BIOPROCESS DEVELOPMENT AND OPTIMIZATION OF MELANIN FROM *PSEUDOMONAS STUTZERI*

Thesis

Submitted in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

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DECLARATION

I hereby *declare* that the Research Thesis entitled **"Bioprocess development and optimization of melanin from *Pseudomonas stutzeri*"**, which is being submitted to the **National Institute of Technology Karnataka, Surathkal** in partial fulfilment of the requirements for the award of the Degree of **Doctor of Philosophy** in the Department of 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 **"Bioprocess development and optimization of melanin from *Pseudomonas stutzeri*"** submitted by **Mr. Harsha Thaira (Register Number: 138013CH13F06)** as the record of the research work carried out by him, is *accepted as the Research Thesis submission* in partial fulfilment of the requirements for the award of degree of **Doctor of Philosophy**.

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DEDICATED TO

THAIRE

ABSTRACT

Melanins are water insoluble polyphenol compounds that are responsible for black, brown and grey pigmentation in eukaryotes and prokaryotes. The presence of indole ring and many functional groups, such as, =O, -OH, -NH, and –COOH makes melanin an ideal choice for many medical, cosmetic and environmental applications. The current market price of melanin is about USD 500 because of unavailability of sustainable manufacturing process. Microorganisms are reported to produce melanin; however, sustainability of a microbial bioprocess depends on raw material requirements and water footprint. A non-pathogenic microorganism capable of growing in seawater and require raw materials, which do not compete for human consumption is ideal for any bioprocess development. Therefore, a marine bacterium known as *Pseudomonas stutzeri*, which readily grows in seawater and does not require any tyrosine addition was selected for this work on melanin production. The best operating conditions were identified for melanin production. Inoculum age of 12 hours and 10% inoculum gave the best melanin production. Different carbon, nitrogen sources and trace elements were screened and glucose and coconut cake meal were selected as the best carbon and nitrogen sources, respectively. Sulphates of copper, magnesium and iron enhanced melanin production. All these media components were optimized using central composite design and melanin production was increased to 3.4 folds to about 520 mg/L. However, melanin demonstrated biomass growth inhibition as well as feedback inhibition on to the enzyme tyrosinase. An alumina adsorbent based novel adsorptive bioprocess was developed to reduce the feedback inhibition by melanin, which resulted in 8.8 fold enhancement in the production of melanin by the marine bacteria *Pseudomonas stutzeri* (153 mg/L to 1349.03 mg/L). Further, an adsorption based downstream process was developed using alumina, at acidic pH (< 3) which resulted in 98% recovery of melanin from the fermentation broth. Particle size analysis of the biosynthesized melanin indicated that they are nanoparticles with a size of 32 ± 0.98 nm and preliminary investigation indicated that melanin nanoparticles adsorbed different heavy metals at very low metal concentrations.

Keywords: natural pigment, melanin, nanoparticles, adsorptive bioprocess

TABLE OF CONTENTS

PAGE NO.

LIST OF FIGURES

FIGURE NO. FIGURE CAPTION PAGE NO.

LIST OF TABLES

TABLE NO. TABLE CAPTION PAGE NO.

CHAPTER 1

INTRODUCTION

CHAPTER 1

1 INTRODUCTION

Pigments are colorful chemical compounds, which absorb light in the region of visible spectrum. Certain molecules known as chromophores which are present in the pigments absorb energy, leading to the excitation of an electron from its external orbital to a higher orbital; the non-absorbed energy, which is reflected and/or refracted is captured by the eye resulting in the generation of neural impulses; as a result of which the color is produced (Hari et al. 1994). Pigments are mainly of two types, synthetic and natural, and are extensively used in cosmetics, textiles, foodstuffs, furnishings, lenses, drugs, and in other products. The natural pigments are classified into different groups based on their structural characteristics. They are Benzopyran derivatives, Isoprenoid derivatives, Tetrapyrrole derivatives, Quinones and Melanins (Hari et al. 1994).

Melanins are class of polyphenol compounds whose monomeric unit is an indole ring. They are accountable for most of the brown, black and grey pigmentations in plants, animals, and microbes. Melanins are usually classified into three groups and they are;

Eumelanins, which are brown or black in color and are the most commonly occurred melanins. They are distributed widely in vertebrates and invertebrates.

Pheomelanins, which are yellow or red in color and are found in birds and mammals.

Allomelanins, which are present in fungi, spores and seeds.

The most common melanin is Eumelanin, which is also known as DOPA (Dihydroxyphenylalanine) melanin. Henceforth, the word melanin will represent eumelanin in this thesis.

Figure 1.1 shows the structure of melanin. The different properties and functions of melanins are being explored for various applications. The high reactivity of melanin due to the presence of =O, -OH, -NH, and –COOH groups is the reason behind growing interest in melanin research. Melanins have a broad spectrum of biological applications. This includes inhibition of Human Immunodeficiency Virus (HIV)

replication, antivenin activity, antimicrobial activity and antioxidant activity (Arun et al. 2015).

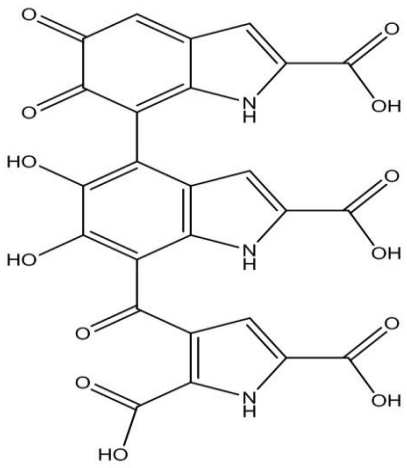

Figure 1-1: Structure of eumelanin.

It has been reported that synthetic soluble melanins can inhibit replication of HIV type 1 and 2 in two human lymphoblastoid cell lines (MT-2 and H9) and in phytohemagglutinin-stimulated human T cells. Effective concentrations of melanin (0.15-10 μg/ml) blocked the infection and interfered with the HIV-induced symplasm formation and cytopathic effects when uninfected cells were mixed with chronically infected cells. It also destroyed the HIV-1 envelope surface glycoprotein suggesting representation of a new class of pharmacologically active substances against HIV by soluble melanins. The author suggests further investigations for the treatment of Acquired Immune Deficiency Syndrome (AIDS) by soluble melanins (Montefiori and Zhou 1991).

Melanin has also shown antivenin activity in mice. Melanin extracted from black tea has shown neutralization effect against all venoms tested. Maximum antivenom effect was found against venom obtained from Japanese mamushi snake. The antivenin activity of the melanin extracted from black tea was likely due to chelating of Ca^{+2}, which is an important co-factor of phospholipase A2, a major component of snake venom. The low toxicity of melanin along with its antagonistic activity against different venoms can lead to effective life-saving treatment against snakebites when identification of the snake is impossible or if specific treatment is unavailable (Hung et al. 2004).

It has been reported that crude pigment from an actinomycete strain, *Streptomyces hygroscopicus* had shown antimicrobial activity against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella* sp. (Balagurunathan et al. 2009). Melanin pigment extracted from *Streptomyces* had also shown antibacterial activity against *Lactobacillus Vulgaris* and *Escherichia coli* (Vasanthabharathi et al. 2011).

Melanin from *Aspergillus nidulans* (Goncalves and Pombeiro-Sponchiado 2005), *Thea sinensis* (Hung et al. 2003), *Cryptococcus neoformans* (Jacobson and Tinnell 1993) and *Ophiocordyceps sinensis* (Dong and Yao 2012) have been proved to be a powerful antioxidant. It was found that the antioxidant activity of melanin was due to its scavenging or chelating properties. Melanin from *Aspergillus nidulans* was found to be a potential anti oxidant against hypochlorous acid (HOCl), which is regarded as the most toxic and abundant oxidant capable of doing considerable tissue damage. The melanin extracted from tea leaves had protective effects against hepatic injury induced by hydrazine (Goncalves and Pombeiro-Sponchiado 2005). Melanin obtained from the fungus, *Cryptococcus neoformans* protected against the negatively charged oxidants, hypochlorite and permanganate and melanin obtained from *Ophiocordyceps sinensis* showed scavenging activity on 1,1-diphenyl 2-picrylhydrazyl (DPPH) (Dong and Yao 2012).

Melanins also have physical and chemical applications. This includes their use in cosmetics and lenses. It has been reported that L-DOPA-melanin can be used as paint-additives for the development of anti fungal paints. The paint, acrylic distemper mixed with melanin preparations showed anti fungal activity against *Aspergillus sp*. (Apte et al. 2013). Melanin is used in cosmetic preparations to protect the skin from harmful effects of ultraviolet radiation and to provide a substantive, natural appearing tan (Riley 1997). Sunscreens are mainly used to block the excessive UV radiation from the sun that affects the skin. They act by scattering and deflecting the incident light that produces burning effect and tanning of the skin or by absorbing this light. Exposure to the long wavelength UVA radiations leads to oxidative stress that causes darkening of existing melanin in the skin. To prevent this, the skin has to be protected widely across the UV range of about 320-400 nm (Brenner and Hearing 2008). This

photoprotective property of melanin is used for developing lenses. Infusion of synthetic melanin into the lens are filtering out up to 98% of the high energy visible light, thus providing the ultimate eye protection. This can be used to reduce the probable damage that contributes to age-related macular degeneration and cataracts (Nosanchuk and Casadevall 2006).

The numerous functional groups such as =O, -OH, -NH, and –COOH makes melanin an ideal choice for heavy metal remediation. Use of melanin for heavy metal removal is at its nascent stage. Squid melanin was used to remove Lead (Pb(II)) and Cadmium (Cd(II)) from water (Xue et al. 2009). Hydrophobic polymer polyvinylidene difluoride (PVDF) coated with synthetic and hair melanin was used for the adsorption of Pb(II) (Sono et al. 2012). Melanin synthesized using tyrosinase enzyme was used for the adsorption of uranium which showed good uptake capacity over a broad range of pH (Saini and Melo 2013). Studies show that the biosynthesized melanin from *Pseudomonas stutzeri* can be used as a potential adsorbent for efficient removal of Hg(II), Cr(VI), Pb(II) and Cu(II) ions from drinking water (Thaira et al. 2018; Manirethan et al. 2018).

In spite of so numerous medical, environmental and biotechnological applications, the large scale use of melanin is limited due to nonavailability of a sustainable and cost-effective method of melanin production. The current market demand of melanin is about 250 tonnes per year, which is produced through non-sustainable chemical route or biological extraction route.

The major commercial sources of melanins are human hair and cephalopod ink. A form of melanin makes up the ink used by many cephalopods. Among the cephalopods, melanin's structure and synthesis are best characterized for cuttlefish, *Sepia officinalis* (Palumbo 2003). Extraction of melanin from human hair is a lengthy process and it requires highly reactive chemicals such as hydrogen peroxide. Since the cephalopod ink is a complex mixture of two glandular secretions, the melanin is associated with impurities such as saliva, mucus and other secretions (Madaras et al. 2010). Due to this complexity, the purification of melanin from cephalopod ink becomes difficult. Current market price of melanin is about USD 500 per gram due to these costly production processes hence, production of melanin from microorganisms

can provide a sustainable alternative for melanin production (M P Biochemicals n.d.; Sigma Aldrich n.d.).

Scientists studied melanin biosynthesis in microorganisms. Two pathways of melanin biosynthesis are found in microorganisms. They are 1,8-dihydroxynaphthalene (DHN) pathway or L-3,4-dihydroxyphenylalanine (L-DOPA) pathway. Overall, reactions of both pathways are represented in Figure 1-2 (Eisenman and Casadevall 2012).

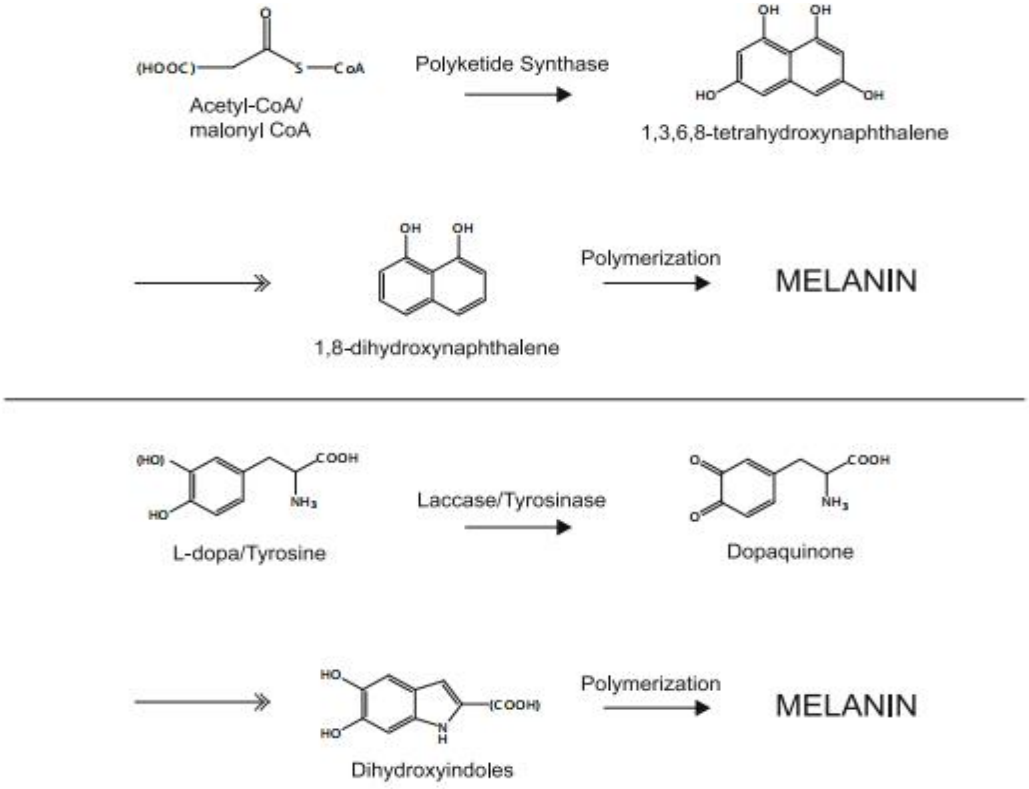

Figure 1-2: DHN (top) and L-DOPA (bottom) pathways of melanin biosynthesis.

The DHN pathway is predominant in fungi. In this pathway, the precursor molecule, known as acetyl-CoA or malonyl-CoA, is produced autogenously. The formation of 1, 3, 6, 8-tetrahydroxynaphthalene (1,3,6,8-THN), from the precursor, is catalyzed by the enzyme called polyketide synthase (PKS). After the production of 1,3,6,8-THN, a series of reduction and dehydration reactions takes place which leads to the production of intermediates scytalone, 1,3,8-trihydroxynaphthalene, vermelone, and finally DHN. Polymerization of DHN leads to the formation of melanin (Langfelder et al. 2003).

The L-DOPA pathway is predominant in bacteria for melanin biosynthesis. In this second pathway, there are two possible precursors, L-DOPA or tyrosine. If L-DOPA is the precursor, it is oxidized to dopaquinone by the enzyme laccase. If tyrosine is the precursor, it is converted to L-DOPA first and then to dopaquinone by the enzyme tyrosinase which carries out both the steps. Dopaquinone being a highly reactive molecule undergoes intramolecular nucleophilic addition by the amino group to yield cyclo DOPA, which is oxidized to form dopachrome. Tautomerization of dopachrome forms dihydroxy indoles that polymerize into melanin (Langfelder et al. 2003; Riley 1997).

Although melanin production pathways are known, there is a dearth of a sustainable production process for melanin. However, it is possible to produce melanin using the microbial source. Major advantages of the microbial melanin production are, constant supply of melanin, not susceptible to seasonal variation, relatively easy process scale-up and plausible use of raw materials, which are not competing for the human food chain. Considering all these positive factors for melanin production, a novel sustainable and easily implementable adsorptive bioprocess is developed in this research work.

CHAPTER 2

REVIEW OF LITERATURE

CHAPTER 2

2 REVIEW OF LITERATURE

2.1 Melanin biosynthesis

As mentioned in the introduction section, the melanin obtained from microorganisms has great advantages over melanin from plants and animals. The very first report of melanin production was in *Pseudomonas aeruginosa* producing pyomelanin (Osawa et al. 1963) followed by bacteria of the *Morganella-Proteus-Providencia* group which produced melanin like pigment (Müller 1985). Homogentisic acid was identified as the product of melA gene activity in a marine bacterium *Shewanella colwelliana* which produced homogentisic acid – melanin complex from tyrosine (Coon et al. 1994). Characterization of the melanogenic system was done in *Vibrio cholera* and melanin was found to be produced from L-tyrosine through homogentisic pathway (Ruzafa et al. 1995). The melanin biosynthesis using homogentisic acid as a precursor was first reported in *Vibrio cholerae*, *Hyphomonas* species and *Shewanella colwelliana*, in which the enzyme p-hydroxyphenylpyruvate hydroxylase was involved (Kotob et al. 1995). The synthesis of melanin and its characterization such as solubility and free radical nature were studied in *Proteus mirabilis* where eumelanin was produced by tyrosinase enzyme. The bacterial melanin produced was able to act as a free radical trap (Agodi et al. 1996). A novel marine bacterium *Alteromonas* strain MMB-1 was isolated from the Mediterranean sea and its melanin synthesis ability was studied using L-tyrosine as a precursor. It contained two polyphenol oxidases, catecholase and laccase and these two polyphenol oxidases were involved in melanin production (Solano et al. 1997). Melanin biosynthesis was studied on a UV-resistant mutant of *Bacillus thuringiensis* subsp. and its UV-protection ability for insecticidal crystals was investigated (Saxena et al. 2002). The thermotolerant strains of *Bacillus thuringiensis* were also reported for melanin production (Ruan et al. 2004). Another important melanin-synthesizing bacterium *Marinomonas mediterranea*, was reported to produce black eumelanin from L-tyrosine (Lucas-Elío

et al. 2012). Thus, most of the literature available on melanin from various microbial sources is melanin identification and application oriented.

The reports on melanin production in large quantities are scarce. Cabrera-Valladares et al. (2006) reported 0.41 g/L melanin in recombinant *Escherichia coli* in M9 media supplemented with isopropyl-d-thiogalactopyranoside (IPTG) and 0.4 g/L tyrosine. This strain was further optimized by Lagunas-Muñoz et al. (2006) to obtain 6 g/L melanin from 4.2 g/l tyrosine. Sajjan et al. (2010) reported 0.18 g/L of melanin production in human pathogenic soil bacteria *Klebsiella* sp. GSK, which was grown in minimal media supplemented with 2 g/L tyrosine. Jalmi et al. (2012) reported 6.6 g/L melanin in fungus *Gliocephalotrichum simplex* in malt extract broth consisting of 1% peptone and 2.5% tyrosine. However, the fungus is a plant pathogen and hence cannot be used for commercialization. Surwase et al. (2013) reported 6.8 g/L melanin in bacteria *Brevundimonas* sp. SGJ with the intermittent supplement of 7.5 g/L tyrosine. Since this bacterium is an opportunistic pathogen and requires growth medium containing non economic constituents such as beef extract, peptone, tryptone and tyrosine, it cannot be used for the large scale production.

In all of the above mentioned literature on melanin production, authors reported an additional L-tyrosine supplement for the production of melanin and process water was being used for preparing growth media. A detailed research on the effect of various operational, biological and nutritional parameters on melanin production is not available. Thus, an optimized bioprocess for sustainable large-scale melanin production is not reported, to date. Considering the melanin economy, there is a need to develop a sustainable bioprocess. Raw material cost and water footprint are major hurdles for successful commercialization of any bioprocess today. Selection of appropriate carbon and nitrogen sources is vital for any bioprocess since they influence the growth of microorganisms and melanin production. The nitrogen and carbohydrate-rich agro-industrial wastes, which are not competing for human food chain can be used as a substrate for production of melanin. The nitrogen and carbohydrate-rich agro-industrial wastes (hull of soy, corn, rice, coconut cake meal, cottonseed meal, molasses, bagasse of sugarcane, etc.) which are widely used as animal feed and organic manure have been used as a substrate for the growth of

microorganisms and production of bio pigments in the recent years (Dhillon et al. 2011b; Mohan et al. 2016; Panesar et al. 2015; Reddy Tadi et al. 2017; Ugalde et al. 2018). The effective utilization of these agro-industrial by-products can produce melanin in a cheaper way and provide value addition to the agriculture by-product.

The bioprocess should also be sustainable. Use of process water makes the bioprocess less sustainable. When the world is suffering from scarcity of drinking water, replacing the process water with non-process water as a medium for large-scale bioprocesses will play a major role in water conservations (DomínguezdeMaría 2013). There is a presence of vast quantity of seawater (96.5%) on earth as compared to freshwater (2.5%) and brackish water (1%). The sea water is a habitat for a large variety of microorganisms; which further adds interest in developing bioprocesses in seawater medium (Thomas 1994). The South Canara region where National Institute of Technology Karnataka is located is blessed with the rich coastline. Moreover, there are numerous food processing industries nearby which are growing at a rate of 10-12%. These industries produce by-products not fit for human consumption. Considering all these factors, we chose marine organism, *Pseudomonas stutzeri*, which does not require tyrosine addition and can be grown in seawater based medium. Ganesh Kumar et al. (2013) reported melanin production from *Pseudomonas stutzeri*, which was isolated from the red seaweed, *Hypnea musciformis*. Since this particular strain can grow in seawater, it can make the bioprocess sustainable and it may provide an opportunity for micro or medium scale bioprocess industry development near the coastal area.

