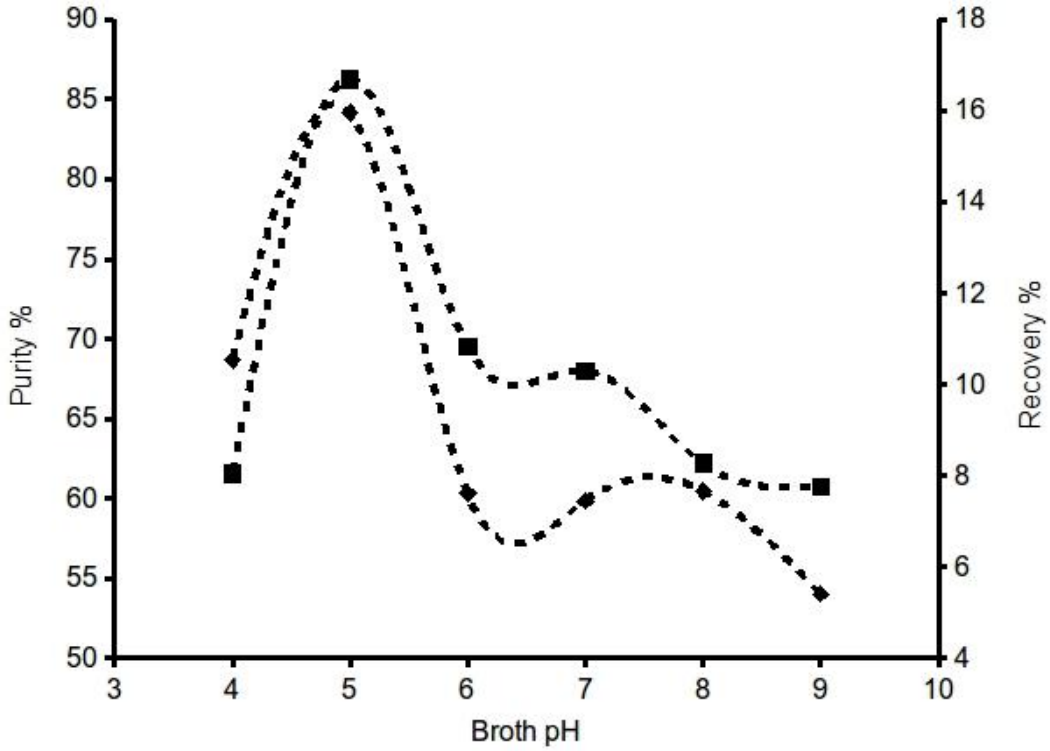


Figure 4.5 Effect of broth pH on PHA purity % - ■ and Recovery % - ◆.

However, basic pH leads to the exposure of hydrophobic domains of the protein and consequently their interaction with the micelles leads to the precipitation of proteins in the micellar phase. Most of the membrane proteins attain net negative charge while few others attain positive charge owing to the change in pH and with

respect to the pI of the proteins. Thus the change in hydrophobicity on maintaining base pH ultimately reduces the purity and recovery of PHA.

4.5.5 Effect of Mixed Surfactants

Effect of mixed surfactants was studied by adding varying wt% of AOT/ CTAB/TX114 with TX100 whose concentration was fixed as 3 wt%. Figure 4.6 (a,b) indicates that purity and recovery % was found to decrease with increasing concentrations of AOT and CTAB, while the purity of PHA was found to increase initially with increasing concentrations of TX114 and further increase in TX114 concentration lead to a steady decline in the PHA purity.

Owing to neutral charged head groups and hydrophobicity exerted by TX100 tails, its interaction with the other surfactants in the mixture is purely based on hydrophobic interactions. TX100+TX114 surfactant mixture imparts strong hydrophobic interactions with that of PHA, while the presence of charged surfactant head group (AOT and CTAB) in the mixture TX100+AOT and TX100+CTAB imparts electrostatic interaction between micelles and the solute molecules within the system (Browne et al. 2011). Additionally, HLB value of surfactant mixtures are different than that of HLB value of individual TX100 concentration, which is as a result of mixing different surfactants at various concentrations (Kunieda and Shinoda 1985). Thus, increasing concentration of TX114 in the surfactant mixture of TX100+TX114 offers maximum hydrophobic specificity towards PHA and resulted in maximum PHA purity of 89.98 %.

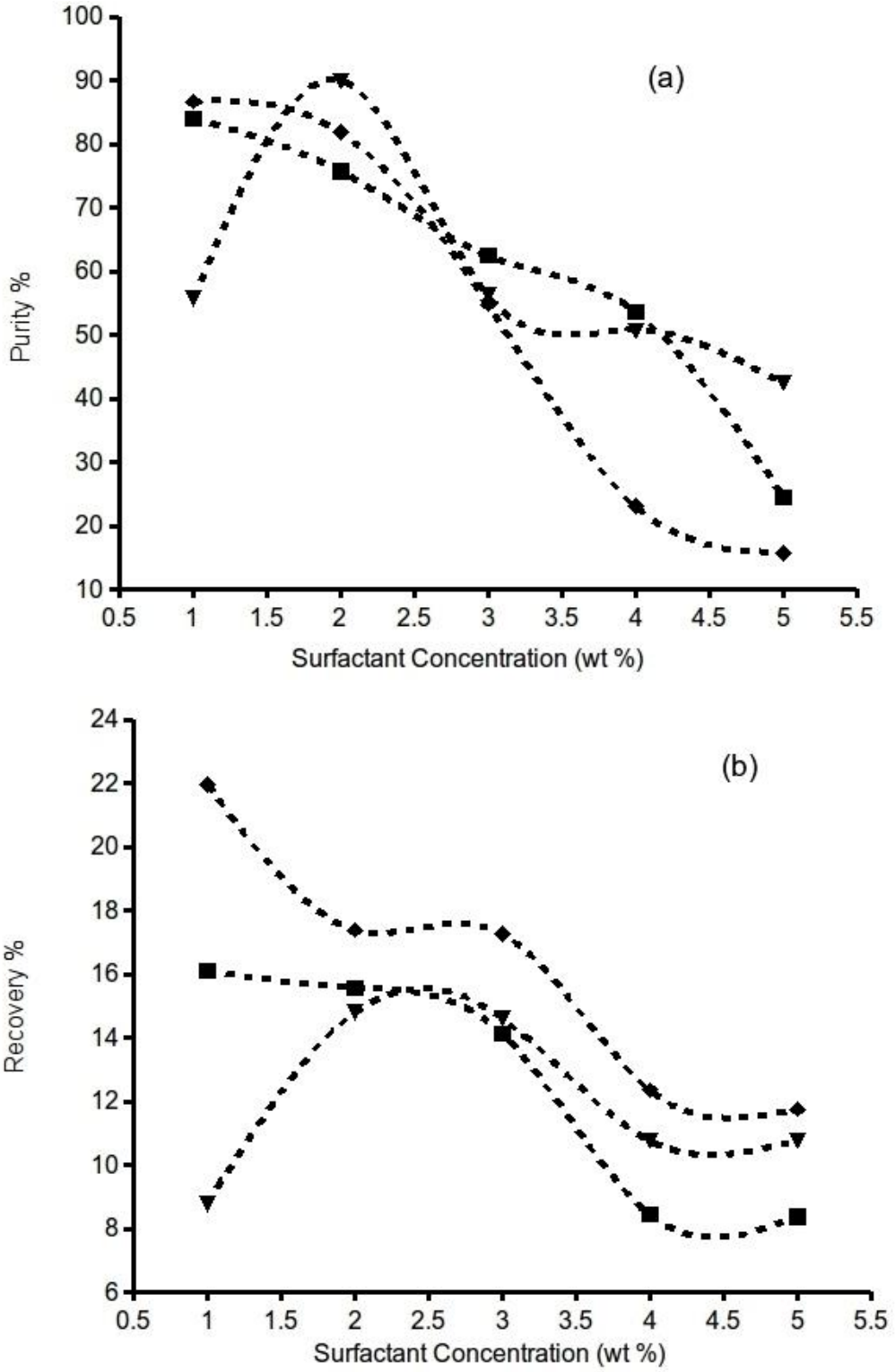


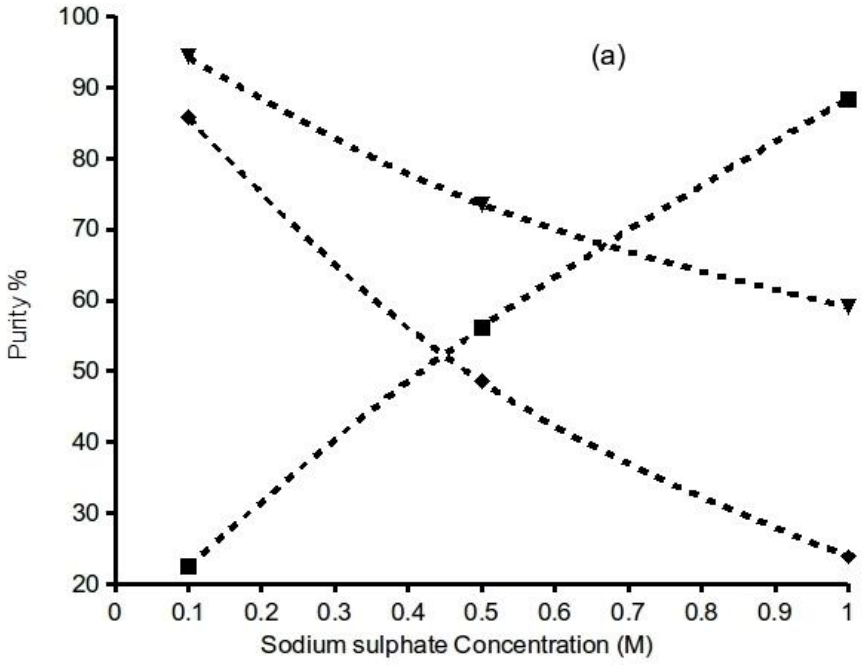
Figure 4.6 Effect of mixed surfactant concentrations at a fixed TX100 concentration of 3 wt. % and varying concentrations of AOT - ■ / CTAB - ◆ / TX114 - ▼ on PHA (a) Purity % (b) Recovery %.

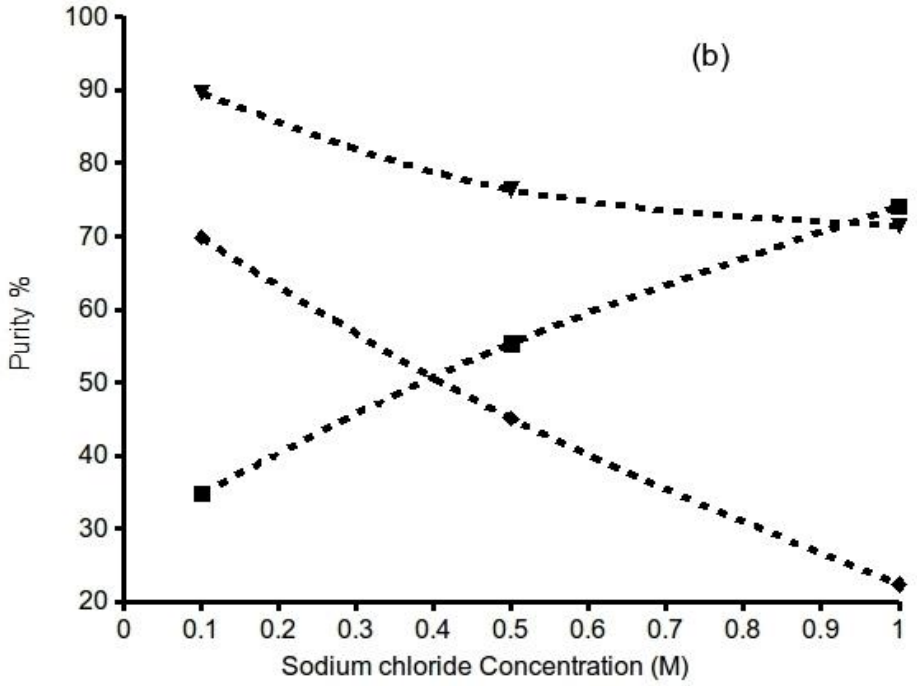
4.5.6 Effect of Additives

Effect of additives were studied by adding different electrolytes (Na_2SO_4, NaCl, $(NH_4)_2SO_4$, NH_4Cl) and polymer of different molecular weights (PEG 4000, PEG 6000, PEG 8000) at varying concentrations. The mixed surfactant systems (TX100+AOT, TX100+CTAB, TX100+TX114) and their respective concentrations which derived maximum PHA purity were used to study the effect of additives.

4.5.6.1 Effect of salts

It can be inferred from figure 4.7a, Maximum PHA purity of 94.28 % was obtained with the addition of 0.1 M sodium sulphate in the presence of TX100 (3 wt%) + TX114 (2 wt. %) surfactant mixture, while variation in recovery of PHA is represented as figure 4.8a. In case of TX100+AOT mixture, increasing concentration of sodium sulphate from 0.1 to 1 M lead to increase in the purity of PHA, while PHA purity was found to decrease with increasing sodium sulphate concentration for TX100+CTAB and TX100+TX114 systems. With increasing concentrations of sodium chloride, purity and PHA recovery were found to increase in the presence of AOT within the surfactant mixture, while purity and recovery was found to decrease in the case of CTAB and TX114 present within the surfactant mixture. Presence of stronger salt such as ammonium sulphate and its increasing concentration resulted in increasing purity and recovery of PHA until 0.5 M ammonium sulphate and with further increase in salt concentration both purity and recovery were found to decline in the presence of AOT and TX114 in the surfactant mixture. The increasing concentration of ammonium sulphate in the presence of TX100+CTAB leads to decline in extraction efficiency. Presence of anionic surfactant AOT in the surfactant mixture and with increasing ammonium chloride concentration, purity and recovery was found to decrease, while in the case of cationic CTAB and nonionic TX114 present in the surfactant mixture, purity and recovery were found to increase until 0.5 M and with further increase in salt concentration, extraction efficiency was found to decline.


(a)
Purity %
100
90
80
70
60
50
40
30
20
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Sodium sulphate Concentration (M)


(b)
Purity %
100
90
80
70
60
50
40
30
20
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Sodium chloride Concentration (M)

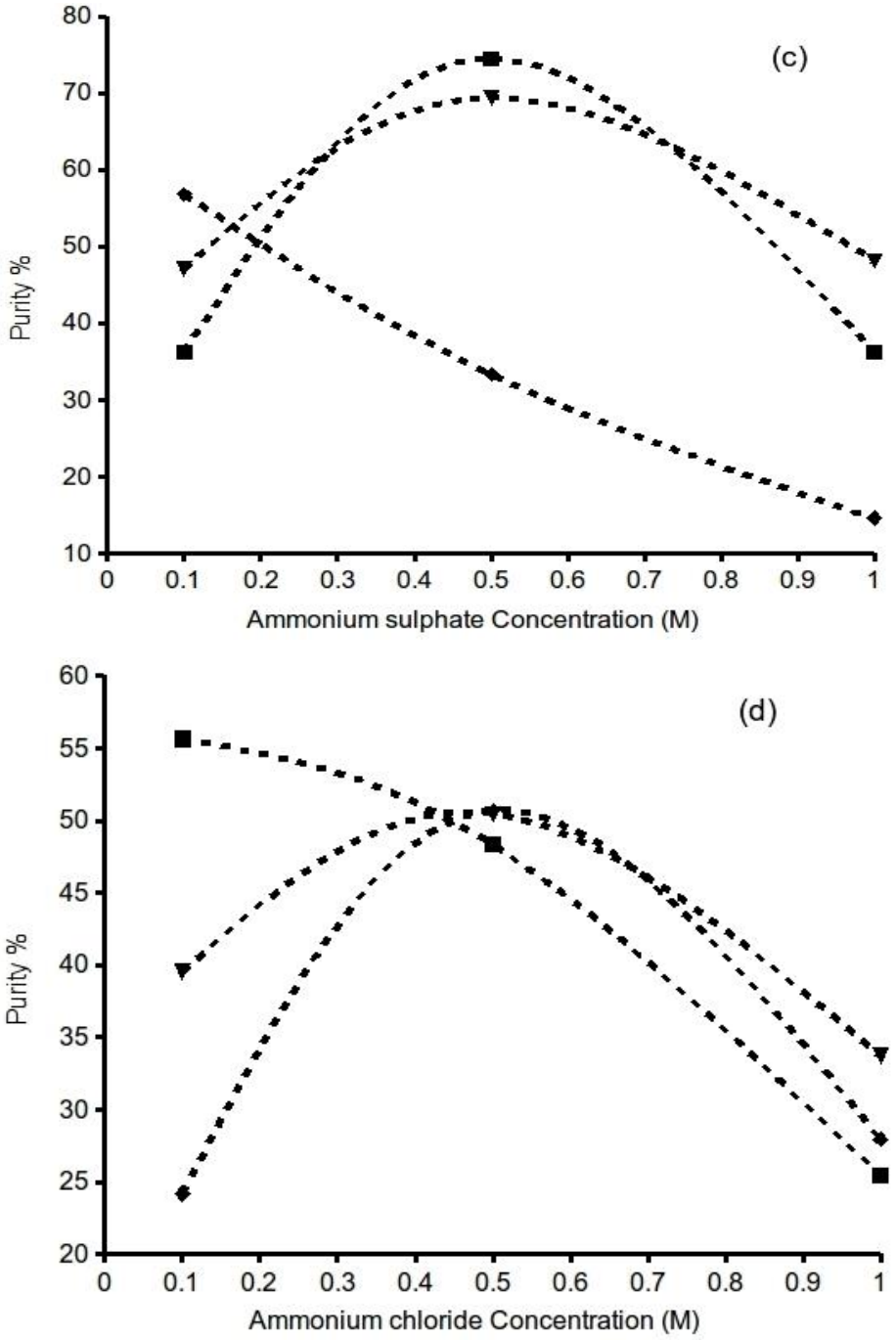


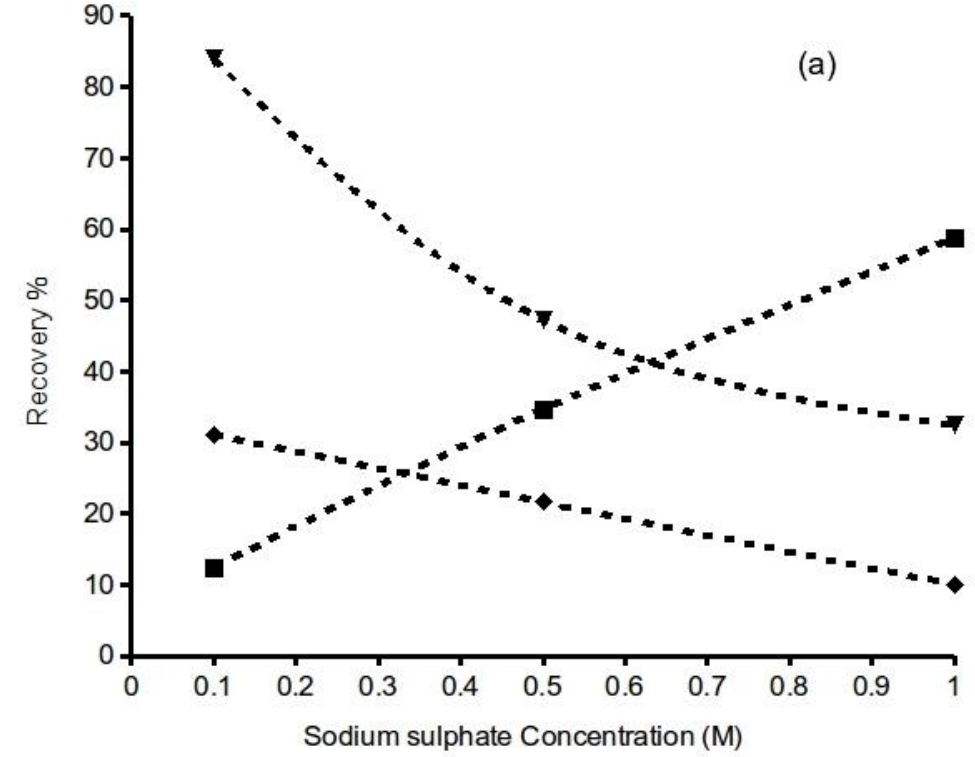
Figure 4.7 Effect of electrolytes and their varying concentrations on PHA Purity % in the presence of mixed surfactants comprising 3 wt% TX100 and AOT - ■ / CTAB - ◆ / TX114 - ▼ (2 wt%).

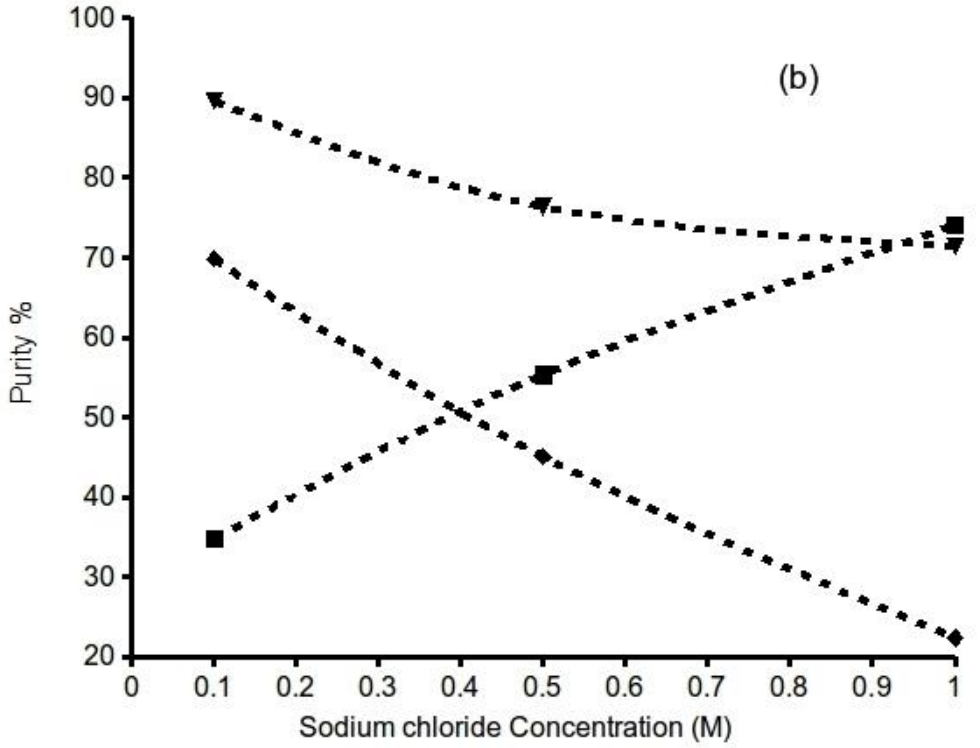
Presence of electrolytes, enhance the cavitation effect on hydrophobic surfaces compared to hydrophilic surfaces, as a result of which increased cell rupture and leakage takes place (Bunkin et al. 1997, Browne et al. 2011). Stronger cations induce

the surface tension and there by decline the extraction efficiency during ultrasonication assisted extraction process while weaker cations decreases the surface tension of the system and induce the extraction of solute into the coacervate phase. However, most of the salts reduce the bubble coalescence rate while a few have no effect (Craig et al. 1993, Bunkin et al. 1997). Thus a combined effect of surfactants, electrolytes and ultrasonication affect the purification of PHA from the broth. Similar results have been reported in UACPE of Polybrominated diphenyl ethers from water and soil sample using TX100, TX114 and PONPE 7.5, and addition of salt lead to increase in micelle size and its interaction with ethers via hydrophobic interaction. In another study involving extraction of estrogens in urine sample using TMN 6 has been reported to result in the addition of salt enhances phase separation by displacing free water present between the micelles and results in coalescence of micelles (Zhou et al. 2012).

Addition of salts to a surfactant solution shields the electrical layer formed by the charged surfactant head groups, thus reducing the surface oscillation of a bubble. This effect diminishes the microstreaming of the bubbles and as a result low shear force acts upon the microbial cell surface thereby declining the cell rupture. In the presence of AOT (negatively charged surfactant) within TX100+AOT mixture, sodium ions interact with the surfactant head group forming Gouy Chapman layer (Stokes and Evans 1997), while free sulphate interacts with the negative sites on the protein and repels them into the top aqueous phase. However, in the case of CTAB (positively charged) within TX100+CTAB mixture, surfactant head group interacts with sulphate and as a result negatively charged cellular impurities settle down over the coacervate phase. The interaction between TX100+TX114 micelles and sodium sulphate is based on hydrophobicity, where charged ion species involves in hydrogen bonding with surfactant head groups and tails. Increasing concentration of salt leads to precipitation of proteins and cellular impurities which settle over coacervate phase that results in lower purity and recovery of PHA. Larger ionic radius of ammonium ions enables it's interaction with the surfactant as well as cellular impurities compared to smaller ionic radius of sodium (Israelachvili 2011, Parikh et al. 2013). As a result,

presence of ammonium and combination of anionic species lead to reduction in the purity and recovery of PHA.





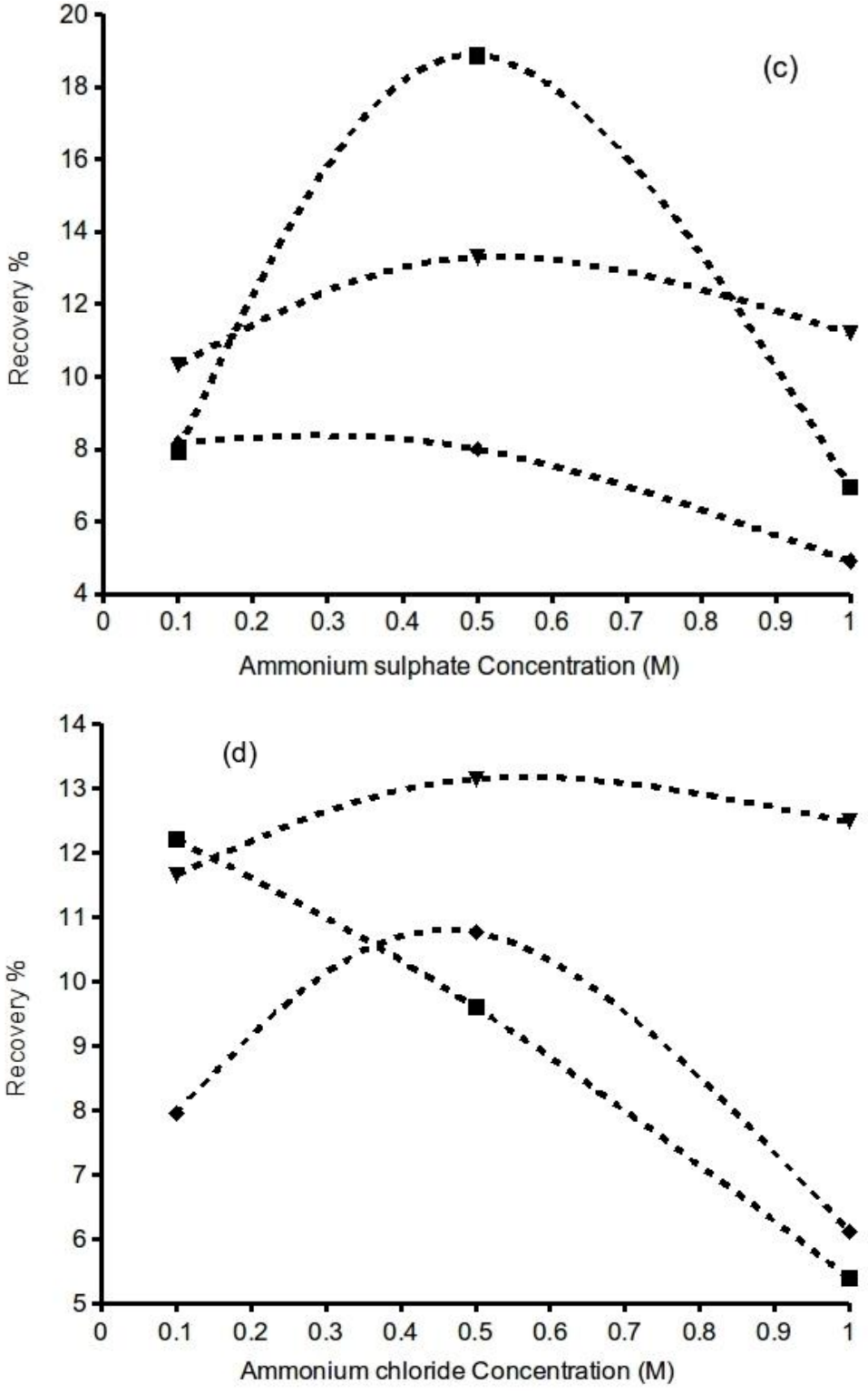


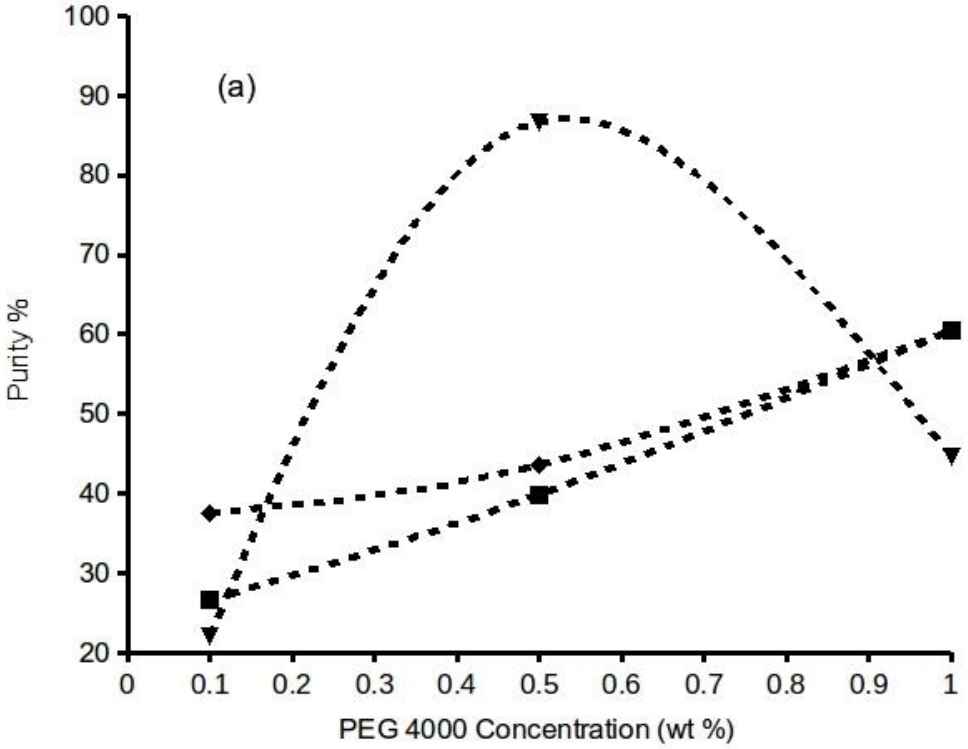
Figure 4.8 Effect of electrolytes and their varying concentrations on PHA Recovery % in the presence of mixed surfactants comprising 3 wt% TX100 and AOT - ■ / CTAB - ◆ / TX114 - ▼ (2 wt%).