Optimization of a bioprocess is indispensable for the maximum product formation and hence effective utilization of raw materials. Optimization can be done by using either conventional or statistical methods. In the conventional method, known as one variable at a time method, a single variable is considered at a time while keeping all the other variables at a fixed level. But to detect the frequent interactions that are occurring between the different variables, statistical experimental designs such as response surface methodology (RSM) may be employed. RSM, which is a collection of mathematical and statistical techniques for building empirical models is widely used for optimizing medium components and process parameters for the production of

industrially important products (Chen 1996; Kumdam et al. 2012; Mohan et al. 2016; Patra and Srivastava 2014; Prakash and Srivastava 2005). It helps in the understanding of the interactions between the variables at different concentrations and also in calculating the optimal concentration of each variable for a particular response (Pareek et al. 2011). Marova et al. (2012) optimized the media by using agricultural waste for pigment producing yeast, *Sporobolomyces roseus*, *Rhodotorula glutinis* and *Rhodotorula mucilaginosa*, where the highest yields were obtained in the cells cultivated on whey medium and potato medium. *Rhodotorula glutinis* cells cultivated on whey medium yielded 46 mg/L of beta-carotene whereas, *Rhodotorula mucilaginosa* cells grown on potato medium yielded 56 mg/L of beta-carotene The production of a green pigment from *Bacillus cereus* was optimized by Banerjee et al. (2014). Peptone-beef extract medium at pH 7 and 37ºC temperature produced maximum pigment (25 mg/ml) at 72 hours. Dikshit and Tallapragada (2014) optimized media and process parameters for the production of natural pigments from *Monascus sanguineusin* under stress conditions. *Monascus sanguineusin* grown in media containing glycerol, peptone and NaCl produced 19 color value units (CVU)/ml of pigment. After the statistical optimization, the yield was increased by almost three times (55.67 CVU/ml).

Nano size of any material enhances its properties and hence is more effective for a given application. Pigments like hematite and Fe_2O_3 in the nano range has been used to obtain transparent paints. These have good stability to temperature strong absorbers of ultraviolet radiation (Mohapatra and Anand 2011). Nejad et al. (2011) mixed ceramic nano pigments including cyan, magenta, TiO_2 (yellow) and $CoFe_2O_4$ together with different ratios for ceramic tile decoration, which was efficient over micro pigments. Abdel-Mohsen and Emira (2015) reported the application nano pigments to produce paints of non black color, which exhibit selective solar absorber properties. These literatures show that the nano pigments has found a wide range of industrial applications in recent years.

2.2 In situ Product Removal (ISPR) technique

Because of inhibitions at product levels, many biotechnological processes using whole cell biocatalysts yield less productivity than chemical processes. Many times,

the product stream is diluted to reduce the inhibitory effect, which increases time and volume requirements for the successive isolation and purification of the product, thereby reduces productivity. The productivity of the bioprocesses can be increased by either strain improvements or by process engineering solutions. In order to remove the toxicity or to reduce the inhibition and to simplify the downstream purification of the product formed, several process engineering solutions are being developed for immediate extraction or removal of the product from the fermentation broth. These techniques are collectively called as in situ product removal (ISPR) technique. There are different ISPR based fermentation methods such as dialysis, flash, extractive and vacuum fermentations, adsorption and ion-exchange resins for the removal of products from large scale fermentation process (Dhillon et al. 2011a; Roffler et al. 1984; Maiti et al. 2016; Prakash and Srivastava 2011; Stark and von Stockar 2003;). Some examples of these methods include evaporation, which occurs via stripping, distillation or by membrane supported techniques such as pervaporation and trans-membrane distillation. Extraction of the product into another phase includes the use of supercritical fluids, water-immiscible organic solvents or an aqueous two-phase system. The size selective permeation techniques such as reverse osmosis, dialysis, electrodialysis or nanofiltration take advantage of membranes. The immobilization procedures include the adsorption on hydrophobic carriers and affinity adsorption techniques.

Adsorptive bioprocess, which is also a class of ISPR technique, involves usage of adsorbents in the bioprocess for the removal of the product as it is produced in the fermentation. Adsorbents with large surface areas, ranging from activated carbon to polymeric resins, can be used to remove products from the fermentation broth. These adsorbents can be added directly to the bioreactor or they can be placed in a separate container with fermentation broth circulating to and from it. The in situ removal of the product in a bioreactor can take place either inside (internal) or in an external loop. While the contact between the microorganisms and the product separation phase can be either direct or indirect (Stark and von Stockar 2003).

Activated carbon was used for the enhancement of ethanol production from yeast. After the removal of the toxic end products, the yeast cells were capable of producing

135 g/L ethanol in 8 hours (Wang et al. 1981). Payne et al. (1988) observed increased alkaloid production from *Catharanthus roseus* when a neutral polymeric resin was used in situ. With in situ product removal, 2.5 fold increase in the total alkaloid production was observed. Different polymeric resins such as amberlite and bonopore were investigated as adsorbents for in situ product removal for the fermentative production of acetone and butanol by *Clostridium acetobutylicum*. Adsorption of 43 mg butanol/g sorbent was observed when bonopore was used, giving a final concentration of butanol in the broth of 0.2% which was well below the toxic limit (Nielsen et al. 1988). Vicenzi et al. (1997) used polymeric adsorbent resins for the removal of 3,4-methylene-dioxyphenyl acetone which allowed the reaction concentration to be increased from 6 g/L to 40 g/L. A non-ionic exchange resin XAD-4 was used for the enhanced production of taxol from *Taxus cuapidata* cell culture. The biosynthesis of taxol was enhanced by 40-70% by adding XAD resin into the culture medium (Kwon et al. 1998). Bae et al. (2001) used hydrophobic polyaromatic Diaion HP-20 resin for the in situ recovery of prodigiosin-like pigment produced from *Serratia* sp. With the addition of internal adsorbent, the pigment production was increased by 1.8 fold. Jia et al. (2006) reported 1.25 fold increase in the production of pristinamycin from *Streptomyces pristinaespiralis* when different adsorbent resins were used in fermentation. Pristinamycin fermentation with the addition of resin JD-1 increased the production from 0.36 g/L to 0.8 g/L. Van Den Berg et al. (2008) used solvent impregnated resins and commercial resin (XAD-4) as an in situ product recovery tool for phenol recovery from *Pseudomonas putida*. Addition of solvent impregnated resins resulted in an increase in the phenol production by 4 fold while the commercial resin gave a 2.5 fold increase in the volumetric production. Liu et al. (2011) used D152 resin for the in situ removal of poly-L-lysine produced by *Streptomyces* sp resulting 6.2 fold increase in the poly-L-lysine production in a bioreactor. López-Garzón and Straathof (2014) has described the use of different resins for the in situ removal of carboxylic acids from the fermentation broth.

However, resins are prone to disintegration due to the effect of fluid shear stress in stirred tank bioreactors, which is a major bottleneck for a successful adsorptive bioprocess (Stark and von Stockar 2003). Therefore, all of the above ISPR projects were done with a complicated configuration setup. In above reported ISPR

techniques, the separation of the product took place outside of the bioreactor and the cells were not in direct contact with the separative force. Such type of solutions reduces the interaction between the cells and the separating device, but, additional equipment and control units makes the system more complex, which is only applicable for the production of a dedicated single product and not a multipurpose plant. Therefore, even if they reported a good yield at small scale, the processes would not be successful at large scale due to the complexity of fluid transfer operations and difficulties in sterility maintenance (Stark and von Stockar 2003).

Implementation of ISPR technique in a bioprocess should be done in a manner that it should not affect the regular bioprocess operation. Easily implementable and adoptable ISPR technique can lead to enhancements in terms of overall productivity and yield. Reduction in energy consumption and maximum product recovery are the essential requirements for a successful implementation of ISPR technique. The economic and scalability considerations of a particular ISPR process are the decisive factor. Addition of complex extensions is the reason for the failure of many ISPR projects. Hence ISPR techniques, which require no plant modification will be always an advantage.

2.3 Purification of melanin

To date, there are only a couple of methods reported for melanin extraction at laboratory scale. The melanin is extracted and purified at laboratory scale by first treating with an alkali and then precipitating with an acid. It is extracted with an alkali like NaOH or KOH to dissolve it completely and then treated with acids like HCl to precipitate it out (Schmaler-Ripcke et al. 2009). Another method of extraction and purification of melanin includes precipitating with 1 M acetic acid or flocculating with alum (Jalmi et al. 2012). However, the percentage recovery and efficiency of both these methods are not yet ascertained. Thus, there is a need for an efficient extraction and purification method that can increase the efficiency and reduce extraction time.

Considering all the bottlenecks mentioned above, following objectives are formulated for this research work.

1. To develop the process for the production of melanin nanopigments from *Pseudomonas stutzeri*.
2. To optimize the process for the production of melanin nanopigments from *Pseudomonas Stutzeri*.
3. To develop the downstream process for the purification of melanin nanopigments produced from *Pseudomonas stutzeri*.
4. To develop the adsorptive bioprocess for the enhancement of melanin production.

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3

3 MATERIALS AND METHODS

All the experiments were carried out in triplicates and mean values are plotted in the thesis, unless otherwise stated in the caption of a figure. The data tables for figures are given in the appendix and table numbers are mentioned in the caption of the figure.

3.1 Chemicals used

Nutrient Broth (NB) and Tryptic Soy Broth (TSB), (Himedia chemicals, Mumbai, India) were used for the maintenance of the organism. Inorganic salts such as NH_4NO_3, $(NH_4)_2SO_4$, $NaNO_3$, KNO_3, NaCl, $CaCl_2$, $CuSO_4$, KCl, K_2HPO_4, KH_2PO_4, $MgSO_4$, $MnSO_4$, $FeSO_4$, $CuSO_4$, and $ZnSO_4$ were used from Nice laboratories, India. Bushnell Hass Broth (BHB), Luria-Bertani broth (LB), Glucose, sucrose, starch, peptone, yeast extract, tryptone, beef extract, agar powder and oat meal powder were obtained from Hi-Media laboratory, India. Corn steep liquor and synthetic melanin were obtained from Sigma Aldrich, India.

3.2 Selection of microorganism for the production of melanin

Based on the literature review, a marine bacteria, *Pseudomonas stutzeri* [MTCC 11712] was selected for the production of melanin.

3.3 Subculturing of the microorganism on agar medium

The bacterial strain obtained from Microbial Type Culture Collection and Gene Bank (MTCC) Chandigarh, India was retrieved under laboratory conditions. The pure culture of *Pseudomonas stutzeri* [MTCC 11712] was maintained on the nutrient agar slants. All cultures were stored at 4°C and subcultured after every 4 weeks. Periodical subculture at every four weeks interval was carried out to maintain the viability of the strain. Melanin production was investigated after each subculture to study the efficiency of strain to produce melanin.

3.4 Growth of the microorganism and production of melanin in shake flask

The nutrient broth prepared in seawater with pH 7 was used as a medium for the culture of microorganisms. Shake flasks of 250 ml volume with a filling volume of 50 ml were used at an agitation rate of 150 rpm and at a temperature of 37°C in an incubator shaker. Samples were taken at regular time intervals to study the growth and melanin production. One flask was kept for each time interval and the experiments were carried out in triplicates. The Optical Density of the samples was measured at 660 nm after every 6 h using appropriate blank. Biomass dry weight of the culture was measured by centrifuging (5000 rpm, 5 min) and drying the pellet for 8 h or until the dry weight reached steady state at 60^0C in a hot air oven. The supernatant obtained after centrifugation was filtered with 0.22 µm syringe filters and their absorbance was measured at 400 nm to quantify the melanin.

3.5 Effect of the operating conditions on melanin production

3.5.1 Effect of different flask filling volume

Effect of different flask filling volumes such as 10 ml, 20 ml, 50 ml and 100 ml was investigated in 250 ml shake flasks at an agitation rate of 150 rpm and at a temperature of 37°C in an incubator shaker.

3.5.2 Effect of shaking frequency

The effect of shaking frequency on melanin production was investigated by incubating the flasks in an incubator shaker at 100, 150, 200 and 250 rpm.

3.5.3 Effect of initial pH

The initial pH effect on the production of melanin was determined by setting initial pH of the medium to 5, 6, 7, 8 and 9 by using 0.2 N HCl and 0.2 N NaOH.

3.5.4 Effect of temperature

The effect of temperature for melanin production was investigated by incubating the culture flasks at 25º, 30º, 37º, 40º and 45ºC in incubator shaker.

3.6 Optimization of biological & nutritional parameters on melanin production

All the experiments conducted used a constant media volume of 50 ml in 250 ml Erlenmeyer flasks at 150 rpm and 37°C. NB was used as the control medium in all optimization experiments.

3.6.1 Effect of the different growth media

The effect of growth media on melanin production was studied by inoculating the culture into different growth media such as Nutrient Broth (NB), Bushnell- Haas broth (BHB), Luria-Bertini (LB) broth and Trypticase Soy broth (TSB).

3.6.2 Effect of inoculum percentage

The effect of inoculum percentage on melanin production was studied by changing the inoculum concentrations of 0.5%, 5%, 10%, 15%, 20%, 30% and 40%. Shake flasks of 250 ml volume with a filling volume of 10 ml were used at 150 rpm and 37°C in an incubator shaker.

3.6.3 Effect of inoculum age

An inoculum of age 6, 12, 18 and 24 hours was used to study its effect on melanin production. Shake flasks of 250 ml volume with a filling volume of 10 ml were used at 150 rpm and 37°C in an incubator shaker.

3.6.4 Effect of carbon and nitrogen sources

The effect of different carbon sources such as glucose, starch, glycerol and sucrose were investigated by adding different carbon source to the medium at a concentration of 5 g/L. A medium similar to the TSB medium was prepared by mixing all individual components except the carbon source. The carbon source, which was originally present in the TSB medium was replaced by the investigated carbon source.

Similarly, the effect of different complex nitrogen sources such as corn steep liquor, soyabean meal, coconut meal, cottonseed meal and oat meal was investigated by adding different nitrogen source to the medium at a concentration of 5 g/L. A medium similar to the TSB medium was prepared by mixing all individual components and the nitrogen source, which was originally present in the TSB medium was replaced by the investigated nitrogen source.

3.6.5 Effect of trace minerals

To study the effect of various mineral salts on the melanin yield, mineral salts were added to the medium. Trace minerals such as $MgSO_4$, $MnSO_4$, FeSO4, $CuSO_4$, and $ZnSO_4$, were used. The concentration of each trace element was varied between 0.005 g/L to 0.1 g/L.

3.7 Effect of constant pH on melanin production

Three different buffers of pH 7.5 were used to maintain constant pH of the culture medium in shake flask. A 0.1 M Tris-HCl stock solution was prepared by dissolving 121.1 g of Tris base in 600 ml distilled water to which 40.3 ml of 0.1 N HCl was added and final volume was made up to 1 L by using distilled water. Sodium citrate buffer was prepared by mixing 5 ml of 0.1 M citric acid solution with 95 ml of 0.1 M Trisodium citrate solution. Phosphate buffer was prepared by dissolving 1.92 g Potassium phosphate monobasic anhydrous and 22.53 g of Sodium phosphate dibasic in 1L of distilled water. To study the effect of constant pH on melanin production, the cells were cultured in the media which was prepared using the buffer solutions. Shake flasks of 250 ml volume with a filling volume of 10 ml were used at 150 rpm and 37°C in an incubator shaker.

3.8 Statistical optimization for melanin production

After selection of important media constituents for melanin biosynthesis using one factor at a time approach, optimization of the significant variables (Glucose, Coconut meal cake, $FeSO_4$, $CuSO_4$ and $ZnSO_4$) was carried out by the central composite design (CCD) for enhancing the melanin production. Five independent variables were studied at three different levels (−1, 0, +1) in a set of 50 experiments as mentioned in Table 3.1. All the experiments were conducted in triplicates and the mean value of melanin yield (mg/L) was reported as the response (Y).

Following generalized polynomial model was used for regression analysis of the data and linear, quadratic and polynomial terms were selected for optimization. StateEase 7 was used for statistical optimization.

$$Y = \beta_0 + \sum_{i=1}^{5} \beta_i \cdot x_i + \sum_{i=1}^{5} \beta_{ii} \cdot x_i^2 + \sum_{i,j=1}^{5} \beta_{ij} \cdot x_{ij} \qquad (3.1)$$

Standard order	Run	Factor 1 A:Coconut cake meal (g/L)	Factor 2 B:Glucose (g/L)	Factor 3 C: $CuSO_4$ (g/L)	Factor 4 D:$FeSO_4$ (g/L)	Factor 5 E: $MgSO_4$ (g/L)	Response :Melanin yield(g/L) (actual)
48	1	25.50	25.50	0.05	0.05	0.05	
45	2	25.50	25.50	0.05	0.05	0.05	
9	3	1.00	1.00	0.00	0.10	0.00	
25	4	1.00	1.00	0.00	0.10	0.10	
17	5	1.00	1.00	0.00	0.00	0.10	
8	6	50.00	50.00	0.10	0.00	0.00	
46	7	25.50	25.50	0.05	0.05	0.05	
12	8	50.00	50.00	0.00	0.10	0.00	
30	9	50.00	1.00	0.10	0.10	0.10	
47	10	25.50	25.50	0.05	0.05	0.05	
34	11	83.77	25.50	0.05	0.05	0.05	
24	12	50.00	50.00	0.10	0.00	0.10	
22	13	50.00	1.00	0.10	0.00	0.10	
42	14	25.50	25.50	0.05	0.05	0.17	
27	15	1.00	50.00	0.00	0.10	0.10	
5	16	1.00	1.00	0.10	0.00	0.00	
23	17	1.00	50.00	0.10	0.00	0.10	
26	18	50.00	1.00	0.00	0.10	0.10	
11	19	1.00	50.00	0.00	0.10	0.00	
10	20	50.00	1.00	0.00	0.10	0.00	
37	21	25.50	25.50	0.00	0.05	0.05	
35	22	25.50	0.00	0.05	0.05	0.05	
15	23	1.00	50.00	0.10	0.10	0.00	
40	24	25.50	25.50	0.05	0.17	0.05	
1	25	1.00	1.00	0.00	0.00	0.00	
19	26	1.00	50.00	0.00	0.00	0.10	
32	27	50.00	50.00	0.10	0.10	0.10	
41	28	25.50	25.50	0.05	0.05	0.07	

28	29	50.00	50.00	0.00	0.10	0.10	
2	30	50.00	1.00	0.00	0.00	0.00	
6	31	50.00	1.00	0.10	0.00	0.00	
38	32	25.50	25.50	0.17	0.05	0.05	
33	33	0.00	25.50	0.05	0.05	0.05	
14	34	50.00	1.00	0.10	0.10	0.00	
36	35	25.50	83.77	0.05	0.05	0.05	
44	36	25.50	25.50	0.05	0.05	0.05	
39	37	25.50	25.50	0.05	0.00	0.05	
16	38	50.00	50.00	0.10	0.10	0.00	
20	39	50.00	50.00	0.00	0.00	0.10	
21	40	1.00	1.00	0.10	0.00	0.10	
50	41	25.50	25.50	0.05	0.05	0.05	
49	42	25.50	25.50	0.05	0.05	0.05	
13	43	1.00	1.00	0.10	0.10	0.00	
4	44	50.00	50.00	0.00	0.00	0.00	
7	45	1.00	50.00	0.10	0.00	0.00	
3	46	1.00	50.00	0.00	0.00	0.00	
43	47	25.50	25.50	0.05	0.05	0.05	
18	48	50.00	1.00	0.00	0.00	0.10	
29	49	1.00	1.00	0.10	0.10	0.10	
31	50	1.00	50.00	0.10	0.10	0.10	

Table 3-1: Experimental design matrix for optimization of melanin production using CCD.

From the 50 experiments, the responses for each run were subjected to multiple nonlinear regressions to obtain the coefficients of the polynomial equation. The F-test was employed in order to evaluate the statistical significance of the quadratic polynomial model.

3.9 Stirred tank bioreactor studies

Bioreactor studies were carried out in a 3 L stirred tank bioreactor (Scigenics, India). The dimensions of the bioreactor are listed in the following table (Table 3-2). The bioreactor was equipped with a sterilizable dissolved oxygen (DO) probe (Mettler Toledo) and pH probe (Mettler Toledo). The DO and pH probes were calibrated before use (APPENDIX 7.1).

Dimension	Description
Total Volume, V_T(L)	3
Working Volume, V_L(L)	2.5
Vessel Height, H_L(m)	0.23
Liquid Height, H_L(m)	0.19
Vessel Diameter, D_T(m)	0.16
Aspect ratio (H_T/D_T)	1.44
Impeller type	Rushton turbine
Impeller diameter (m)	0.052

Table 3-2: List of the dimensions of the 3 L stirred tank bioreactor.

The experiments were conducted under the optimized process parameters and media was prepared by adding optimized media components. The working volume of the bioreactor was set as 2.4 L. The dissolved oxygen level was maintained above 50% of the saturation by controlling aeration and agitation and the pH was maintained at 7.5±0.2 using 0.1 N NaOH and 0.1 N HCl. The temperature was maintained at 37ºC by using the heater and circulating cold water through the cooling jacket during the fermentation. Aseptic sampling was done periodically to measure the concentration of melanin and biomass during the fermentation process.

3.10 Identification of melanin biosynthesis pathway

To identify the melanin synthesis pathway, the melanin synthesis inhibitors such as arginine and sodium azide were added into the microbial culture. Shake flasks of 250

ml volume with a filling volume of 10 ml were used at 150 rpm and 37°C in an incubator shaker. Arginine is an inhibitor of tyrosinase enzyme (L-DOPA pathway) and sodium azide is an inhibitor of laccase enzyme (DNB pathway). 1 mM concentration of the inhibitors was used.

3.11 Effect of feedback inhibition by melanin

The feedback inhibition of melanin was studied by adding different concentrations of melanin (100 mg/L, 200 mg/L, 500 mg/L and 1000 mg/L) into the culture medium at the start of the bioprocess. Shake flasks of 250 ml volume with a filling volume of 10 ml were used at 150 rpm and 37°C in an incubator shaker. Biomass growth, the enzyme activity of the cells and melanin produced were measured as the protocol described in section 3.17.