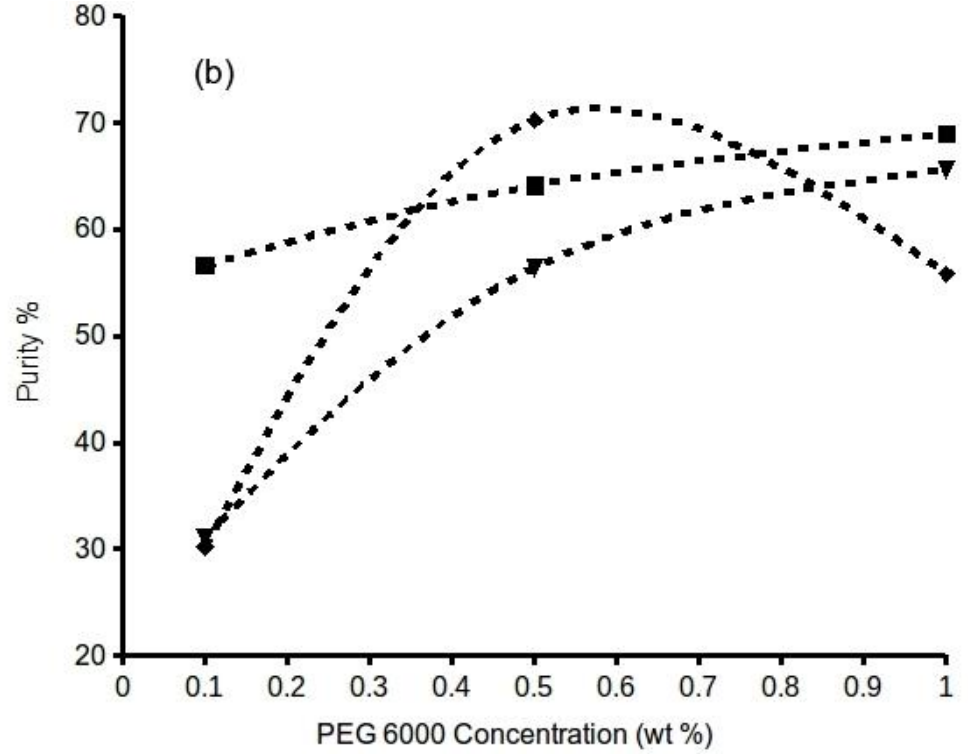
4.5.6.2 Effect of polymer

Effect of polymer was studied by considering increasing molecular weight of PEG and its varying concentrations. The results obtained were in accordance to the fact that with increasing molecular weight, hydrophobicity of the solution increases. However, in the presence of surfactants, PEG and surfactant micelles form a complex that interacts with the solutes present in the feed. Results obtained by studying the effect of PEG molecular weight and its varying concentrations on purity % and recovery % are represented as figure 4.9 and 4.10 respectively.

It can be seen that with addition of PEG 4000 to the different surfactant mixtures, presence of AOT and CTAB lead to increase in the purity and recovery of PHA, while presence of TX114 in the mixture of TX100+TX114 enhanced PHA purity and recovery until PEG concentration of 0.5 wt% and with further increase to 1 wt%, purity and recovery were found to decline. With increasing concentrations of PEG 6000, purity and recovery of PHA was found to increase for all the surfactant mixtures studied. On adding PEG 8000, purity and recovery was found to increase in the presence of charged surfactants within the surfactant mixture, while presence of TX114 initially increased PHA purity until 0.5 wt%. With further increase in PEG 8000 concentration, purity was found to decline, though recovery was found to increase with increasing concentration of PEG 8000.

Introduction of polymer to micelle system leads to micelle-polymer interaction via hydrophobic interaction that in turn alters partitioning of PHA into the coacervate phase. Higher concentrations and molecular weights of PEG in the micelle system impart strong hydrophobic interactions with hydrophobic PHA molecules and repulse the protein into the top aqueous phase thereby increasing the purity of PHA.


(a)
Purity %
100
90
80
70
60
50
40
30
20
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
PEG 4000 Concentration (wt %)


(b)
Purity %
80
70
60
50
40
30
20
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
PEG 6000 Concentration (wt %)

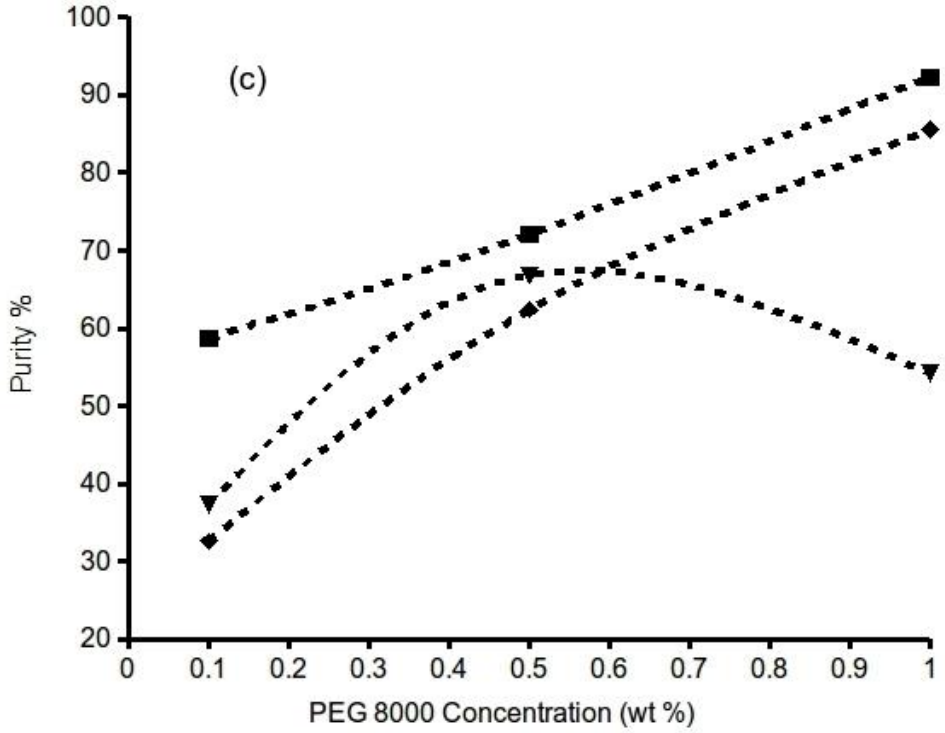
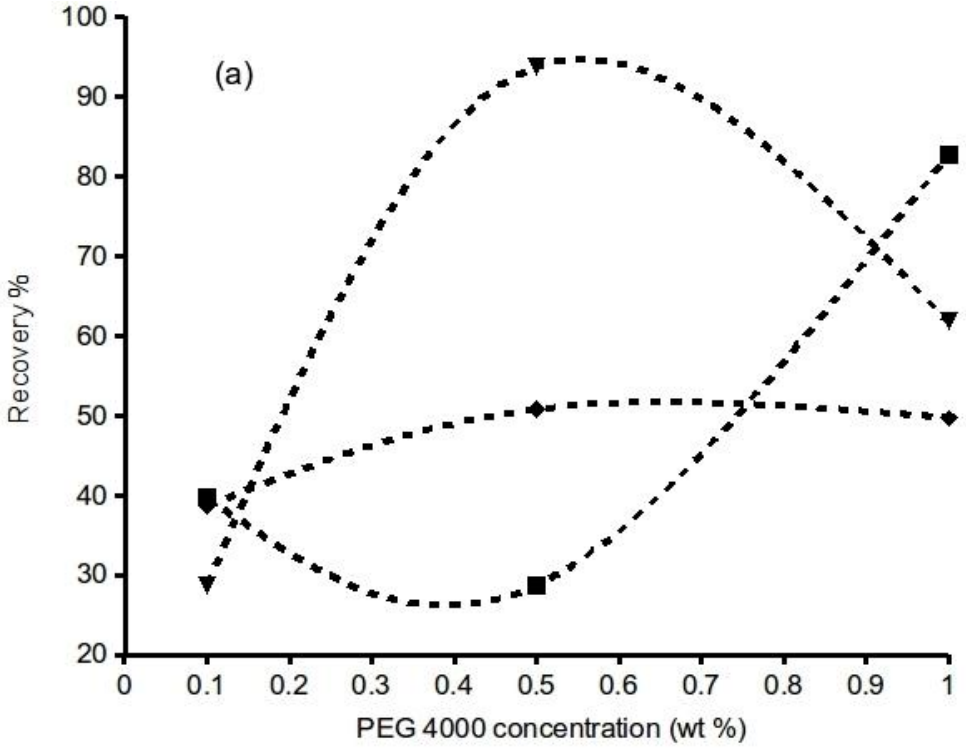
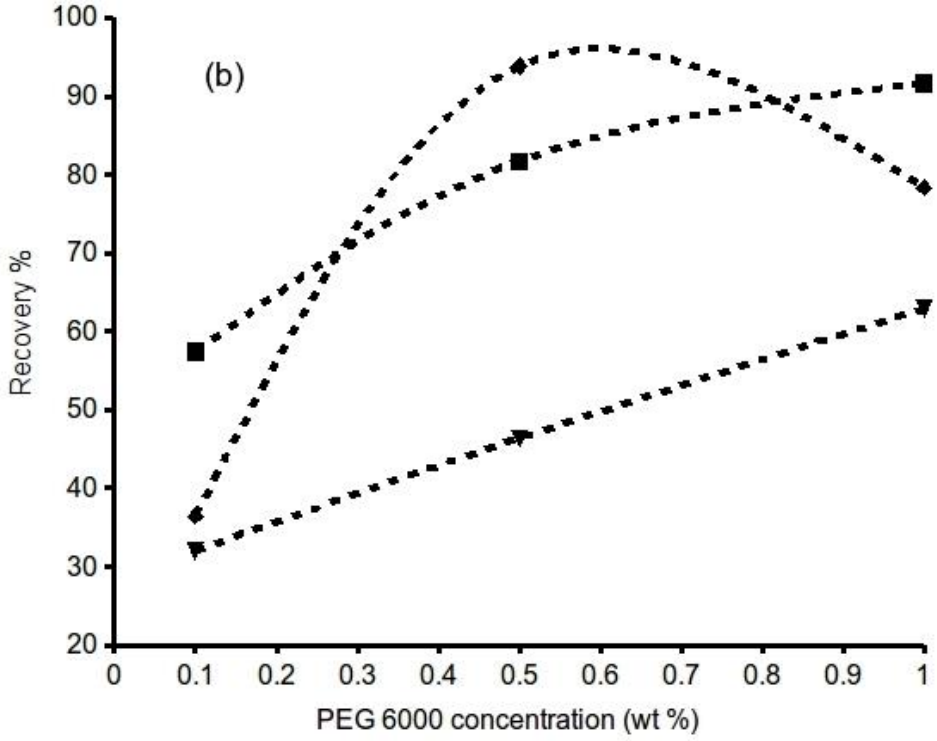


Figure 4.9 Effect of different molecular weights of PEG and their varying concentrations on PHA Purity % in the presence of mixed surfactants comprising 3 wt% TX100 and AOT - ■ / CTAB - ◆ / TX114 - ▼ (2 wt%).

Presence of polymer reduces the surface tension of the solution that enhances cavitation and generation of even sized bubbles that implode and improvises cell disruption and leakage. However, at lower concentrations and molecular weights of PEG the hydrophobic interaction is relatively low with proteins (Naqvi and Khatoon 2011). Conversely, the presence of free PEG molecules present in the system at higher PEG concentration competes with PHA in the formation of stable polymer-surfactant complexes, explained by necklace bead model (Hansson and Lindman 1996), that precipitates as coacervate phase and reduces the recovery of PHA extracted.


(a)
Recovery %
100
90
80
70
60
50
40
30
20
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
PEG 4000 concentration (wt %)


(b)
Recovery %
100
90
80
70
60
50
40
30
20
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
PEG 6000 concentration (wt %)

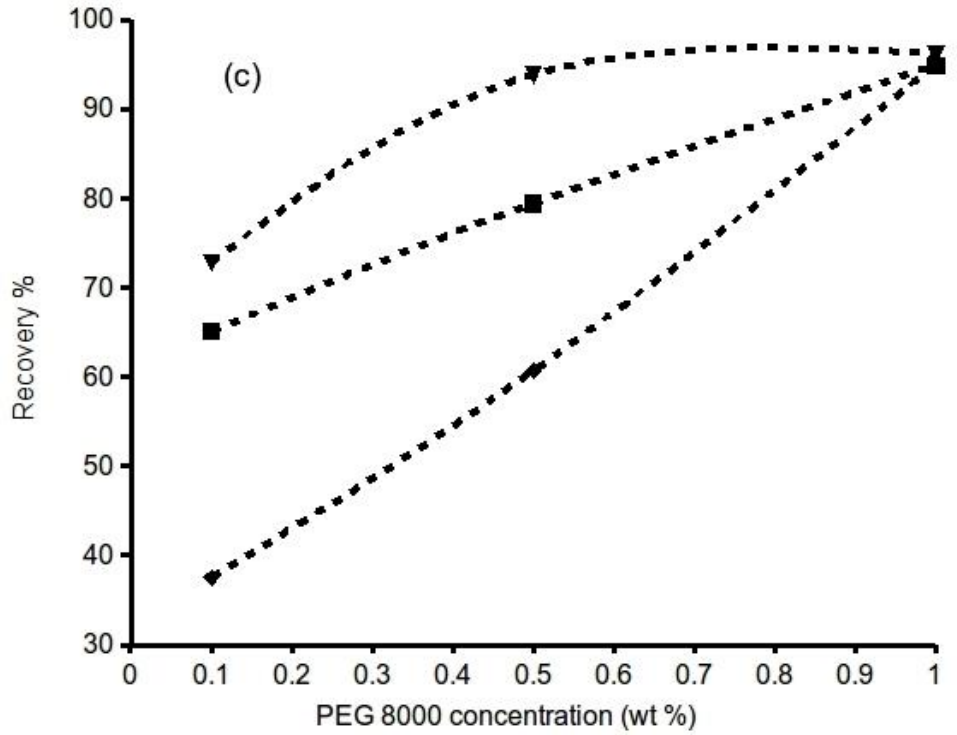


Figure 4.10 Effect of different molecular weights of PEG and their varying concentrations on PHA Recovery % in the presence of mixed surfactants comprising 3 wt% TX100 and AOT - ■ / CTAB - ◆ / TX114 - ▼ (2 wt%).

4.5.7 Chromatographic analysis of purified sample

LCMS analysis of the sample which gave maximum purity of PHA (3 wt% TX100 + 2 wt% TX114, broth pH – 5, in the presence of 0.1 M sodium sulphate performed at 6 kHz of sonication frequency for 6 minutes) was performed in a reverse phase HPLC column. 20µl of the sample was injected to the column run at appropriate conditions as mentioned above and the obtained chromatograms for standard PHBV and sonication assisted CPE extracted PHA were compared as shown in figure 4.11. It is inferred from the peaks obtained, that the retention time of the standard PHBV peak with the highest intensity was around ~ 4.8 minutes while that of the extracted PHA sample with maximum purity was about ~ 6.2 minutes.

Positive m/z peaks obtained during mass spectral analysis of standard PHBV and PHA chromatographic peaks are represented in supplementary material. Different m/z peaks represent the oligomers of PHBV and PHA molecule that are obtained as a result of partial pyrolysis during ionization within the MS unit. Pyrolysis of polymer

samples results in oligomer formation that is random in their structure and so the base peak shifts according to the ions that are generated. Occurrence of increasing m/z peaks represents the formation of monomer to oligomeric units (dimer, trimer, tetramer and so on). It is deduced from the figure 4.12 that most of the peaks fall on the same m/z value; however, pyrolysis resulted in highest base peak at 88 m/z for standard PHBV while it was 495 m/z for the PHA sample extracted in this work. m/z peaks < 400 m/z in the case of extracted PHA denotes that the molecular weight of PHA extracted is higher than that of standard PHBV.

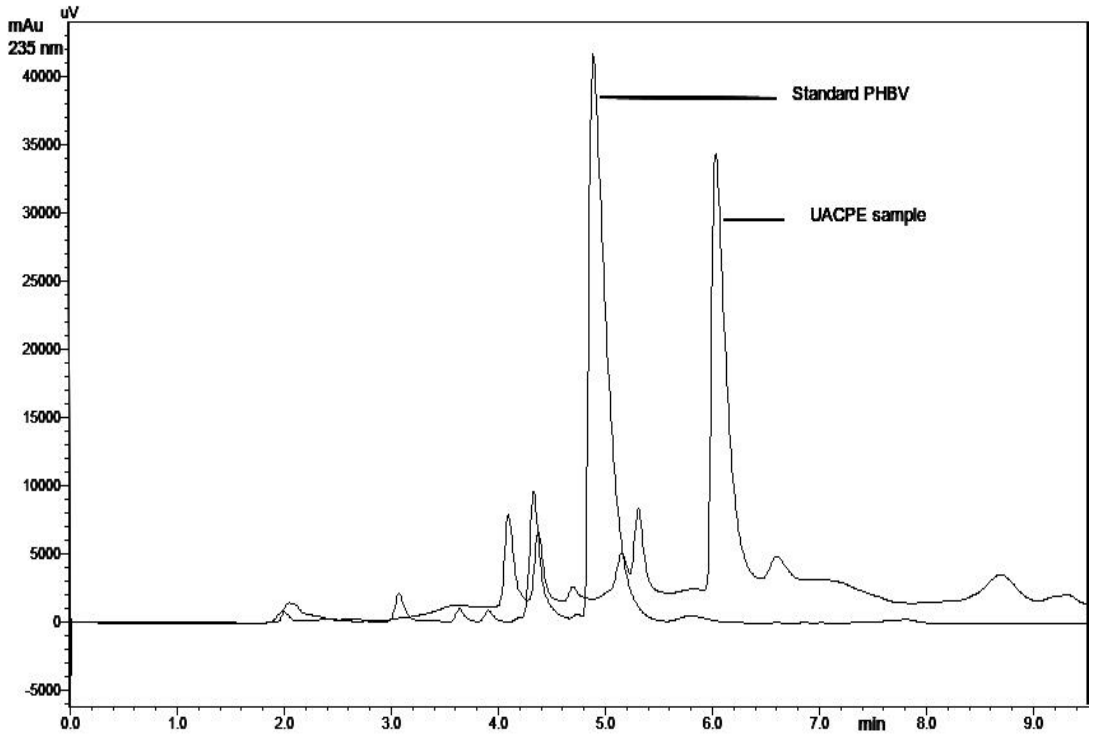


Figure 4.11 Comparative chromatogram of Standard PHBV and UACPE sample with highest purity of PHA.

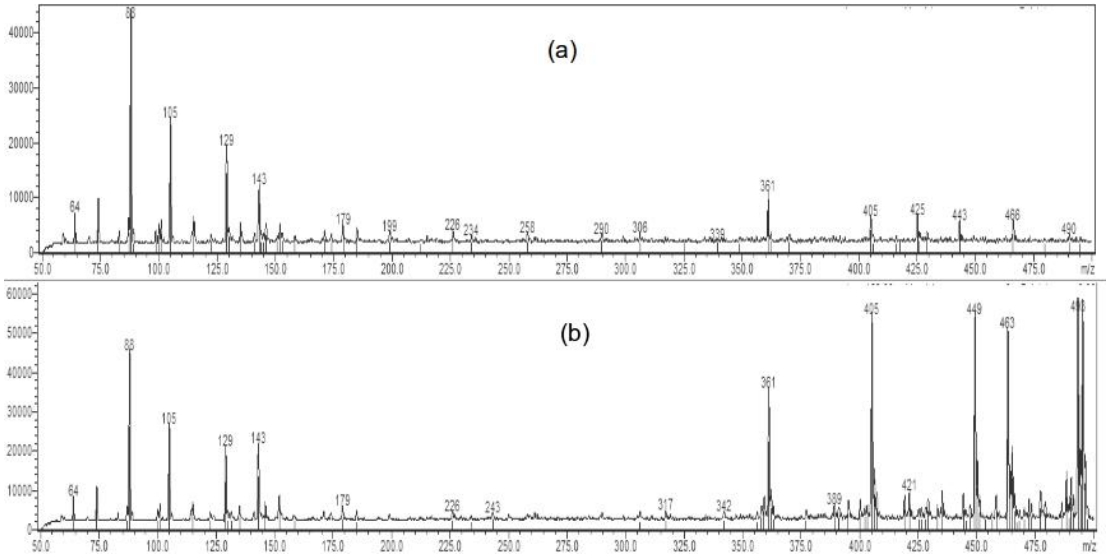


Figure 4.12 Mass spectrum – positive m/z peaks of (a) standard PHBV and (b) PHA obtained via UACPE.

4.6 Summary

PHA purity was found to increase by introducing low frequency sonic waves with respect to those obtained via cloud point extraction of PHA as explained in chapter 3. The developed technique also enhanced the utilisation of ionic surfactants whose cloud point > 100ºC and is difficult to maintain, that in turn increases the design and operational cost during process scale up. Sonic waves enhance the rupture of cell wall which highly influences the entry of micelles into the cells and encapsulation of PHA apart from inducing the formation of two phases. sonication assisted CPE method developed in this work enhances the purity and recovery of PHA from biomass and is easy to scale up to handle bulk volumes of feed. It was observed that presence of mixed surfactant TX100+TX114 resulted in higher purity of 89.98 % compared to that obtained in the presence of individual surfactant, TX100 (purity % - 84.7), as a result of increasing hydrophobicity imposed by mixed surfactant system. Studies on the effect of addition of salts on extraction efficiency imply that weaker salt, sodium sulphate improvised PHA purity to 94.28 % unlike purity obtained in the presence of ammonium chloride during CPE of PHA, as described in chapter 3. Presence of charged surfactant head groups within the

surfactant mixture highly influenced the extraction efficiency with the addition of salts to the feed. High molecular weight PEG (PEG 8000) added to the feed was found to enhance PHA purity to 92. 3 % compared to low molecular weight PEG molecules. Reverse phase chromatographic analysis of purified sample and standard PHBV revealed that the nativity of PHA was retained.

CHAPTER 5

GUM ARABIC - MIXED MICELLE COACERVATE ASSISTED MEMBRANE EXTRACTION OF PHA

Although, cloud point extraction and ultrasonication assisted cloud point extraction resulted in high PHA purity, difficulties such as maintenance of constant cloud point temperature during continuous operation and parallel separation of strongly hydrophobic cellular impurities including membrane proteins and lipids as a result of enhanced exposure of hydrophobic cores of the micelles, put ahead a need to design a separation process that offers enhanced specificity towards PHA. To enhance PHA specificity and to simplify the separation process design by eliminating the addition of heat and sonication, which are difficult to maintain during large scale operations, commercially well-established downstream processing technique, membrane separation process, is considered in the present study. Selectivity of PHA can be improvised with the usage of surfactants while membrane separation has been industrially employed for separation of proteins. Since the novel separation techniques such as membrane distillation, membrane pervaporation offer both specificity and high purity and recovery of the solute molecules, similar ideology has been put forth in extending one such process integrated technique, micellar enhanced membrane filtration towards selective separation and purification of PHA from homogenized crude broth.

5.1 Membrane Filtration

Reducing the operational cost while achieving higher purity and recovery of a certain product of interest is a major goal in any industry, membrane extraction owing to its ease in operation and handling has been employed chiefly in the bio-industries. The discovery and invention of novel materials lead to the usage of membrane separation process as a main downstream processing technique worldwide. Semipermeable or selectively permeable material is used as membrane that enhances the diffusion of solute across the membrane. During membrane filtration, pressure is applied on the membrane side which acts as a driving force to create chemical potential difference and as a result of which solute transfer is enhanced. Selective

permeability of membrane enhances the diffusion of solute while other solute molecules in the feed are rejected back into the feed stream. The solutes that diffuse across the membrane are known as permeate while those rejected by the membrane are known as retentate. Membrane filtration is generally classified based on the size of the solute, membrane used and type of operation. Depending upon the membrane pore size, membranes are subdivided into microporous and nonporous membranes, microporous membranes works on the principle of size exclusion, microfiltration and ultrafiltration are based on the difference in the size of the solute molecules and their diffusion across a membrane of fixed pore size. Nonporous membranes are dense membrane made up of bulk packing of polymer or ceramic material that works on the principle of diffusion of solution rather than the solute; reverse osmosis, pervaporation and nanofiltation are classified under dense porous membranes. Based on the mode of operation, membrane filtration is carried out as dead end filtration or cross-flow filtration. During dead end filtration, feed is fed perpendicular to the membrane, by applying pressure solution with selective solutes diffuse across the membrane while other solutes are rejected and form a layer of cake over the membrane which in turn reduces the membrane flux and deteriorates the membrane separation process. In cross flow filtration, feed is left to flow parallel to the membrane, with continuous flow of the feed solution; solutes that are rejected from the membrane are washed away and as a result of which transmembrane pressure and membrane fouling are reduced. Cross flow filtration is commonly known as tangential flow filtration and is industrially employed as a result of its high performance compared to dead end filtration process.

As the name suggests, microfiltration is employed to separate particles of larger size usually in the range of 0.1-10 μm while a maximum pressure of 3.5 bar is maintained. Microfiltration is commonly used in the separation of suspended solids, bacteria and colloids. Microfiltration membranes have the in-definitive pore size distribution and are often classified based on their molecular weight cut off to separate solutes of varying size. Different materials involving both inorganic and organic molecules have been employed to form a membrane and are classified into two types, synthetic and polymeric membranes. Synthetic membranes are cast by including both inorganic and organic materials and are often used under harsh

conditions such as high temperature, acid filtration etc. Ceramic and metal membranes fall under this category and so are able to with stand high temperatures. Polymeric membranes are cast by coating a layer of polymeric material on a porous support; such membranes are less expensive and are often employed for generic to industrial filtration. Depending upon the polymeric material, high selectivity, permeability, and mechanical strength during operation can be obtained, which classifies its application. Porosity, even pore size distribution, low cost and fouling tendency, mechanical strength and flexibility, surface charge, hydrophobicity/ hydrophilicity are a few parameters taken into account to choose and consider a membrane for an appropriate separation process. Membrane extraction is employed primarily to purify water and waste water, separation of biological molecules such as proteins, nucleic acids, organic acids and other industrially and medically important enzymes from the biological feed stream, clarification of alcoholic beverages, clarification of fruit juices, vinegar and separation of cheese from whey, clarification of fine chemicals, in paints and adhesives, petroleum refineries, separation of metal ore etc. Ease in scale up/down of the overall process, comparatively low operational cost, ability to integrate process integrates with other unit operations are advantages of membrane separation process (Rathore and Shirke, 2011). Although, achieving specificity during membrane extraction is a difficult task apart from membrane fouling, lower flux with increasing operation time and maintenance of membrane units are few common problems encountered during membrane extraction.