3.12 Extraction and purification of melanin

The melanin was extracted and purified by first treating with an alkali and then precipitating with an acid. Melanin was extracted by NaOH to dissolve it completely and then the solution was acidified using HCl to precipitate it out (Schmaler-Ripcke et al. 2009).

Here, the fermentation broth was centrifuged at 5,000*g* for 5 min to remove the biomass. The cell free supernatant was extracted with 1 M NaOH and further autoclaved at 120° C for 20 minutes to denature proteins and phospholipids. The autoclaved solution was centrifuged at 5,000*g* for 5 min to collect the supernatant. The alkaline pigmented supernatant was acidified to pH less than 3 with 1N HCl to precipitate melanin. The precipitated melanin was harvested by centrifugation (12,000 *g*, 20 min). It was then washed with distilled water (3 times) and ethanol (3 times) followed by drying overnight at room temperature. Melanin powder thus obtained was stored in the refrigerator at 4ºC.

3.13 Development of the adsorptive bioprocess

Adsorptive bioprocess is a type of In situ Product Removal (ISPR) technique. As mentioned in the literature review, in this method, the target molecule in the bioprocess is removed as it is biosynthesized during the bio fermentation process.

Here, adsorption was used to remove the melanin from the broth as it is produced to reduce feedback inhibition as well as for recovery.

3.13.1 Effect of different adsorbents

Different adsorbents such as Fuller's earth (white in colour, particle size 100-150 μm), Zeolite (white in colour, particle size <45 μm), Celite (white in colour, particle size <45 μm), alumina (white in colour, particle size 50-150 μm) and activated carbon (black in colour, particle size 100-150 μm) were used to identify the best adsorbent for melanin adsorption from the broth. These adsorbents were added to the fermentation broth of pH 8 at 1 g/L concentration and kept in a rotary shaker at 150 rpm for 12 hours. The broth was centrifuged to separate the adsorbents and the absorbance of the supernatant was measured at 400 nm using a spectrophotometer to measure the extent of adsorption.

The melanin bound adsorbents were treated with 0.1N NaOH for 3 times to desorb melanin. This solution was centrifuged and the absorbance of the supernatant was measured at 400 nm to quantify the amount of adsorbed melanin.

3.13.2 Effect of dosage time of the adsorbent

The best adsorbent for melanin adsorption was identified from above study. This adsorbent was added into the fermentation broth at different times to identify the best dosage time for the maximum melanin production and recovery.

3.13.3 Effect of amount of adsorbent dosage

Different concentrations of the best adsorbent (0.5, 1, 2, 4, 6, 8 and 10 g/L) were added to the cultures at the time identified by the above mentioned study. The growth medium containing free melanin and melanin bound on adsorbent was harvested at the end of the bioprocess. The broth was centrifuged at 4000 rpm for 5 minutes. The supernatant was collected and amount of melanin was calculated by measuring absorbance at 400 nm. The pellet containing adsorbent was washed two times with distilled water followed by two times washing with ethanol to remove cells and other compounds attached to the adsorbent. Later, a 0.1 M NaOH solution was added to the washed adsorbent to desorb melanin. The mixture was centrifuged at 5000 rpm for 10 minutes and melanin amount in the supernatant was measured by a

spectrophotometer. The desorption process was repeated thrice. This concentrated desorbed melanin solution was acidified to pH less than 3 to precipitate melanin. The melanin thus obtained was washed three times with distilled water, dried and stored at 4ºC.

3.14 Mathematical modeling of growth and melanin formation

3.14.1 Biomass growth kinetics

The biomass growth rate was quantified using modified Verhulset model, as described by the equation given below (Vázquez and Murado 2008),

$$\frac{dx}{dt} = \mu_m \cdot x \cdot \left(\frac{x_m - x}{x_m}\right) \quad (3\text{-}2)$$

where,

x= biomass concentration (g/l)

x_m = maximum biomass concentration (g/l)

μ_m= maximum specific growth rate (1/h)

Vazques et al. (2008) further derived following equation for biomass concentration from equation (3-2)

$$x = \frac{x_m}{1+exp\left[2+\frac{4 \cdot v_x}{x_m} \cdot (\tau_x - t)\right]} \quad (3\text{-}3)$$

Where,

v_x= biomass growth rate (g/l/h) = $\mu_{obs} \cdot x$

μ_{obs} = observed specific growth rate (1/h)

τ_x=lag time (h)

t=time (h)

Equation (3-3) was used to simulate growth profile of *pseudomonas stutzeri* [MTCC 11712].

3.14.2 Substrate consumption kinetics

The substrate is used for biomass growth, product formation and maintenance in a bioprocess. The rate of change of substrate can be written as,

$$-\frac{ds}{dt} = \frac{1}{Y_{P/S}} \cdot \frac{dp}{dt} + \frac{1}{Y_{X/S}} \cdot \frac{dx}{dt} + m_s \cdot x \qquad (3\text{-}4)$$

The majority of the glucose was used for biomass generation, therefore, for simplification, neglecting substrate consumption for product formation will reduce equation (3-4) to,

$$-\frac{ds}{dt} = \frac{1}{Y_{X/S}} \cdot \frac{dx}{dt} + m_s \cdot x \qquad (3\text{-}5)$$

Where,

s= substrate concentration (g/l)

$Y_{P/S}$ = product yield coefficient, i.e., melanin yield coefficient (g/g)

p= product concentration (g/L), i.e., melanin concentration (g/L)

$Y_{X/S}$= biomass yield coefficient (g/g)

m_s= maintenance coefficient (g/g/h)

integrating above equation from time t=0 to time t=t, and substituting value of x from equation (3-3) gives following equation for substrate concentration profile,

$$s = s_0 - \frac{1}{Y_{X/S}}[x - x_0] - \left(\frac{m_s \cdot x_m^2}{4 \cdot v_x}\right) ln \left[\frac{x_0 \cdot \left(exp^{\frac{4 \cdot v_x}{x_m} \cdot t} - 1\right) + x_m}{x_m}\right] \qquad (3\text{-}6)$$

Where, s_0 = initial substrate concentration (g/l) and x_0 = initial biomass concentration (g/l)

3.14.3 Melanin production kinetics

Melanin formation can be described by the Ludeking-Pirate equation. However, melanin was growth associated and therefore, the non-growth associated product formation term was neglected and the simplified equation can be written as,

$$\frac{dp}{dt} = \alpha \cdot \frac{dx}{dt} \quad (3\text{-}7)$$

Where,

α = Ludeking-Pirate constant linked to growth associated product formation (g/g)

Integrating equation (3-7) result in the following equation for product concentration profile

$$p = \alpha \cdot (x - x_0) \quad (3\text{-}8)$$

3.14.4 Kinetic parameter estimation

All parameters mentioned above were estimated by the nonlinear least square method of parameter estimation using Microsoft Excel solver tool. Batch experiment data were used and the sum of squared error (SSE) was minimized using Solver tool, as given by the following equation,

Minimize,

$$SSE = \sum_{i=1}^{n} \left(x_{sim} - x_{exp}\right)^2 \quad (3\text{-}9)$$

Where,

x_{sim}= is simulated value and x_{exp} = experimental value

3.15 Heavy metal adsorption studies

Biosynthesized melanin was used in adsorption studies for the removal of heavy metals such as Hg(II), Pb(II), Cr(VI) and Cu(II) at neutral pH. Studies on removal of heavy metals were conducted for 24 hours with 25 mg melanin and 10 mg/L heavy metal solution. Batch adsorption was carried out in 100 ml conical shake flasks with 25 ml filing volume and 150 rpm.

3.16 Melanin characterization

3.16.1 Fourier Transformed Infra Red (FTIR) spectroscopy studies

FTIR is considered to be the most informative, non-destructive and well-resolved method, which will provide information on various functional groups and detailed structural analysis of melanin (S. Sajjan et al., 2010). The purified dark brown powder

and standard synthetic melanin were analyzed by Fourier transform infrared (FTIR) spectroscopy (Bruker Alpha, Massachusetts, US) with potassium bromide (KBr) as blank. The FTIR analysis was carried out at Department of Chemistry, NITK Surathkal.

3.16.2 Particle size analysis

Particle size analysis of the biosynthesized melanin from *Pseudomonas stutzeri* was done using nanoparticle analyzer (Nanopartica SZ–100, HORIBA Scientific, Kyoto, Japan). Before introducing into the instrument, pure samples of biosynthesized melanin were dispersed in distilled water using an ultrasonicator. The particle size analysis was carried out at Department of Chemical Engineering, NITK Surathkal.

3.16.3 Nuclear Magnetic Resonance spectroscopy (NMR) studies

Nuclear Magnetic Resonance spectroscopy (NMR) studies were carried out using Bruker Avance III (400MHz, Massachusetts, US). NMR spectrum with respect to ^{1}H (hydrogen-1) nuclei within the molecules were analyzed. The NMR studies were carried out at Sophisticated Test and Instrumentation Centre, CUSAT, Cochin.

3.16.4 Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) analysis

The Scanning electron microscope (JSM-6380, JEOL, Japan) and Transmission Electron Microscope (JEM-2100, JEOL, Japan) were used to study the size and morphological characteristics of the biosynthesized melanin. The SEM and TEM analysis were carried out at Department of Chemical Engineering, NITK Surathkal.

3.17 Analytical techniques

3.17.1 Melanin quantification

Quantification of melanin produced in the cell-free culture supernatant was estimated by UV-Visible spectrophotometer (Genesys 10S, Thermofisher Scientific, USA) at 400 nm using a standard curve of melanin (Ganesh Kumar et al. 2013).

3.17.2 Biomass quantification

Samples were taken at regular time intervals to study the growth of the microorganism. The OD of the samples was measured at 660 nm after every 4 h using appropriate blank. Biomass dry weight of the culture was accounted for by centrifuging (5000 rpm, 5mins, and 4°C) and drying the pellet for 8 h at 60^0C in a hot air oven.

3.17.3 Tyrosinase assay

3.17.3.1 Extraction of tyrosinase enzyme from *Pseudomonas stutzeri*

The protocol for tyrosinase enzyme assay by Ren et al. (2013) was followed to determine the enzyme activity of tyrosinase extracted from *Pseudomonas stutzeri*. To estimate the tyrosinase activity of *Pseudomonas stutzeri* cells, the bacterial culture was first centrifuged at 5,000 rpm to collect the cells. Tyrosinase enzyme extraction was done by sonicating the cells in cold 100 mM phosphate buffer of pH 6.5. The homogenate was centrifuged at 10000 rpm for 15 min and the supernatant was collected which was used as a source of enzyme. Tyrosinase activity was assayed by using L-tyrosine as substrate. 0.2 ml of the enzyme solution was added to a 2.8 ml of 0.1M sodium phosphate buffer (pH 6.5) containing 1mM L-tyrosine and the formation of dopachrome was monitored by measuring the absorbance at 475 nm. One international unit (IU) of tyrosinase activity is defined as the amount of enzyme required to oxidize 1 µmol of L-tyrosine to dopachrome per minute under the above conditions, which was calculated using the molar extinction coefficient of dopachrome (3600 L M^{-1} cm^{-1}) by the following equation,

$$:\frac{U}{mL} = \frac{\left(\frac{Absorbance}{minute}\right)\cdot(Assay\ volume(ml)).(dilution factor).(1000)}{(3600\ L\ .\ M^{-1}.cm^{-1}).(1\ cm).(enzyme\ volume\ (ml))} \qquad (3.10)$$

3.17.3.2 Assay for the tyrosinase enzyme obtained from Mushroom

The mushroom tyrosinase was purchased from Sigma Aldrich, India. The tyrosinase enzyme activity was measured by the spectrophotometric method described as per Sigma-Aldrich protocol by measuring the absorbance of the product, dopachrome at 280 nm. Following equation explains the reaction between the tyrosine substrate and the tyrosinase enzyme.

$$L - tyrsoine + O_2 \xrightarrow{tyrosinase} L - DOPA$$

$$L - DOPA \xrightarrow{tyrosinase} dopachrome + H_2O$$

Reagents Used - Reagent A (50 mM Potassium Phosphate Buffer, pH 6.5), Reagent B (1 mM Tyrosine solution in deionized water), Reagent C (solution containing 500-1,000 units/ml of Tyrosinase in cold Reagent A) and deionized water.

Procedure: A reaction cocktail was prepared by pipetting 9 ml of deionized water, 10 ml of reagent A and 10 ml of reagent B into a test tube and the pH was adjusted to 6.5 at room temperature with 0.1 M HCl or 0.1 M NaOH. It was oxygenated by bubbling 99.9% pure air for 5 minutes immediately before use. Blank was prepared by mixing 2.90 ml of reaction cocktail and 0.10 ml Reagent A and the Test samples were prepared by mixing 2.90 ml of reaction cocktail and 0.10 ml of tyrosinase enzyme. The samples were mixed immediately by inversion and increase in absorbance was recorded at 280 nm until the absorbance at 280 nm become constant, using a UV-Visible spectrophotometer. The enzyme activity was calculated by following equation, (Duckworth and Coleman 1970).

$$\frac{U}{mL} = \frac{(\Delta A_{280\,test} - \Delta A_{280\,blank}) \cdot dilution factor}{(0.001) \cdot (0.1)} \qquad (3.11)$$

Where,

$\Delta A_{280\,test}$ = absorbance change of a given sample at 280 nm per minute

$\Delta A_{280\,blank}$ = absorbance change of a blank solution at 280 nm per minute

0.001 = change in absorbance per minute per unit of Tyrosinase at pH 6.5 at 25° C in a 3 ml reaction mixture

0.1= Volume (in milliliters) of enzyme used

3.17.4 Estimation of Total Reducing Sugar (TRS)

TRS was measured by dinitrosalicylic (DNS) method (Miller 1959). The DNS solution was prepared by dissolving 1 g of DNS in 50 ml of distilled water. To this solution, about 30 g of sodium potassium tartarate was added in small lots, the solution developed milky yellow color. Then 20 ml of 2 N NaOH was added, which

converted the solution color to transparent orange yellow. The final volume was made to 100 ml with the distilled water and solution was stored in an amber colored bottle.

Glucose working solution (0.01 M) was prepared by dissolving 180 mg of glucose in 100 ml of distilled water in a standard flask.

Procedure: Standard Glucose solution was pipetted out in the range of 0.2, 0.4, 0.6, 0.8 and 1 ml, into 5 separate test tubes. A test tube containing distilled water was used as a blank solution. Using distilled water, the volume in each test tube, including the test tube containing the blank solution was made up to 2 ml. 1 mL of DNS reagent was added to each tube and the test tubes were covered with aluminium foil. The contents in the test tubes were heated in a boiling water bath for 5 minutes. The test tubes were cooled to room temperature and 9 ml of distilled water was added to each test tube and mixed well. The test tube solution was added into different cuvettes and optical density was measured at 575 nm using a spectrophotometer. A calibration chart was prepared with the amount of glucose on X-axis vs OD at 575 nm (A_{575}) on the Y axis.

3.17.5 Estimation of protein in the broth

The Bradford protein assay is a quick, inexpensive and simple way to determine protein concentration. A protein is reacted with the Bradford reagent and absorbance is measured at 575 nm (Kruger 2002).

Procedure: Different volumes [0 µl (0 µg), 10 µl (5 µg), 20 µl (10 µg), 30 µl (15 µg), 40 µl (20 µg), 50 µl (25 µg)] of 0.5 mg/ml Bovine Serum Albumin (BSA) was pipetted out into separate test tubes. The distilled water was used as a blank. 1.5 ml of Bradford reagent was added to each test tube and covered with plastic paraffin film and gently mixed by inverting the test tube. The test tubes were incubated at room temperature for 10 minutes and the absorbance of each sample was measured at 595 nm using a spectrophotometer. A calibration chart was prepared using above values. To measuring the unknown quantity of protein in the test sample, a 50 µl of the broth that has to be quantified was pipetted into a cuvette. 1.5 ml of Bradford reagent was added to the cuvette, gently mixed by inverting the cuvette and incubated at room temperature for 10 minutes. The absorbance of the sample was measured at 595 nm.

3.17.6 Estimation of nitrogen

The nitrogen content was estimated by using MColor test kit (Merck), in which ammonium ions in solution are converted into blue colored complex. In strong alkaline solutions, ammonium nitrogen gets converted into almost entirely as ammonia, reacts with a chlorinating agent to form monochloramine. This, in turn, reacts with thymol to form a blue indophenol derivative. The concentration of ammonical nitrogen was obtained by comparing the color of the sample with the color of the standards.

3.17.7 Estimation of DNA

The DNA content was estimated by diluting the sample with distilled water or TE buffer (Tris HCl- EDTA) and measuring the optical density at 260 nm. The following formula was used to determine the concentration of DNA:

$$Total\ DNA\ (\mu g) = \frac{(A_{260}).\left(50\ \frac{\mu g}{ml}\right).(dilution\ factor).(0.1\ ml)}{1} \quad (3.12)$$

Where,

A_{260} = Absorbance at 260 nm

0.1 = Volume of the sample

1 = Absorbance of DNA standard solution of concentration 50 µg/ml, at 260 nm

CHAPTER 4

RESULTS AND DISCUSSION

CHAPTER 4

4 RESULTS AND DISCUSSIONS

4.1 Acclimatization of microbial culture under laboratory conditions

Nutrient agar slants and plates were prepared for maintenance of the *Pseudomonas stutzeri* obtained from MTCC Chandigarh, India.

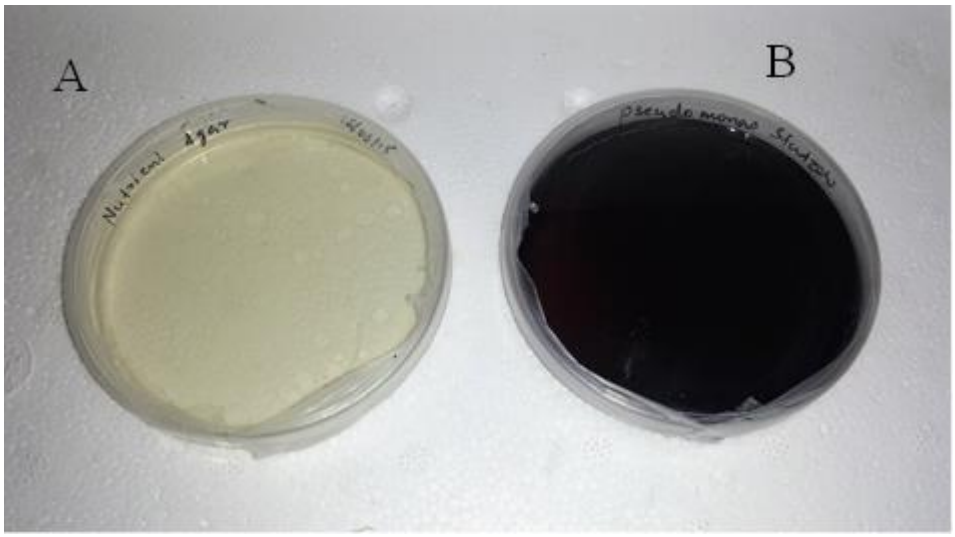

Figure 4-1: A) Nutrient agar plate (blank). B) *Pseudomonas stutzeri* grown on nutrient agar plate.

Figure 4.1B shows melanin pigmentation in agar plate on day 5. Melanin production efficiency was measured by growing the culture in NB medium for 72 hours and measuring the melanin content after 72 hours of incubation. Melanin production was in the range of 150 to 155 mg/L after 72 hours of incubation. Results revealed that melanin production efficiency, i.e., melanin production did not reduce up to 6 subcultures. However, after eight subcultures, i.e., eight months, melanin production efficiency reduced to 80% of the original value of 150-155 mg/L. Therefore, after every six months, the old culture was discarded and a new microbial culture was initiated from the glycerol stock maintained at -80ºC.

4.2 Melanin production and growth profile in basal medium

Figure 4-2 shows growth and production profile in basal medium, i.e., NB medium. Figure 4-3 demonstrates color changes in *Pseudomonas stutzeri* culture with respect to time. The cells attained exponential growth phase within 12 hours of inoculation and biomass concentration reached to 1.7 g/L on 12th hour. Biomass growth was gradual after 12th hour and the maximum biomass produced was 2.27 g/L at 66th hour. Melanin production was growth associated and the maximum amount of melanin produced was 153 mg/L at 72 hours.

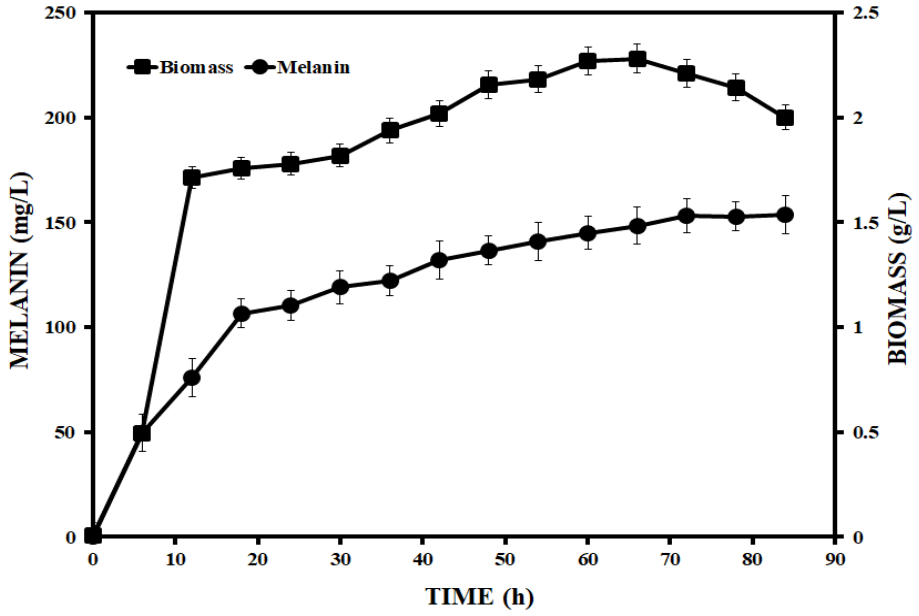

Figure 4-2: Growth of the microorganism and melanin production in shake flask (APPENDIX Table 7-1).

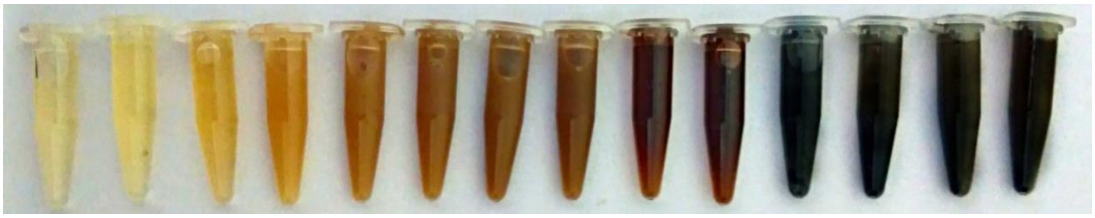

Figure 4-3: Change of color of culture broth due to melanin production from 0th hour to 84 hour at 6 hour interval of time.

As shown in Figure 4-2, the melanin biosynthesis increased gradually up to 72 hours and then remained constant. There was marginal increase in melanin concentration

after 60 hours of cultivation. The marginal increase in melanin concentration in the medium coincided with cell mass decrease, which could be due to the release of intracellular melanin. The volumetric productivity of melanin decreased gradually with time. At 24th hour, the volumetric productivity was 4.6 mg/L.h but after 72 hours, the volumetric productivity was decreased to 2.13 mg/L.h which is about 46% lesser. Melanin did not degrade after 72 hours and its concentration remained constant. The yield coefficient of melanin over biomass was about 64 mg/g dry weight of biomass. The pH of the culture increase from 7 at the beginning up to 8.8 at the end of the bioprocess.

4.3 Optimization of nutritional, operational & biological parameters for melanin production

Usually, bioprocess development and optimization are carried out at shake flask level first and then scaled-up to stirred tank bioreactor. Operating parameters such as, temperature, pH, type of medium used, shaking frequency of the conical flask etc., play important role in bio-product formation due to their influence on cell metabolism. Optimization of operating parameters has increased the productivity of microbial, animal and plant cell based bioprocesses (Giese et al. 2014; Gómez-Pazarín et al. 2016; Kumdam et al. 2013; Raval et al. 2003).

4.3.1 Effect of growth media

Figure 4-4 and Figure 4-5 demonstrate melanin and biomass production in different growth media. As shown in Figure 4-4, the TSB medium exhibited the maximum melanin production (238 mg/L) and maximum biomass production (3.96 g/L) at 60th hour, followed by LB and NB and BHB media. Carbon and nitrogen sources are major components for the growth of microorganisms as well as product formation (Bailey et al, 1976). NB and LB contains carbon and nitrogen sources whereas BHB contains only minerals and salts. That may be the reason for less biomass and melanin production in BHB medium. TSB medium has the highest amount of carbon and nitrogen sources out of all the media selected, which enhanced the cell growth and melanin biosynthesis. After 60th hour of incubation, there was no significant increase in both the biomass and melanin production. Surwase et al. (2013) observed that the

Brevundimonas sp. SGJ produced melanin at a concentration of 401 mg/L at the 48th hour. While Chávez-Béjar et al. (2013) observed 66 mg/L of melanin production in LB broth. *P. stutzeri* produced about 150 mg/L melanin in BHB medium without the presence of any carbon or nitrogen source.

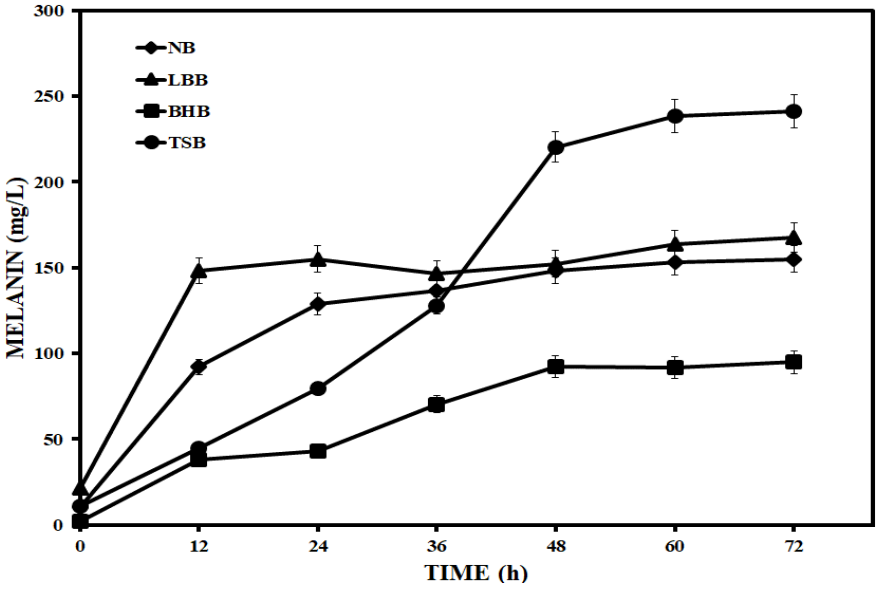

Figure 4-4: Melanin production in different growth media (APPENDIX Table 7-2).

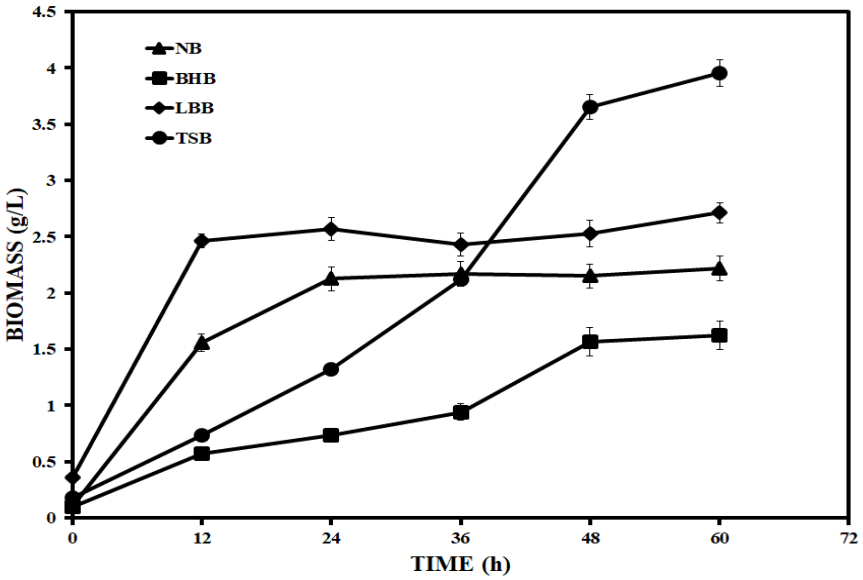

Figure 4-5: Biomass production in different growth media (APPENDIX Table 7-2).

Inoculum for this study was prepared in the NB medium and the inoculum was transferred at 12 hours of age. The complex carbon and nitrogen source availability from the inoculum may be the reason of production of melanin in BHB medium.

The melanin yield coefficient over biomass remained almost constant in the range of 60 to 65 mg/g dry weight in all media used in this study. This proved that the melanin production was growth associated and any medium, which produce more biomass would produce more melanin. Since TSB medium demonstrated maximum melanin production it was used for further studies.

4.3.2 Effect of operating temperature

Microorganisms, such as bacteria are more tolerant to the various environmental conditions than other organisms. However, each species has its own characteristic range of values in which it grows and reproduces best. This range of upper and lower temperature is a function of cell metabolism. For lower temperatures, the molecules move slower and enzyme activity decreases. As the temperature increases, the molecules move faster, enzyme activity increases which speed up the metabolism and cells rapidly increase in size. But, above a particular temperature, enzymes start to denature, and the total effect is detrimental.

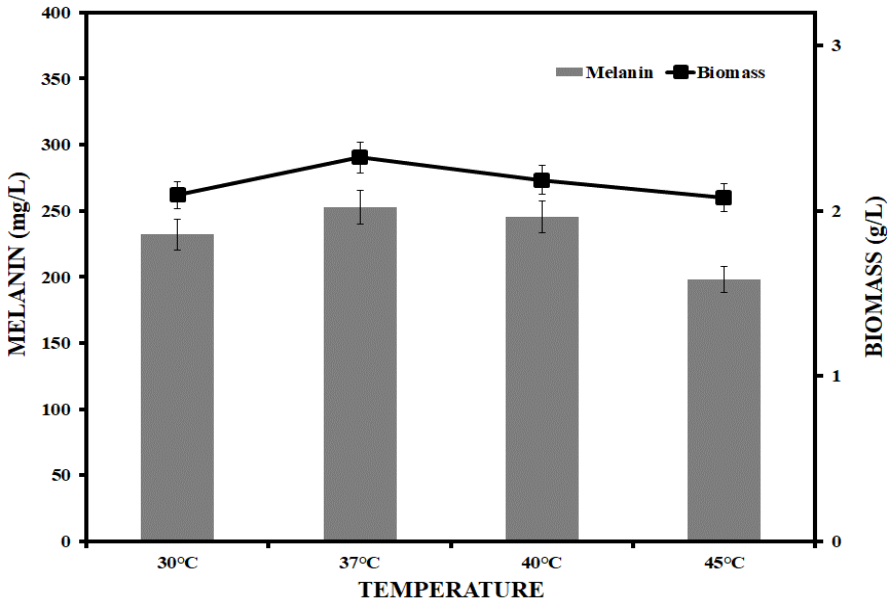

Figure 4-6: Melanin production and biomass production at different temperatures (APPENDIX Table 7-5).

Since *Pseudomonas stutzeri* grows in sea water having an average temperature of 25º to 32ºC, it was necessary to study the effect of temperature on growth and melanin production. Effect of cultivation temperature on melanin production is mentioned in

Figure 4-6. The maximum melanin yield, 253.08 mg/L was obtained at 37ºC at the 48th hour. The increase in the incubation temperature resulted in a significant decrease in the melanin production. The biomass yield also decreased with increase in the temperature.

The temperature optimization for melanin production was reported for *E. coli* W3110 and *Aeromonas media* for which optimum incubation temperatures were 30º and 37ºC, respectively (Lagunas-Muñoz et al., 2006). In the earlier reports, the temperature used for melanin synthesis was 30ºC for *Bacillus thuringiensis*, *Bacillus thuringiensis* subsp. Galleriae strain K1 and H-14 (Chen et al., 2004), whereas 37ºC was used for *Burkholderia cenocepacia* and *Klebsiella* sp. GSK (Keith et al., 2007; Shrishailnath Sajjan et al., 2010)

4.3.3 Effect of initial pH

Microorganisms are sensitive to the hydrogen ion concentration in their surrounding environment. Microbial metabolism is governed by enzyme activities. These enzyme activities are profoundly affected by pH. Change in pH beyond a certain level alters their shape or denatures the enzymes. Also, it alters the ionic charges on the molecule. Therefore, it was necessary to identify the optimum initial pH for melanin production.

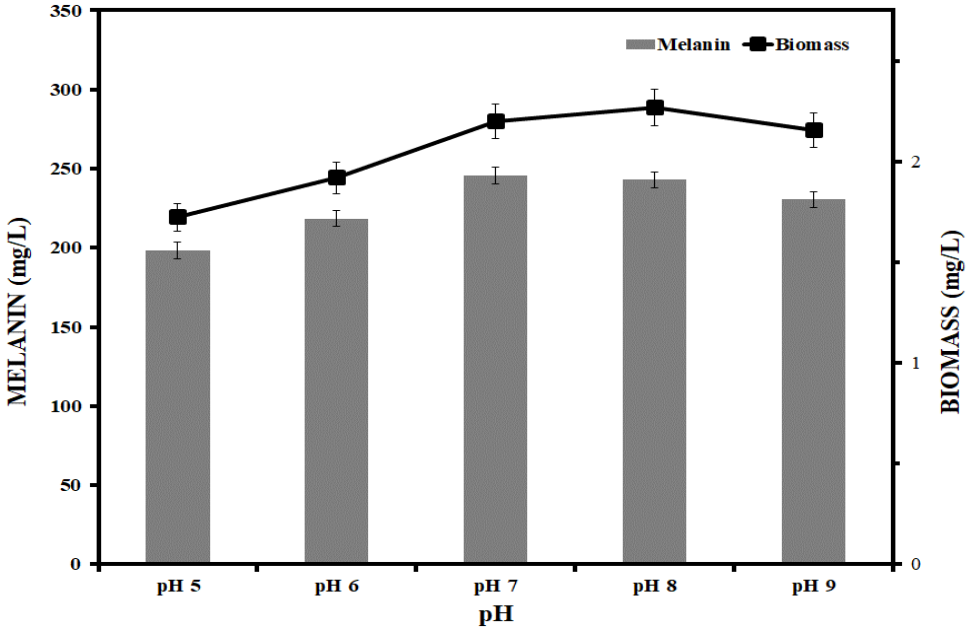

Figure 4-7: Melanin production and biomass production at different pH (APPENDIX Table 7-6).

When the initial pH of the medium was changed to different pH levels, at neutral and basic pH (pH 7 and pH 8) both the melanin and biomass production were more. The melanin and biomass production decreased in acidic pH. The maximum melanin (246 mg/L) was obtained at initial pH of 7 (Figure 4-7). Since *Pseudomonas stutzeri* is a marine organism it grew well in the pH range of 7-8. This could be the reason for good growth and melanin production in basic pH as compared to acidic pH.

4.3.4 Effect of shaking frequency

As explained above, oxygen transfer rate controls the overall growth rate of any microorganism. Shaking frequency also influences the oxygen transfer rate from air to the liquid medium. The oxygen transfer rate in shake flask is directly proportional to the shaking frequency with an exponent of 1.12 (Meier et al. 2016). As shown in Figure 4-8, the highest production of melanin was obtained at 200 rpm (248 mg/l at 48th h). The melanin production and biomass production decreased at higher and lower shaking frequencies (250 rpm and 100 rpm), respectively.

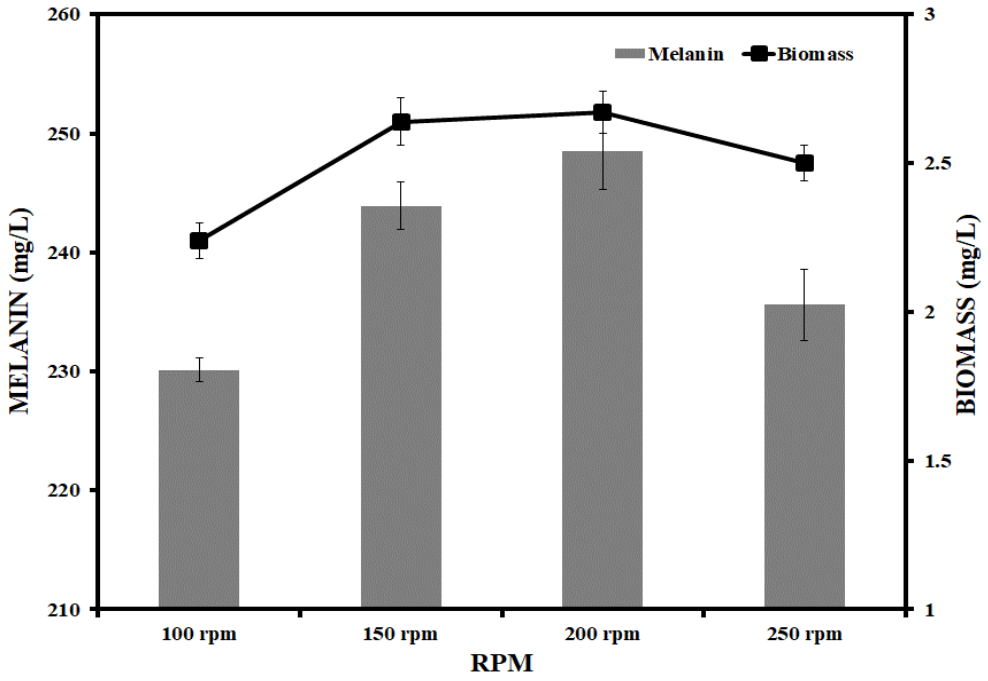

Figure 4-8: Melanin production and biomass production at different shaking frequencies (APPENDIX Table 7-7).

The decrease at lower shaking frequency was due to less oxygen transfer rate. Whereas at higher shaking frequency (250 rpm), the mechanical instability of the

shaker increased vertical wobbling motion, which generated excessive foam. This effect reduced oxygen transfer rate from air to liquid and hence affected the growth and melanin biosynthesis (Kar et al. 2012).

4.3.5 Effect of flask filling volume

Aerobic microorganisms require oxygen for growth and product formation. Usually, oxygen is the least soluble medium component out of all medium components. Therefore, most of the aerobic processes are controlled by the oxygen transfer rate from air to the liquid medium. In shake flasks, the oxygen transfer rate is inversely proportional to culture volume of filling volume with an exponent of 0.74. The oxygen transfer can be thus be increased by lowering the culture volume or filling volume (Giese et al. 2014; Meier et al. 2016; Peña et al. 2007). Figure 4-9 shows the effect of filling volume on melanin production.

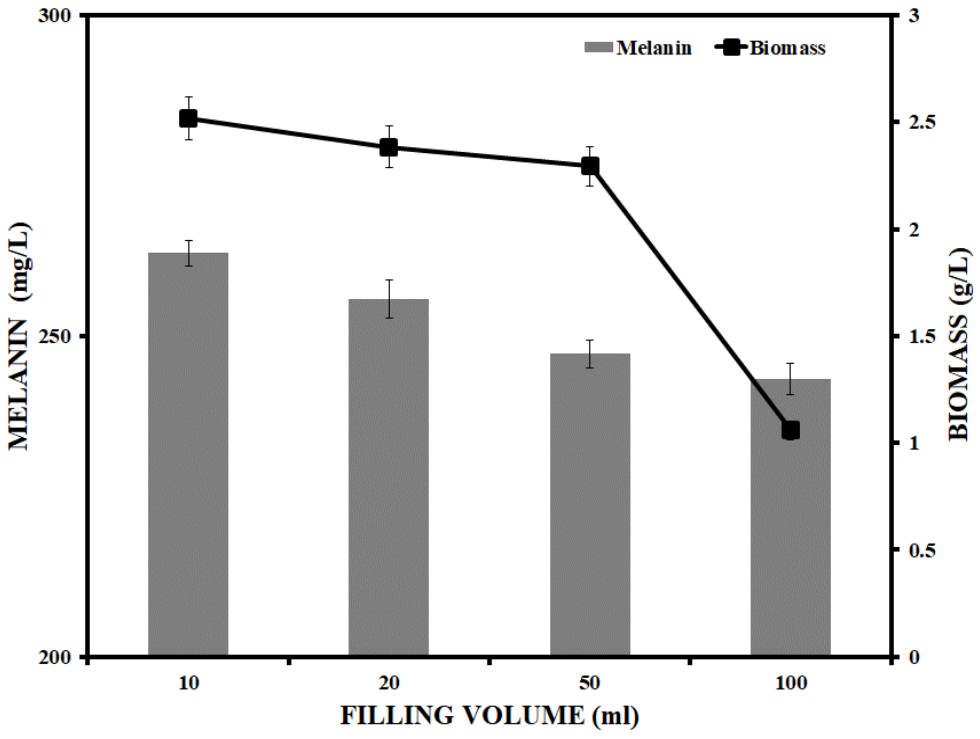

Figure 4-9: Effect of filling volume on melanin production and biomass production (APPENDIX Table 7-8).

As oxygen transfer rates were relatively higher at low filling volumes, the lower filling volumes produced more biomass and melanin, which was due to higher aeration and the availability of more surface area, which increased overall oxygen

transfer rate. Oxygen is also required to oxidize tyrosine by tyrosinase enzyme, which is the melanin biosynthesis initiation reaction. Moreover, the time to reach the maximum melanin production reduced substantially, from about 120 hours with 100 ml filling volume to only 48 hours at 10 ml filling volume.

4.3.6 Effect of inoculum percentage

Microbial concentration plays an essential role in many microbial systems. A low inoculum size may increase lag phase or non-productive phase of a bioprocess. The large inoculum size may reduce the productive phase of a bioprocess due to relatively fast depletion of nutrients (Kumdam et al. 2013). Inoculum percentage has played an essential role in regulating bioluminescence, antibiotic biosynthesis, virulence determination, catalase activity and initiation of chromosomal replication (Caipo et al., 2002). Whether due to the interaction between cells, statistical effects, or sensitivity of microbiological methods, initial inoculum levels can drastically affect experimental results. Higher inoculum concentrations may exhaust the medium nutrients at a much faster rate without increasing productivity and thus optimum inoculum percentage is essential for a successful bioprocess.