5.1.1 Micelle assisted membrane Filtration

Process intensification, a sustainable approach towards selective separation of valuable products in the chemical and biochemical industries is of current research interest owing to several advantages over conventional unit operations (Stankiewicz and Moulijn, 2000). Efficient and sustainable process designs, integration of multiple unit operations into a single operation, miniaturization of process equipment, low level of waste generation and maximum utilisation of raw materials, reduction in overall cost of the process are a few perks it offers. Micellar Enhanced Membrane Filtration (MEMF), a novel process integrated unit operation that has been wide topic of research over a decade since the research work of Leung in combining both

surfactant based micellar extraction and membrane separation towards separation of metal ions from the feed (Leung, 1980). Addition of surfactant micelles to the crude offers higher specificity towards the solutes while pressure driven filtration enhances the effective separation of solutes associated with the micelles from the feed across the membrane barrier. Addition of surfactant concentration beyond the CMC to the feed stream results in formation of micelles, which interact hydrophobically and/or electrostatically with the solute molecules (Blankschtein et al., 1986). On applying pressure, based on stronger or weaker interactions between micelles and solutes, solute molecules which are strongly bound to the micelles are retained as retentate owing to the larger hydrodynamic radius of the micelles that cannot diffuse across smaller pore diameters; while loosely bound solutes pass through the membrane barrier as permeate. Hydrophobic solutes have a greater affinity towards the surfactant tails spread across the micellar core made up of nonionic surfactant. The engrossed solutes in the hydrophobic micellar core are retained as retentate stream during the membrane process owing to large size of the micelles. When ionic surfactants are used, solute molecules interact with the charged surfactant head group as a result of strong electrostatic interactions and are retained in the retentate stream. During Membrane enhanced filtration, pressure of about 97 to 587 KPa is maintained, while the pore size of the membrane is varied from micro to nano range to screen the surfactants from diffusing across the membrane. Most of the hydrophobic solutes are encapsulated within the micellar core while charged solutes remain interacting with the charged surfactant head groups of a micelle. On applying pressure, micelles are rejected into the retentate stream and so the solute molecules are also rejected into the retentate. Only unbound solutes pass through the membrane and are usually of very low concentration when compared to the bulk concentration present in the retentate. The technique has been used to concentrate metal ions and other compounds from waste stream, so that can be reused or disposed appropriately than to treat a larger volume of waste stream. Presence of high surfactant concentration or an apparent change in the CMC of a surfactant solution enhances the formation of micelle to a larger extent while the diffusion of surfactant monomers across the membrane is considerable reduced. Mixed micelles are the easiest and most commonly adopted method to reduce the CMC of the surfactant solution while operating MEMF.

Mixed micelles are the easiest and most commonly adopted method to reduce the CMC of the surfactant solution while operating MEMF. Even though the micelles are formed at ambient pressure, the destabilization of micelles was noticed at higher pressures during the membrane separation process and hence loses the selectivity offered by the micelles. Generally the micelles are preserved at the intact form by emulsifying the mixture or forming micelle complexes with the emulsifier. The emulsifiers not only emulsify the surfactant-crude mixture but also lead to higher stability through the molecular interaction between solute and emulsifier molecules. Such mixtures/complex aggregates are found to be stable at high pressure during the membrane separation processes. This method also improves the selectivity of a specific solute in the separation process.

5.2 Literature review

5.2.1 Micelle assisted membrane Filtration

Several solute molecules have been reported to be separated from the feed stream by employing MEMF. MEMF has been generally used in the separation of heavy metals and other organic pollutants from the water stream. Table 5.1 briefly indicates the use of micelle enhanced filtration of various compounds from its source. Literature review on MEMF suggest that membrane type and molecular weight cut off, surfactant type and concentration, presence of chelating agent and pH are a few common operational parameters that have been studied by the authors. The preceding section briefly elaborates the process and effect of different operational variables on the separation of solute molecules.

Table 5.1 Application of Micelle assisted membrane Filtration.

Solute	Type of Filtration and membrane used	Surfactant used	Reference
Eosin	Polyamide ultrafiltration membrane with MWCO - 1000 Da	Cetylpyridinium chloride (CPC)	Purkait et al. 2004
Trichloroethylene (TCE) and chromate	Ultrafiltration membrane made up of regenerated cellulose, MWCO -10000	Cetylpyridinium chloride (CPC) and Tween 80	Lee et al. 2005

	Da		
Aromatic alcohols	Polyamide membrane, MWCO – 1000 Da	cetylpyridinium chloride (CPC)	Purkait et al. 2005
Methylene blue	Polysulfone hollow fibre with a MWCO-10,000 Da	SDS	Bielska and Szymanowski 2006
Safranin T	Ultrafiltration membrane made up of regenerated cellulose with a MWCO of 10,000 Da	SDS	Zaghbani et al. 2008
Di-butyl phosphate (DBP) and tri-butyl phosphate (TBP)	Ultrafiltration membrane of varying MWCO (3000 to 10,000 Da).	SDS	Misra et al. 2009
Sugars	Millipore, YM-10, regenerated cellulose (MWCO – 10,000 Da)	CTAB, SDS, TX100 and Aliquat 33	Mehling et al. 2012
Hexavalent and trivalent chromium	Polysulfone hollow fibre ultrafiltration cartridge with a MWCO-10,000 Da	Rhamnolipid JBR 425	Abbasi-Garravand et al. 2014

Abbasi-Garravand et al. (2014) studied MEMF of Hexavalent and trivalent chromium using Polysulfone hollow fibre ultrafiltration cartridge with a MWCO-10,000 Da by employing Rhamnolipid JBR 425 as surfactant. The authors have reported that rhamnolipid concentration of 0.05% (vol/vol) was used to avoid clogging. On studying effect of transmembrane pressure (40, 70, 100 and150 KPa), the membrane flux was found to increase with transmembrane pressure. The flux was 13.6 L/m^2 h at transmembrane pressure of 40 KPa and increased to 63.5 L/m^2h at transmembrane pressure of 150 KPa. Rhamnolipid leads to biofouling of membrane, however both polysulfone membrane and rhamnolipid are negatively charged by maintaining system pH at 6, which results in electrostatic repulsion and low concentration polarization on the membrane side, thus fouling was insignificant. As rhamnolipid concentration was increased (265, 530 and 1060 mg/L) the number of micelle increases and amount of chromium encapsulated increases which leads to higher rejection of Chromium as 96.2% at rhamnolipid concentration of 1060 mg/L.

Methylene blue was successfully separated from water by employing Polysulfone hollow fibre with a MWCO-10,000 Da and Sodium Dodecyl Sulphate as surfactant in MEMF study conducted by Bielska and Szymanowski (2006). The authors found that dye rejection was found to increase with increasing dye concentration (2-24 mg/L), as a result of distribution of methylene blue among the solution and the micelles. The dye rejection was found to increase linearly with increasing SDS concentration (1.4 - 72 mM), as a result of increasing number of micelles and its interaction with the dye molecules. Presence of sodium ion (NaCl - 0 to 300 mM) decreases CMC of SDS micelle, increases number of micelles as a result of screening of electrostatic repulsion between surfactant head groups by sodium ions.

Di-butyl phosphate (DBP) and tri-butyl phosphate (TBP) were extracted using SDS during MEMF with Ultrafiltration membrane of varying MWCO (3000 to 10,000 Da). Removal of organic compounds with uranium was studied and it was found that, 300 ppm of TBP along with 720 ppm of DBP was extracted in the presence of uranium, while 300 ppm of TBP and 1340 ppm of DBP was extracted in the presence of 67 ppm of uranium. As the solution pH was adjusted towards acidity (pH 8.0 to pH 2.0), rejection of organic compounds were found to increase. At surfactant concentration less than CMC, rejection % was less which is as a result of concentration polarization of organic compounds and gel layer formation. As SDS concentration was increased, rejection of organic compounds increased and reached a maximum of 90% at SDS concentration higher than 50mM. It has been reported that the rejection of 93 % was observed by employing MWCO – 3000 and 5000 Da, while with increasing MWCO rejection % was found to decrease, as a result of diffusion of micelles through membrane of high MWCO 10000 Da. It was also reported that solute rejection was found to decrease from 93 % at 720 ppm to 85 % at 3880 ppm of DCP concentration which is attributed towards the effect of poor solubilisation by micelles at high concentrations of DBP. Rejection of DBP remained unchanged (93%) while TBP rejection was observed to be 99% as a result of encapsulation of organic compounds within hydrophobic micellar core. Rejection % of TBP and DBP was insignificant while 80 % rejection of uranium was observed as a result of electrostatic interaction of metal with surfactant head group (Misra et al. 2009).

Lee et al. (2005) studied MEMF of Trichloroethylene (TCE) and chromate using ultrafiltration membrane made up of regenerated cellulose, MWCO -10000 Da while employing Cetylpyridinium chloride (CPC) and Tween 80 as surfactants. The authors reported that 96 % chromate removal happened at 5mM CPC, while increasing concentration of Tween 80 from 10 to 24 mM within the surfactant mixture; however chromate removal was decreased from 93.7 % to 84.8 %, as a result of reduction in decrease in micelle surface charge density with the addition of nonionic surfactant. The effect of mixed surfactants and its concentrations on removal of TCE was studied and it was found that presence of Tween 80 in the mixed surfactant system enhanced the rejection of TCE to about 88%. Simultaneous removal of TCE and Chromate was studied and it was found that chromate was rejected to about 83 % in the presence of TCE and TCE was removed by about 87.9 % in the presence of chromate.

Purkait et al. (2005) studied MEMF of Aromatic alcohols - para nitro phenol (PNP), meta nitro phenol (MNP), phenol (P), catechol (CC), beta- napthol (BN) and ortho chloro phenol (OCP) by employing cetylpyridinium chloride (CPC) and Polyamide membrane, MWCO – 1000 Da.. It has been reported that in the absence of surfactant, retention of solute was found to be lesser than 30 %, as a result of concentration polarization and diffusion of solute across the membrane. However the presence of surfactant had improved the retention % and it is higher than 65 % which is as a result of solubilisation of alcohols by surfactant micelles. Hydrophilic alcohols (PNP, MNP, CC and P) are solubilised in the periphery of the surfactant micelles and maximum solubilisation was observed in the case of para nitro phenol while it was minimum for phenol. Hydrophobic alcohols are solubilised within the micellar core and are highly retained by the micelles (retention % - 97). Permeate flux was more in the case of hydrophobic and slightly hydrophilic solutes compared to that of highly hydrophilic alcohols. Alcohol retention was found to increase with increasing surfactant-solute ratio. Increasing solute concentration leads to unbound solute and are readily diffused across the membrane thus declining the retention %. The increasing surfactant concentration results in increased retention of PNP, retention reached a maximum of 94 % at CPC/PNP ratio of 110. Retention of OCP and PNP was found to increase from 89 % to 98%, with increasing surfactant concentration, while membrane flux was found to decrease as a result of membrane blocking by micellar aggregates.

Safranin T was successfully extracted by ultrafiltration membrane made up of regenerated cellulose with a MWCO of 10,000 Da and SDS as surfactant (Zaghbani et al. 2008). The retention of the dye was found to be less than 5 % in the absence of surfactant, while presence of surfactant increased the retention of dye to about 99%. Membrane flux was found to decrease, while it was more prominent in the presence of surfactant. In the presence of SDS, membrane flux decline remains constant after a certain concentration of SDS. Retention of dye was independent with variation in transmembrane pressure. The retention % remained unchanged with increasing salt concentration, however, membrane flux was found to decrease with increasing salt concentration. Membrane flux was found to increase when pH was changed from 3 – 11 as a result of hydrophilicity offered by deprotonation of membrane, while change in dye retention was insignificant.

Cetylpyridinium chloride (CPC) and Polyamide ultrafiltration membrane with MWCO -1000 Da were used in the extraction of dye - Eosin (Purkait et al. 2004). It was observed that the retention of dye was low and decreased with increasing filtration time in the absence of surfactant, while presence of surfactant had less significant variation in the retention of dye as a result of dye solubilisation within the micellar core. It was found that increase in pressure resulted in decline in dye retention. Membrane flux was found to increase with increasing pressure as a result of pressure driven solute diffusion across the gel layer and the membrane pores. However, for a given pressure, membrane flux was found to decline steadily as a result of concentration polarization of surfactant micelles over the membrane. Membrane flux was found to decline with increasing surfactant concentration for a given pressure, while retention was found to increase with increasing surfactant concentration. Retention of dye was found to increase until solubilisation capacity of the micelles at a fixed surfactant concentration and further retention decreased with increasing dye concentration.

Iqbal et al. 2007 studied the effect of different process variables such as surfactant and its varying concentrations, pH (4.0,7.0 and 8.5), effect of anions (Nitrate and Phosphate) during MEMF of Arsenic using ultrafiltration membrane made up of regenerated cellulose (MWCO-10000 Da) and hexadecylpyridinium chloride (CPC), hexadecyltrimethyl ammonium bromide (CTAB), octadecylamine acetate (ODA) and

benzalkonium chloride (BC) as surfactants. The authors have reported that arsenal removal increased from 85 to 96 % in the presence of increasing concentration of CPC from 5 to 10mM, while the arsenal removal was enhanced to 94%, 81%, and 55% in the presence of CTAB, ODA and BC at 10mM, respectively. ODA showed significant decrease in relative membrane flux compared to other surfactants; however, relative flux was decreased by increasing surfactant concentration. Arsenate exists in monoionic form at system pH of 4 and so removal was found to decrease compared to its di-ionic form that exists at pH 7 and 8.5. CPC, ODA and CTAB showed similar effects on arsenal removal for a fixed pH and were insignificant even at low pH as a result of increasing interaction offered by large number of surfactant micelles.

Sugars (arabinose, cellobiose, glucose, and sucrose) were successfully filtered from its source using millipore, YM-10, regenerated cellulose (MWCO – 10,000 Da) and surfactants CTAB, SDS, TX100 and Aliquat 33 (Mehling et al. 2012). The authors found that in the presence of anionic/cationic surfactant mixture with addition of sugar carrier, recovery was found to remain constant. For cationic and nonionic/cationic surfactant mixture with phenylboronic acid as sugar carrier, rejection was found to increase. For cationic surfactant and its mixtures, rejection of monosaccharide was higher than that of disaccharide. The increasing glucose/CTAB ratio lead to decrease in rejection of glucose and after a ratio of 2, rejection was found to remain constant.

Jalaei Salmani (2016) studied the extraction of solid solutes from dairy wastewater using polyacrylonitrile ultrafiltration membrane (PAN-350) (MWCO – 20000Da) and linear alkyl benzene sulfonate (LAS) and TX100 as surfactants. On studying different process variables, membrane flux was found to increase with increasing transmembrane pressure as a result of driving force enhancement. Membrane flux was less in the case of nonionic surfactant compared to anionic surfactant as a result of increasing viscosity by TX100, while smaller size of nonionic micelles lead to pore blocking. Permeate flux was found to decrease with increasing surfactant concentration as a result of concentration polarization of micelles. Surfactant concentration below CMC and above CMC leads to increased rejection as a result of

concentration polarization of solutes and entrapment of solutes by large number of micelles with increasing surfactant concentration, respectively.

5.2.2 Gum Arabic as emulsifier

Gum Arabic is an exudate gum obtained from acacia trees mostly growing in African and Asian countries. Being an arabinogalactan type polysaccharide is amphiphilic in nature and exhibits increased solubility in water even at its higher concentrations (50 % w/v) forming low viscous solution. GA is a branched globular molecule, made up of central protein core, rich in proline, hydroxyproline and serine, which are coated by carbohydrate units made up of β,1-3 galactose molecules. Carbohydrate unit often varies and is composed of D-galactose, L-arabinose, L-ramnose and D-glucoronic acid, variation in the sugar moiety and their degree of cross linking decides the molecular weight of GA, average molecular weight of the GA is ~ above 2MDa (Mahendran et al., 2008). β,1-3 galactopyranose units forms the backbone of GA which are linked with 1,6 – galactopyranose units that ends with 4-O-methyl-β - glucuronopyranose and β - glucuronopyranose units, 1,3-α-L-arabinofuranose and 1,4 α-L-rhamnopyranose units are bonded to the main chain. Three different types of GA have been reported till date, arabinogalactan (AraG), arabinogalactan-protein (AraGP) and a Glycoprotein (GP) (Akiyama et al. 1984, Goodrum et al. 2000).

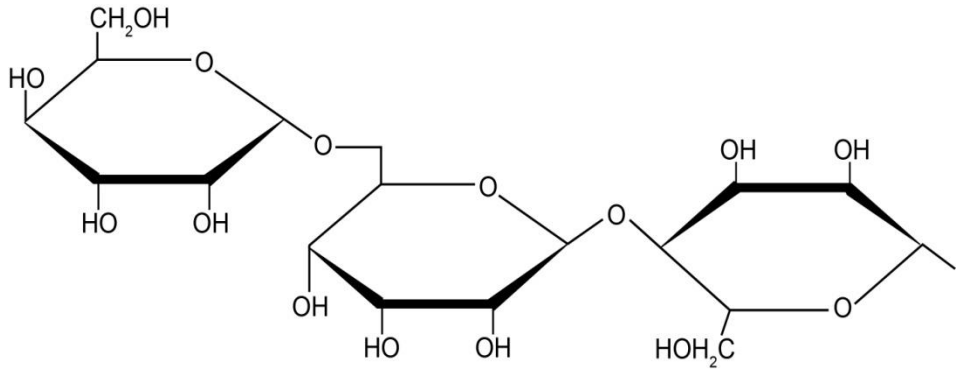


Figure 5.1 Structure of gum arabic.

GA exhibit Newtonian behaviour at lower concentrations and are readily soluble in aqueous medium (Williams et al., 1990). Carbohydrate part of the GA orients into the aqueous phase and aids to maintain the stability of emulsions via electrostatic repulsion from one another, while the protein part of the GA assembles on oil-water

interface and maintains rigidity of the emulsion formed (Montenegro et al. 2012). GA monomers undergo interfacial adsorption as an individual emulsifier and form interfacial bilayer when used with proteins (Bouyer et al., 2013). Devoid of system conditions, GA forms emulsion as a result of steric repulsion unlike proteins stabilized emulsions (Chanamai and McClements, 2002). GA is used in the formation of stable emulsions with or without protein additives that are used in encapsulation of food flavouring agents, oils etc. (Krishnan et al., 2005a , Krishnan et al., 2005b, Madene et al. 2006, Gharsallaoui et al. 2007, Jun et al. 2011, Rascón et al., 2011).

Matsumura et al. (2000) studied the interaction of emulsifying agents with lipids within an emulsion and found that with increasing time the interfacial tension of the emulsion system was found to decline in the presence of GA as a result of its increased surface activity represented by the peptide moieties present in GA. Further the authors observed that the viscosity at interface was found to increase, there by GA forms a viscoelastic film and prevents coalescence of emulsions. However, increase in viscosity of the solution was slow with respect to time, which is attributed to the time taken by GA molecules to be adsorbed at the interface of the emulsion. The emulsions formed by encapsulating oil droplets were of the size 1-4 μm. During rheological studies, with application of stress, the emulsions are destabilized and GA molecules come in contact to each other and forming an elastic polymer network. Sanchez et al. (2002) found that the rheological properties of GA emulsions are time dependent, GA emulsions viscosity increased with time as the time taken by GA to get adsorbed on the oil-water interface is large and viscoelastic property of GA varied from liquid to solid properties with increasing time and they have been reported to form network at air-liquid and liquid-liquid interfaces. Rheological studies by Bouyer et al. (2011) discuss that the stability of the emulsion increases with increasing GA concentration when emulsion stability was studied in terms of days of storage. Increasing number of GA molecules on the oil-water interface forms a steric film around the oil droplets thereby causing steric repulsion and prevents coalescence of the emulsions. The authors observed that the diameter of the emulsion was larger at reduced GA concentrations. At higher concentrations the adsorption time is reduced and as a result smaller droplets are formed. They also found that addition of protein – β-lactoglobulin and GA together or addition of GA to oil to form emulsion and then adding β-

lactoglobulin did not favour emulsion formation, addition of protein to oil and then adding GA resulted in the formation of stable emulsions, the authors hypothesize that it might be as a result of interaction of protein and GA which nullifies the surface activity of GA molecules. Klein et al. (2010) studied the effect of addition of whey isolate to GA and the emulsions formed. The authors found that mixture of biopolymers than individual polymer enhanced the stability of the emulsions, although, they had negligible effect on the emulsion size. At low pH, the complexes formed coacervates while at higher pH, the biopolymers underwent electrostatic repulsion and adsorbed onto the oil water interface in a complimentary fashion. The emulsions formed were reported to be of less temperature sensitive and addition of biopolymer in a consecutive manner formed stable emulsions than mixing both the biopolymers together. Membrane fouling studies implicated while using GA by Manning et al. (2016) reports that 0.1 and 0.8 µm membrane pore size had lower fouling rate compared to 0.5 µm pore sized polysulfone membrane. The size of GA is reported to be similar to that of the pore size as a result they clog the pores resulting in increased fouling, while smaller pore size prevents entering of GA into the pores and leads to cake formation., which is the main fouling mechanism compared to that of pore blocking. Smaller pore size membranes have been found to reject GA into the retentate stream making the separation process more intensive.

5.3 Aim and scope of the work

Though, membrane extraction or membrane filtration is an advantageous actively employed industrial separation method used in the purification of biological molecules from their feed mixture, major drawbacks such as cake formation and flux decline reduce the process efficiency while increasing the operational cost of the process. Process integrated approach of micelle assisted membrane extraction offers enhanced separation while lowering flux decline compared to conventional membrane filtration process. This chapter aims to examine the efficiency of GA in forming coacervate complex with surfactant micelles and their encapsulation efficiency of PHA. Several membrane and micellar extraction process variables such as inlet pressure, GA concentration, surfactant concentration, mixed surfactants, broth pH, addition of salts and alcohols are to be studied to evaluate their extraction efficiency.

5.4 Materials and Methods

5.4.1 Materials

Surfactants -Triton X114, AOT and CTAB were purchased from Sigma Aldrich, India. Gum arabic (GA) with an average molecular weight of ~ 2.5MDa was procured from Sigma Aldrich, France. Methanol, ethanol and propanol were purchased from Merck India Ltd., India. Sodium sulphate (Na_2SO_4), sodium chloride (NaCl), ammonium sulphate ($(NH_4)_2SO_4$) and ammonium chloride (NH_4Cl) were purchased from CDH, India. PTFE membrane (pore size – 0.45 µm, Diameter – 100 mm) was procured from AXIVA, India.

LABINDIA analytical UV 3000 + UV/Vis spectrophotometer, India was used for UV spectral analysis. Deionised water was used during the protocols and the experiments were conducted at room temperature unless and otherwise stated. Fermentation broth with maximum PHA production 11.96 g/L as described in 2.4.5 was used to conduct the study, fermentation broth was homogenized at high speed for 10 minutes and was used for the extraction protocol as such.

5.4.2 Membrane extraction protocol

Batch dead end membrane filtration module made up of stainless steel, was used during the extraction protocol (figure 5.2). Module is made up of three detachable parts; top flange with two separate inlets for nitrogen gas and solution, metal rod with a magnetic bead at its end is attached to the top flange; middle cylindrical shell and a bottom grooved membrane support with permeate outlet. PTFE membrane was placed over the grooved bottom support and fitted to the cylindrical shell. Membrane resistance (R_m) was found by filtering deionised water at different inlet pressure through the membrane and the calculated R_m was found to be 4.496 x 10^{10} m^{-1}. The whole set up was placed above a magnetic stirrer and constant mixing of the solution was maintained. GA emulsion solution (25 wt. %) was prepared by diluting known volume of gum arabic stock solution with deionised water. Prepared GA emulsions and known volume of homogenized fermentation broth was added to the mixing tank, the added solutions were subjected to mixing for about 30 minutes at room temperature to ensure proper interaction of GA micelles and the broth components. Desired pressure was applied to the solution by passing nitrogen from

the pressurized cylinder and the permeate flowrate corresponding to specific pressure was noted down. Samples were withdrawn from permeate and the same was subjected to crotonic acid assay (Law and Slepecky, 1961) to estimate the amount of PHA extracted.

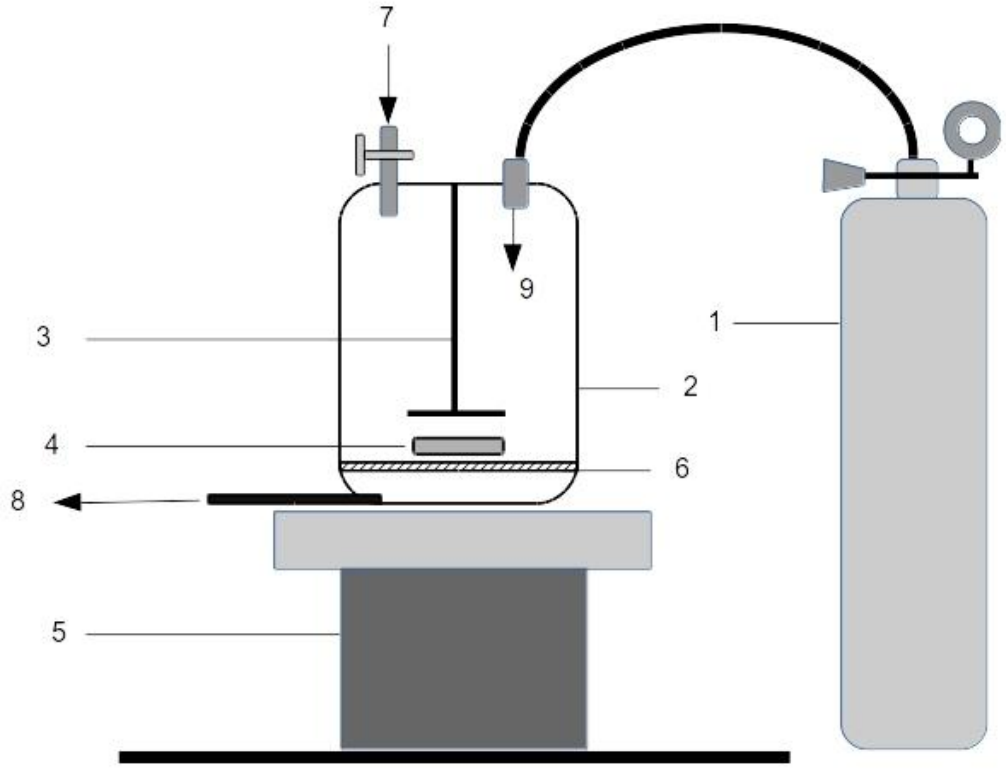


Figure 5.2 Schematic representation of dead end filtration module. 1 – Nitrogen cylinder, 2 - filtration cell, 3 – stirring rod, 4 – magnetic bead, 5 – magnetic stirrer, 6 – membrane on support, 7 – liquid inlet, 8 – permeate outlet, 9 – Gas inlet.

Extraction efficiency parameters were calculated using the following formulas

$$Membraneflux\left(\frac{L}{m^2h}\right) = \frac{Permeateflowrate\left(\frac{L}{h}\right)}{Membranesurfacearea(m^2)} \qquad (5.1)$$

$$Rejectionfactor = \left(1 - \frac{C_p}{C_F}\right) \times 100 \qquad (5.2)$$

Where, C_p – Concentration of PHA in permeate and C_F – Concentration of PHA in feed.

Purity and recovery were calculated using equations 3.1 and 3.2 respectively.

5.4.3 Effect of process variables

GA emulsion with/without addition of alcohol was tested and the system which gave maximum recovery of PHA was fixed to conduct further experiments. Effect of addition of surfactant was studied by adding individual surfactants (TX114, AOT, CTAB) and its varying concentrations (0.1 M to 1.0 M) to the GA emulsion system and the above explained extraction protocol was followed. Extraction process parameters were calculated and the system with minimum rejection of PHA into the retentate stream was chosen to study the effect on membrane extraction of PHA. Combination of mixed surfactants (TX114/AOT/CTAB) was added to the mixture of GA emulsion and homogenized crude fermentation broth, the same was subjected to membrane extraction protocol, and the results obtained were used to calculate extraction efficiency parameters. Mixed surfactant system which gave maximum recovery of PHA was chosen to study the effect of pH (4.5 to 6.5), addition of salts (Na_2SO_4, NaCl, $(NH_4)_2SO_4$ and NH_4Cl) at varying concentrations from 0.1 to 0.5 M, addition of alcohol (ethanol and propanol and their varying concentrations, vol%). The above explained extraction protocol was repeated for each process variable and their effect on extraction efficiency was analysed to screen the system that imparts maximum recovery of PHA in the permeate stream.

5.5 Results and Discussion

Gum arabic – micelle complex coacervate enhanced membrane extraction of polyhydroxyalkanoate from crude fermentation broth was studied by considering different process variables. During membrane extraction, it is assumed that the hydrophobic PTFE membrane facilitates diffusion of hydrophobic solutes present in the feed such as PHA molecules and lipids while hydrophilic solutes such as cellular proteins are rejected from the membrane and are retained within the filtration cell as retentate. It is considered that during membrane extraction, all the GA molecules and surfactant monomers added to the feed mixture interact to form stable GA-micelle coacervate and the monomer concentration of GA/surfactant passing through membrane pores are lesser than the CMC of GA/surfactant and are considered negligible. The overall effectiveness of the cocaervate complex enhanced membrane

extraction process was assessed in terms of distribution coefficient of PHA among the GA-surfactant coacervate complex, PHA rejection %, flux across the membrane, purity and recovery of PHA in the permeate stream.