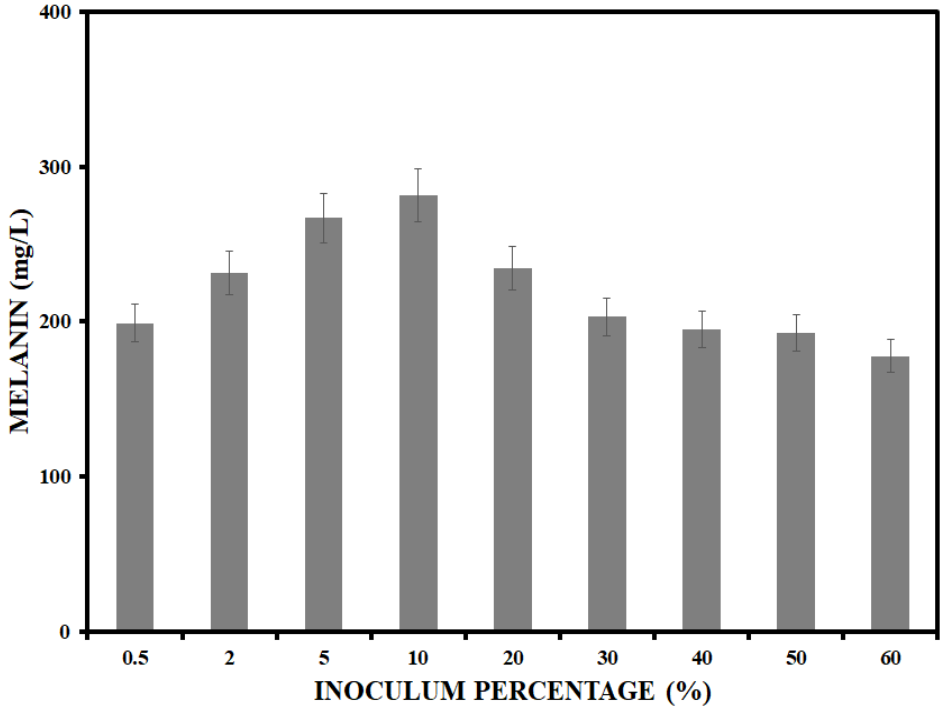

Figure 4-10: Melanin production at different inoculum percentages (APPENDIX Table 7-3).

As Figure 4-10 depicts, the highest melanin production of 272 mg/l was achieved when 10% inoculum was added to 50 ml of TSB medium. Further increase in inoculum percentage (20%, 30%, 40%) did not increase the melanin yield significantly. As a result of which, 10% inoculum volume was selected to be the optimum inoculum volume to be used for further investigations.

4.3.7 Effect of inoculum age

Age of microorganisms also called as the age of inoculum play crucial role in the health of any bioprocess. If microorganism age is too young or too old then the biosynthesis of any compound will not occur at an optimum level. Usually, optimum inoculum age is different for different bioproducts synthesis and therefore needs to be optimized separately for each bioproduct (Shuler and Kargi 1992). Therefore, the potential of growth of bacteria in the starter culture with respect to the time duration and production of melanin was investigated by using the inoculum grown for 6, 12, 18, and 24 hours.

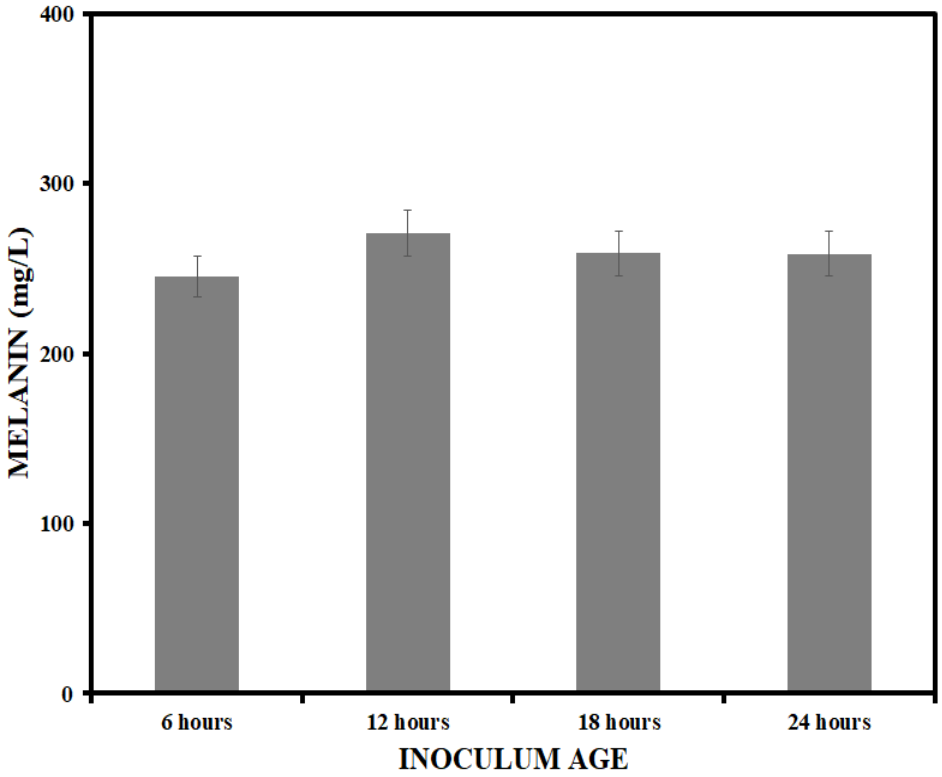

Figure 4-11: Melanin production at different inoculum age (APPENDIX Table 7-4).

After investigating different inoculum age for their melanin production, the 12 hour old inoculum gave the highest melanin concentration (282 mg/L) at the 48th hour (Figure 4-11).

After optimizing the process parameters, the melanin production was increased from 153 mg/L to about 280 mg/L. Optimization of operating conditions reduced bioprocess time from about 100 hours to 48 hours. Table 4-1 highlights the optimized operational, nutritional and biological parameters. 10% inoculum of a 12 hour old culture at 37°C temperature, pH 7 at 200 rpm produced maximum melanin.

Process Parameters	**Optimum value**
Medium	TSB
Temperature	37ºC
Initial pH	7
Filling volume	10 ml
Shaking frequency	200 rpm
Inoculum age	12 h
Inoculum percentage	10%

Table 4-1: Optimized process parameters for melanin production

Among the different media used, Tryptic Soy Broth (TSB) proved to be the best. However, the medium pH increased beyond 8.5 after 48 hours of bioprocess operation in shake flask experiments, which might have ceased biomass growth. The pH of the medium plays important role in growth and product formation. There are reports where pH maintenance in shake flask increased the productivity of a bioprocess (Maralingannavar et al. 2018). Therefore, we investigated the effect of constant pH by

maintaining the pH of the culture in the range 6.5 to 7.5 by using buffers such as Tris-HCl, Citrate buffer and Phosphate buffer.

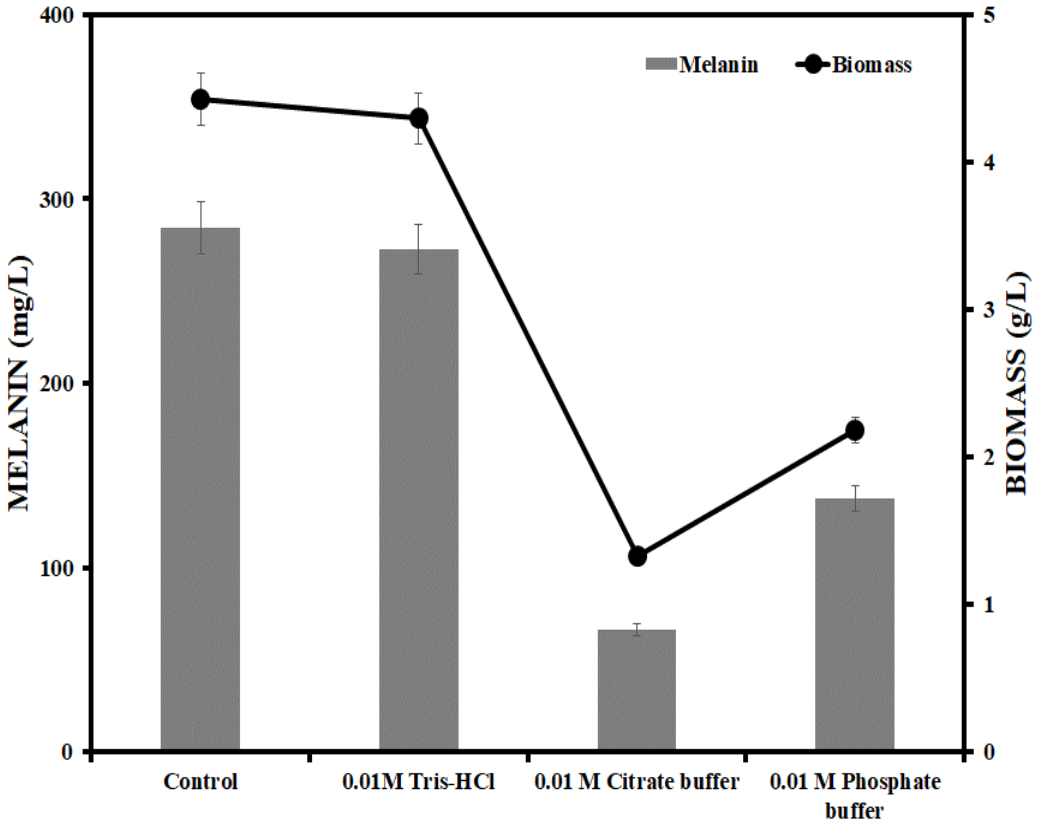

Figure 4-12: Effect of buffers on biomass and melanin production (APPENDIX Table 7-13).

As Figure 4-12 indicates, out of all the three buffers, Tris-HCl buffer produced better results but melanin production was similar to that of control. Citrate buffer and phosphate buffer were reducing the growth of microorganisms and hence melanin production. Therefore, out of these three buffers, Tris-HCl was used for further studies

Different concentrations of Tris-HCl were studied. However, there was no significant increase in melanin production. Lower concentration of the buffer had less influence on the cell growth and melanin production. Whereas, the melanin production and the cell growth decreased with increase in the buffer concentration, which is shown in Figure 4-13. This was due to the negative effect of Tris surfactant on the *Pseudomonas stutzeri*.

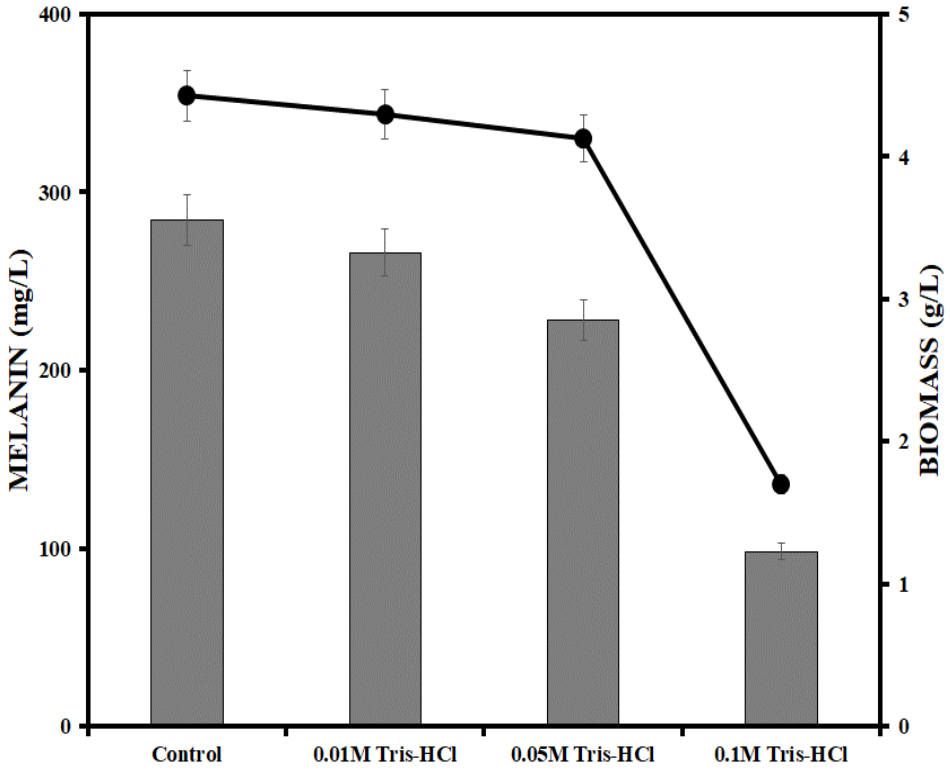

Figure 4-13: (A) Melanin and biomass production at different buffer concentrations (APPENDIX Table 7-14).

Above results indicate an increase in medium pH did not reduce melanin production much and therefore, the addition of buffers in shake flask experiments was avoided in further studies. With a view of identification of suitable nutritional source for melanin production, TSB was modified with different carbon sources, nitrogen sources and trace elements.

4.4 Identification of important media constituents for melanin production

4.4.1 Effect of trace elements

Trace minerals are essential components of microbial nutrition. A lack of essential trace minerals increases the lag phase and may decrease the specific growth rate and yield. They are essential for many enzymatic activities which control the metabolism of a living cell (Shuler and Kargi 1992). Important trace elements, such as zinc, manganese, copper, magnesium and iron, which impart growth were selected to study their effect on biomass growth and production of melanin. Trace minerals such as $MgSO_4$, $MnSO_4$, $FeSO_4$, $CuSO_4$, and $ZnSO_4$, were studied at various concentrations.

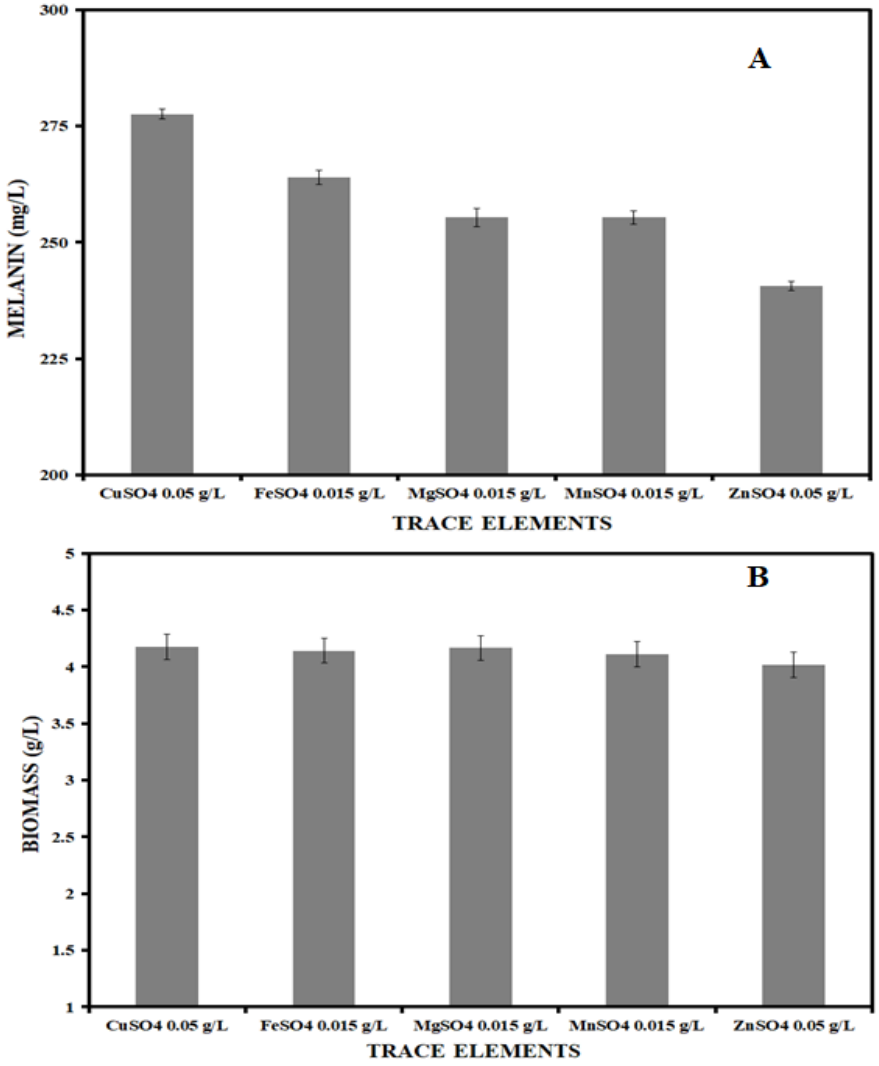

Figure 4-14: Effect of trace elements on (A) melanin production and (B) biomass production (APPENDIX Table 7-9).

Among them $CuSO_4$ (0.05 g/L), $MgSO_4$ (0.015 g/L) and $FeSO_4$ (0.015 g/L) yielded more biomass and melanin (Figure 4-14). Zinc and manganese are reported to inhibit tyrosinase activity in few microbial species. This could be the reason for less melanin production in presence of zinc and manganese. Higher concentrations of copper inhibit bacterial growth and hence, it was not used in a concentration greater than 0.05 g/L.

4.4.2 Effect of different nitrogen sources

Considering the economy and increasing the yield of melanin production, there is a need for the selection of economically feasible media components. To achieve this it

was necessary to select the appropriate carbon and nitrogen sources since they profoundly influence the growth of microorganisms and hence melanin production. The nitrogen and carbohydrate rich agro-industrial wastes (hull of soy, corn, rice, coconut cake meal, cottonseed meal, molasses, bagasse of sugarcane, etc.), which do not compete for human food chain have been used as a substrate for the growth of microorganisms and production of bio pigments in the recent years (Panesar et al. 2015).

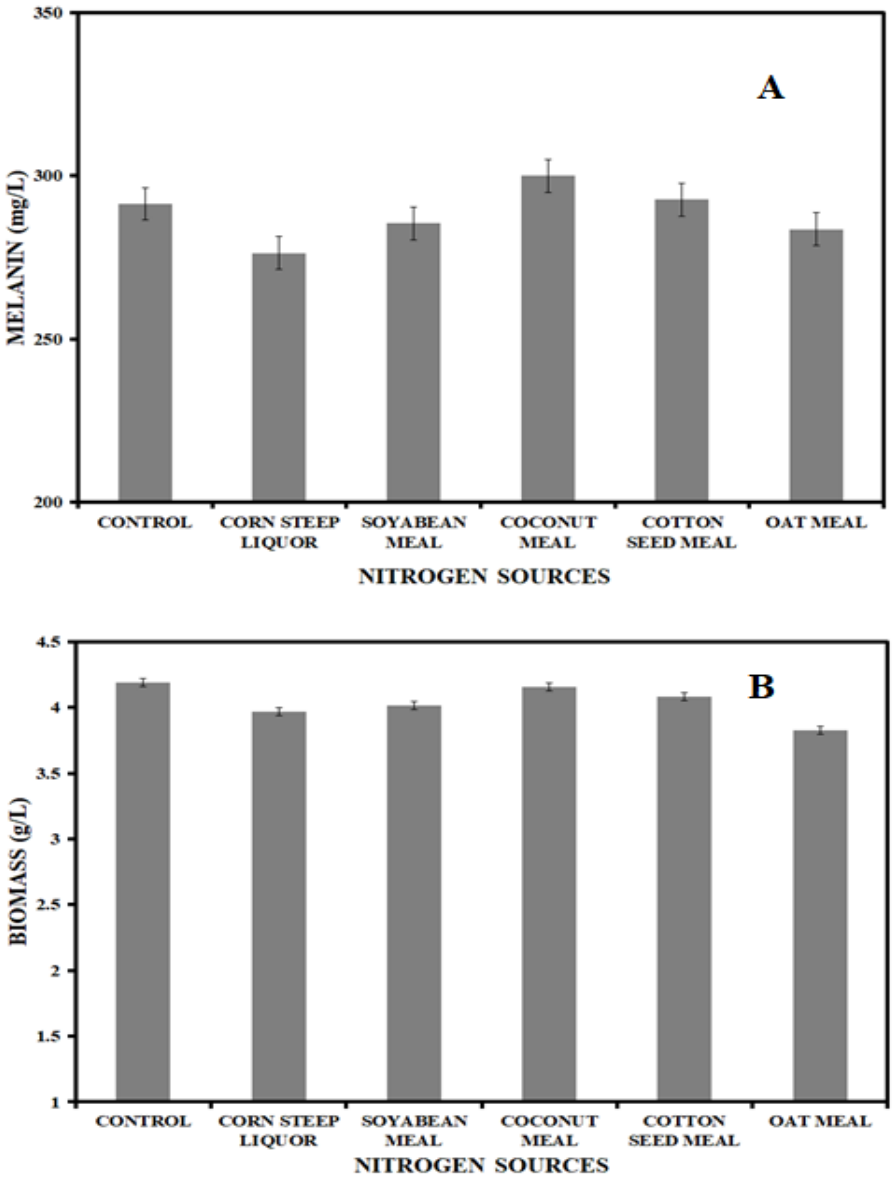

Figure 4-15: Effect of different nitrogen sources on (A) melanin production and (B) biomass production (APPENDIX Table 7-10).

Figure 4-15 shows the effect of different sources of nitrogen such as corn steep liquor, soya bean meal, coconut meal, cottonseed meal and oat meal on biomass and melanin production. Maximum melanin yield of about 300 mg/L was observed in the medium supplemented with coconut meal in the 44th hour. Melanin contains only about 6 to 7% of its weight as nitrogen and therefore we did not observe marked differences in melanin yield with respect to different nitrogen sources. This is a positive aspect of bioprocess development as any bioprocessing industry can use above-mentioned industrial nitrogen rich by-product from nearby food processing industries. Since, nearby regions of National Institute of Technology of Karnataka has high growth of coconut processing industries, which produce coconut meal as a by-product, it was used as the nitrogen source.

Researchers have reported the usage of wheat flour and yeast flour for the production of melanin in *Bacillus thuringiensis* subsp. *Galleriae* strain K1 (Aghajanyan et al. 2005) while maize flour and yeast extract were used for melanin production in *Bacillus thuringiensis L*-7601 (Zhang et al. 2007), whereas bactotryptone and casein were used for *Bacillus cereus* 58 (Zhang et al. 2007). A mixture of peptone and yeast extract was used as a nitrogen source for melanin production in *Kluyveromyces marxianus* and *Streptomyces chibaensis* (Hewedy and Ashour 2009).