Available literature on GA emulsions suggests that the GA emulsion droplet size depends on the concentration of GA added to the feed and is reported that the emulsion drop size decreased with increasing GA concentration. However, prevailing the same conditions, the number of stable emulsion complex droplets formed was found to increase (Jafari et al., 2013). On visual observation, it was found that addition of lower concentrations of GA (< 15 wt%) to the feed resulted in highly unstable emulsion-solute complex, although the solution was highly viscous in nature; increasing the GA concentrations higher than 15 wt% formed stable GA-solute complex as a result of smaller eddies generated during solution mixing owing to high viscosity of GA solution (Panagiotou and Fisher, 2012). Higher concentrations of GA is required to form stable emulsions, in case of concentrations lesser than solute to be encapsulated, GA emulsions formed flocculate and coalesce with each other (Williams et al., 1990). In general, biomacromolecules undergo slow diffusion from bulk solution to the oil-water interface and usually the proteinaceous part of the polymer are folded in such a way that hydrophobic sites are hidden while hydrophilic sites are exposed towards the surrounding water molecules. Bulk concentration of GA in water undergoes structural reformation to align over the oil-water interface, exposure of hydrophobic domains of GA emulsion droplet with adjacent droplets results in the formation of network of stable GA emulsions and prevents re-coalescence of emulsion droplets as an effect of steric stabilization (Jafari et al., 2013).

Available literature on the particle size of GA emulsion suggests that the surface-volume mean diameter remains constant around 0.82 μm on maintaining GA concentrations above 10 wt% (Nakauma et al., 2008). As 0.45 μm pore size membrane was used for microfiltration, it is assumed that all the GA emulsions formed are rejected back into the retentate. It has been previously observed by researchers (McNamee et al., 1998) that the average emulsion size was found to grow with the ratio of hydrophobic solute/GA and solely depends on the size of the hydrophobic solute molecule encapsulated. The authors further reported that the

encapsulation efficiency of GA emulsions were high at low hydrophobic solute/GA ratio and was found to decrease with increasing ratio. Since, the experimental work explained in this article emphasize on the effect of different process variables on membrane extraction of PHA from the feed, studies on encapsulation of PHA by GA emulsions and their physical characterization are not of main focus of this manuscript.

5.5.1 Effect of Pressure

Effect of pressure on flux and rejection % of PHA was initially examined in the presence of fixed higher concentrations of GA (25 wt%). Available literatures suggest that addition of ethanol elevates the mean drop size of the gum arabic emulsions (Burgaud and Dickinson, 1990) while lowering emulsification property of GA and its adsorption over oil-water interface (Jafari et al., 2013). Hence, increasing chain length of alcohols – ethanol and propanol which are readily solubilised in water were added at fixed concentration (25 vol%) along with GA (25 wt%) to understand the effect of increasing chain length of alcohol on the membrane flux and membrane diffusion of PHA. Even at low pressure maintained (0.2 bar), inclusion of alcohol to the GA emulsion feed mixture containing homogenized broth lead to increased rejection of PHA into the retentate stream compared to that of pure GA (25 wt%) emulsion system as shown in figure 5.3a. Flux across the membrane and rejection % of PHA in the presence of GA (25 wt%), Ethanol (25 vol%) + GA (25 wt%) and Propanol (25 vol%) + GA (25 wt%) mixed with homogenized crude fermentation broth at different pressure conditions were studied and the results obtained are graphically represented as figure 5.3a. However, minimum variation in the flux was observed for all the three systems studied (figure 5.3b) on maintaining low pressure conditions (0.2 bar). Membrane flux and PHA rejection % was found to increase with elevating pressure and PHA rejection reached a maximum of ~ 92 % for all the systems studied on maintaining maximum inlet pressure of 1 bar, while flux of all the three systems varied drastically from one another on maintaining pressure of 1 bar. Increasing pressure leads to pressure driven solute diffusion across the membrane in turn promotes membrane flux.

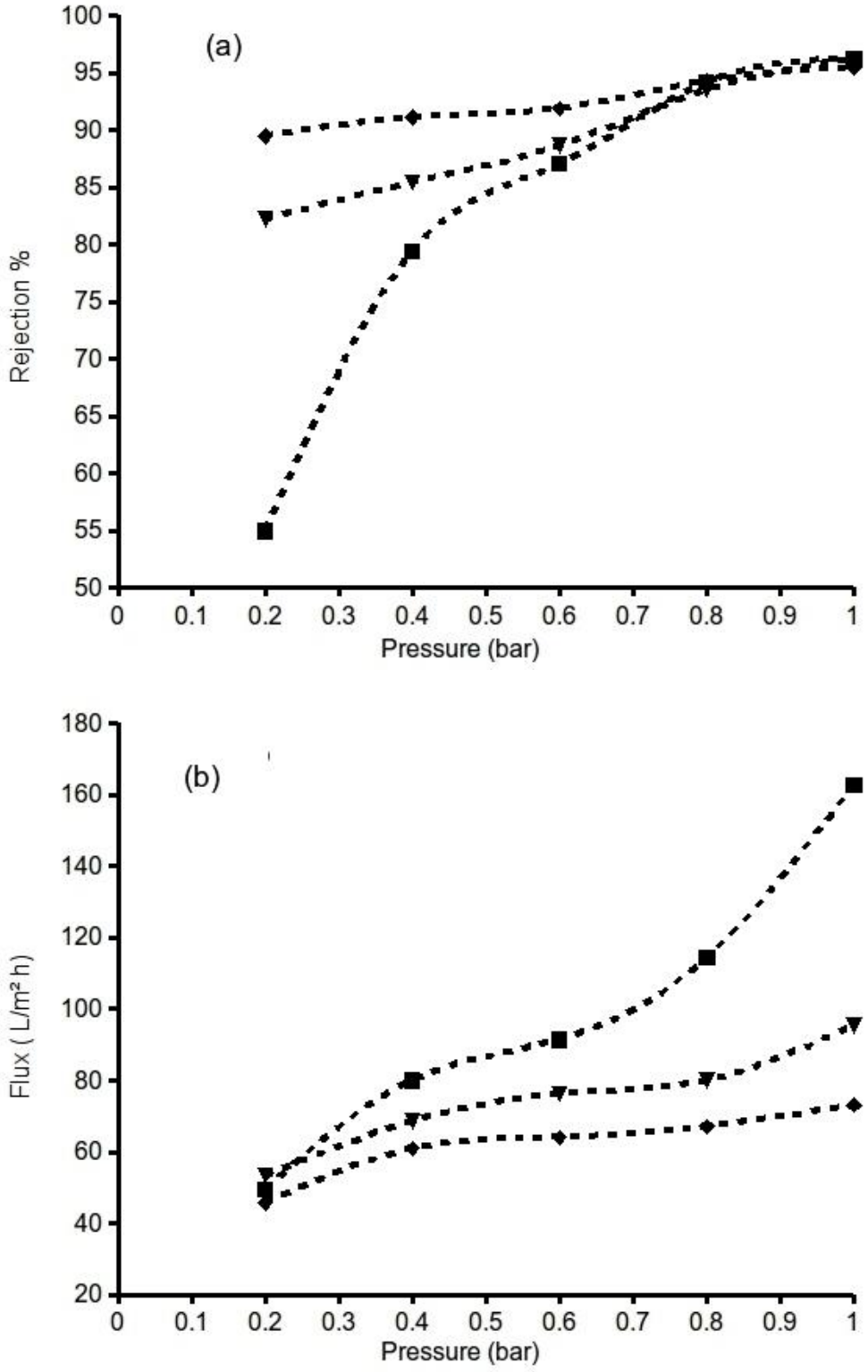


Figure 5.3 Effect of pressure on PHA rejection % (a) and flux (b). ■ - Gum arabic (25 wt%), ▼ - Gum arabic (25 wt%) + Ethanol (25 vol%), ◆ - Gum arabic (25 wt%) + Propanol (25 vol%).

As a result of expansion of GA emulsion with the addition of alcohol, hydrophobic and hydrophilic sites of GA molecules are exposed which might cause hydrophobic interaction of protein part of GA with PHA, while carbohydrate part of GA electrostatically interacts with charged cellular impurities. On applying pressure, weaker hydrophobic bonds are easily interrupted compared to stronger electrostatic interactions; hence, more PHA molecules diffuse across the membrane at low pressure (0.2 bar). On maintaining high pressure, concentration polarization of GA emulsions with attached cellular impurities takes place as a result of emulsion droplet coalescence resulting in the formation of bigger emulsions that blocks the membrane pore and reducing the flux across the membrane (Renard et al., 2012). Membrane flux was found to decrease in the order - GA (25 wt%) > Ethanol (25 vol%) + GA (25 wt%) > Propanol (25 vol%) + GA (25 wt%). On prevailing high pressure, as a combined effect of concentration polarization, restructuring of emulsion complex, membrane blocking and separation of smaller sized impurities from the emulsion complex takes place. Such impurities diffuse across the membrane pores while large sized PHA molecules are rejected into retentate stream. To study the effect of other process variables on the extraction efficiency, further experiments were conducted at an inlet pressure of 0.2 bar.

5.5.2 Effect of surfactants

Effect of addition of different surfactants and its varying concentrations to GA emulsions were studied and their effect on PHA rejection and flux across the membrane are represented as figure 5.5 a and b respectively. Addition of emulsifying agent to micellar solution imparts significant effect on the interactive forces between the surfactant micelles, such developed high interactive forces are said to overcome the steric and hydration forces existing between the coacervate complexes (Chanamai and McClements, 2002). Bhattacharyya and Argillier (2005), report that increasing surfactant concentrations in GA emulsions, declines the surface tension of solution and forms an insoluble bulk complex at certain concentration of surfactant known as critical aggregation concentration (CAC), such CAC values are lesser than the CMC of a given pure surfactant solution.

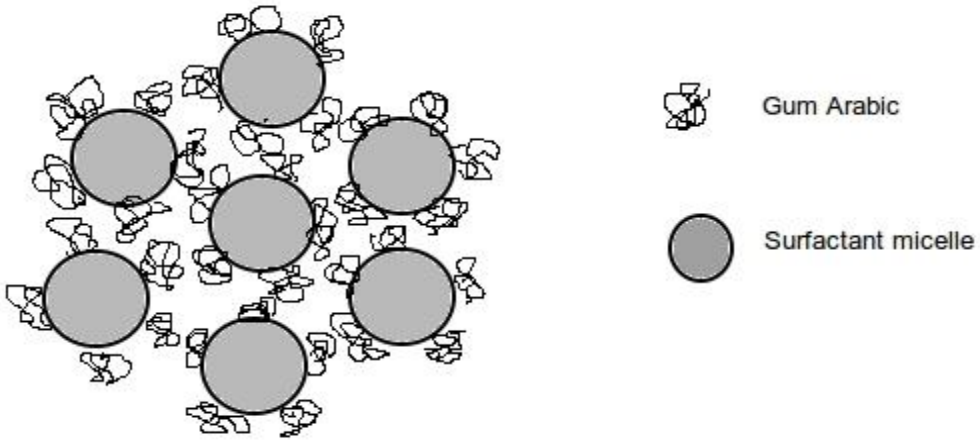


Figure 5.4 Stable micelles formed as a result of steric repulsion between Gum arabic on oil-water interface.

Effect of addition of individual surfactants and its varying concentrations to homogenized fermentation broth and GA (25 wt%) mixture on PHA rejection and membrane flux was studied at a constant pressure of 0.2 bar. GA is strongly negatively charged and addition of charged surfactants to the emulsion system results in electrostatic interaction/repulsion between charged surfactant head group with that of carbohydrate moiety of GA, while addition of nonionic surfactant results in hydrophobic interaction with that of the proteinaceous part of the GA emulsions. It can be inferred from figure 5.5a, that addition of CTAB to emulsion system significantly decreased the rejection of PHA until 0.5 M surfactant concentration and PHA rejection was found to increase with further increasing CTAB concentrations. Similar results have been reported on increasing rejection % with increase in surfactant concentration, when SDS was used during MEUF of Methylene blue from the feed and di-butyl phosphate (DBP) and tri-butyl phosphate (TBP) from the aqueous stream.

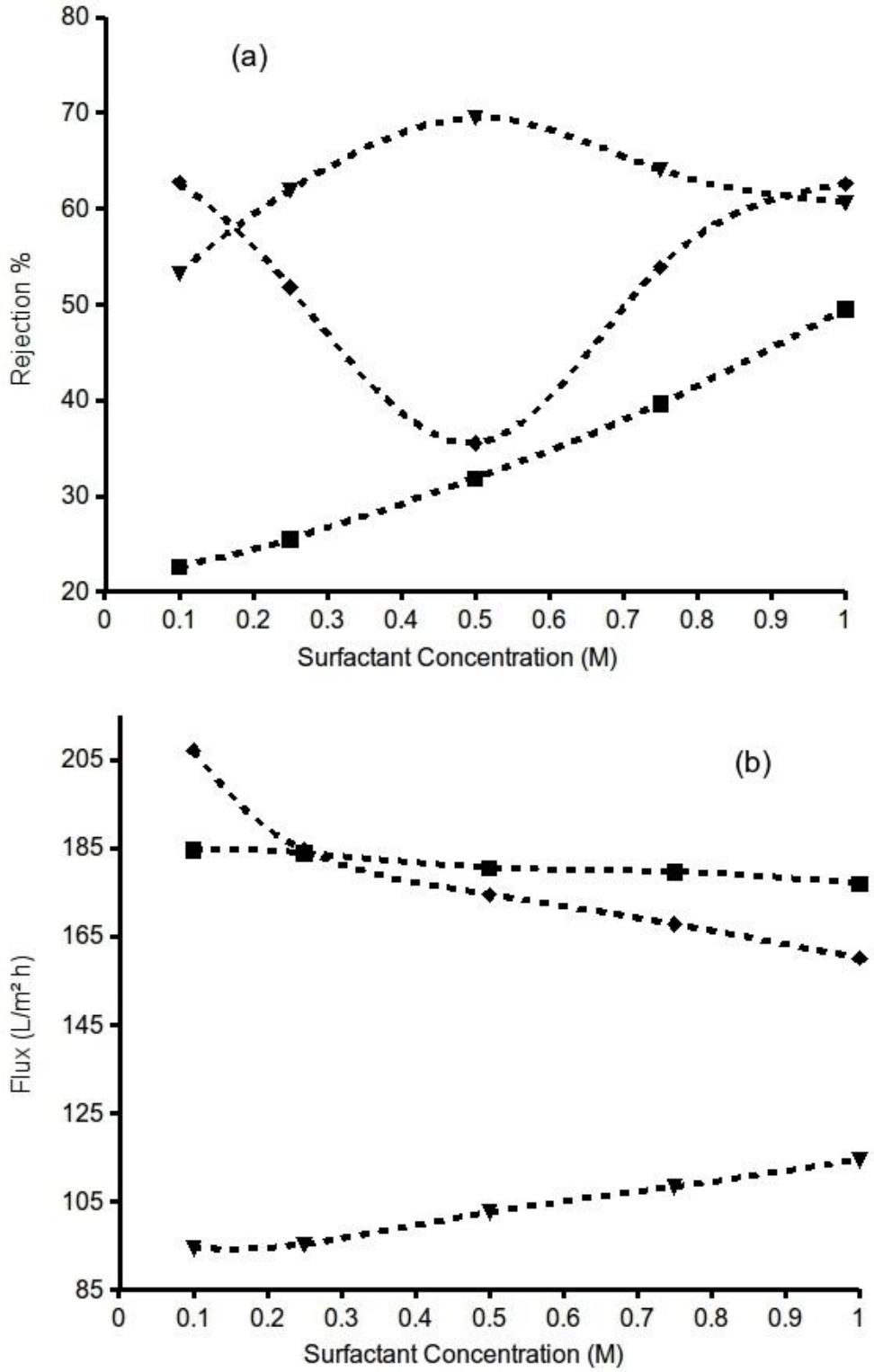


Figure 5.5 PHA rejection % (a) and flux (b) as an effect of individual surfactant concentration. ■ - TX114, ▼ - AOT, ◆ - CTAB.

Earlier report suggest that the formation of GA-micelle complex takes place at a wide concentration of surfactant and the net interaction depends on the overall charge of GA and alkyl chain length of surfactant (Yomota et al., 1986). Cellular impurities present in the feed mixture interact with the coacervate complex via electrostatic interaction due to the presence of positively charged CTAB while PHA molecules interact with coacervate complex through hydrophobic interaction. On applying pressure, weaker hydrophobic interactions are interrupted causing PHA to diffuse across the membrane while cellular impurities remain attached to the coacervate complex, which is evident from the lower PHA rejection % observed at 0.5 M CTAB concentration (figure 5.6 a).

Higher concentrations of CTAB in the feed mixture leads to stronger interaction between negatively charged GA and positively charged CTAB micelles that in turn settle down as coacervate complex over the membrane blocking the pores (Yomota et al., 1986), which results in increased rejection of PHA into retentate stream. Introduction of nonionic surfactant (TX114) leads to hydrophobic interaction between the surfactant tail and hydrophobic polypeptide chain of GA which leads to formation of stable coacervate hydrophobic core (Ribeiro et al., 2004). With increasing TX114 concentrations, PHA rejection was found to increase as a result of stronger encapsulation of PHA within the hydrophobic core of coacervate complex. Thus, addition of inclining concentrations of nonionic (TX114) and cationic (CTAB) surfactants to GA emulsion lead to decline in flux while addition of anionic (AOT) surfactant lead to increase in flux as shown in figure 5.5b. Addition of negatively charged AOT surfactant monomers to the feed induces inter-micelle-emulsion repulsion. With concentrations up to 0.5 M AOT, PHA rejection was found to increase and further increase in AOT concentration might lead to GA emulsion and micelles precipitation; as a result of which most of the PHA molecules are expelled from the coacervate complex and are diffused across the membrane pores, while proteins are retained by the surfactant micelles.

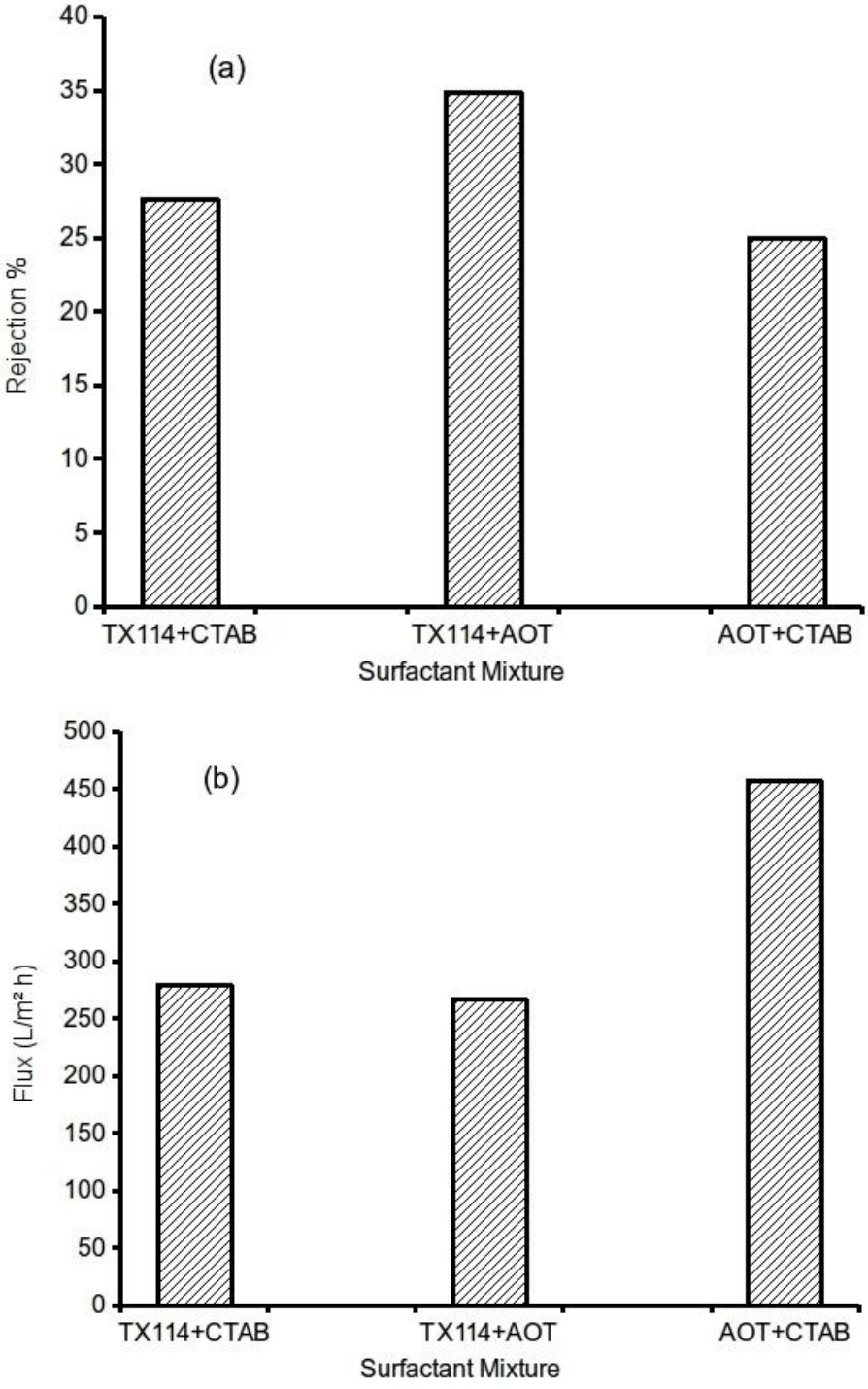


Figure 5.6 Effect of Different combinations of surfactants and their respective weight % on PHA rejection % (a) and flux (b). TX114 (0.1%) + CTAB (0.5%); TX114 (0.1%) + AOT (0.1%); AOT (0.1%) + CTAB (0.5%).

Combination of individual surfactant concentrations that gave higher recovery of PHA in the above study was considered to verify their effect as mixed surfactants on PHA rejection and membrane flux. Coacervate complex made up of mixed micelle consisting of AOT (0.1 wt%) and CTAB (0.5 wt%) and GA emulsion resulted in minimum rejection of PHA into retentate (figure 5.6a) and a higher membrane flux (figure 5.6b) among the surfactant mixtures studied. As discussed earlier, presence of TX114 in the coacervate complex induces stronger hydrophobic interaction with PHA molecules retaining them within the retentate stream, declining PHA extraction. Cationic-anionic surfactant mixture (AOT+CTAB) exhibits stronger electrostatic interaction with cellular impurities and weaker hydrophobic interaction with PHA, thus increasing the diffusion of PHA and membrane flux. Similar results have been reported on addition of nonionic surfactant- tween 80 to charged surfactant cetylpyridinium chloride on MEUF of trichloroethylene and chromate (Lee et al. 2005).

5.5.3 Effect of broth pH

Effect of pH was studied by varying the pH of the homogenized crude broth and adding it to the coacervate complex - GA emulsion with mixed surfactant (AOT+CTAB) micelle that gave maximum extraction of PHA. From figure 5.7, it can be observed that increase in the broth pH results in decreasing PHA rejection and increasing membrane flux until pH value of 6; on further approaching neutral pH, rejection % increased while membrane flux was found to decline. At broth pH - 6, net negative charge of the GA emulsions will undergo strong electrostatic repulsion from AOT head groups while they interact with CTAB head groups in the presence of mixed cationic-anionic micelles. Presence of large sized AOT head groups will impose stronger inter-micellar repulsion exposing the hydrophobic sites of the coacervate complex which interact with PHA and other hydrophobic solutes. Since, electrostatic interaction is stronger than the weaker hydrophobic interactions, proteins are strongly attached to the GA-micelle complex while weakly bonded PHA molecule diffuses across the membrane on application of pressure.

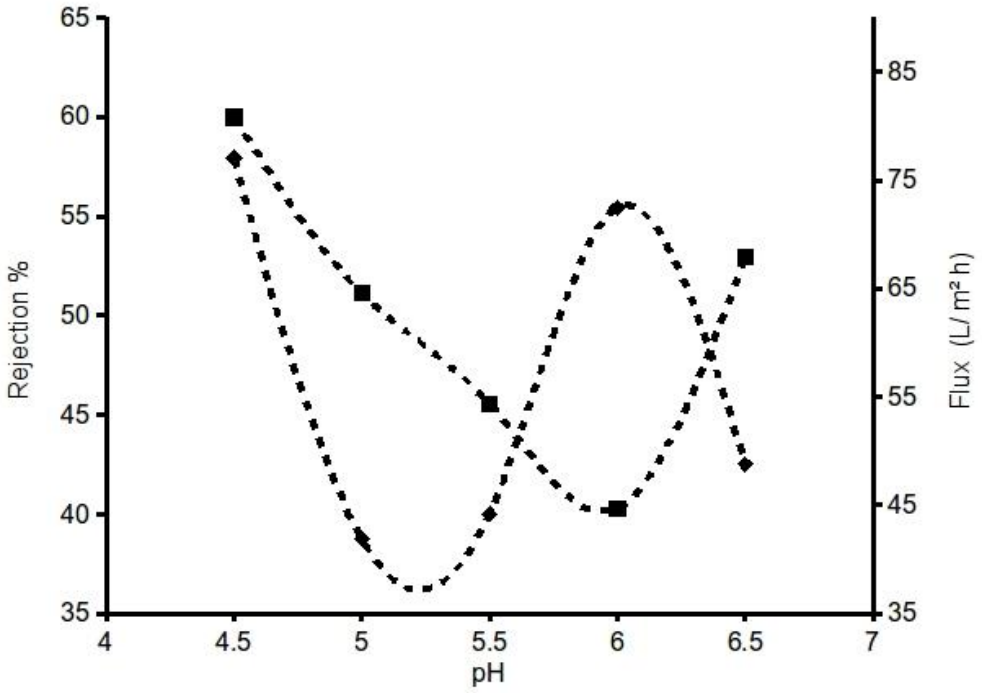


Figure 5.7 Effect of broth pH in the presence of mixed surfactants on PHA, ◆ - rejection % and ■ - membrane flux.

GA possess strongly acidic isoelectric point and increasing pH above 3, results in increase in the net negative charge of the emulsion as a result of protonation of amine groups or deprotonation of carboxyl groups of GA that results in the exposure of charged sites (Chanamai and McClements, 2002). Charged cationic-anionic micelles can readily interact with the charged sites of GA and form stable coacervate complex that in turn encapsulates most of the cellular proteins. Espinosa-Andrews et al. (2007) suggest that GA emulsion formation was high between pH of 3.0-6.0 and with further decrease/increase in pH, formation of emulsion was found to decline. Results obtained are in accordance to the above cited reference indicating that minimum PHA rejection was obtained at pH of 6.0 while further increase in pH should result in dissolution of coacervate complex, deposition over the membrane and membrane pore blocking.

5.5.4 Effect of Electrolyte

Four different electrolytes from Hofmeister series namely ammonium sulphate, ammonium chloride, sodium sulphate, and sodium chloride at varying concentrations were considered to study its effect on coacervate complex enhanced membrane extraction of PHA. Experiments conducted reveal that PHA rejection was

less when electrolytes with weaker cations were added to the feed mixture compared to electrolytes with stronger cation (figure 5.8). As shown in figure, rejection of PHA and membrane flux was minimum in the presence of 0.5 M ammonium chloride among the different salts studied. With the addition of ammonium sulphate and sodium chloride, PHA rejection was found to decrease and then increase, while with increasing concentrations of ammonium chloride and sodium sulphate, PHA rejection was found to increase and then decrease. For increasing concentrations of all the salts studied, it was observed that membrane flux was found to decrease steadily; while ammonium sulphate offered maximum flux across the membrane, minimum flux was obtained for ammonium chloride.

Earlier reports suggest that (Anderson et al., 1990, Jayme et al., 1999) increasing salt concentration screens the electrical double layer over the emulsion there by reducing the zeta potential and also reduces the overall viscosity of the solution, which enhances interaction of coacervate complex and charged cellular impurities. Electrolytes containing stronger cation induce surface tension while presence of weaker cations decline surface tension, there by leading to formation of weaker and stronger coacervate complex respectively. Presence of larger ionic radius of ammonium leads to electrostatic repulsion between coacervate complexes in the feed mixture there by exposing electrostatic and hydrophobic binding sites that can bind with cellular proteins and PHA respectively (Israelchvili, 2011). Increasing the ammonium concentration in the feed mixture leads to disruption of surfactant micelles and release of solute molecules attached to the coacervate complex. At high electrolyte concentrations, ammonium and chlorine ions present in the solution interacts with charged cellular impurities leading to precipitation of coacervate complex with encapsulated proteins over the membrane pores while PHA molecules are expelled from the complex which leads to decline in PHA rejection.

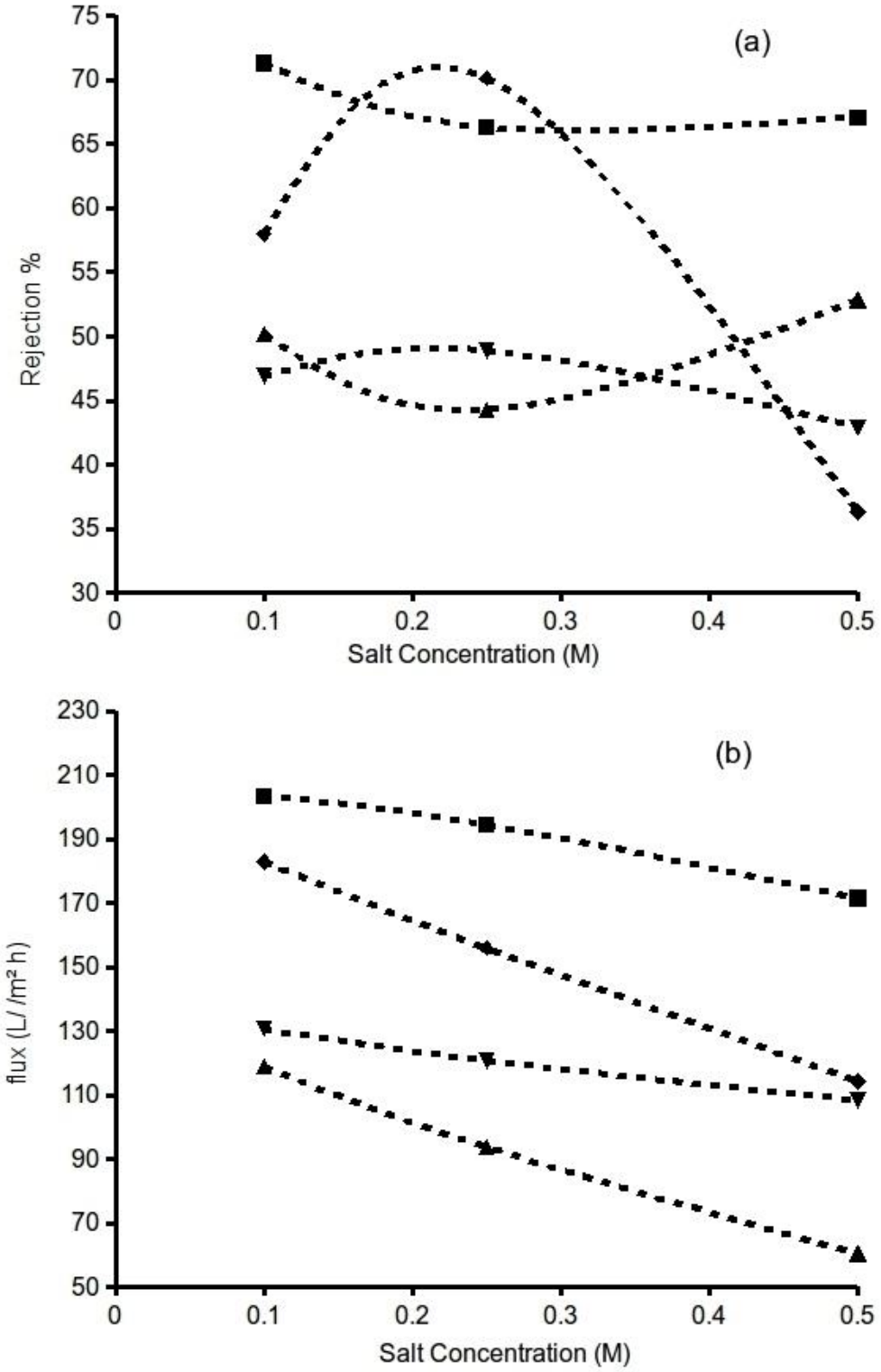


Figure 5.8 Effect of Electrolytes and its varying concentrations on PHA rejection % (A) and membrane flux (B). ■ - Ammonium sulphate, ▲ - Ammonium chloride, ▼ - Sodium sulphate, ◆ - Sodium chloride.

Concentration polarization of coacervate complex along with solutes leads to gel layer formation and membrane blocking, there by membrane flux is gradually

reduced. Earlier report by Mantell (1954) suggests that monovalent cations increase the viscosity of the GA solution while divalent cations decrease the viscosity of the solution. Considering the fact, presence of sodium in the salts studied should enhance the viscosity, however presence of sulphate ion will lead to electrostatic repulsion on interacting with the head groups within the coacervate complex and a result settle down over the membrane and block the membrane pores. Chloride ion being a weaker anion does not significantly affect the interaction among the coacervate complex and as a result of sodium influences the flux, when sodium chloride is added to the coacervate complex.

Ammonium owing to its larger ionic radius and divalent charge decreases the viscosity; however, presence of ammonium ions lead to steric repulsion among coacervate complex and leads to precipitation over the membrane surface and membrane pore blocking, when ammonium chloride is added to the feed mixture. On addition of ammonium sulphate, ammonium tends to decrease the viscosity of the solution while presence of sulphate counter acts to maintain the integrity of coacervate complex as a result of which membrane flux is comparatively high.

5.5.5 Effect of alcohol

Addition of alcohol imposes a significant effect on the GA emulsion system as discussed in section 3.1. Hence experiments were conducted to understand the effect of addition of alcohol (ethanol and propanol) to the coacervate complex containing mixed cationic-anionic surfactants. It was observed that addition of ethanol increases PHA rejection into the retentate stream and decline the flux across the membrane compared to addition of propanol at the concentrations studied as shown in figure 5.9 (a,b).

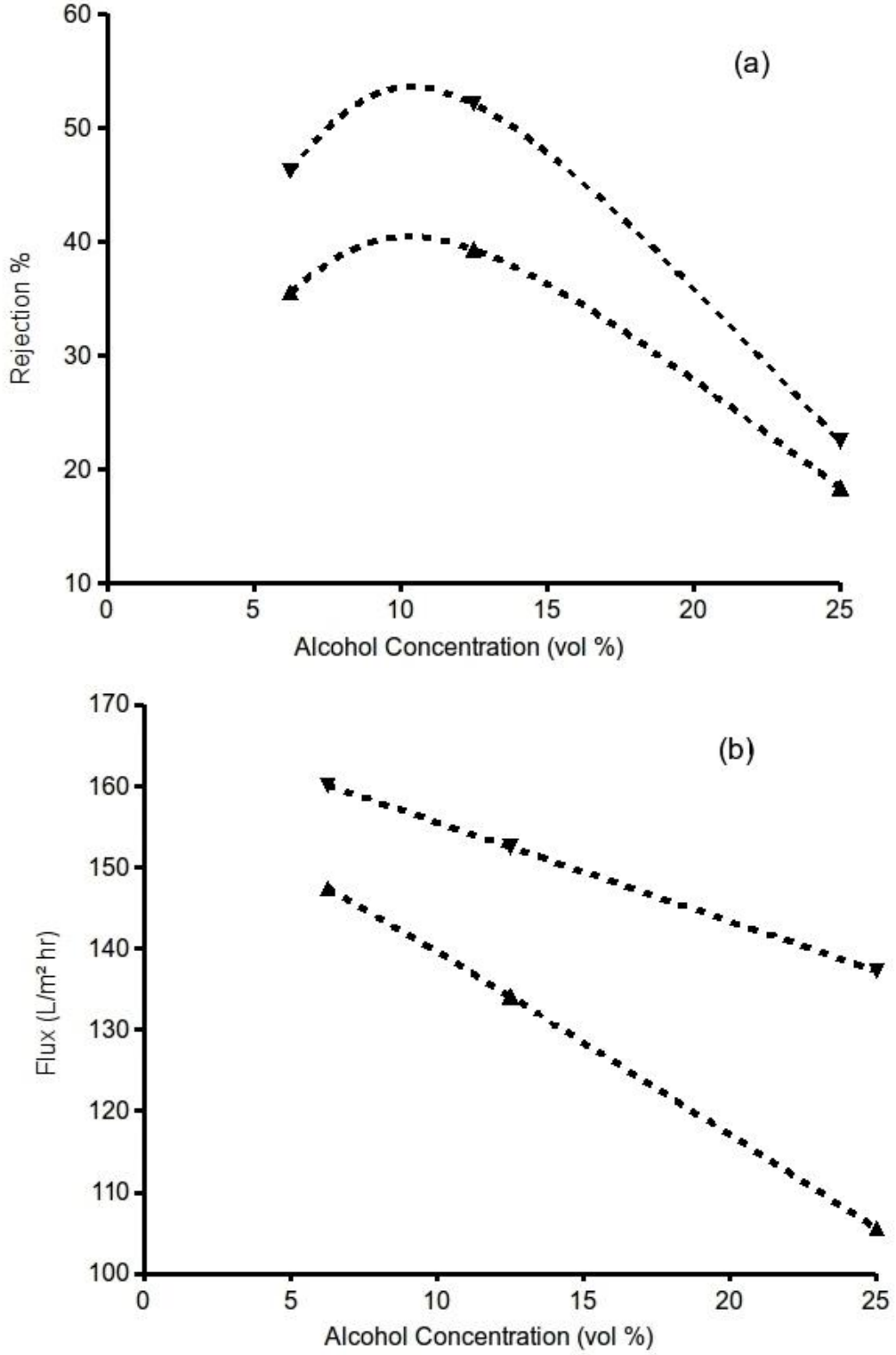


Figure 5.9 Effect of alcohol in the presence of mixed surfactants and broth pH - 6 on PHA rejection % (a) ▼ – Ethanol, ■ - Propanol and membrane flux (b) ◆ - Ethanol, ▲ - Propanol.

The obtained results are concurrent with literature reference (Burgaud and Dickinson, 1990), addition of alcohol lowers the interfacial tension of GA on oil-water interface thereby causing larger emulsion size. The authors have reported that short chain length alcohol (Ethanol) decrease the micellar size and CMC while higher chain length alcohol (Propanol) initially reduce the micellar size and then enhance micellar growth at higher concentrations. Thus, addition of high concentrations of propanol to coacervate complex results in enlarging the size of complex. Enlarged size leads to exposure of hydrophobic sites of the coacervate complex, thereby causing maximum rejection of PHA and its diffusion across the membrane while cellular proteins are retained by the coacervate complex as a result of stronger electrostatic interaction.

5.6 Summary

Addition of surfactants to the feed mixture increases the number of GA-micelle complex which are formed at a lower CAC. Hence, large number of micelle complex formed impose stronger hydrophobic interaction with PHA which is reflected as higher distribution coefficient and recovery compared to that of pure GA emulsions. However, individual ionic surfactants and its respective concentrations failed to increase the distribution coefficient of PHA as they indulge in stronger electrostatic force interaction with cellular proteins, as a result of which purity of PHA extracted decreases. TX114 at a minimum concentration of 0.1 M possess higher distribution coefficient compared to other individual surfactant systems studied and enhanced purity and recovery of PHA. Experiments on mixed surfactants were conducted to utilise the combined effect of electrostatic interaction/repulsion offered by ionic surfactants (CTAB, AOT) and hydrophobicity offered by non-ionic surfactant (TX114). Mixed surfactant system with TX114 failed to provide a significant recovery and distribution coefficient as a result of stronger hydrophobicity imparted by TX114 surfactant tail; while mixed ionic surfactants exhibit combined inter-GA-micellar complex electrostatic interaction or/and repulsion and exposure of hydrophobic sites of GA and its interaction with PHA. The electrostatic interaction of the molecules in the mixture were modified by changing the pH of the system and by adding electrolytes. It was found that the distribution coefficient and recovery of PHA

was found to decrease in comparison to native GA-micelle complex whose pH is unaltered.

Table 5.2 Extraction efficiency and coefficients obtained as a result of effect of process variables.

Process variable	Distribution Coefficient (D)	Rejection %	Flux (L/m^2 h)	Purity %	Recovery %
Gum arabic 25 wt%, 0.2 bar	0.8197	54.954	49.571	84.835	45.046
Gum arabic 25 wt%, TX114 0.1 M, 0.2 bar	3.4270	22.589	184.637	96.146	75.826
Gum arabic 25 wt%, AOT (0.1M) + CTAB (0.5 M), 0.2 bar	2.8433	26.024	457.579	89.234	73.976
Gum arabic 25 wt%, AOT (0.1 M) + CTAB (0.5 M), 0.2 bar, pH – 6	1.4821	40.291	72.450	71.010	59.709
Gum arabic 25 wt%, AOT (0.1 M) + CTAB (0.5 M), 0.2 bar, Ammonium chloride 0.5 M,pH-6	1.7528	36.328	114.395	75.723	63.672
Gum arabic 25 wt%, AOT (0.1 M) + CTAB (0.5 M), 0.2 bar, Propanol 25 vol%, pH -6	4.4464	18.362	137.274	97.089	81.638

Addition of electrolyte screens the micelles by forming Gouy Chapman layer that reduces the exposure of hydrophobic sites available for PHA interaction. Addition of propanol at higher volume increases the emulsion size thereby increasing the interaction of GA-micelle complex with proteins and impurities and diffusion of PHA. Hence, highest purity, recovery and distribution coefficient with lower rejection % of PHA was obtained in the emulsion system containing GA 25 wt%, AOT (0.1 M) + CTAB (0.5 M) with 25 vol% propanol at an inlet pressure of 0.2 bar; interestingly, flux was found to also increase from 49.57 (Gum arabic 25 wt% emulsion system) to 137.274 L/m^2 h. The results indicate that the complex emulsion mixture is an effective extraction system for membrane assisted enrichment of PHA from homogenized fermentation broth yielding higher PHA recovery and flux.

Complex coacervates formed by mixing GA and surfactant micelles are large enough to be screened by micro filters and so were successfully employed to encapsulate release PHA from crude homogenized fermentation broth. As a charged biopolymer GA actively interacts with mixed ionic surfactants compared to surfactant mixtures containing nonionic surfactants. Though lower chain length alcohols doesn't show significant effect on encapsulation efficiency of surfactant micelles, its addition to the feed mixture had a drastic influence on the structural properties of GA and as a result rejection % was lowered with the addition of propanol. Developed GA mixed micelle coacervate assisted membrane filtration process assists in separation and purification of PHA, while separating co-products from the fermentation broth such as enzymes and other microbial proteins that adds up to the process economy.

CHAPTER 6

MICELLAR LIQUID CHROMATOGRAPHIC SEPARATION OF PHA

High purity and retaining native form of PHA after purification is a prerequisite, if the PHA is to be utilised for the medical and pharmaceutical applications. The purified PHA should be free from pyrogens to the maximum extent. Although, developed CPE, UACPE and MEMF techniques offers selective partitioning/purification of PHA, an industrially employed unit operation which involves easy operation and maintenance that reflects low operational cost with high purity is in high demand. The requirement of high pure PHA intensifies the search and development of a novel separation process, which offers the highest purity of PHA in a single step. Chromatography is said to be the polishing step in transforming the separated and purified product to marketable form. Hence, this chapter discusses the implementation of simple yet highly efficient chromatographic process which may provide superior quality of PHA. It is well known that surfactant based micellar extraction offers high specificity towards solutes of interest, ease in preparation and handling, reusability of micelles and low concentrations of surfactants required to form micelles, which are ultimately lowers the raw material cost and operational cost. Hence the present work is focused to implement the nonionic surfactant based micellar phase as mobile phase in chromatography to purity PHA. The developed chromatographic process enable to load the crude fermentation broth directly to the column without any pre-treatment and concentration steps for the solute, unlike the requirement of any conventional chromatographic separation technique. Thus nonionic surfactant based micellar liquid chromatography emphasizes direct separation and detection of PHA from crude broth, while reducing the usage of highly volatile, organic solvents.

6.1 Chromatography

Chromatography is an industrially employed downstream processing technique widely utilised for the selective purification of solutes from feed, based on difference in solutes adsorption on to an adsorbent packed column. The technique has

been accepted and licensed to separate and purify bio molecules of interest from the feed by several regulatory bodies and is often included as the final purification step (product polishing), which offers maximum purity of about 99 %. Adsorption of solute within the pores of solid adsorbent packed inside the column is achieved by liquid mobile phase (Witkiewicz 2000). During chromatographic operation, two different mobile phases are employed; one of the mobile phase which aids to carry the injected solute into the column and its adsorption on to the column packing, while another mobile phase is used to elude the solute from the column. Mobile phases in general can be anything ranging from buffer to salt solution or an organic solvent, for HPLC operation, organic solvents are used as the base to which salts or buffers are added. During reverse phase HPLC, polar solvents are used to elute nonpolar solutes adsorbed onto nonpolar column material, while in normal phase HPLC operation polar solutes are eluted using nonpolar solvents from polar adsorbent column bed. Isocratic elution employs mixing of two different mobile phases at a fixed composition ratio and is used to adsorb and elute the solutes; whereas gradient elution is employed to increase the elution time difference between the solutes by running two different mobile phases in varying proportions with respect to time. Stationary phase is usually the mild steel or glass column packed with silica and the adsorption capacity of the column varies with the chemical composition of silica packing.

During chromatographic separation, the solutes that are strongly adsorbed onto the column bed are bound to the silica while the unbound solutes elute out of the column at a shorter time right after sample injection by diffusing across the void volume present in the column bed. Among the various chromatographic techniques developed, reverse phase high performance liquid chromatography is given high importance owing to its ability to separate hydrophobic solutes, when water itself can be employed as a mobile phase apart from utilising most of the polar solvents such as acetonitrile, methanol etc. (Neue 1997, Curling 2007, Synder et al. 2012). Although, chromatography offers various advantages, a few drawbacks such as usage of petrochemical based solvents and its disposal stands a major issue. Industrial chromatographic operations involve the usage of large volumes of solvent which in turn increases the overall operation cost and has reusability issues.

Peak efficiencies highly depend upon the mobile phase composition, apart from column conditions maintained during chromatographic separation. The partitioning of solutes from stationary phase to mobile phase depends upon the solubility of the solutes in the mobile phase and its composition and most of the solutes have same partitioning coefficient and elute at the same retention time leading to poor resolution (Kirkland et al. 1977, Neue 2005). Direct injection of biological samples have been strongly avoided when using solvent based chromatographic separations, as the complex feed of bio molecules are believed and reported to disrupt the column environment and contaminates the column as they are strongly retained within the column and are not completely eluted. With increasing demand towards green chemistry and sustainable process development and operation, identification and usage of novel mobile phases is major topic of interest among chromatographic researchers.

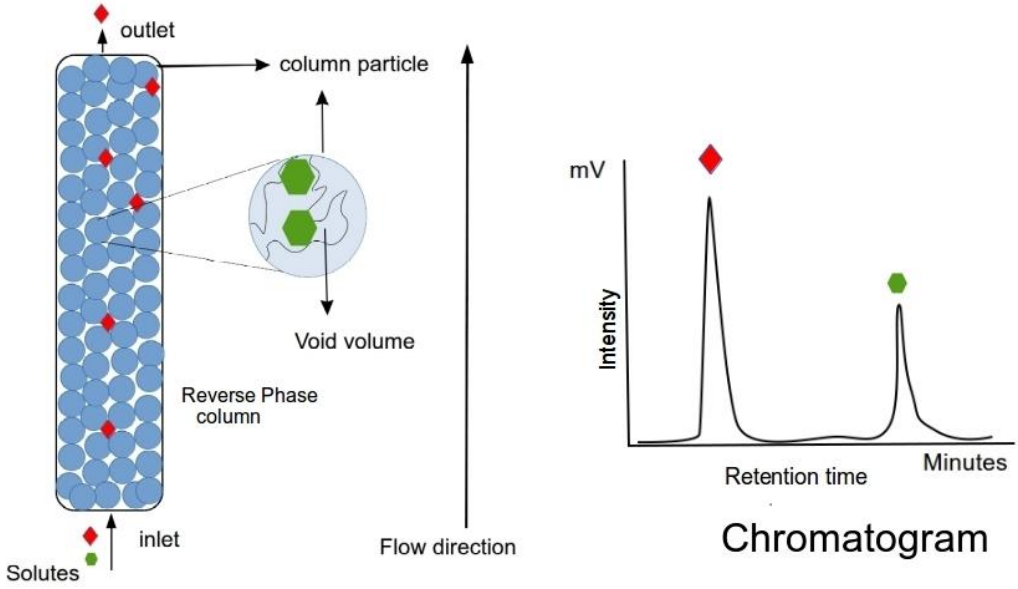


Figure 6.1 Working principle of Chromatography.

6.2 Micellar liquid chromatography

As it is well known that surfactant based micellar extraction offers high specificity towards solutes of interest, ease in handling and operation of a micellar system towards extraction of solute and reuse of used surfactant micelles, very low concentrations of surfactants required to form micelles which ultimately lowers the raw material cost and operational cost. Armstrong and Henry (1980) introduced the

usage of micelles as mobile phase in high pressure liquid chromatography, since then several researchers have attempted to elaborate its usage towards detection of several solute molecules of interest present in the biological feed. During RP HPLC, non-polar solutes interact strongly with the non-polar stationary phase, while polar solutes are easily eluted from the column on introducing polar solvents as mobile phase and spend very little time interacting with the column. Micelles alter the interactions between the strongly bound hydrophobic solute with the column by solubilising the solute within the hydrophobic micellar core and elute them out of the column particles successfully. As surfactant solution is run through the column, surfactant monomers present in the solution and those that detach from the micelles are adsorbed on to the column bed. Hydrophobic tails interact with the column packing forming a stronger hydrophobic layer while the hydrophilic tail protrudes out that can interact with the solutes and polar solvent.

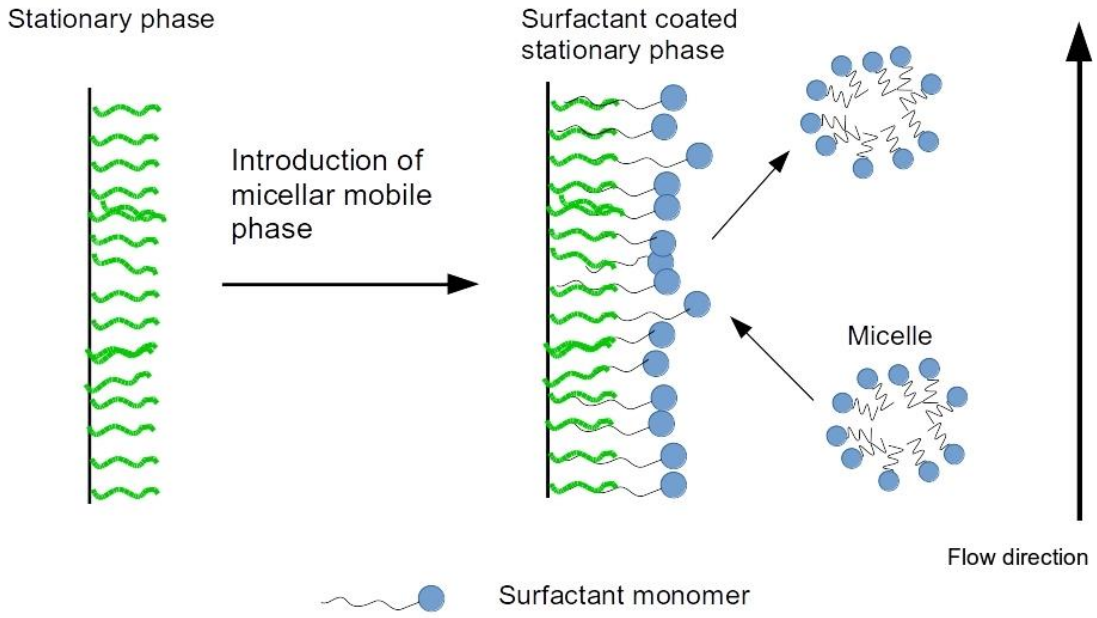


Figure 6.2 Introduction of micellar mobile phase to a reverse phase column.

When a mixture of solutes are injected in to the column, water soluble solutes partition between bulk mobile phase, micelles in the mobile phase (micellar pseudo phase) and the stationary phase. Nonpolar solutes get partitioned between micellar pseudo phase and the stationary phase. When a hydrophobic solute interacts with the surfactant coated stationary phase, micelles passing across the column solubilize the

solute and encapsulates the same within its hydrophobic micellar core and elutes it out of the column. Partitioning of solute between micellar pseudo phase and the surfactant coated stationary phase is influenced by altering several process variables such as surfactant concentration, addition of organic solvent to the micellar mobile phase and its varying concentration, pH of the micellar mobile phase, change in ionic strength etc. (Basova et al. 1999, Berthod 2000).

6.3 Review of Literature

6.3.1 Selection of surfactant to form micellar mobile phase

The selected surfactant to form mobile phase should possess a low CMC, since higher CMC values denote higher surfactant concentration requirement to form mobile phase that impart viscosity to the solution. Such surfactants and its concentrations lead to high back pressure and serious damage to the column by eroding silica with it and also induce background noise in the UV detector (Kalyankar et al. 2014). Presence of organic solvent causes a change in the CMC of a surfactant that not only lowers the amount of surfactant required to form micelle but also influences the interaction of such organic solvent with the solutes.

Peak broadening is a common problem that occurs during operation of MLC and is attribute to several factors such as poor wetting of the column by the surfactant micelles, partitioning of solutes to and from micelles, bulk mobile phase and the stationary phase column, micelle interaction with solutes bonded to stationary phase column pores that makes it a complex process (Kalyankar et al. 2014).

Addition of organic solvents to micellar mobile phase results in hybrid mobile phase that helps to reduce the retention time of a solute during MLC and to increase the peak efficiency. Competitive binding of alcohol and surfactant onto the stationary phase results in reduction of surfactant adsorption onto the column, thus reducing the amount required to coat the stationary phase. With increasing solvent concentration in the mobile phase, the amount of surfactant required to coat the stationary phase drastically reduces. Higher chain length alcohols such as butanol and pentanol in lower concentrations reduce the CMC of a micellar solution compared to short chain length alcohols such as methanol, ethanol and propanol. However, use of long chain

length alcohols lead to formation of reverse micelles and interrupts the micelle based separation process (Lopez-Grio 1998, 2000).

As surfactant mobile phase is run through the column, surfactant monomers detach from the bulk micellar phase, fill the surface of pores present within the column particle, altering the column's polarity, pore volume and surface area of the pores present for solute interaction. Presence of surfactant reduces the surface area and pore volume of the column and imparting weaker interactions between surfactant coated column and the solutes in the feed. Presence of micelle brings about a change in solute solubility, acidity and reaction rates unlike conventional organic solvent based mobile phases. Surfactant charge and tail length influence the column parameters. With the addition of SDS and CTAB, amount of surfactant adsorbed was found to increase in the order of silica < cyanopropyl < methyl < octyl < octadecyl while the polarity was found to decrease. Presence of nonionic surfactants impart polarity change of the column while mobile phase made up of charged surfactants induce net positive or negative charge to the column. NMR studies on SDS coated stationary phase revealed that the hydrophobic tail bonds with the C18 - alkyl chain of silica stationary phase and the sulphate group of the surfactant protrudes out forming a negatively charged bed which can interact with the solutes (Lavine et al.1996). In case of CTAB, the head group interacts with the silanol group of the stationary phase while the hydrophobic tail protrudes out for interaction with solutes creating a hydrophobic column bed. Solutes are solubilised in one among the three following regions of a micelle (i) hydrophobic micellar core (ii) hydrophilic surfactant head group (iii) palisade region – region between the surfactant head group and the tail (Ruiz-Ángel et al. 2009, Kalyankar et al. 2014).

6.3.2 Effect of stationary phase

Effect of stationary phase have the effect of stationary phase physical parameters on the peak efficiency during micellar liquid chromatography also been studied and reported in the literature. To elucidate the stationary phase physical parameters on the peak efficiency during micellar liquid chromatography. Reduced peak efficiency during micellar liquid chromatography has been overcome with the use of short columns and columns packed with short chain length column materials.

Excluded micelles will not be able to interact with the solutes unless they are eluded out of the stationary phase pores, higher surfactant concentrations are required to elute strongly hydrophobic solutes. Nonionic surfactants form large sized micelles unlike ionic surfactant based micelles and are easily excluded out of the pore as a result of steric repulsion. Previous studies on pore size of the column and its effect on exclusion of solutes by micelles revealed that the larger pore size of column allow better penetration of micelles and elution of solutes in less time, however, increasing the stationary phase pore size reduces the volume and specific surface area of the stationary phase (Borgerding et al. 1989). Silanol groups which are ionized in acidic or basic conditions are coated on to the porous silica column and, positively charged solutes interact with the silanol group and cause peak tailing. Effect of silanol on the elution efficiency is altered with change in pH (Nawrocki 1997). Studies conducted with SDS and CTAB as micelle mobile phase caused stronger adsorption of monomers onto the column, formed Donnan like potential on the surface of the pores that repel like charged species (Ruiz-Ángel et al. 2009). On continuous run of mobile phase, surfactant monomers coated on the pore surface repel the micelles entering the column. Studies on various pore size of the column and usage of different surfactants as mobile phase lead to a conclusion that increasing porosity of the column lead to reduced surface area and stationary phase volume (Ruiz-Ángel et al. 2009). Thus, surfactant micelles can easily penetrate through the larger pore sized columns and elute out the bound solutes in shorter elution time.