Since coconut meal used in this study is not fit for human consumption, it has an advantage over other reported complex nitrogen sources.

4.4.3 Effect of different carbon sources

Microorganisms react differently to different carbon sources. Large carbohydrate molecules are broken down to the simplest six-carbon sugar, glucose, which is used by microorganisms in different metabolic pathways for growth (Shuler et al, 2002). Although glucose is a preferred carbon source, it may inhibit the growth through catabolite repression and therefore, it was necessary to identify a suitable carbon source for the growth of a given microorganism.

Figure 4-16 shows the effect of different carbon sources such as glucose, starch, glycerol and sucrose at a concentration of 5 g/L concentration on biomass and melanin production. From the results, glucose proved to be the best carbon source for

melanin biosynthesis, yielding 386.66 mg/L melanin, while starch was a poor carbon source. Similarly glucose gave higher biomass yield whereas starch and glycerol resulted in comparatively lower biomass production. Starch and glycerol increased medium viscosity to about 50 mPaS, which decreased oxygen transfer rate. This reduced the melanin and biomass production. Helan et al. (2013) also reported higher melanin production in presence of glucose.

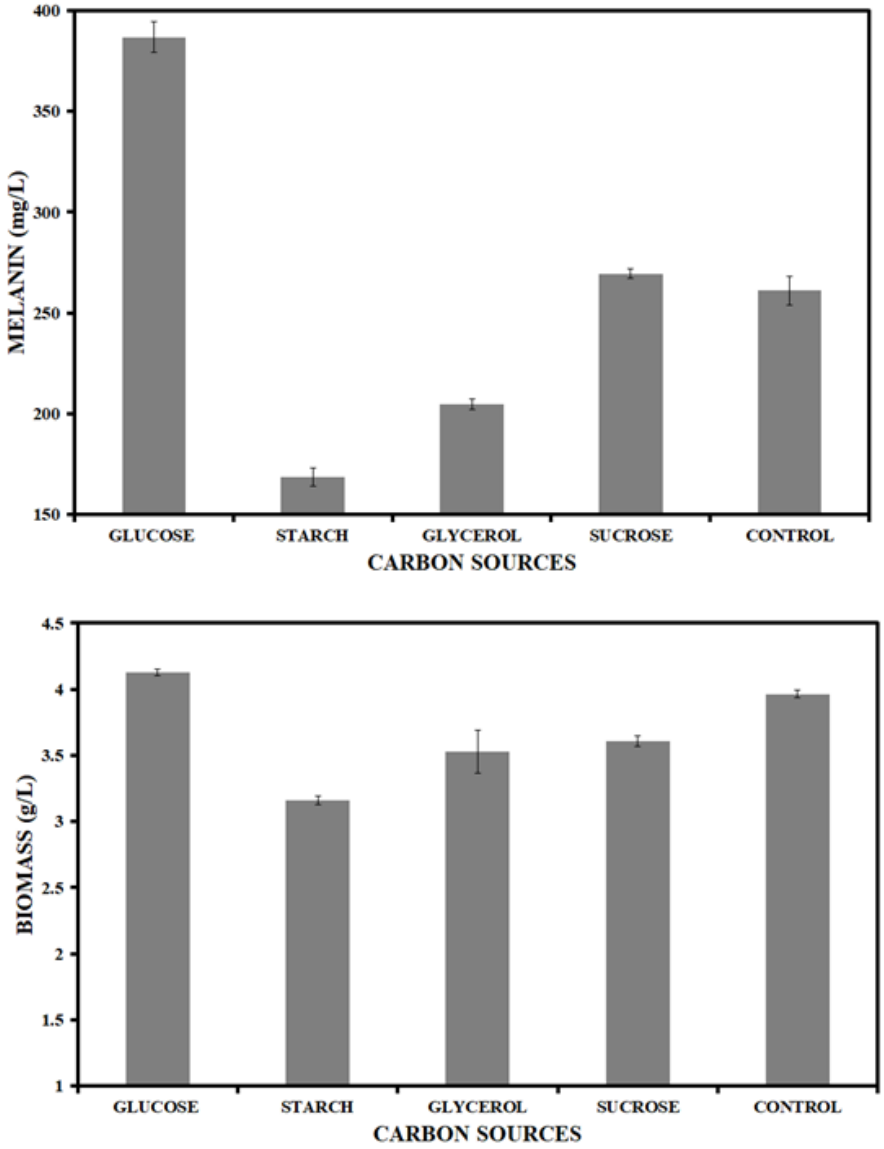

Figure 4-16: Effect of different carbon sources on (A) melanin production and (B) biomass production (APPENDIX Table 7-11).

Thus, one factor at a time approach identified, glucose and coconut meal as sources of carbon and nitrogen, respectively, while $CuSO_4$, $MgSO_4$ and $FeSO_4$ were identified as important trace elements for melanin biosynthesis.

After the optimization of media components, the melanin yield was increased by 2.48 fold that is from 153 mg/L (basal media) to 380 mg/L. The optimized operating conditions reduced completion time of bioprocess from about 100 hours to 48 hours at shake flask level.

4.5 Statistical optimization of melanin production using CCD

CCD was performed using above mentioned five independent variables at three different levels (−1, 0, +1) in a set of 50 experiments for optimization of melanin production. The experiments were conducted in triplicates and the mean value of melanin yield (mg/L) was recorded as the response (Y). The data were fitted to linear, quadratic and two factor interaction model. As mentioned in Table 4-2, the quadratic model gave the best fit out of three models chosen for optimization.

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value**	**R^2**	**Adjusted R^2**	**Predicted R^2**
Linear	1.76E+5	37	4747.72	872.72	< 0.0001	0.78	0.76	0.73
2FI	1.69E+5	27	6255.22	1149.83	< 0.0001	0.79	0.70	0.66
Quadratic	11075.97	22	503.45	92.54	< 0.0001	0.99	0.98	0.96

Table 4-2: Model summary statistics.

Usually, lower F value suggests better predictability of the model to the actual data. Insignificant lack of fit demonstrated a very high significance for the regression model. A p value less than 0.0001 suggest that there is only a 0.01% chance that a Lack of Fit F-value could occur due to noise. The predicted R^2 of 0.96 is in reasonable agreement with the adjusted R^2 of 0.98; confirms the goodness of the model. The

following figure compares model predicted values of melanin with actual values of melanin obtained in statistical design experiment.

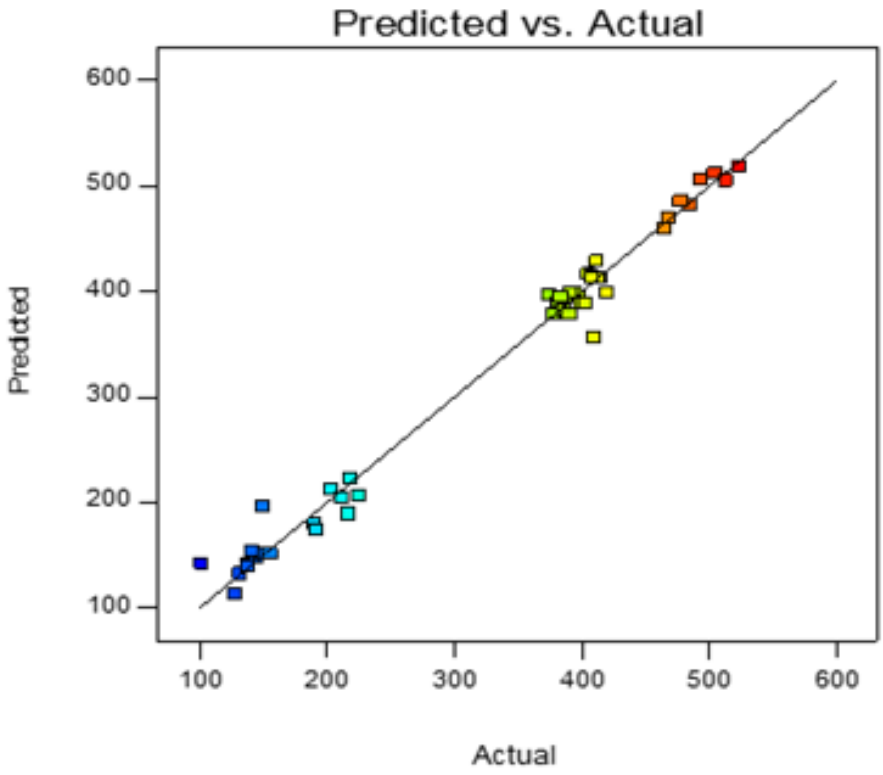

Figure 4-17: Quadratic model predicted response to the actual response for melanin nanopigment production.

As Table 4-3 and Figure 4-17 explains, a very good approximation of the actual data points using quadratic model was obtained.

		Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Response 1	
Std	**Run**	**A:coconut cake meal g/L**	**B: glucose g/L**	**C: $CuSO_4$ g/L**	**D: $FeSO_4$ g/L**	**E: $MgSO_4$ g/L**	**melanin yield g/L (actual)**	**melanin yield g/L (predicted)**
48	1	25.50	25.50	0.05	0.05	0.05	410.7	417.6
45	2	25.50	25.50	0.05	0.05	0.05	412.5	408.8
9	3	1.00	1.00	0.00	0.10	0.00	131.9	132.4
25	4	1.00	1.00	0.00	0.10	0.10	141.7	156.8
17	5	1.00	1.00	0.00	0.00	0.10	138.5	139.9
8	6	50.00	50.00	0.10	0.00	0.00	504.7	511.8

46	7	25.50	25.50	0.05	0.05	0.05	410.3	403.4
12	8	50.00	50.00	0.00	0.10	0.00	485.3	480.9
30	9	50.00	1.00	0.10	0.10	0.10	419.7	397.9
47	10	25.50	25.50	0.05	0.05	0.05	414.3	421.3
34	11	83.77	25.50	0.05	0.05	0.05	399.6	388.5
24	12	50.00	50.00	0.10	0.00	0.10	513.3	504.9
22	13	50.00	1.00	0.10	0.00	0.10	407.8	415.5
42	14	25.50	25.50	0.05	0.05	0.17	383.8	394.6
27	15	1.00	50.00	0.00	0.10	0.10	225.9	206.0
5	16	1.00	1.00	0.10	0.00	0.00	138.8	142.4
23	17	1.00	50.00	0.10	0.00	0.10	211.9	204.6
26	18	50.00	1.00	0.00	0.10	0.10	390.2	395.6
11	19	1.00	50.00	0.00	0.10	0.00	190.8	179.3
10	20	50.00	1.00	0.00	0.10	0.00	374.5	396.9
37	21	25.50	25.50	0.00	0.05	0.05	43.2	393.7
35	22	25.50	0.00	0.05	0.05	0.05	409.9	356.9
15	23	1.00	50.00	0.10	0.10	0.00	204.1	211.9
40	24	25.50	25.50	0.05	0.17	0.05	386.7	383.0
1	25	1.00	1.00	0.00	0.00	0.00	128.4	113.3
19	26	1.00	50.00	0.00	0.00	0.10	192.3	174.2
32	27	50.00	50.00	0.10	0.10	0.10	493.9	505.8
41	28	25.50	25.50	0.05	0.05	0.07	390.6	402.3
28	29	50.00	50.00	0.00	0.10	0.10	477.8	485.3
2	30	50.00	1.00	0.00	0.00	0.00	398.5	394.2
6	31	50.00	1.00	0.10	0.00	0.00	412.1	428.1
38	32	25.50	25.50	0.17	0.05	0.05	378.4	379.2
33	33	0.00	25.50	0.05	0.05	0.05	149.6	150.0
14	34	50.00	1.00	0.10	0.10	0.00	405.3	416.1
36	35	25.50	83.77	0.05	0.05	0.05	381.9	390.1
44	36	25.50	25.50	0.05	0.05	0.05	408.7	413.9
39	37	25.50	25.50	0.05	0.00	0.05	391.3	407.9
16	38	50.00	50.00	0.10	0.10	0.00	524.3	518.3
20	39	50.00	50.00	0.00	0.00	0.10	468.4	469.7
21	40	1.00	1.00	0.10	0.00	0.10	148.1	152.2
50	41	25.50	25.50	0.05	0.05	0.05	408.0	411.5
49	42	25.50	25.50	0.05	0.05	0.05	413.6	412.2
13	43	1.00	1.00	0.10	0.10	0.00	144.9	146.7
4	44	50.00	50.00	0.00	0.00	0.00	465.6	459.8
7	45	1.00	50.00	0.10	0.00	0.00	217.4	189.1
3	46	1.00	50.00	0.00	0.00	0.00	102.2	141.9
43	47	25.50	25.50	0.05	0.05	0.05	408.5	412.1
18	48	50.00	1.00	0.00	0.00	0.10	394.5	398.5
29	49	1.00	1.00	0.10	0.10	0.10	156.0	150.9
31	50	1.00	50.00	0.10	0.10	0.10	218.4	221.8

Table 4-3: CCD experimental design matrix with actual and predicted responses

The analysis of variance (ANOVA) of quadratic model terms is given in Table 4-4.

Source	Sum of Squares	Mean Square	F Value	p-value Prob > F
Model	8.02E+5	40123.40	104.69	< 0.0001
A-coconut cake meal	**6.91E+5**	**6.91E+5**	**1803.20**	**< 0.0001**
B-glucose	**39916.22**	**39916.22**	**104.15**	**< 0.0001**
C- $CuSO_4$	**5152.27**	**5152.27**	**13.44**	**0.0010**
D- $MgSO_4$	790.36	790.36	2.06	0.1617
E- $FeSO_4$	1080.19	1080.19	2.82	0.1039
AB	**2736.35**	**2736.35**	**7.14**	**0.0122**
AC	46.03	46.03	0.12	0.7314
AD	529.76	529.76	1.38	0.2493
AE	1001.07	1001.07	2.61	0.1169
BC	659.44	659.44	1.72	0.1999
BD	679.12	679.12	1.77	0.1935
BE	64.84	64.84	0.17	0.6839
CD	429.89	429.89	1.12	0.2983
CE	565.36	565.36	1.48	0.2343
DE	62.82	62.82	0.16	0.6886
A^2	**1.228E+5**	**1.228E+5**	**320.39**	**< 0.0001**
B^2	**9559.22**	**9559.22**	**24.94**	**< 0.0001**
C^2	**3308.78**	**3308.78**	**8.63**	**0.0064**
D^2	1322.79	1322.79	3.45	0.0734
E^2	**1673.41**	**1673.41**	**4.37**	**0.0455**

Table 4-4: ANOVA for response surface quadratic model.

The Model p-value of less than 0.0001 implies that the model is significant. There is only a 0.01% chance that an F-value could occur due to noise. From the regression analyses, it is clear that among the five variables, three highlighted variables (coconut cake meal, glucose and $CuSO_4$) are most significant in terms of melanin production. Model terms having values of probability of failure ('Prob > F') less than 0.05 were considered very significant, whereas those greater than 0.1 are insignificant. In this case, A, B, C, AB, A^2, B^2, C^2, E^2 were significant model terms. Interestingly, $MgSO_4$ and $FeSO_4$ were insignificant model terms. This effect could be due to the presence of coconut meal. Coconut meal is known to contain a good amount of iron and

magnesium, which reduced the importance of the two factors, namely, $MgSO_4$ and $FeSO_4$. This also indicates the importance of the statistical design of experiments. The media components which seemed significant during one factor at a time approach became insignificant due to the presence of coconut meal. Moreover, a p value of interaction terms suggests that all interaction terms except AB were also insignificant. This fact is graphically visible in interaction contour plots (Figure 4-18).

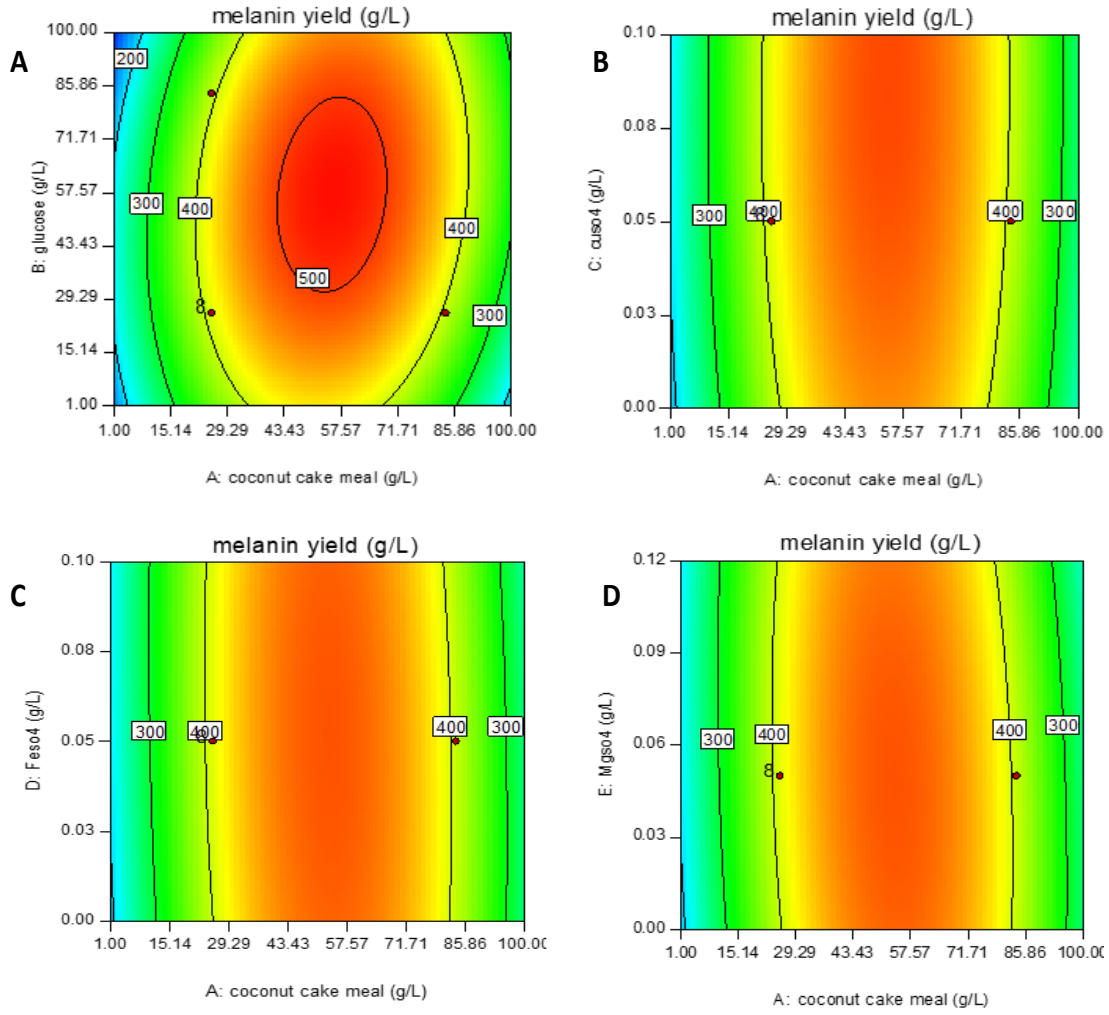

Figure 4-18: Contour plots of melanin production as a function of (A) glucose and coconut cake meal. (B) $CuSO_4$ and coconut cake meal. (C) $FeSO_4$ and coconut cake meal. (D) $MgSO_4$ and coconut cake meal.

Parallel lines in interaction plots of coconut meal with trace minerals (Figure 4-18 B, C and D) indicate that there is no interaction effect between two factors for melanin production whereas contours between coconut meal and glucose (Figure 4-18 A)

indicate that there is a strong interaction effect between two factors for melanin production.

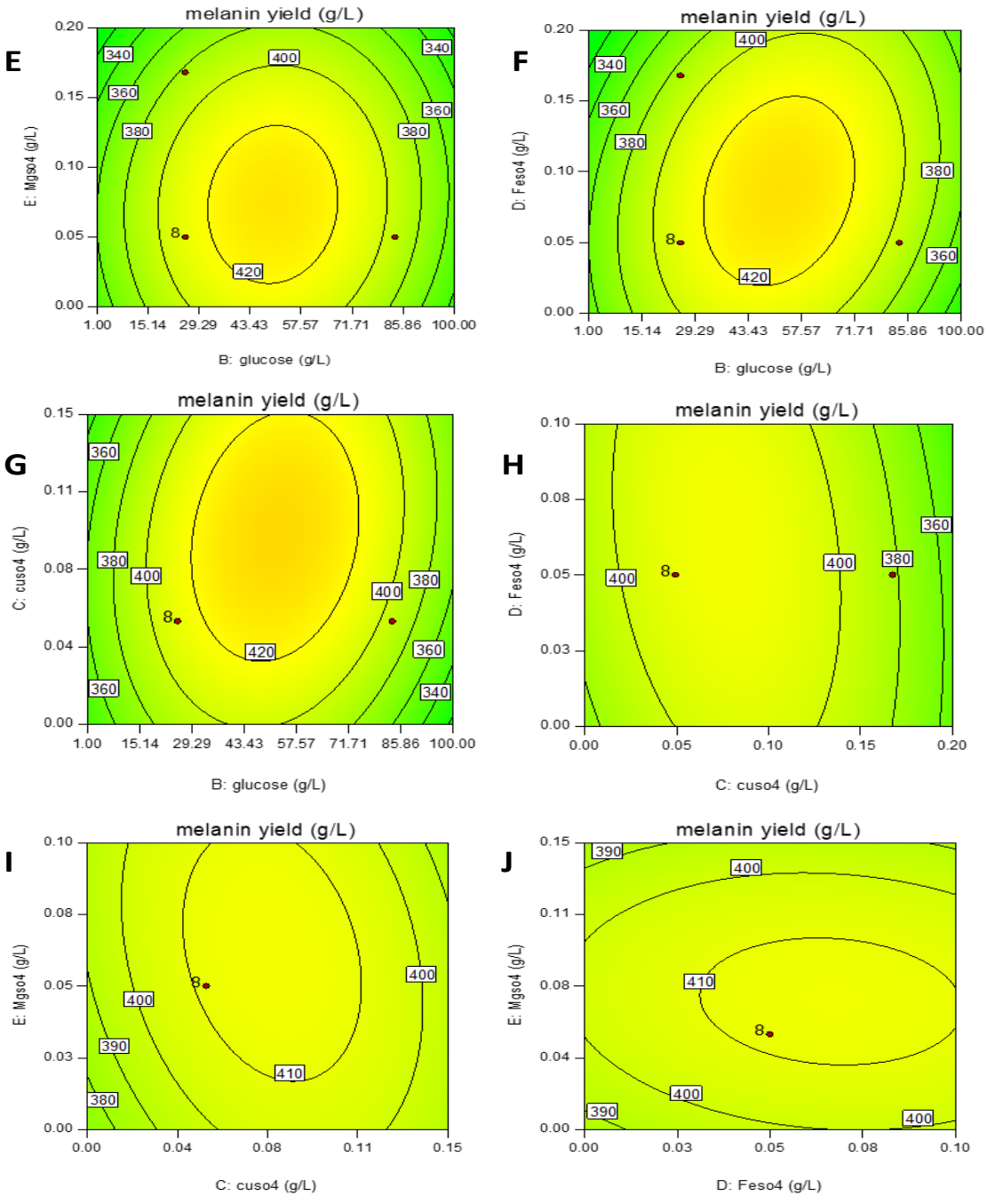

Figure 4-19: Contour plot on melanin production as a function of (E) $MgSO_4$ and glucose, (F) $FeSO_4$ and glucose, (G) $CuSO_4$ and glucose, (H) $CuSO_4$ and $FeSO_4$, (I) $CuSO_4$ and $MgSO_4$, (J) $MgSO_4$ and $FeSO_4$.