6.3.3 Effect of mobile phase parameters

Ionization state of the solute plays a vital role in its interaction with the column material during RPLC, the peak efficiency can be altered by varying the pH of the mobile phase. PH shift brings about a change in solute retention which also has a parallel effect on solute specificity when mixture of solutes are mixture of solutes is injected into the column, making it a complex phenomenon. However, such problems have been resolved with the usage of pH adjusted hybrid micellar mobile phases, several models have been developed to predict and analyse the effect of pH on peak efficiency (Ruiz-Ángel et al. 2009, Kalyankar et al. 2014). On utilising anionic SDS to form micellar mobile phase, pH range of 2.5-3.0 has been maintained to increase

the separation of solutes and its resolution to separate weak acids which works based on the presence of protonated groups of the acidic solute. While maintaining such low pH for basic solutes does not affect the retention of the solutes, it only intends to increase the peak efficiency (Kalyankar et al. 2014). By maintaining basic pH of the micellar mobile phase, acidic solutes are easily eluted out from the column there by increasing the column life and offers higher separation and selectivity of the solutes. In recent studies, carboxylic acids with carbon atoms of up to 6, were tested for their efficacy to modify the micellar mobile phase pH and offers lower viscosity to the mobile phase unlike addition of aliphatic alcohols with similar carbon atoms. Addition of carboxylic acids to SDS was found to impart lower hydrophobicity unlike its aliphatic alcohol counterpart and so its addition influenced the peak resolution but not the other peak efficiency parameters (Boichenko and Bertho 2010).

6.3.4 Solute partitioning in Micellar liquid chromatography

Partitioning coefficient of solutes are explained based on three coefficients, partitioning between water and stationary phase (P_{WS}), partitioning between water and micellar mobile phase (P_{WM}) and partition between micellar mobile phase and stationary phase (P_{MS}). As P_{WS} increases, retention of solute increases while increasing PWM leads to elution of solute by the micelle mobile phase (Hernández and Alvarez-Coque 1992). When a solute interacts with surfactant micelle, change in the micellar mobile phase alters the retention time, such as increasing surfactant concentration reduces the retention time or remains unaltered with change in micellar mobile phase composition. Physiochemical models and empirical equations have been developed to explain the solute interaction in micellar liquid chromatography. Physiochemical models have been developed based on surfactant concentration, pH of the mobile phase, organic solvent concentration (Ref). Developed equations have been for micellar mobile phases and hybrid micellar mobile phases containing organic solvents.

Several models have been developed to predict retention behaviour of solute during micellar liquid chromatography. Armstrong and Nome (1981) proposed a model that considers the partitioning of solute between bulk water, micelles in the

mobile phase and the stationary phase. The following equation is used to describe the partitioning behaviour

$$\frac{V_e - V_0}{V_s} = \frac{k}{\varphi} = \frac{P_{ws}}{1 + \upsilon(P_{wm} - 1)[M]} \qquad (6.1)$$

where V_e – Volume of mobile phase required to elute a solute from the column

V_s – Volume of surface on the stationary phase

V_o – Void volume of the column

φ – phase volume ratio, V_s/V_o

κ - Retention factor

υ – partial specific volume of surfactant in micelle-micelle

P_{ws} – Partition coefficient between solvent and stationary phase

P_{wm} – Partition coefficient between solvent and micelle

M – monomeric surfactant available to form micelle (Surfactant concentration - CMC)

while the equation is reduced to the following equation (6.2) in the absence of micelles

$$V_e = V_0 + V_s P_{ws} \qquad (6.2)$$

Arunyanart and Cline-Love (1985) developed a model to describe the association equilibria existing between bulk water in the mobile phase and the stationary phase binding sites with that of monomeric surfactants present in the micelle. The described equation develops a hyperbolic correlation.

$$k = \varphi \frac{[A]}{[A] + [AM]} = \frac{\varphi K_{AS}[S]}{1 + K_{AM}} = \frac{K_{AS}}{1 + K_{AM}[M]} \qquad (6.3)$$

where K_{AS} and $K_{AM\ are}$ binding constant of solute with stationary phase and that of solute with surfactant monomer within the micelle respectively. K_{AM} is multiplied with aggregation number to obtain binding constant of a whole micelle. [S] – activity of stationary phase.

$$\frac{1}{k} = \frac{1}{K_{AS}} + \frac{K_{AM}}{K_{AS}}[M] = C_0 + C_1[M] \qquad (6.4)$$

Foley (1990) developed a model to describe the importance of association equilibria between the micelles and solutes in terms of retention factor

$$k = k_0 \frac{1}{1 + K_{AM}[M]} \qquad (6.5)$$

where κ_0 - Retention factor obtained in the absence of micelles

The above models explain that with increasing surfactant concentration, retention of solute decreases and at high surfactant concentrations, solutes are eluted near the dead volume of the column. It is to be noted that the experimental models fit the predicted models in terms of surfactant types and the micelles that are formed and different column materials used during the study, while maintaining a constant organic modifier concentration.

Equations 6.6 and 6.7 represent that increasing surfactant concentration leads to lower retention of solute within the column. Addition of charged surfactants leads to increased electrostatic interaction with the solute molecules within the stationary phase pore, retaining it from being eluted out of the column while nonionic surfactants involve in hydrophobic interaction with the solutes that are less favourable than electrostatic interaction and enhance the solute elution. Equation 6.8 has been developed for charged solutes and the influence of pH, it can be seen that the variation of retention factor with pH is sigmoidal (Arunyanart and Love 1985). Equation 6.10, includes the effect of organic solvent and its effect on retention factor as a result of change in competing interaction of alcohol with that of surfactant micelles and stationary phase, the equation is an extended form of equation 6.6 (Boichenko et al. 2006). The equation can be used to predict linear, non-linear and quadratic variance in retention factor by maintaining surfactant concentration or organic solvent concentration. Empirical equations are the easiest way to predict the solute retention devoid of performing experiments to analyse their influence. However, results

obtained from empirical equations are non-linear and show a significant difference from that of experimental results. Empirical equations have the potentiality to predict the effect of multi variables on the peak efficiency in a single run, which can be cross verified for its variation by performing experimental run with the obtained conditions.

$$logk = C_0 + C_1[m] + C_2\varphi \quad (6.6)$$

$$\frac{1}{k} = C_0 + C_1[M] \quad (6.7)$$

$$\frac{1}{k} = C_0 + C_1[M] + C_2\varphi \quad (6.8)$$

$$\frac{1}{k} = C_0 + C_1[M] + C_2\varphi + C_3[M]\varphi \quad (6.9)$$

$$\frac{1}{k} = C_0 + C_1[M] + C_2\varphi + C_3[M]\varphi + C_{11}\varphi^2 \quad (6.10)$$

Where φ – volume fraction of organic modifier; C_0, C_1, C_2, C_3 and C_{11} are coefficients of fitting

6.3.5 Peak efficiency parameters

Mobile phase related parameters such as surfactant concentration, organic solvent concentration, pH and column parameters such as column packing material and its pore size, packing, column length etc. The first and fore most parameter is peak resolution, peak resolution denotes the efficient identification of two peaks as two single distinct peaks from one another. A resolution value of above 1.0 or 1.5 will make sure that the peaks are separated from one another in such a way that the peak area and height can be calculated without any distortion in the values ((Ref). As resolution of peaks increases, time taken for the solutes separation also increases. Resolution of peaks is interconnected to other peak efficiency parameters such as separation factor (S), efficiency and retention factor (K). Retention factor (K) of a

solute is the measure of retention of solute within the column or time spent by the solute within the column. Higher K values indicate that the solute has spent considerable time within the column and/or is strongly bound to the column while lower K values indicates that the solute had eluted out with very less interaction towards the column packing. K is calculated as ratio of retention time of solute to that of retention time of unretained compound estimated by disturbance in the baseline prior to the elution of solute or is often taken as retention time of uracil in the column (Rodenas-Montano et al. 2014). K value can be altered by increasing the surfactant concentration in the micellar mobile phase, higher surfactant concentration often leads to influence of solute-column interaction by the micelles and are eluted out from the column at a lower elution time. Presence of short chain length alcohols reduce the retention time of solute as a result of decrease in hydrophobicity of the micellar mobile phase, whereas increasing chain length of alcohol results in stronger hydrophobic interaction of solute with that of alcohol added micelle solution and the interaction of solute with the column (Boichenk and Berthod 2010). However, increasing concentration of alcohol results in inclining hydrophobicity there by inducing stronger interaction with the column. When retention factor is very high or low, resolution of the peaks are reduced and lower retention factor leads to lower retention factor and poor separation of peaks.

Separation factor (S) often referred to as selectivity is the ability to chemically distinguish two different solutes from one another. S value is measured in terms of distance between apices of two distinctive peaks. Higher S value indicates increased separation of solutes and when S value equals one, two solutes are said to elute at the same retention time. Addition of organic solvent, its type and concentration, micellar mobile phase pH and surfactant concentration influences the selective interaction of solute to the surfactant coated column pores, such influences are majorly based on the solute properties. Surfactant coated column material offers higher specificity unlike conventional RPLC (Kalyankar et al. 2014). Mobile phase pH is a key factor in influencing the specificity of separation towards solute. Stationary phase physical parameters such as polarity, hydrophobicity of the silica material offers varying specificity towards solutes in the feed mixture injected to the column, while

increasing the column temperature leads to higher separation of solutes(Ref). Efficiency of a chromatographic separation is achieved in terms of plate height and plate number. Theoretical plate is the equilibrium attained by the solute molecule as it transcends between the stationary phase and the mobile phase, as the number of theoretical plates increase better separation is achieved. Larger peaks compared to narrow peaks are expected to have a large number of theoretical plates and offer higher efficiency (Kraak et al. 1976).

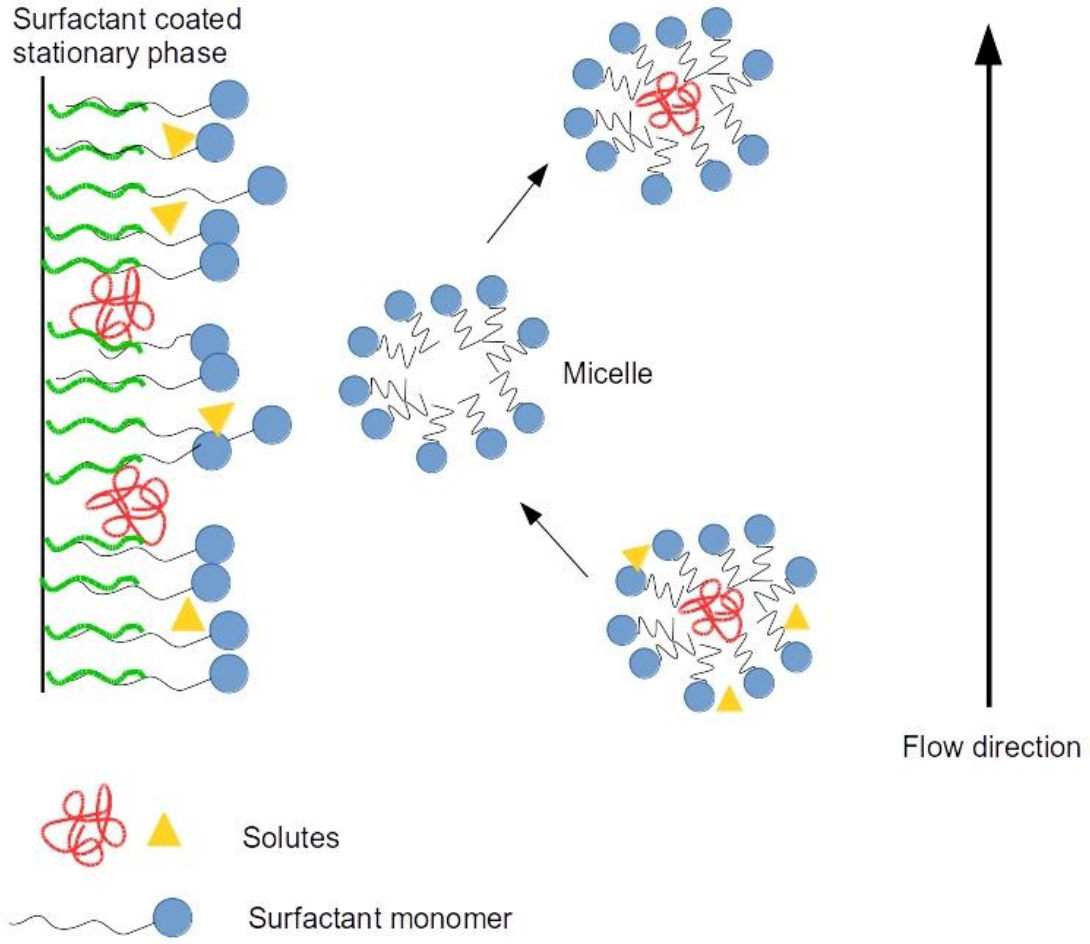


Figure 6.3 Separation of solutes in Micellar liquid chromatography.

6.3.6 Literature survey on solutes analysed by micellar liquid chromatography

Since the invention of micellar liquid chromatography technique, several biological samples have been tested for the presence of different solutes. In general, most of the literatures detail the effect of surfactant concentration, type of surfactant, effect of organic modifier – type and concentration, system pH and the presence of

buffer and its concentration on the peak efficiencies. Table 6.1 enlists a few biological and other samples that have been effectively been analysed in MLC. The preceding section elaborates the effect of various operational parameters on the peak efficiencies and effective separation of solutes.

Table 6.1 Literature survey on Micellar liquid chromatographic separation of solutes.

Source	Solute	Micellar mobile phase	Reference
Sunflower, corn and olive oils, margarine, lard and butter oil	Phenolic antioxidants	0.1M SDS, 2.5% n-propanol and 10mM phosphate of pH 3	Noguera-Ortí et al. 1999
Pharmaceutical preparations	antihistaminic drugs	CTAB 0.04M, 3% 1-butanol, pH 3, CTAB 0.04M, 3% 1-butanol, pH 5, CTAB 0.02M, 3% 1-propanol, pH 6, CTAB 0.02M, 3% 1-propanol, pH 7, CTAB 0.04M, 10% 1-butanol, pH 3	Martinez-Algaba et al. 2006
Fish muscle	Quinolones	0.065M SDS, 12.5% propanol, 0.5% TEA-pH 3	Rambla-Alegre et al. 2010
Olive extract	Hydroxytyrosol	0.05M SDS, 4% methanol, pH 7	Rambla-Alegre et al. 2011
Human serum	diltiazem hydrochloride (DI), metoprolol tartrate (ME) and isosorbide mononitrate (ISMN)	0.0045 M SDS, 0.02 M NaH_2PO_4, 10 % (V/V) 1-propanol, pH adjusted to 7	Li et al. 2014
Plasma	Anti-retroviral drugs	0.05 M SDS, pH 7	Garrido-Cano et al. 2015
Waste water	Pesticides	0.15 M SDS, 6% 1-pentanol, pH 3	Romero-Cano et al. 2015
Human serum albumin	Ampicillin	0.06 M SDS/CTAB, 20 % (V/V)	Stepnik et al. 2016

Garrido-Cano et al. (2015) studied the chromatographic separation of Tyrosine kinase inhibitors (erlotinib, imatinib, sunitinib, sorafenib and lapatinib) from plasma. The authors found that weakly alkaline solutions resulted in slow and continuous hydrolysis of C18 column, reduces the lifespan and gives a poor performance. Retention time of the solutes increased at lower SDS concentration and it was found that retention time reduced in the order butanol < pentanol, with addition of alcohol as organic modifier. Anti-retroviral drugs (Abacavir, lamivudine, raltegrvir) were separated and analysed with direct injection of plasma to the column by Peris-Vicente et al. (2014). Different process variables were studied by the authors to optimize the micellar mobile phase. The authors have reported that Lamivudine elution time was less than 4 minutes and Lamivudine was found to overlap with the other protein peaks. Micellar mobile phase made up of 0.05 M SDS, pH 7 was successfully employed to separate the drugs. Stepnik et al. (2016), studied the chromatographic separation of Ampicillin from human serum albumin using the micellar mobile phase consisting 0.06 M SDS/CTAB, 20 % (V/V) Acetonitrile at pH value of 7.4. Organic compounds such as diltiazem hydrochloride (DI), metoprolol tartrate (ME) and isosorbide mononitrate (ISMN) were separated successfully from human serum by performing MLC with optimized micellar mobile phase made up of 0.0045 M SDS, 0.02 M NaH_2PO_4, 10 % (V/V) 1-propanol, pH adjusted to 7 by Li et al. (2014). The authors studied different process variables such as effect of pH (3 to 7), effect of organic modifier (Methanol, Acetonitrile and Propanol), effect of propanol concentration, effect of Sodium to design an efficient MLC process. Retention behaviour was found to be same in the presence of methanol and acetonitrile when used as organic modifier, while resolution was better in the presence of acetonitrile, separation was better in the presence of propanol.

Pharmaceutical Preparations were tested for their presence of antihistaminic drugs (brompheniramine, chlorcyclizine, chlorpheniramine, doxylamine, flunarizine, guaifenesin, promethazine, terfenadine, triprolidine, caffeine, dextromethorphan, diphenhydramine, hydroxyzine, paracetamol, pyridoxine and tripelennamine) by performing MLC (Martinez-Algaba et al. 2006). Five different micellar mobile phases were developed to separation the solutes based on their pharmaceutical preparations. Different mobile phase compositions such as CTAB 0.04M, 3% 1-butanol, pH 3,

CTAB 0.04M, 3% 1-butanol, pH 5, CTAB 0.02M, 3% 1-propanol, pH 6, CTAB 0.02M, 3% 1-propanol, pH 7, CTAB 0.04M, 10% 1-butanol, pH 3 gave better peak resolutions and enhanced separation of solutes. Quinolones - difloxacin (DIF), flumequine (FLU), enrofloxacin (ENR), oxolinic acid (OXO), and sarafloxacin (SAR) was chromatographically separated with direct injection of supernatant from homogenized fish muscle dissolved in buffer. It has been reported that the usage of triethylamine (TEA) reduces peak tailing and retention time. Micellar mobile phase composition of 0.065M SDS, 12.5% propanol, 0.5% TEA-pH 3 resulted in better peak resolution and separation (Rambla-Alegre et al. 2010).

Waste and sewage water was directly injected to the reverse phase column, RPC-18 to separate pesticides - Thiabendazole (TBZ), 4-tert-octylphenol (4-tOP), Chlorpyrifos (CPF) with an optimized micellar mobile phase composed of 0.15 M SDS, 6% 1-pentanol, pH 3 (Romero-Cano et al. 2015). Rambla-Alegre et al. (2011) studied the chromatographic separation of Hydroxytyrosol present in Olive extract. Higher concentrations of SDS (0.1 and 0.15 M) resulted in elution of solute with other impurities at a low elution time. Introduction of methanol as organic modifier to the micellar mobile phase, increased the peak efficiency and reduced the elution time to 3.5 minutes. An optimized micellar mobile phase composed of 0.05M SDS, 4% methanol, pH 7 resulted in enhanced peak efficiencies.

Imidazole dipeptides - Anserine and carnosine present in homogenized meat samples were successfully separated by Gil-Agustí et al. (2008). The authors studied different process variables such as effect of stationary phase (Kromasil C18 column, a Hypersil phenyl-type column and a Kromasil amino column), Effect of pH (3, 5 and 7), Effect of SDS concentration (0.05 to 0.15M). Low surfactant concentration lead to better resolution and higher retention times, while concentrations as high as 0.15 M lead to overlapping of solute peaks. Micellar mobile phase composed of 0.1 M SDS, pH 7 gave optimum retention time of 12 minutes and better resolved peaks. Sunflower, corn and olive oils, margarine, lard and butter oil were tested for the presence of phenolic antioxidants (propyl and octyl gallates, tert-butylhydroquinone and 3-tert-butyl-4-hydroxyanisole) by performing MLC (Noguera-Ortí et al. 1999). Better resolution of peaks were obtained in the presence of micellar mobile phase composed of 0.1M SDS, 2.5% n-propanol and 10mM phosphate of pH 3.

Kulikov (2007) developed MLC method to separate Derivatized selenium (IV) from pharmaceutical products (multi-vitamin tablets, syrups) and animal premixes by studying the effect of organic modifier (butanol and pentanol) as the main influential factor. The micellar mobile phase composing 0.05M SDS, 10% (v/v) 1-butanol that resulted in enhanced separation and linearity of detection of solutes. In a similar study conducted by Carda-Broch et al. (2007), Trazodone was identified in urine. It was found that change in system pH enhanced the interaction of protonated solute with SDS coated stationary phase.

Rambla-Alegre et al. (2010) studied the identification and separation of melamine present in Milk. Authors have reported that on studying the effect of pH (3 and 7), at neutral pH, melamine was found to elute as a very low time of 2.5 minute, while pH 3 lead to protonation and its interaction with SDS coated stationary phase and also resulted in better solution of peak. Effect of organic modifier (propanol and butanol) was examined and it was found that the addition of propanol improved peak separation of melamine from other milk proteins while butanol lead to overlapping of peaks. Chin-Chen et al. (2010) found that retention factor was found to decrease, while peak asymmetry was found to increase with increasing surfactant concentration, on studying MLC separation of active compounds - curcumin, capsaicin and piperine from turmeric, red pepper and black pepper, respectively.

Pharmaceuticals and biological fluid was injected to identify and separate nicotine, the authors found that with increasing SDS concentration, retention time of nicotine was found to decrease. The increasing volume % of pentanol leads to about 40 % reduction in retention factor. Tayeb-Cherif et al. (2016) studied the presence of Quinolones - oxolinic acid, flumequine, marbofloxacin and enrofloxacin in Honey and reported that Retention time of the quinolones were found to increase with increasing surfactant concentration as a result of stronger electrostatic attraction and optimized mobile phase composing 0.05M SDS/12.5% 1-propanol/0.5% triethylamine at pH 3 gave better resolution of peaks.

6.4 Aim and scope of the work

Chromatography is employed as a final purification process in bioindustries that involve separation and purification of high value low volume products.

Considering its advantages of ease in handling and operation of process, high purity of solutes, ease in design and scale of the process, this current chapter aims to study chromatographic separation of PHA. As a result of paradigm shift towards green chemistry and green chemistry based process development, this chapter aims to develop an effective and efficient chromatographic process that utilises micelles as mobile phase and involves direct detection of biological samples, while reducing the usage of highly volatile, replenishable high cost solvents. The chapter aims to study the effect of nonionic surfactant based micelles as mobile phase and the effect of micelle related influential parameters such as surfactant concentration, organic modifier concentration, effect of salts and broth pH. Different process variables were optimized via one variable at a time approach to attain higher separation of PHA from cellular impurities.

6.5 MATERIALS AND METHODS

6.5.1 Materials

TritonX 100 (TX100), Standard Poly(3-Hydroxybutyrate–co-3 hydroxyvalerate) PHBV (12 %) were purchased from Sigma Aldrich, India. Sodium dihydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4) was purchased from CDH, India. Methanol, Ethanol and Isopropanol were purchased from Merck India limited, India. Deionised water was used during the experiments.

Fermentation broth with maximum PHA production of 11.96 g/L as described in chapter 2, was used as such for the chromatographic run. Crude broth as such was injected to the column as the feed mixture. Shimadzu HPLC LC 20 A, Japan was used to conduct chromatography experiments.

6.5.2 Micellar liquid chromatography protocol

Micellar mobile phase was prepared by dissolving a known concentration of surfactant in deionized water and after complete solubilisation of surfactant; volume was made up using deionised water and was stored in capped glass bottles as stock solution. To prepare particular concentration of surfactant solution, required volume of stock was taken and was the required volume was made up using deionised water. Surfactant solutions prepared were degassed by letting them in a sonicating water bath for 10 minutes, to remove air bubbles trapped inside and to ensure complete solubility

of surfactant. Effect of surfactant concentration on separation of PHA was studied by varying the concentration of TX100 in the mobile phase (0.05 M, 0.1 M and 0.5M). Each individual concentration of surfactant was used as mobile phase in a single run and the chromatograms obtained were compared and the raw data obtained was processed to analyse the surfactant concentration with maximum separation and minimum tailing. The surfactant concentration with maximum peak efficiency was fixed to study the effect of other process variables on separation of PHA.

Effect of organic solvent in micellar mobile phase was studied by considering varying chain length of alcohol and its concentrations (vol%). Methanol, ethanol and propanol (2,3 and 4 vol%) were added to the micellar mobile phase concentration which gave maximum peak efficiency in individual sample bottles and the same were degassed before used as hybrid micellar mobile phase. Effect of ionic strength was studied by considering the following two salts NaH_2PO_4 and KH_2PO_4 (0.005 M and 0.01M). Stock solutions of the salts were prepared and required volume was added to the hybrid micellar mobile phase containing alcohol which gave maximum peak efficiency.

To study the influence of direct loading of cells and lysed cells, a known volume of crude broth was subjected to ultrasonication at 6 kHz for 10 minutes, the obtained sonicated sample was loaded as feed while hybrid micellar mobile phase containing alcohol and salt (both Sodium and Potassium and its respective concentration) that gave maximum peak efficiencies were used to elute the solutes. Effect of pH was studied by injecting pH adjusted sonicated broth to the column, sonicated broth samples pH was adjusted between 3 – 9 and the samples were injected as individual runs.

Shimadzu HPLC LC 20A series was used for chromatographic separation, reverse phase column capcell pak C18 MG II was used for elution while the temperature of the column oven was maintained at 30ºC. 20ML sample loop was used to inject the feed mixture to the column, glass hamilton syringe (1 mL) was used to inject the feed into the sample loop. Before and after every wash, syringe was washed with 100% methanol to ensure removal of any particulate matter sticking onto the glass syringe walls. After every three consecutive runs, pressure of the column was checked and the instrument tubing and the column together were washed with 50 %(V/V) Isopropanol

in water to remove any uneluted solute from the column and the tubing. UV analysis of the eluted peaks were done at 235 nm. The raw chromatograms obtained were processed using LABSOLUTIONS software provided by Shimadzu, Japan. Retention time of PHA and other peak efficiency parameters as described below were calculated and analysed.

$$Numberoftheoretical\ plates = 16 \times \left(\frac{t_R}{W}\right)^2 \qquad (6.11)$$

Where, t_R -Retention time, W – Peak width

$$Retention\ time(K) = \frac{t_R}{t_0} - 1 \qquad (6.12)$$

Where, t_0 – unretained peak time

$$Tailing(S) = \frac{W_{0.005}}{2 \times a_{0.005}} \qquad (6.13)$$

Where , $W_{0.005}$ – Peak width at 5 % of the height

$a_{0.005}$ – Width of front half of the peak at 5% height of the peak

$$Separationfactor(a) = \frac{K_1}{K_2} \qquad (6.14)$$

K_1 and K_2 – Retention factor of peak 1 and 2 respectively.

6.6 RESULTS AND DISCUSSION

Micellar mobile phase made up of TX100 was tested for its efficiency towards micellar liquid chromatographic separation of PHA from the crude broth. Nonionic surfactants are mild and are non-denaturing and since the solute of interest, PHA is strongly hydrophobic in nature, utilisation of TX100 influences the hydrophobicity of the column. TX100 has been chiefly employed towards separation of several hydrophobic solutes via cloud point extraction, apart from TX114 and several other non-ionic surfactants. Among the Triton series, TX100 offers lower viscosity while

TX114 offers high viscosity with increasing surfactant concentrations, which is why hence TX100 was chosen to form micellar mobile phase, while TX45 is dispersive in nature and its formation of micelles and encapsulation of solutes remains unclear. TX100 is readily soluble in water which is evident from its HLB value- 13.5, CMC of TX100 is between 0.22-0.24 mM with maximum absorbance at 275 nm and 283 nm. Lowest TX100 concentration of 0.005 M was prepared and was blank run, without sample injection to study the effect of surfactant effect on UV adsorption and variation in pressure. It was observed that TX100 interference at 235 nm was negligible and major peaks were obtained at ~2, 4 and 10 minutes.

6.6.1 Effect of surfactant concentration

Micellar mobile phases were prepared with increasing surfactant concentrations (0.005, 0.01 and 0.025M). Each of the micellar solution was used as mobile phase during individual chromatographic run and 20 µL of the crude broth was injected to the column. Figure 6.4, shows the effect of increasing surfactant concentration on PHA peak efficiency, it can be seen as the surfactant concentration is increased, retention factor was found to decrease significantly. Uncharged solutes react with the surfactant coated stationary phase via hydrophobic interaction and dipole-dipole interaction. When surfactant micelles when are passed through the column, encapsulating them within micellar hydrophobic core and elute them. The number of micelles increased with increasing surfactant concentration and, the number of micelles formed increases, inclining its interaction with PHA, as a result of which resulted the decrease in retention time decreases from 0.005 M to 0.025 M TX100 concentration.

However, it is to be noted that other peak efficiency parameters were also influenced with increasing surfactant concentration. Tailing and separation factor were found to increase with increasing surfactant concentration and the peak efficiencies decreased with further increase from 0.01 M to 0.025 M., the peak efficiencies decreased. 0.01 M surfactant concentration offers sufficient number of micelles to elute PHA from the stationary phase column pores, while at 0.005 M the micelles formed are less and so separation of PHA from other solutes is not effective, whereas increased TX100 concentration of 0.025 M offers a large number of micelles

that elute all the cellular proteins and cellular impurities together with PHA that declines efficient separation.

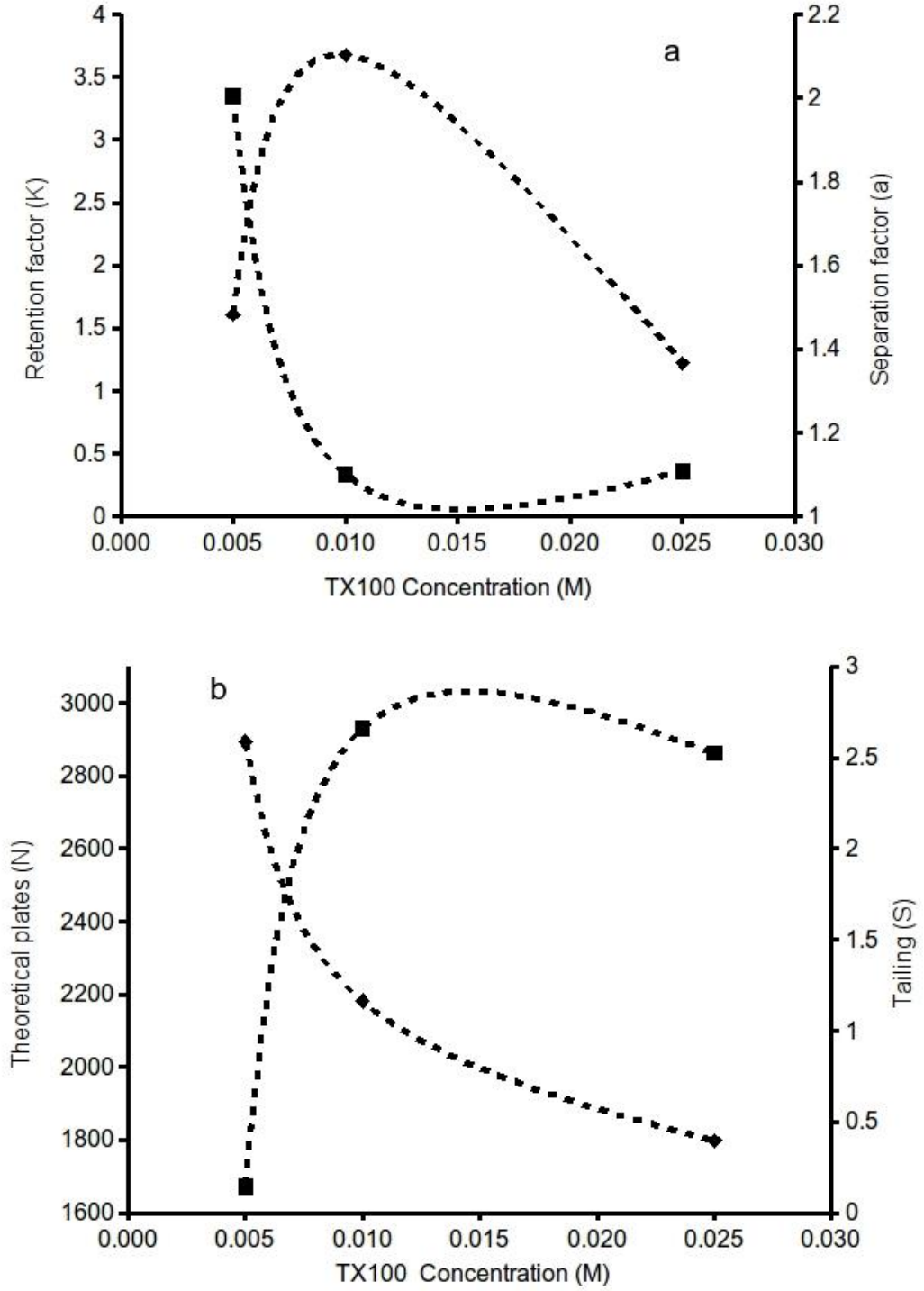


Figure 6.4 Effect of TX100 concentration on PHA peak efficiency (a) Retention factor - ■ , Separation factor - ◆ (b) Theoretical plate - ■, Tailing - ◆.

As a result of increasing mass transfer resistance exerted by partitioning of cellular impurities into micelles than PHA partitioning into micelles, peak broadening increases from 0.005 M to 0.01 M., with The further increase in surfactant concentration at 0.025 M with increasing number of micelles, the peak broadening decreases as there exist large number of micelles required to encapsulate and elute PHA and cellular impurities. However, tailing is higher at 0.01 M and 0.025 M than 0.005 M TX100 concentration. As surfactant concentration is increased, efficiency in terms of theoretical plates was found to decrease as a result of mass transfer resistance offered by competitive binding of cellular impurities to micelles instead of PHA partitioning.

6.6.2 Effect of alcohol chain length and its varying concentration

0.005 M TX100 concentrations which gave minimum tailing was chosen to study the effect of organic solvent. Stronger interaction of surfactants adsorbed onto the stationary phase and hydrophobic interaction between PHA adsorbed onto the surfactant coated column bed can be altered with the addition of organic solvents. In general alcohols of increasing chain length and acetonitrile are added to reduce the retention of a solute within the stationary phase. Addition of polar organic solvent alters the polarity of the column there by eluting highly hydrophobic solutes at lower retention times. Presence of alcohol in the micellar mobile phase alters the CMC of the mobile phase, as a result amount of surfactant required to form micelles is reduced, thereby increasing the number of micelles in the mobile phase. Shorter chain length alcohols are solubilized among the surfactant head groups while longer chain length alcohols are solubilized among the surfactant tails or in the palisade region(between surfactant head and tail). Methanol and ethanol are solubilized among the surfactant head groups while propanol is solubilized in the palisade region. Methanol, ethanol and propanol at different volume % were added to 0.005 M TX100 micellar mobile phases with varying concentration of 2,3 and 4 Vol%.

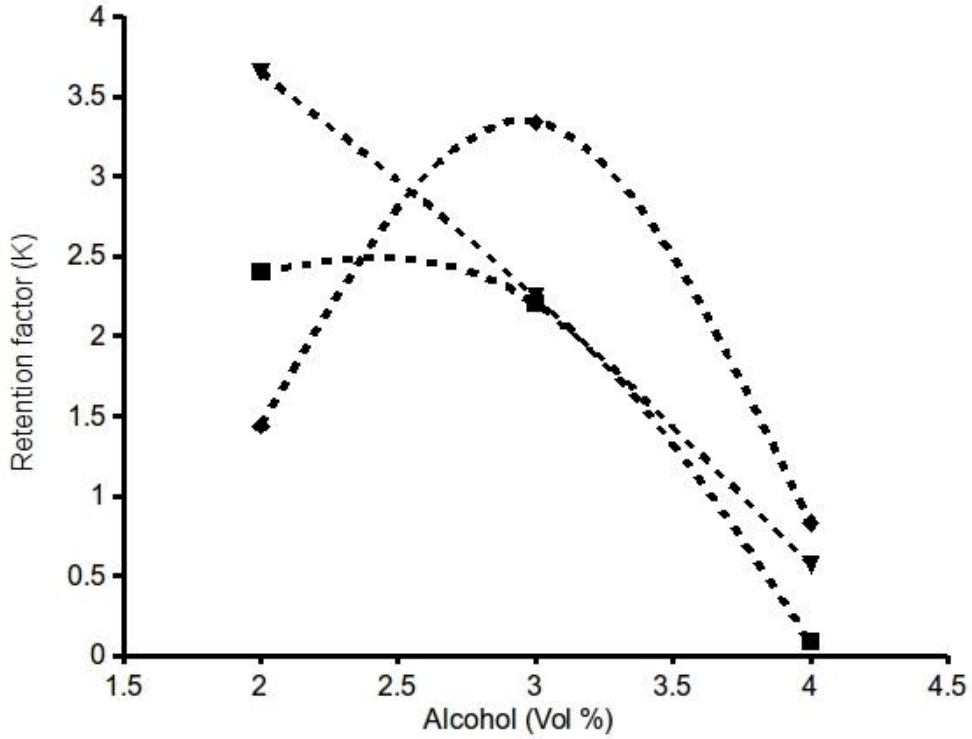


Figure 6.5 Effect of increasing chain length of alcohol and its varying concentrations (vol%) on retention factor ■ - Methanol, ◆ - Ethanol and ▼ - Propanol.

It can be observed from the figure 6.5, that with increasing chain length of alcohol retention time was found to decrease from methanol to ethanol and then increased with propanol, methanol is readily soluble in water and the effect on disruption of hydrophobic interaction is less compared to that of ethanol. However, ethanol imparts hydrophilicity compared to propanol which enhances the hydrophobic interactions; as a result PHA is eluted at a shorter retention time in the presence of ethanol compared to that of propanol. The increasing alcohol concentration lead to decrease in the retention time as a result of reduction in CMC value and the formation of large number of smaller sized micelles. Similar results were obtained for micellar liquid chromatography of hydroxytyrosol from olive extract, in the presence of methanol as organic modifier when SDS was used to form micellar mobile phase.

Table 6.2 Effect of increasing chain length of alcohol and its varying concentrations (vol%) on peak efficiencies.

Alcohol	Concentration (vol%)	Number of Theoretical plates	Tailing (S)	Separation factor (a)
Methanol	2	3696	0.978	1.625
	3	1214	1.303	1.479
	4	1468	1.458	1.486
Ethanol	2	1046	1.439	1.144
	3	3098	2.839	1.136
	4	1050	2.367	1.051
Propanol	2	5953	2.567	1.227
	3	4546	2.89	1.058
	4	922	2.01	1.044

Tailing was found to increase while separation decreased with increasing alcohol concentrations as a result of elution of cellular impurities by hybrid micellar mobile phase containing alcohol. As alcohol concentration increases the number of micelles formed increases there by disrupting the hydrophobic interaction of solutes with that of surfactant coated column bed and eluting them at shorter intervals with that of PHA. Efficiency was found to decrease from methanol to ethanol while it increased with propanol as a result of enhanced hydrophobicity offered by propanol's longer carbon chain compared to that of ethanol and methanol. However with increasing alcohol concentration efficiency was found to decrease as shown in table 6.2, as a result of interaction of micelles with other hydrophobic solutes present in the column along with PHA.

6.6.3 Effect of electrolytes and its concentrations

Electrolytes are added to the micellar mobile phase to improvise peak efficiency and to enhance better elution of solutes from the column. Addition of

electrolyte to the mobile phase reduces the CMC of the micelles to a large extent as a result of incorporation of charged ion species among the surfactant head groups, as the ionic radius increases, larger ions disrupt the micelles resulting in the formation of larger number of smaller micelles. Addition of salt leads to formation of ions, cations interact with the negatively charged silanol groups of bonded silica within the column, these and these interactions are electrostatic in nature hence and so overcome the hydrophobic interaction exerted by surfactants from the micellar phase. The interaction of surfactants is reduced, as a result of which retention of PHA by the surfactant coated stationary phase declines. Effect of addition of electrolyte and its varying concentrations were studied by considering NaH_2PO_4 and K_2HPO_4 at concentrations of 0.005 M and 0.01 M.

From results tabulated in table 6.3, it can be noted that addition of sodium decreased the retention time of PHA compared to potassium, larger head group of potassium on interacting with silanol groups results in steric repulsion and exposure of stationary phase, that results in increase in hydrophobic bonding of PHA with the stationary phase compared to smaller radius of sodium that could easily cover the stationary phase. However, exposure of hydrophobic surfactant tails and the silanol group also leads to interaction of cellular impurities as a result separation of PHA and other solutes from the column takes place within shorter span of time resulting in poor separation factor and increased tailing. Thus 0.01 M sodium resulted in higher separation and lower tailing compared to potassium ions, while increasing salt concentration reduced the efficiency which is reflected as a 12 times reduction in the theoretical plate height.

Table 6.3 Effect of salts and its varying concentrations (M) on peak efficiencies.

	Concentration (M)	**Retention factor**	**Number of Theoretical plates**	**Tailing (S)**	**Separation factor (a)**
Sodium	0.005	0.77	13560	1.263	1.596
	0.01	0.339	1105	2.165	1.726
Potassium	0.005	0.357	1662	2.836	1.363
	0.01	2.202	426	2.19	1.267

6.6.4 Effect of ultrasonication

Effect of ultrasonication was studied to analyse its influence in separation and retention time of PHA compared to that of separation with whole cell. Ultrasonication leads to cell rupture and leakage there by PHA is released into the surrounding medium which can be easily encapsulated by the surfactant micelles, unlike micelles effect on cell rupture and leakage of cellular material. Fermentation broth was ultrasonicated at 6 kHz for 10 minutes and the sonicated crude sample was loaded onto the column, hybrid micellar mobile phase made up of 0.005 M TX100, 2 Vol% Methanol and 0.005 M Na_2HPO_4/K_2HPO_4 was used to study the elution pattern and the corresponding peak efficiencies.

Table 6.4 Effect of salts and sonication of fermentation broth on peak efficiencies.

	Concentration (M)	Retention factor	Number of Theoretical plates	Tailing (S)	Separation factor (a)
Sodium	0.005	1.76	4680	1.458	1.137
Potassium	0.005	2.173	3697	1.042	1.76

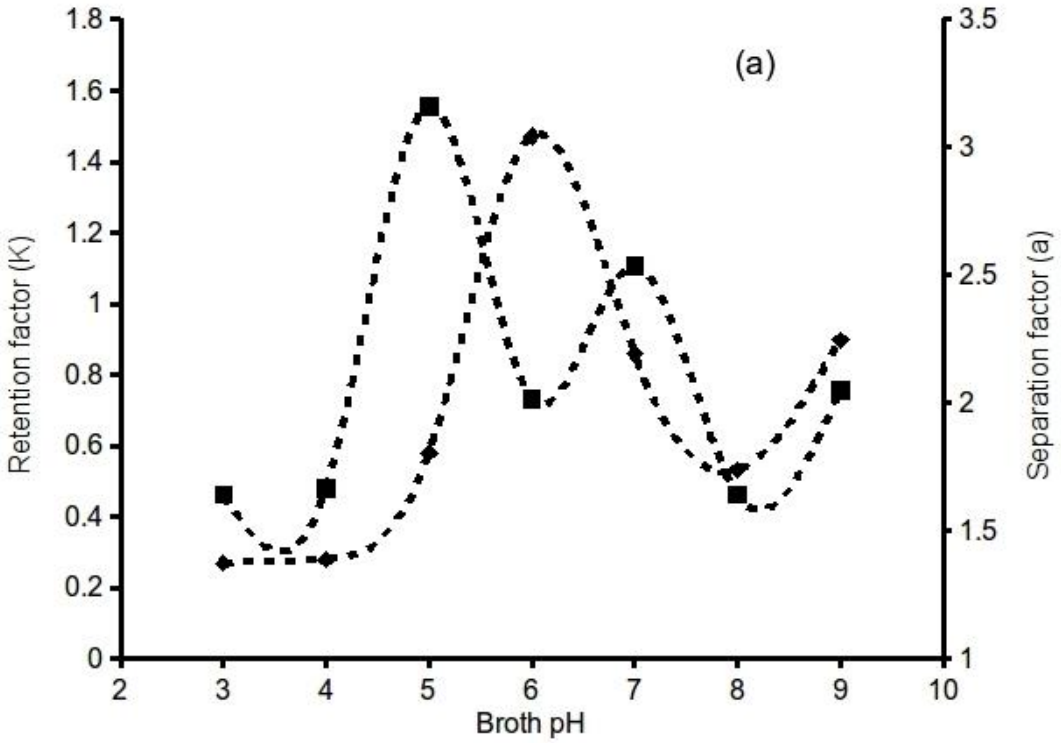
Retention factor of PHA in sonicated broth was less than that of crude broth which should be as a result of enhanced separation and elution of PHA associated proteins from PHA inclusion bodies. As a result of sonication, retention factor and separation factor of PHA was found to increase while tailing decreased.

6.6.5 Effect of pH

The pH has a predominant effect when charged surfactants are used as micellar mobile phase. To study the effect of pH on peak efficiency, broth pH was altered as nonionic surfactant has negligible effect on changing the pH of micellar mobile phase. pH of the sonicated broth was altered between 3 and 9 to study its effect on PHA elution. Change in pH brings a change in the selectivity towards solute molecules by the micelles. The overall effect of pH might alter the electrostatic interaction of the proteins, with respect to change in pH around their respective pI, the net charge of protein molecule varies. This leads to protonation or deprotonation of

amino acid units of the protein which influences protein refolding and exposure of hydrophobic sites of the protein, which can interact with the surfactants coated on to the stationary phase and are also partitioned into the micelle core.

It can be seen from figure 6.6a, as the pH of the broth is increased the retention time is increased until neutral pH and then declines with further increase in pH expressing sigmoidal pattern. Most of the proteins, especially membrane proteins are negatively charged, at pH-3, with respect to their pI, the proteins may attain net positive charge and electrostatically interact with the silanol group while they alter the hydrophobic interaction between surfactant coated column bed and PHA. Efficiency in terms of number of theoretical plates was found to increase on approaching neutral pH and with further increase in pH, efficiency was found to decrease. At neutral pH, maximum equilibrium is attained as there exist net zero charge of cellular proteins as a result of which partitioning of PHA into micelles is enhanced, however, proteins interact via hydrophobic interaction which is expressed in terms of decrease in tailing 2.661 to 1.334 and increase in separation factor from 1.384 to 3.045 at pH 6, as shown in figure 6.6b.



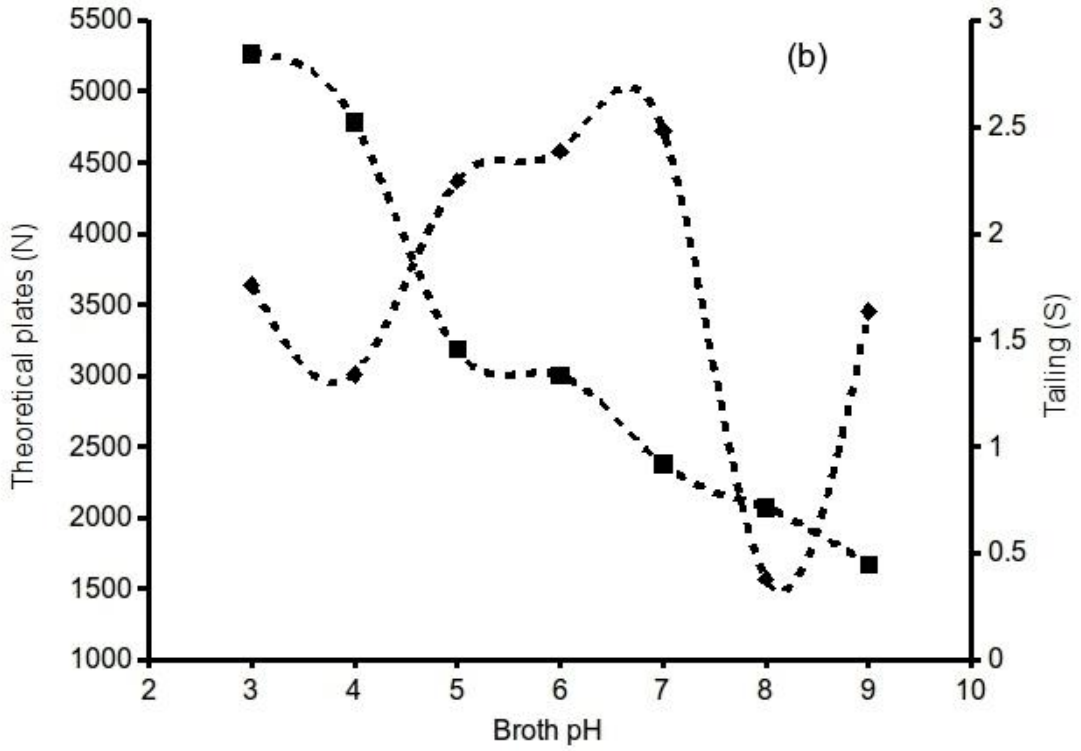


Figure 6.6 Effect of broth pH on peak efficiencies (a) Retention factor - ■ , Separation factor - ◆ (b) Theoretical plate - ■, Tailing - ◆.

6.7 Summary

Developed nonionic surfactant based micellar liquid chromatography is inferred to be effective towards separation of PHA from cellular impurities while crude broth can be as such injected to the reverse phase column. MLC method developed can be further scaled up using preparative column, once the novel PHA identified and separated can be separated in purest form using gel permeation chromatography. It was found that with increasing surfactant concentration, PHA retention was found to decrease from a factor of 3.35 to 0.36, while methanol offfered optimum retention., The separation of PHA peaks from other solute peaks was higher in the presence of lowest concentration of methanol studied (2 vol%) and it was found to increase with the addition of sodium ions in the form of Na_2HPO_4. Micellar mobile phase composed of 0.01 M TX100, 2 vol% Methanol, 0.01 M NaH_2PO_4 and on injecting ultrasonicated crude broth whose pH was adjusted to 6.0, resulted in a maximum separation factor of 3.05 and a lower PHA retention factor of 0.73.

CHAPTER 7

CONCLUSIONS

Considering the growing market for biotechnological products, extensive application and flexible physical properties of PHA has opened up a new horizon towards development and usage of green chemistry based polymers. Ranging from a variety of molecules via random synthesis to tailor made synthesis, PHA molecules of different physicochemical properties that meets up a specific application can be synthesised by fermentation of carbon source. PHA synthesis has also opened up an alternative towards utilisation of waste resources that reduces pollution to a larger scale and paved a way towards product from waste. Crude glycerol a common by-product in the biodiesel industry, as a result of its bulk production and storage and a meagre percentage is utilised in various industries, impose threat to the environment. Current fermentation technique developed in this research work is believed to be an effective method towards scale up and production of PHA in industrial scale. Apart from reduction in overall production cost, further research and development could result in a more tailor made PHA that could serve various purposes. Earlier reports on production of terpolymer PHA from crude glycerol are limited and this research work puts forth a method to synthesise medium chain length terpolymer PHA, which remains in its amorphous and viscous form even after separation from biomass. *Cupriavidus necator*, a common soil microbe has been effectively utilised to synthesise novel PHA - **Poly [3 – Hydroxybutyrate – co – 3 – Hydroxyvalerate – co – 3 – hydroxy – 4 - methoxy phenyl valerate] (P3HB-co-HV-co-MeOPhHV),** from a cheaper and abundant carbon source, crude glycerol in an unaerated and unsterile mode. It is a known fact, that maintaining aseptic conditions and aeration of the medium during fermentation, considerably increases the overall production cost. By adopting the developed fermentation mode, that incorporates a predatory common soil microbe in an unsterile and unaerated mode, PHA accumulation of ~ 85 % has been achieved. Novel mcl-PHA synthesised in this research work has been identified to possess the following physical properties as enlisted in table 7.1

Table 7.1 Physical properties of P3HB-co-HV-co-MeOPhHV.

Glass transition temperature (T_G)	Melting temperature (T_M)	Crystallinity %	Degradation onset temperature (T_D)	Bond strength	Average Molecular weight (M_w)
-14.34 ºC	104.85 ºC	17	250.64 ºC	12.66 MPa (Acrylic)	994 Da

Industrially chloroform extraction is employed to PHA from biomass which not only deteriorates the PHA nativity, but also demerits the use of such extracted PHA in biological applications. Over a decade, nonionic surfactants have been widely documented for its application towards cloud point extraction of membrane proteins and other biomolecules and hence, the technique was employed to cloud point extract PHA from fermentation broth. Extraction efficiency was found to increase with the variation of process parameters. Thus, nonionic surfactant induced cloud point extraction of PHA reduces the overall operating cost of the process by handling larger volumes of broth to be extracted in a minimum time and in a more sustainable way. Promising batch cloud point extraction of PHA from fermentation broth was further extended with insight towards continuous extraction that aids in large scale extraction of PHA.

Process integration of ultrasonication with cloud point extraction lead to the development of an adiabatic micellar extraction system which reduces the operational cost to a great extent and also inflates the extraction efficiency. Considering the advantages of UACPE towards separation of strongly hydrophobic solutes such as biopolyester, it can actively be extended towards separation of any hydrophobic solutes from biological feed as described in this article. UACPE apart from enhancing specificity based extraction, it also retains the nativity of the solute, which is vital in the separation of any bioproduct. PHA from *Cupriavidus necator* was purified by low frequency sonic waves assisted CPE in the presence of mixed surfactants with an overall purity of 94.34 %, higher than purity of 92.49 % obtained by heat induced cloud point extraction of PHA using nonionic surfactants and the current sonication

assisted process design enhanced the usage of ionic surfactants, whose cloud point temperature (>100ºC) is usually difficult to maintain. The effect of sonic waves on biopolymer is screened by the presence of micelles and hence the nativity of the polymer is assumed to be unaltered which could be confirmed with further physiochemical and application based studies. Sonication assisted CPE of biopolymer from the source is first of its kind research, setting a standard towards separation of any such polymer from the source without the necessity to perform complex separation process/equipment.

Although, surfactant micelles have been extensively employed in the effective separation and purification of hydrophobic solutes, considering the complexity of the feed mixture such as fermentation broth and the cellular impurities that also interact with the micelles, innovative process integrated extraction techniques are required to achieve high specificity and sustainable operation in operating feed of biological origin. Considering the pros of process intensification and its effect on the paradigm shift towards sustainable process design and operation, the present research intends to integrate micelle assisted extraction and microfiltration of PHA from the homogenized crude fermentation broth. Addition of surfactant to GA emulsion resulted in the formation of stable GA-surfactant micelle complex that interacts with PHA and other cellular impurities. Stronger interactions imposed by the presence of cationic-anionic surfactants in the micelle complex retain the cellular proteins within the retentate thereby increasing the diffusion of PHA across the membrane. However, concentration polarization studies and physical characterization of used membrane needs to be performed to confirm the diffusion of PHA and gel layer formation and its constituents that leads to membrane pore blocking. MEMF of Polyhydroxyalkanoate is first of its kind approach towards separation of PHA, by combining both micelle induced hydrophobic interaction and pressure driven membrane extraction. Thus, the currently developed process integrated MEMF ensures sustainable process design and its operation towards high recovery of PHA in its native from the crude fermentation broth than industrially employed solvent extraction process.

TX100 was successfully employed as micellar mobile phase in the Chromatographic separation process., it was found that the retention of strongly bound hydrophobic solute was found to decrease with the introduction of organic

solvent, retention of strongly bound hydrophobic solute was found to decrease while efficient separation of PHA was found to increase. The developed chromatographic separation enhances the extraction and separation of PHA from cellular impurities by surfactant micelles, effectively at room temperature. Chromatographic separation is a well-known industrially employed product polishing step which enhances the purity to about 98-100% and is the major step in determining the overall cost of the process. The process integration of micellar extraction and chromatographic separation results in a single step extraction and purification step which reduces the number of unit operations required to extract PHA from its source and separate proteins and cellular impurities from PHA resulting in low cost purification process with maximum purity and yield of PHA. However, considering the novelty of PHA, construction of calibration curve and exact quantification of PHA requires further studies. The following table 7.2 comprises overall purity and recovery of PHA of every single micellar based extraction technique developed in the present study and the conditions that resulted in maximum PHA purity.

Table 7.2 Optimized extraction conditions and their corresponding efficiencies.

Extraction technique	Optimized process conditions	Purity %	Recovery %
Cloud point extraction	TX114 (4.5 wt%) + TMN6 (0.5 wt%), pH -3, 0.1 M ammonium chloride	92.49	84.4
Sonication assisted cloud point extraction	TX100 (3 wt%) + TX114 (2 wt%), broth pH – 5, 0.1 M sodium sulphate, sonicated at 6 kHz for 6 minutes)	94.28	83.94
Gum arabic – mixed micelle coacervate enhanced membrane filtration	Gum arabic 25 wt%, AOT (0.1 M) + CTAB (0.5 M), 0.2 bar, Propanol 25 vol%, pH -6	97.089	81.638
Micellar liquid chromatography	0.01 M TX100, 2 vol% methanol, 0.01 M NaH_2PO_4, sonicated broth pH adjusted to 6	Retention factor – 0.732 Separation factor - 3.045	

Considering the fact that the developed micelle based extraction techniques are of batch scale operation, further studies needs to be performed to scale up the extraction process. Advanced structural analysis of novel mcl-PHA synthesised will put forth an idea on the availability of binding sites and in determining the possible types of interactions that prevails during micellar extraction of PHA. Apart, from such interactions, such structural and interaction studies would aid in the development of novel hybrid separation techniques that could be cost effective while offering high productivity.

Scope for future work

- Further characterization and application based studies of long chain length PHA synthesised in this work.

- Continuous micelle assisted membrane separation of PHA in cross flow filtration unit that could enhance PHA recovery and reduces membrane fouling.