Although contour plots of trace minerals showed good interaction amongst one another as well as with glucose (Figure 4-19), the interaction effect was less than 5% of the optimum value of melanin. This is evident from the difference in melanin production values of two adjacent contour lines in Figure 4-19.

Therefore, the quadratic model was simplified by excluding insignificant model terms. Following are the model terms for prediction of melanin production.

Melanin yield =

+ 95.9 + 11 * coconut cake meal + 2 * glucose + 722.1* $CuSO_4$ + 0.015 * coconut cake meal * glucose - 0.1 * coconut cake meal^2 -0.03 * glucose^2 - 4250 * $CuSO_4^2$ - 3021 * $MgSO_4^2$

The significant model terms were chosen for further optimization using response surface methodology. The optimum values for these parameters from the above model were found to be (g/L): 51.4, 49.8, 0.09, 0.03 and 0.05 for coconut cake meal, glucose, $CuSO_4$, $FeSO_4$ and $MgSO_4$ respectively.

4.6 Validation of the statistical optimization

An experiment was performed in a 3 litre stirred tank bioreactor using the optimum values of factors mentioned above. Figure 4-20 indicates the growth and melanin production profile. We obtained, 518 mg/L of melanin in stirred tank bioreactor, which was 99% identical to the model predicted value of 522.5 mg/L, thus validating the experimental model.

Figure 4-20 shows model predicted values of biomass growth, sugar consumption and melanin production. The model fitted well with experimental data of melanin and biomass growth. After optimization of process parameters and the statistical optimization of media components, the melanin production was increased by 3.38 fold. That is from 153 mg/L to 518 mg/L. Under optimized conditions, the specific growth rate has been increased from 0.013 1/h (basal medium) to 0.029 1/h. The melanin yield over biomass was about 85 mg/g dry weight whereas, melanin yield over glucose was about 10.44 mg/g glucose.

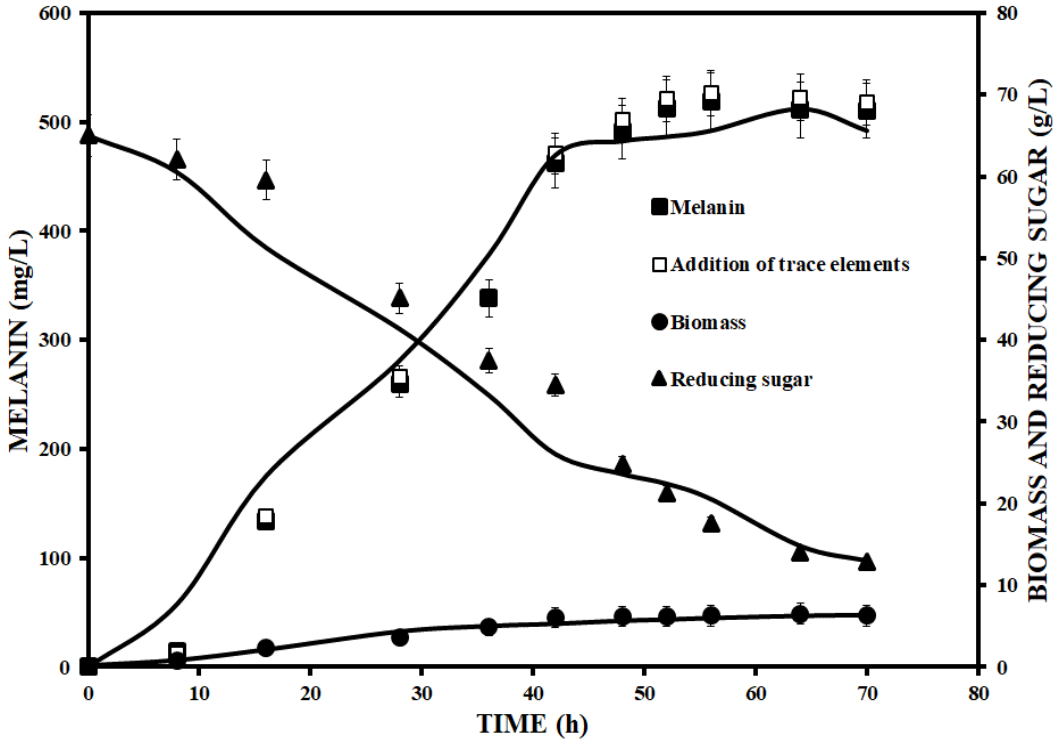

Figure 4-20: Melanin production and reducing sugar concentration in stirred tank fermenter under optimized conditions (— simulation results) (APPENDIX Table 7-12).

Although, a very good agreement was obtained between model predicted optimum value and experimental value of melanin, about 12.8 g/L carbon source was un-utilized (Figure 4-20). Determination of ammoniacal nitrogen in the broth revealed that about 11.9 g/L ammonical nitrogen source also was unutilized at the end of the bioprocess. The melanin production, as well as microbial growth, did not increase beyond a certain level. After assuming that the decline in melanin growth and melanin production may be because of the exhaustion of trace minerals, additional trace elements were supplied after 48 hours and results are plotted as open squares in the above figure. However, as figure depicts, the addition of trace elements did not cause any enhancement in melanin production or biomass production (Figure 4-20).

4.7 Melanin inhibition kinetics

Since melanin has many functional groups, it can bind to the amino acid residues of an enzyme and thereby alter the enzyme activity. However, first, it was necessary to identify the melanin biosynthesis pathway in *P. stutzeri* culture. As mentioned in the

introduction section, melanin is produced either by L-DOPA pathway or laccase pathway in any organism. As shown in Figure 4-21, when the microbial culture was treated with tyrosinase inhibitors such as arginine, the melanin production decreased and laccase inhibitors such as sodium azide had no significant effect in melanin production which confirmed that the bacteria *Pseudomonas stutzeri* follows L-DOPA pathway of melanin production with tyrosinase as the key enzyme.

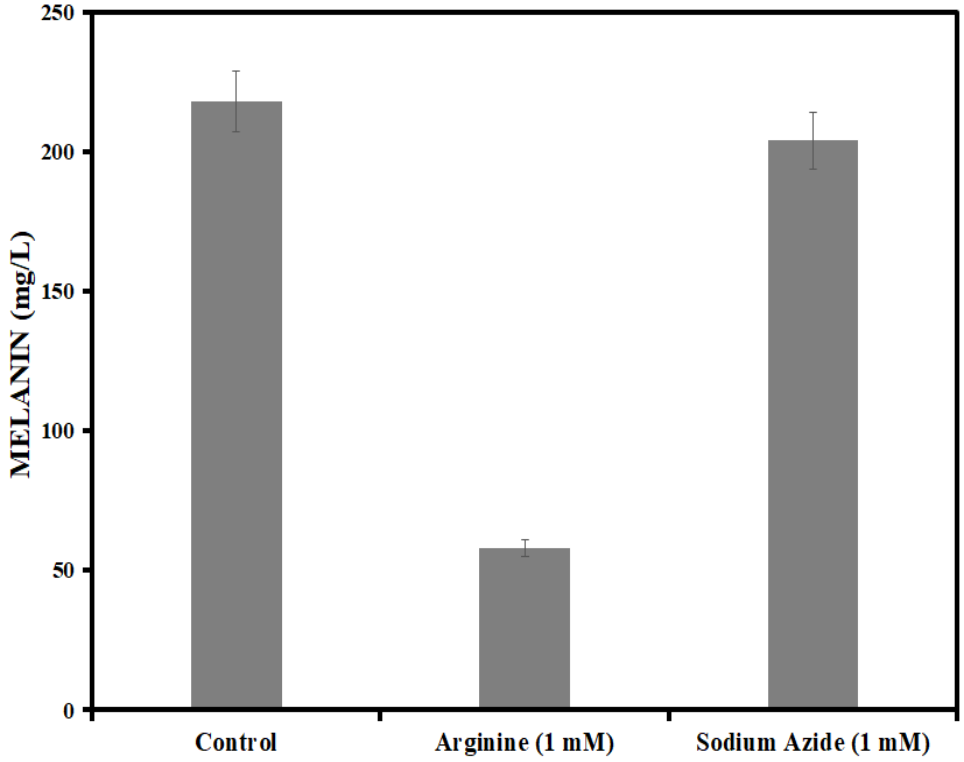

Figure 4-21: Identification of melanin synthesis pathway (APPENDIX Table 7-15).

Melanin is a known antimicrobial agent and therefore may inhibit cell growth. Moreover, the enzyme tyrosinase, which is responsible for producing melanin may be inhibited by melanin.

The feedback inhibition of melanin was investigated by adding different concentrations of melanin (100 mg/L, 200 mg/L, 500 mg/L and 1000 mg/L) into the culture medium. Growth, enzyme activity of the cells and melanin produced were measured. Figure 4-22, 4-23 and 4-24 demonstrate that the cell growth, melanin production and enzyme activity, decreased with increasing concentration of melanin.

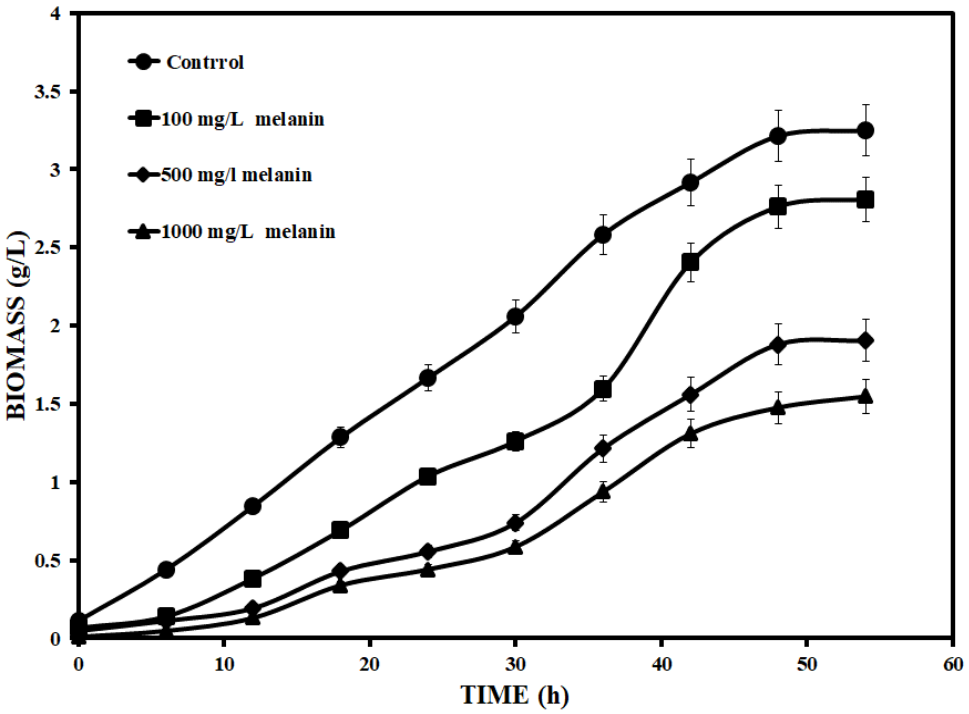

Figure 4-22: The growth of the cells at different melanin concentrations (APPENDIX Table 7-16).

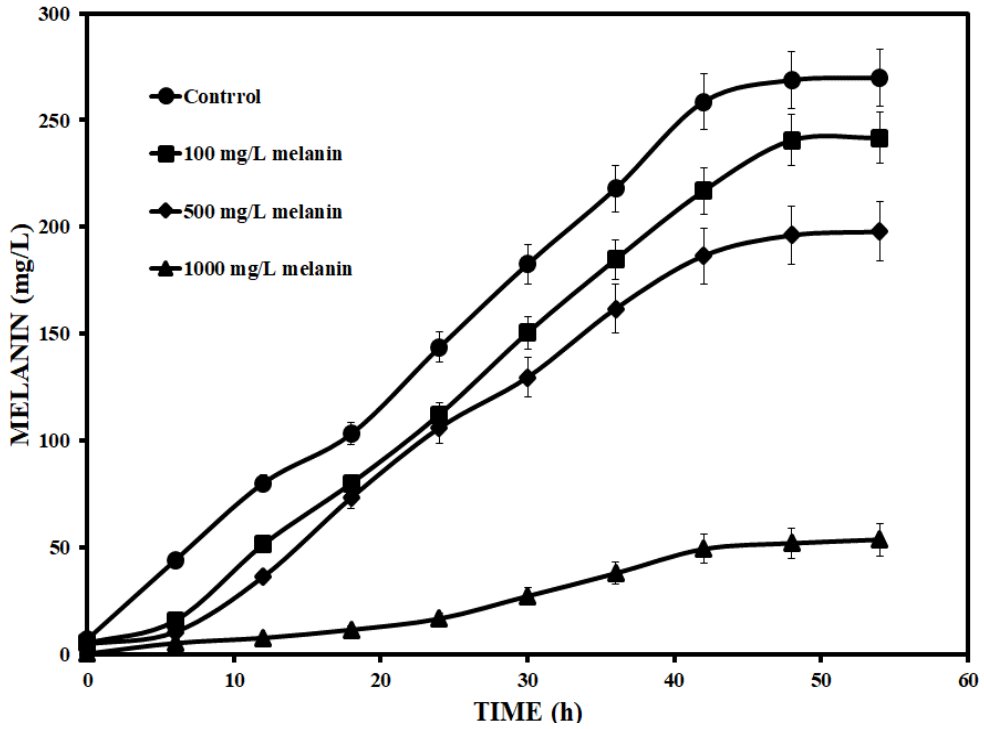

Figure 4-23: The melanin production at different melanin concentrations (APPENDIX Table 7-16).

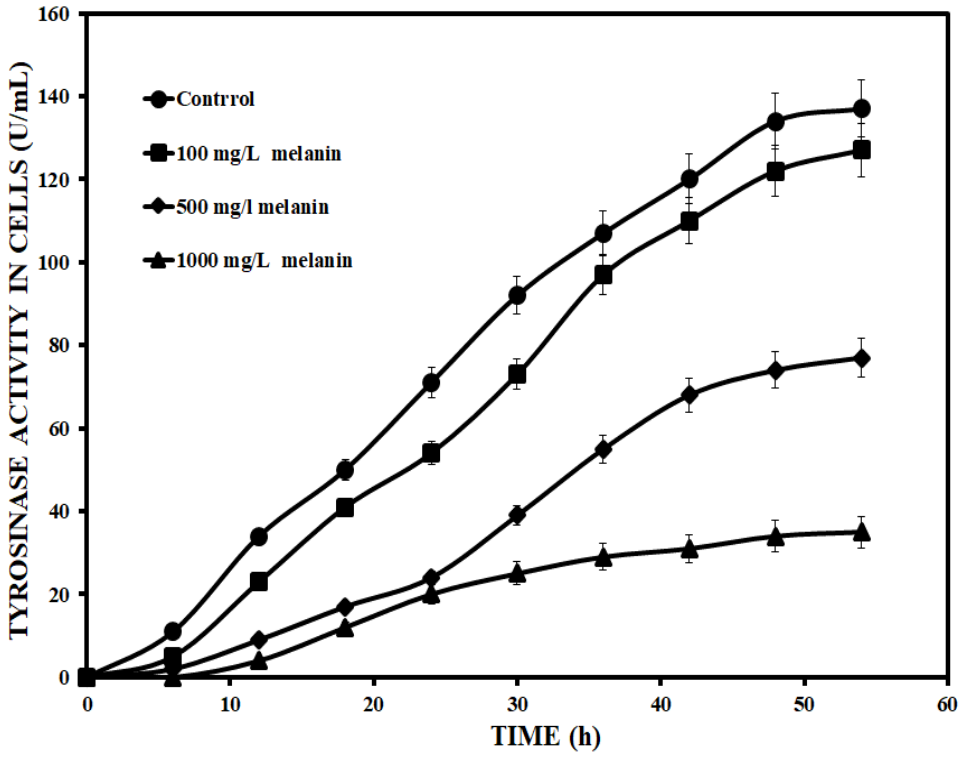

Figure 4-24: The enzyme activity of the cells at different melanin concentrations (APPENDIX Table 7-16).

At 500 mg/L initial melanin concentration in the medium, the biomass as well as melanin reduced almost 50% of the maximum value obtained in the control. At 1000 mg/L of initial melanin concentration, there was marginal melanin production. The biomass growth rate reduced significantly at 1000 mg/L initial melanin concentration. Tyrosinase enzyme activity decreased by a factor of 4 in the presence of 1000 mg/L melanin in the broth.

Researchers reported growth inhibitory effect of melanin on different microorganisms. The extracellular melanin isolated from *S. commune* showed significant antifungal activity against dermatophytic fungi, *Trichophyton simii*, and *T. rubrum* and antibacterial activity against *E. coli, Proteus sp.* (Arun et al. 2015). Melanin produced by *A. auricula* displayed inhibitory activity on biofilm formation of the three bacterial strains, *Pseudomonas aeruginosa* PAO1, *Pseudomonas fluorescens* P-3 and *E. coli* K-12, and there was a proportional reduction in biofilm biomass with the increase in pigment concentration (Bin et al. 2012). Melanin produced by

Streptomyces lusitanus DMZ-3 showed cytotoxic activity on brine shrimps (Madhusudhan et al. 2014).

Although, microbial melanin inhibition is reported in the literature, the finding that melanin also inhibits the source organism itself as well as exhibits feedback inhibition is not reported to date. Figure 4-24 demonstrates the inhibitory effect of melanin on intracellular tyrosinase activity. The maximum activity decreased from about 130 U/mL in control medium to about 30 U/mL in melanin (1000 mg/L) inhibited medium. As shown in the figure, melanin reduced the maximum enzyme activity as well as the rate of enzyme activity.

These studies clearly show that the higher melanin concentration in the culture adversely affects the tyrosinase enzyme activity inside the cell as well as the cell growth. Since no extracellular tyrosinase activity was observed, it is not reported in the results. Melanin also inhibited tyrosinase activity in the eukaryotic source. Tyrosinase from mushroom source also exhibited melanin inhibition.

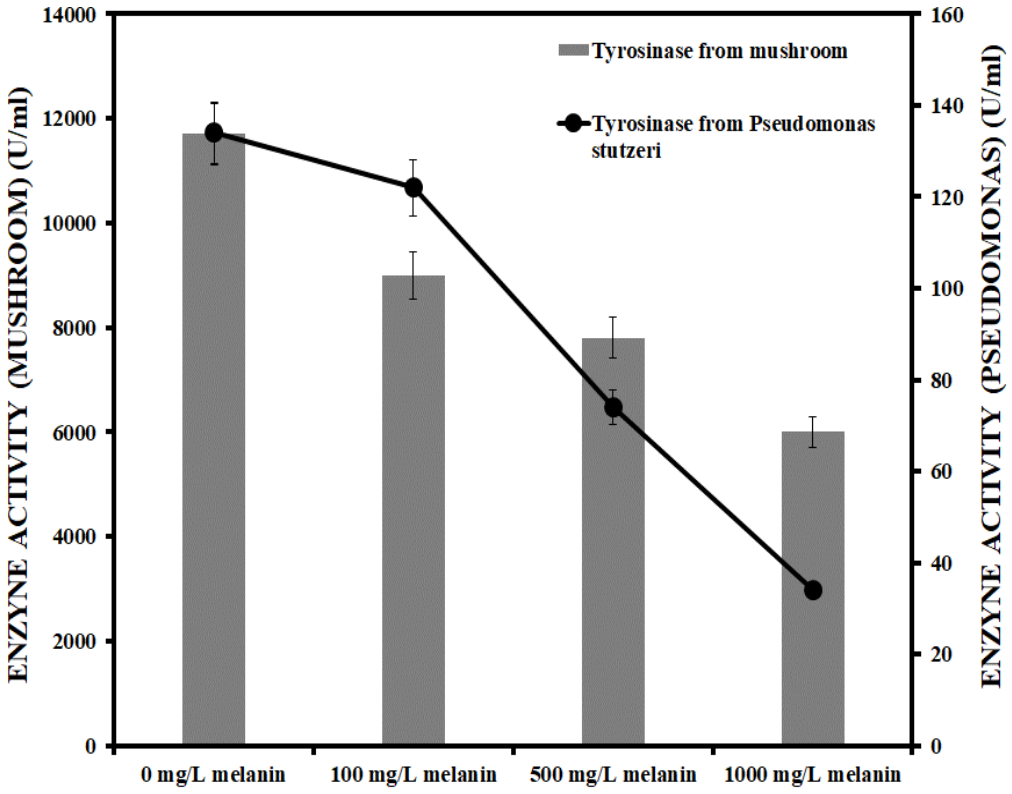

Figure 4-25: Decrease in the enzyme activity with increase in the inhibitor concentration (APPENDIX Table 7-17).