- Development of preparative micellar liquid chromatography that would aid in the continuous chromatographic separation and purification of PHA from the biomass.

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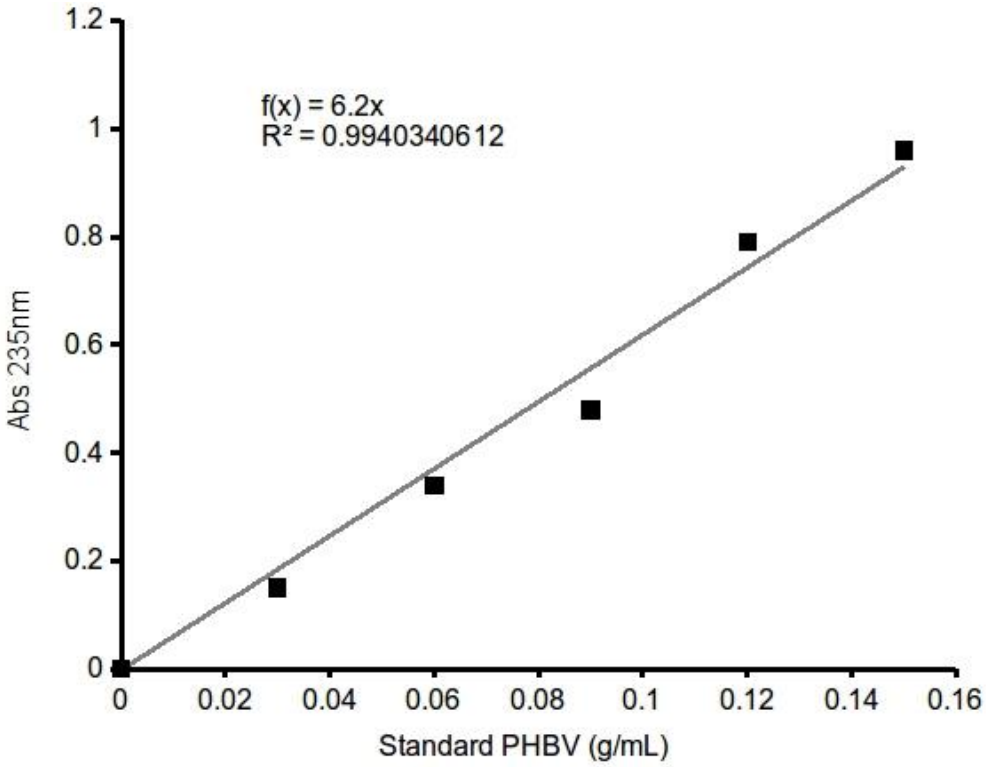
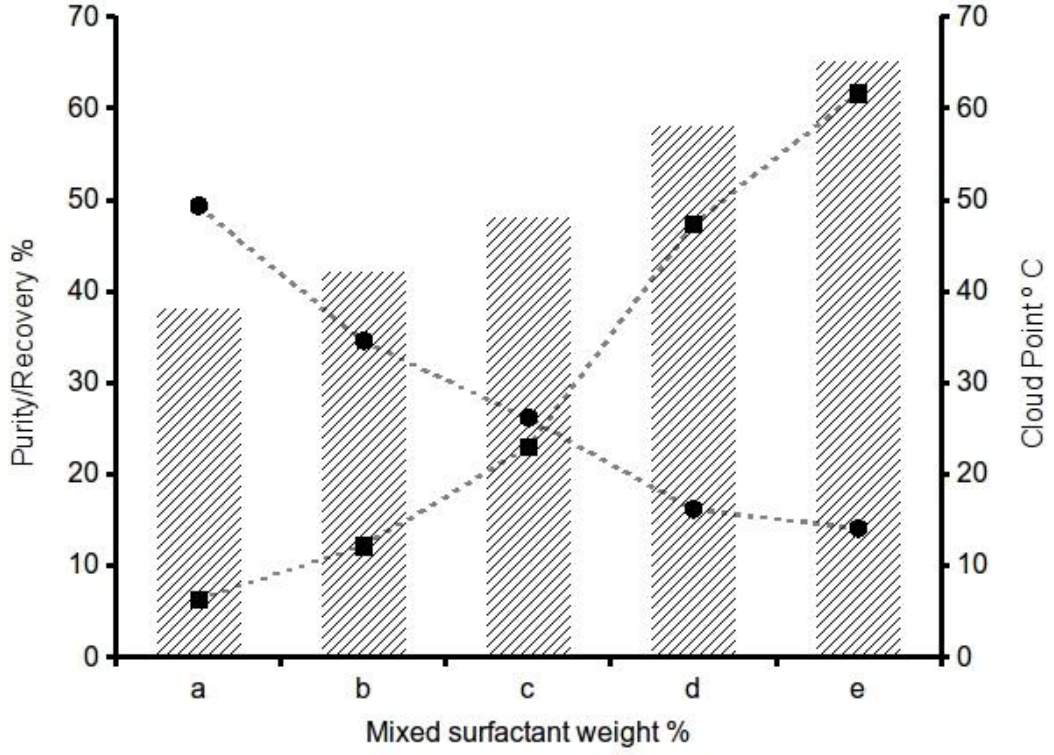
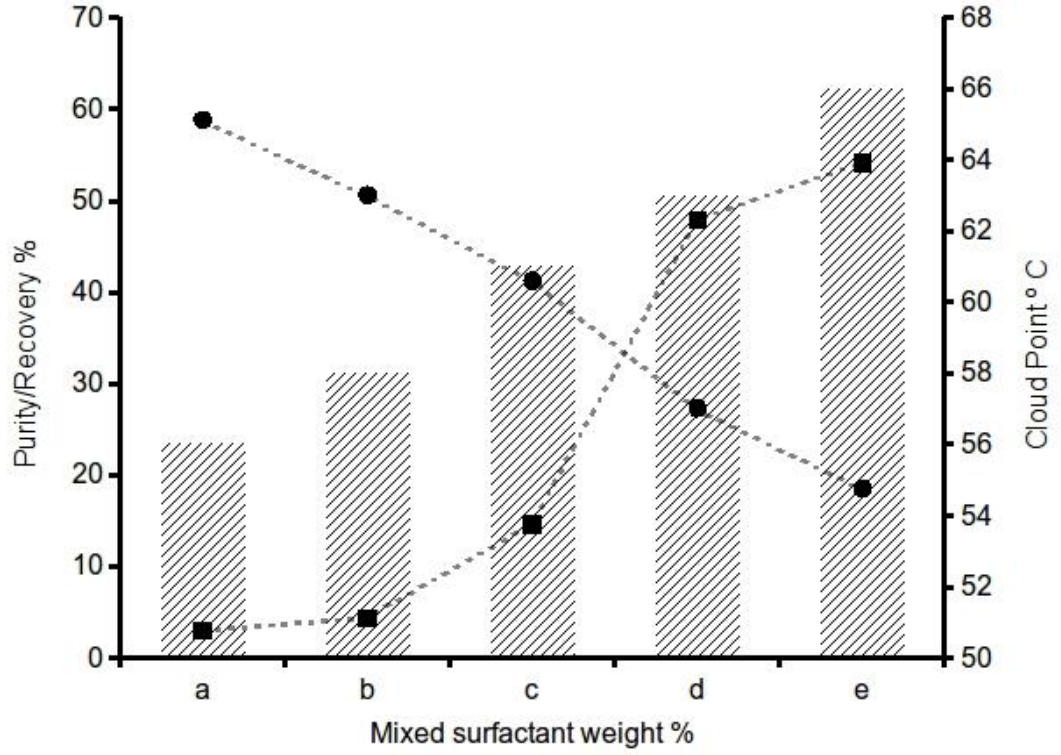
APPENDIX



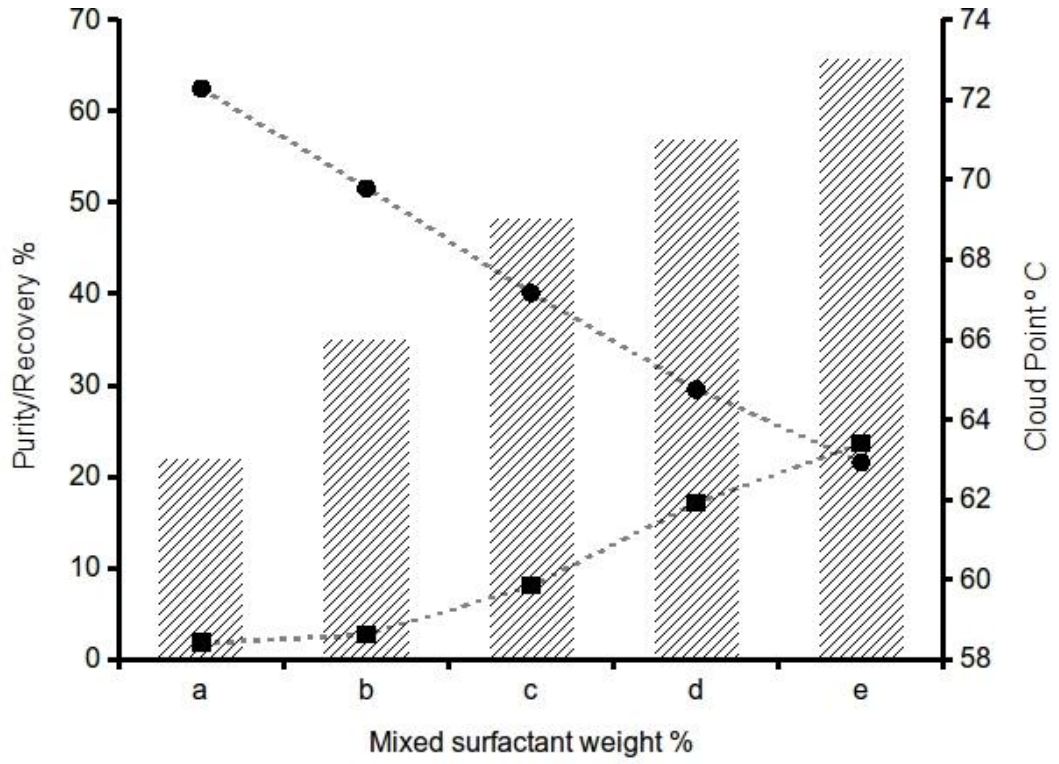
A1. Standard calibration curve for PHA



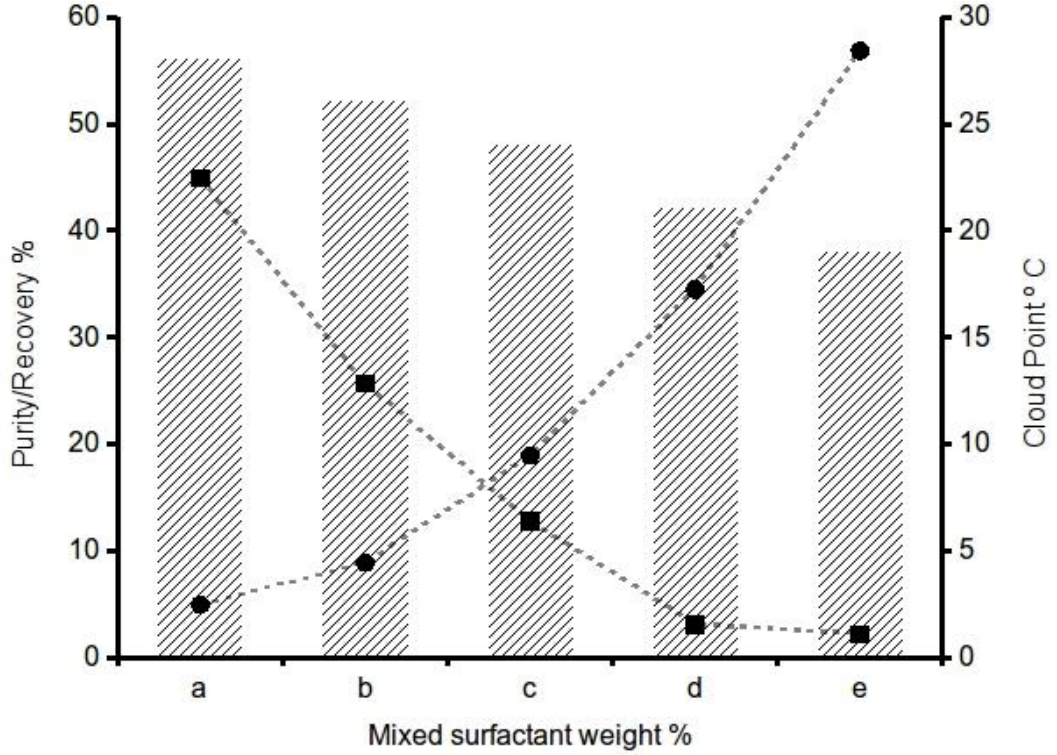
A2. Purity % - ■ **,** recovery % - ● and cloud point temperatures (Diagonal line bars) of mixed surfactant mixture – TX114+TX100 at 1 wt% : a – 0.9 % + 0.1 %, b – 0.75 % + 0.25 %, c – 0.5 % + 0.5 %, d – 0.25 % + 0.75 %, e – 0.1 % + 0.9 %.



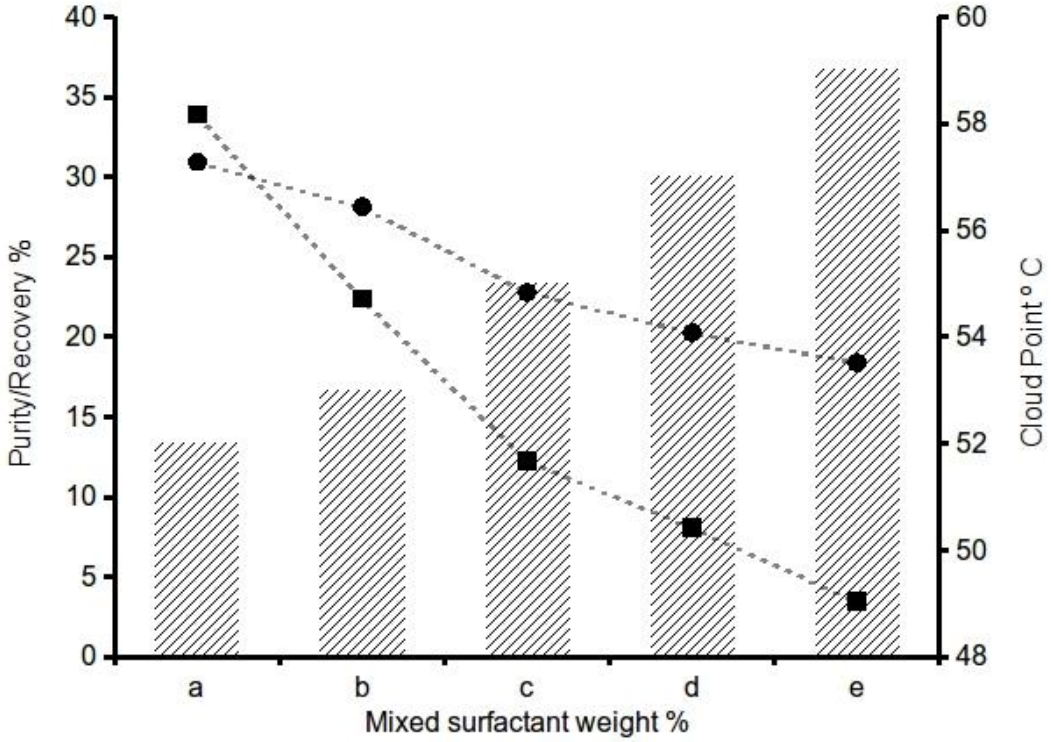
A3 Purity % - ■ **,** recovery % - ● and cloud point temperatures (Diagonal line bars) of mixed surfactant mixture – TX114+TX100 at 5 wt% : a – 4.5% + 0.5%, b - 3.75%+1.25%, c - 2.5%+2.5%, d - 1.25%+3.75%, e - 0.5%+4.5%.



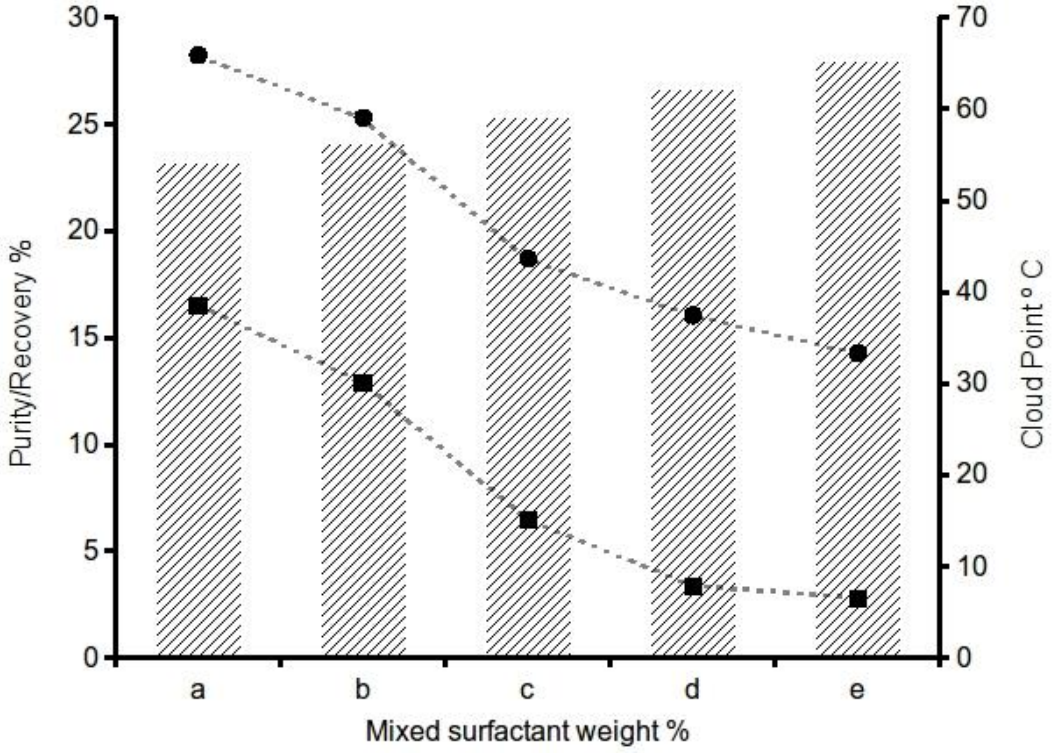
A4. Purity % - ■ , recovery % - ● and cloud point temperatures (Diagonal line bars) of mixed surfactant mixture – TX114+TX100 at 7 wt% : a – 6 % + 1 %, b – 4.75 % + 2.25 %, c – 3.5 % + 3.5 %, d – 2.25 % + 4.75 %, e – 1 % + 6 %.



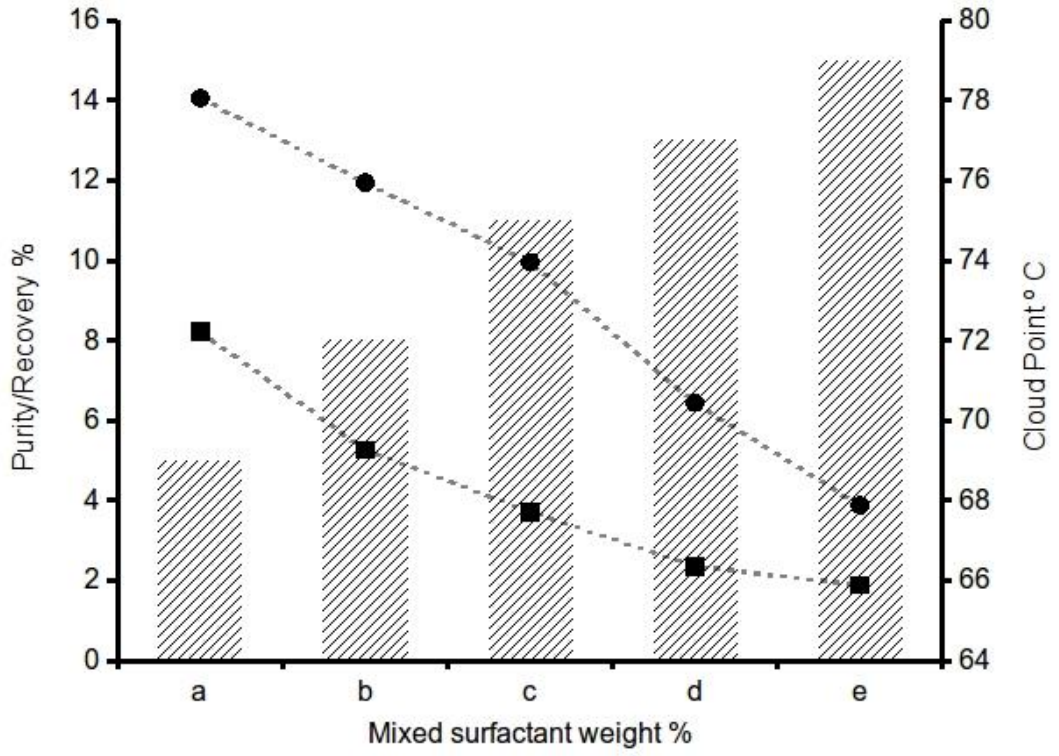
A5. Purity % - ■ , recovery % - ● and cloud point temperatures (Diagonal line bars) of mixed surfactant mixture – TMN6 + TX114 at 7 wt% : a – 6 % + 1 %, b – 4.75 % + 2.25 %, c – 3.5 % + 3.5 %, d – 2.25 % + 4.75 %, e – 1 % + 6 %.



A6. Purity % - ■ , recovery % - ● and cloud point temperatures (Diagonal line bars) of mixed surfactant mixture – TMN6 + TX100 at 1 wt% : a – 0.9 % + 0.1 %, b – 0.75 % + 0.25 %, c – 0.5 % + 0.5 %, d – 0.25 % + 0.75 %, e – 0.1 % + 0.9 %.



A7. Purity % - ■ , recovery % - ● and cloud point temperatures (Diagonal line bars) of mixed surfactant mixture – TMN6 + TX100 at 5 wt% : a – 4.5% + 0.5%, b - 3.75%+1.25%, c - 2.5%+2.5%, d - 1.25%+3.75%, e - 0.5%+4.5%.



A8. Purity % - ■ , recovery % - ● and cloud point temperatures (Diagonal line bars) of mixed surfactant mixture – TMN6 + TX100 at 7 wt% : a – 6 % + 1 %, b – 4.75 % + 2.25 %, c – 3.5 % + 3.5 %, d – 2.25 % + 4.75 %, e – 1 % + 6 %.

Rotor speed (RPM)	Continuous phase flowrate (mL/min)	Dispersed Phase flowrate (mL/min)	Purity %			Recovery %		
			15	30	45	15	30	45
50			82.55	83.87	86.01	67.89	65.87	63.78
100			79.69	79.94	83.10	70.47	66.00	65.79
150	**35**		77.05	79.57	82.54	73.47	69.54	67.84
50			75.78	76.54	79.99	75.87	73.15	70.99
100			73.21	75.47	76.97	76.78	75.98	73.25
150	**45**		70.58	72.15	76.00	79.79	76.88	75.47
50			66.15	69.58	73.55	81.15	79.13	76.84
100			65.47	68.74	72.15	81.55	81.48	80.87
150	**55**		63.59	65.12	66.17	85.48	83.57	81.46

A9. Table representing extraction efficiency on varying rotor speed, continuous and dispersed phase flowrates.

		Dispersed Phase flowrate (mL/min)	15	30	45
Rotor speed (RPM)	**Continuous phase flowrate (mL/min)**				
50			0.02586	0.02266	0.01943
100	**35**		0.02950	0.02566	0.02302
150			0.03419	0.02986	0.02652
50			0.05048	0.04872	0.04627
100	**45**		0.05766	0.05685	0.05482
150			0.06184	0.06128	0.06124
50			0.08596	0.08594	0.08365
100	**55**		0.09716	0.09687	0.09109
150			0.10681	0.10660	0.10434

A10. Table representing Mass transfer coefficient obtained on varying rotor speed, continuous and dispersed phase flowrates.

CURRICULUM VITAE

Educational Qualification

PhD in Biochemical Engineering (July 2012 – July 2017) National Institute of Technology Karnataka, India.

Research topic – Surfactant based extraction of polyhydroxyalkanoate from fermentation broth.

Production of Polyhydroxyalkanoate (Bioplastic) in unsterile, unaerated batch fermentation process and purification by micellar based extraction techniques

Hands on Experience Development of novel Emulsion (o/w) and inverse emulsion (w/o) systems, Development of polymer based aqueous two phase system, Phase separation, development of dye-affinity reverse micelles, biomolecule based micelles, development of novel surfactant systems, Separation and purification of hydrophobic solutes and hydrophilic solutes from feed mixture.

M.Sc Biochemical Engineering (Sep 2007- Sep 2009) Technical University of Delft, The Netherlands.

Thesis - fractionation and characterization of crude fermentation broth for the development of protein purification database.

Chromatographic separation of recombinant *E.coli* proteins and mass spectral detection of separated proteins to reveal protein identity. Development of database with identified proteins and related chromatographic parameters to be used for process modeling.

B.Tech – Industrial Biotechnology (July 2002- May 2006) Kamaraj college of Engineering and Technology, Affiliated to Anna university Chennai, India.

Thesis – Phytochemical Characteriztion, identification and in vitro studies of leaf extracts of Terminalia arjuna via NMR and FTIR studies, modeling and protein docking of characterized flavanone with cellular tumor antigen p53.

Instruments handled High Pressure Liquid Chromatography (HPLC), Liquid Chromatography coupled Mass spectrometry (LCMS), Fast protein Liquid Chromatography (FPLC), Ion Exchange Chromatography (IEC), Hydrophobic Interaction Chromatography (HIC), MS/MS analyzer, PAGE and gel electrophoresis unit, gel documentation unit, UV/Vis Spectrophotometer, digital densitometer, viscometer, refractometer , karl fischer titrator, surface tensiometer, Membrane separation unit, Thermogravimetric analyzer, Lab scale bioreactor, incubator shaker, Gas Chromatography (GC), ultrasonicator, homogenizer, lyophilizer, Cooling centrifuge, Microscope, Rotating Disc Contactor, Bubble column extractor, pulsed liquid liquid extraction column, usage of liquid disposing pumps and other basic lab instruments.

Mini projects *(As a part of course curriculum - Technical University of Delft, The Netherlands).*

- *Development of process integrated design as an alternative to process involved during Bhopal gas disaster.*
- *Product design towards improvisation of baby napkins – product design, market survey and analysis.*

Internship

Studies on the oxygen mass transfer during fed-batch fermentative production of bioethanol from wood waste using fungal strains, DSM food specialities B.V., Delft, The Netherlands. (May 2009 – August 2009).

Software skills: Windows and Linux OS , Microsoft office and Libre office ,ASPEN and Matlab - basics, Design of Experiments – minitab, modeling softwares, accessing and usage of different protein, metabolic pathway and genome data banks.

Publications

Manuscripts published in journals

Murugesan, S. and Iyyaswami, R., 2017. Nonionic surfactants induced cloud point extraction of Polyhydroxyalkanoate from *Cupriavidus necator*. *Separation Science and Technology*, *52*(12), pp.1929-1937.

Murugesan, S. and Iyyaswami, R., 2017. Low frequency sonic waves assisted cloud point extraction of polyhydroxyalkanoate from *Cupriavidus necator*. *Journal of Chromatography B*, *1060*, pp.207-214.

Murugesan, S., Iyyaswami, R., Kumar, S.V. and Surendran, A., 2017. Anionic surfactant based reverse micellar extraction of l-asparaginase synthesized by *Azotobacter vinelandii*. *Bioprocess and biosystems engineering*, *40*(8), pp.1163-1171.

Book Chapter

Murugesan, S., Ambakam, P., Naveen, A., Makkada, A., Solanki, N. and Iyyaswami, R. (2016). Mixed Surfactant Based Reverse Micelle Extraction of Lactose Peroxidase from Whey. Biotechnology and Biochemical Engineering (pp. 111-119). Springer Singapore.

Manuscripts submitted and under review

Sivananth Murugesan and Regupathi Iyyasamy, Hydrocolloid – micelle coacervate complex assisted membrane filtration of polyhydroxyalkanoate from crude fermentation broth.

Sivananth Murugesan and Regupathi Iyyasamy, Valorization of biodiesel derived crude glycerol into PHA terpolymer by *Cupriavidus necator* via a sustainable fermentation process.

Sivananth Murugesan and Regupathi Iyyasamy, Micellar liquid chromatographic separation of polyhydroxyalkanoate synthesized by *Cupriavidus necator*.

Sivananth Murugesan, Palash Khandelwal and Regupathi Iyyasamy, Nonionic surfactant based cloud point extraction of polyhydroxyalkanoate from the fermentation crude in a rotating disc contactor.

Murugesan, S., Veetil, S.M., and Iyyasamy, R., Study on the effect of process variables on hydrodynamics of reverse micelles in rotating disc contactor.

Manuscripts in pipeline: 6