Figure 4-25 compares the effect of initial melanin concentration on the activity of tyrosinase obtained from prokaryotic as well as the eukaryotic source. At 1000 mg/L initial concentration of melanin, the activity of tyrosinase from *P. stutzeri* dropped almost by a factor of four, whereas, the activity of tyrosinase from mushroom reduced to half of that obtained in absence of melanin.

The type of melanin inhibition on tyrosinase was investigated by plotting Lineweaver-Burke plot where data were plotted as the reciprocal of the substrate concentration (1/S) versus the reciprocal of the enzyme velocity (1/V) (Figure 4-26, Figure 4-27).

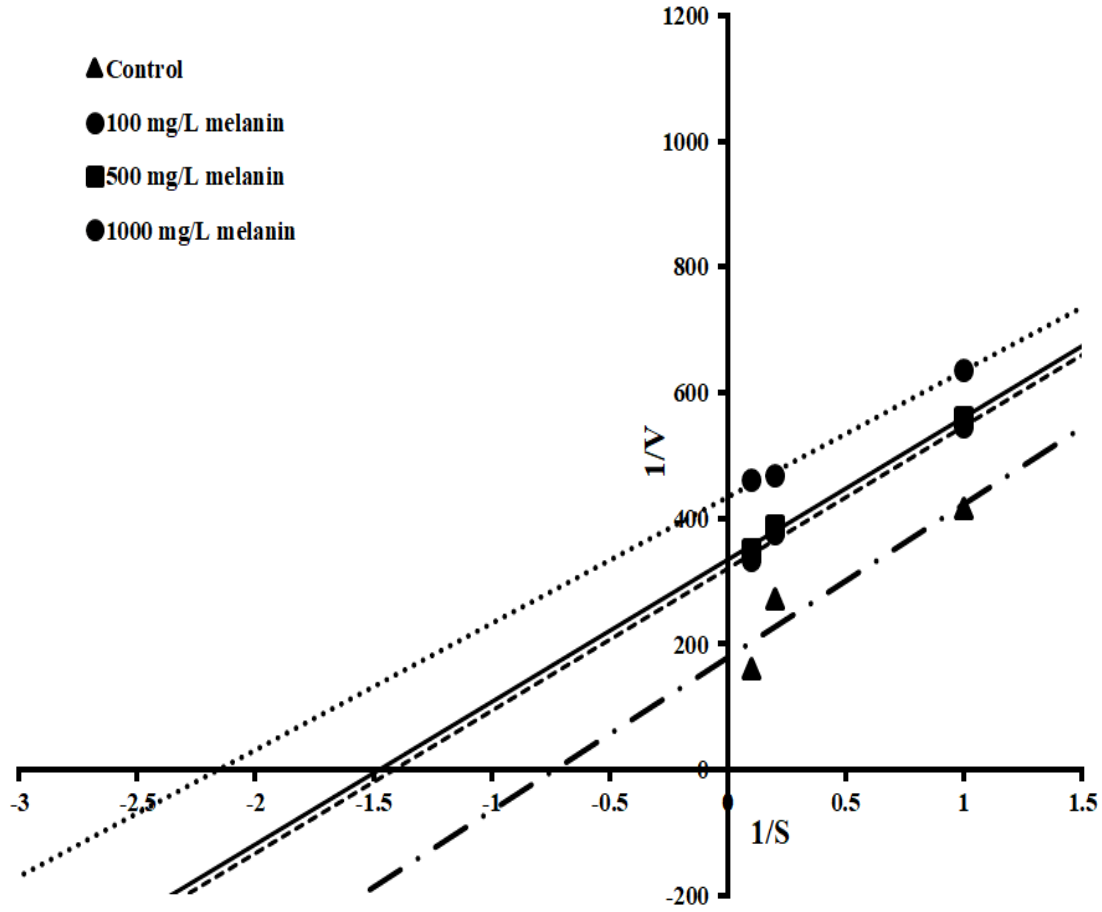

Figure 4-26: The Lineweaver-Burke plot for tyrosinase enzyme from mushroom (APPENDIX Table 7-18).

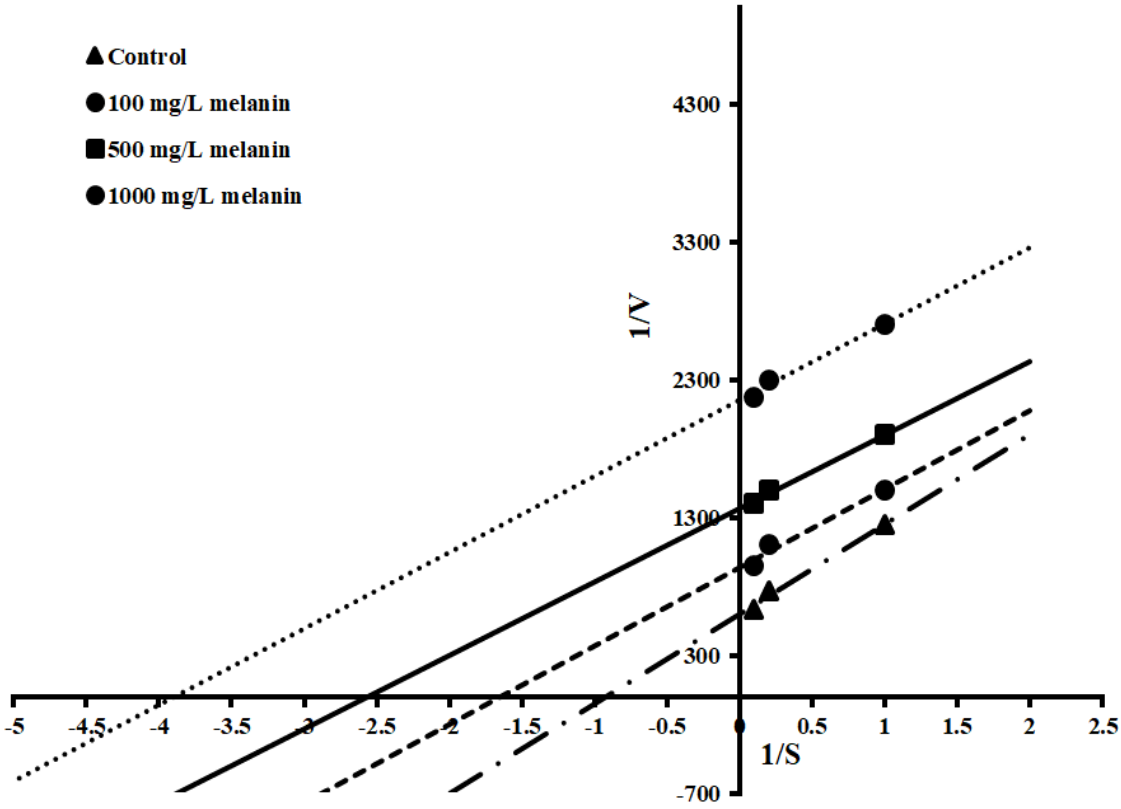

Figure 4-27: The Lineweaver-Burke plot for tyrosinase enzyme from *Pseudomonas stutzeri* (APPENDIX Table 7-19).

The Lineweaver-Burke plot for tyrosinase enzyme from both the sources demonstrated that the particular inhibition is of the uncompetitive type where both the apparent K_m (Michaeli's constant) and apparent V_{max} (Maximum enzyme velocity) decrease with increase in inhibitor concentration.

4.7.1 Modeling melanin inhibition kinetics

Above study revealed feedback inhibition of the melanin on the tyrosinase enzyme. Melanin inhibition kinetics was quantified by the generalized equation of Han and Levenspiel (Han and Levenspiel 1988),

Han & Levenspiel proposed a following generalized expression for inhibition kinetics,

$$\mu = \mu_{max} \cdot \left(1 - \frac{p}{p^*}\right)^n \cdot \frac{s}{\left(s + K_S \cdot \left(1 - \frac{p}{p^*}\right)^m\right)} \qquad (4\text{-}4)$$

Where,

μ=apparent specific growth rate (1/h)

μ_{max} = maximum specific growth rate (1/h)

p= product concentration (g/l)

p^*= critical product concentration at which growth stops (g/l)

s= substrate concentration (g/l)

m,n = parameters

Above equation can be rewritten as

$$\mu = \mu_{obs} \cdot \frac{s}{(s+K_{sobs})} \quad (4\text{-}5)$$

Where,

$$\mu_{obs} = \mu_{max}\left(1 - \frac{p}{p^*}\right)^n \quad (4\text{-}6)$$

and $$K_{obs} = K_s \cdot \left(1 - \frac{p}{p^*}\right)^m \quad (4\text{-}7)$$

Above equation can be rearranged to Lineweaver-Burke plot of 1/μ versus 1/s, as given below

$$\frac{1}{\mu} = \frac{1}{\mu_{obs}} + \frac{K_{obs}}{\mu_{obs}} \cdot \frac{1}{s} \quad (4\text{-}8)$$

The intercept gives μ_{obs} and K_{obs} can be calculated from the slope of above equation.

Equation (4-6) can be written as

$$\ln(\mu_{obs}) = \ln(\mu_{max}) + n \cdot \ln\left(1 - \frac{p}{p^*}\right) \quad (4\text{-}9)$$

Thus, different μ_{obs} can be obtained in presence of different melanin concentrations (p) from the experiments. The actual value of the maximum specific growth rate can be obtained by non-linear equation (4-6) or from the intercept of above equation of the straight line (4-9). Figure 4-28 quantifies the effect of melanin inhibition on the maximum specific growth rate of microorganisms. When the inhibitor concentration

was increased up to 1000 mg/L, the maximum specific growth rate of *P. stutzeri* in TSB medium decreased from 0.125 1/h under control condition to 0.008 1/h. The maximum specific growth rate of the culture in optimized medium containing coconut meal decreased from about 0.25 1/h to about 0.01 1/h. Since melanin was growth associated product, its biosynthesis was proportional to the growth rate. This may be the reason for steeper decrease of the specific growth rate in optimized medium containing coconut meal as compared to that in TSB medium.

The parameters of equation 4.6 were obtained using the Microsoft Excel solver tool and following equations were proposed to quantify the inhibitory effects of melanin on the maximum specific growth rate in TSB medium as well as optimized medium,

$$\mu_{obs} = 0.125\left(1 - \frac{p}{p^*}\right)^{0.89} \text{ for TSB medium} \qquad (4\text{-}10)$$

$$\mu_{obs} = 0.25\left(1 - \frac{p}{p^*}\right)^{0.91} \text{ for optimized medium,} \qquad (4\text{-}11)$$

The values of coefficients m and n were greater than zero, confirming uncompetitive inhibition by melanin.

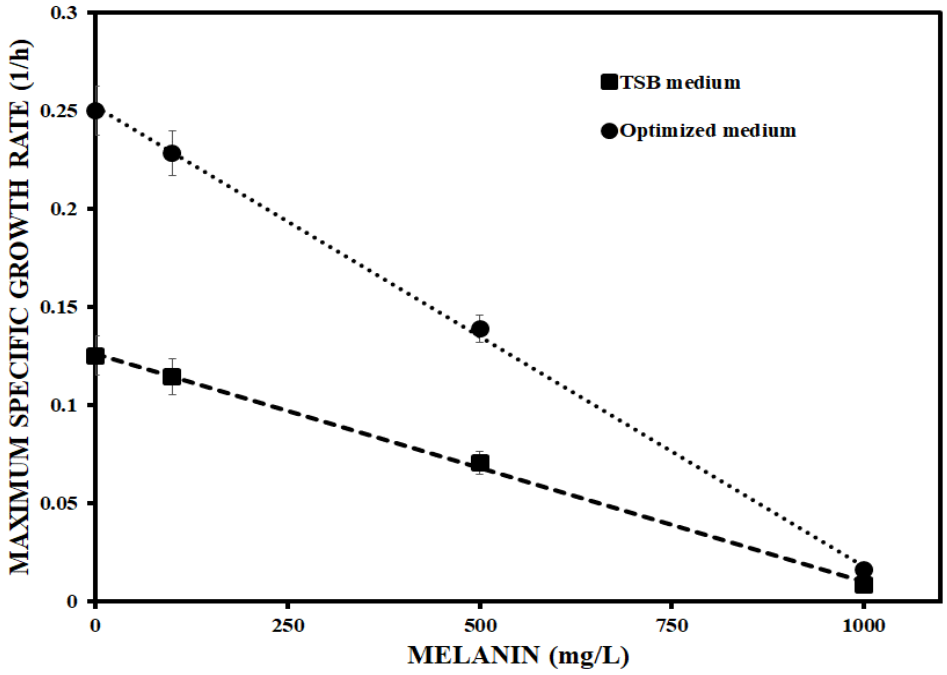

Figure 4-28: Maximum specific growth rate at different melanin concentrations in TSB medium and Optimized medium (APPENDIX Table 7-20).

Above results revealed the substantial inhibitory effect of melanin on growth and production. Hence, it was necessary to remove melanin from the broth to reduce feedback inhibition and increase the productivity of the bioprocess.

4.8 Adsorptive bioprocess development

As mentioned in the literature review, the in ISPR technique the target molecule in the bioprocess is removed as it is biosynthesized during the bio fermentation process. There are numerous reports on ISPR being used in bioprocess development research, however, only a handful of such reports are commercialized due to difficulty in scale-up. Therefore, to increase the commercial feasibility of the melanin production process, in this research work, a simple, effective and easily scalable adsorptive bioprocess was developed. In the first phase, different commercially viable and easily available adsorbents such as Zeolite, Celite, Alumina and Activated carbon were used to study their melanin adsorption potential from the broth. The method used to identify the potential adsorbent is explained in the section 3.13.

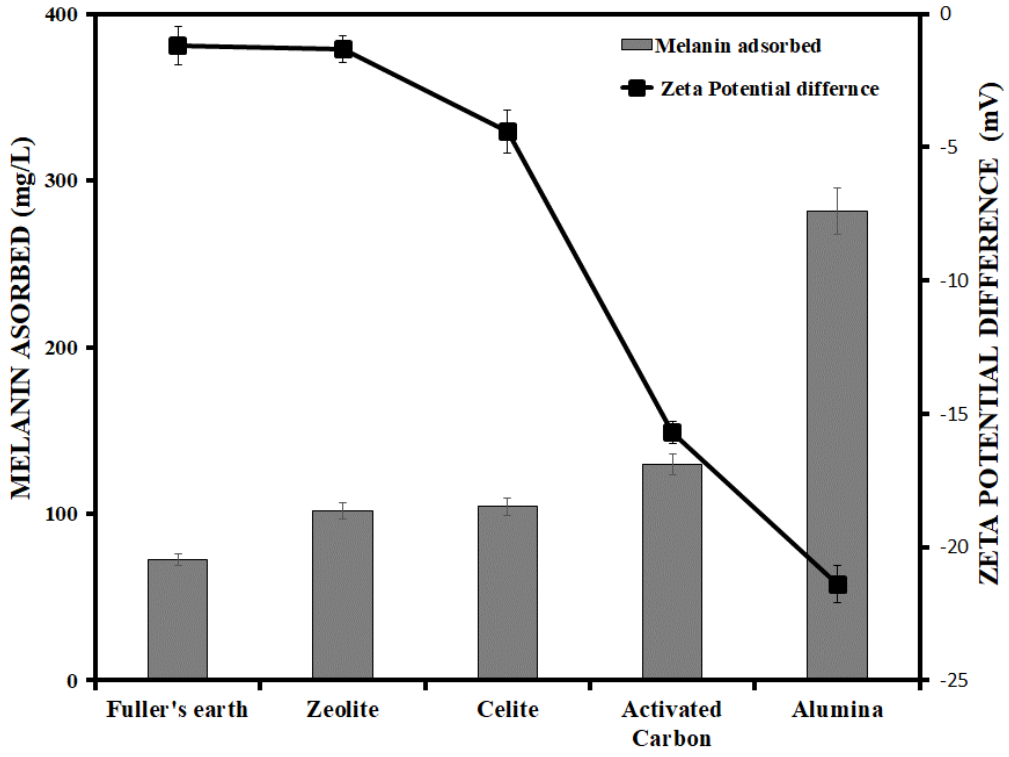

Figure 4-29: Adsorption of melanin by different adsorbents and their zeta potential values (APPENDIX Table 7-21).

As Figure 4-29 indicates, Alumina was the best adsorbent, which adsorbed almost 60% of the melanin produced from the broth at pH 8. Melanin is ionized in anionic form in basic pH of the broth and hence solubilizes in water. Zeolite and fuller's earth are usually cation exchangers, due to which they did not bind large amounts of melanin. Celite and activated carbon are good physical adsorbents, however, they adsorb non-polar compounds. Melanin becomes nonpolar in acidic pH by neutralizing its negative charge with hydronium ions present in the acidic medium. On the contrary, alumina is a polar adsorbent and hence was able to adsorb polar and anionic melanin from the fermentation broth. The maximum adsorption using alumina coincided with maximum difference in zeta potential, confirming the suitability of alumina as the adsorbent for melanin in basic pH.

Therefore, alumina was chosen for ISPR technique. The time of dosage of alumina to the culture broth was investigated in the next step of adsorptive bioprocess development. Alumina was added to the culture at different stages of growth.

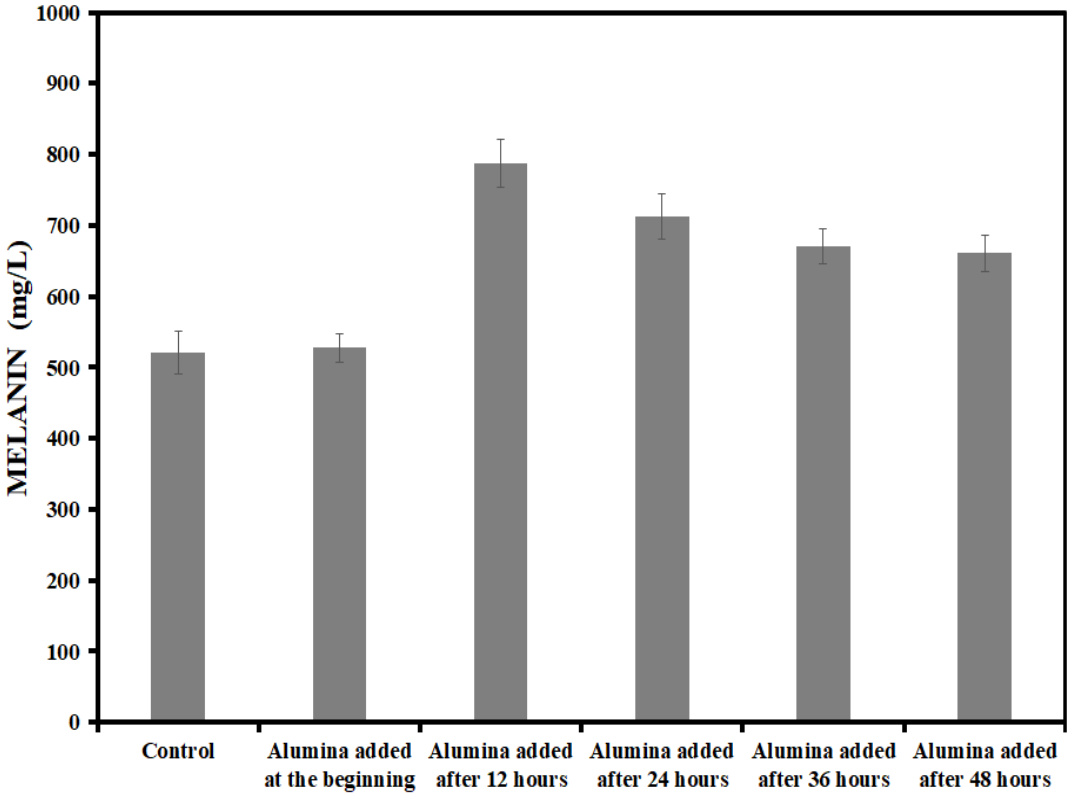

Figure 4-30: Effect of time of adsorbent dosage on melanin production (APPENDIX Table 7-22).

Figure 4-30 shows the effect of dosage time of alumina on melanin production. Dosage time of 12 hours demonstrated the better melanin yield. Cells were in exponential phase of their growth with a biomass concentration of 1.7 g/L. The pH of the medium was 7.3 and melanin concentration was about 140 mg/L. There was about 50% increase in melanin production when adsorbent was added to the culture after 12 hours than the normal culture grown in optimized medium. The melanin production decreased when alumina was added at the start of the bioprocess. Alumina is known to partially inhibit microbial growth. Since melanin was not biosynthesized in the medium just after the inoculation, free alumina might have inhibited the growth and hence production of melanin. Alumina addition at 36 hours after inoculation also did not show improvement in melanin production. By this time, melanin was already produced in quantities, which would have initiated inhibitory effects. These findings indicate that time of addition of adsorbent is crucial for successful adsorptive bioprocess, especially if the adsorbent is inhibiting the microbial growth. The effect of adsorbent concentration on melanin production was investigated in the next step of adsorptive bioprocess development.

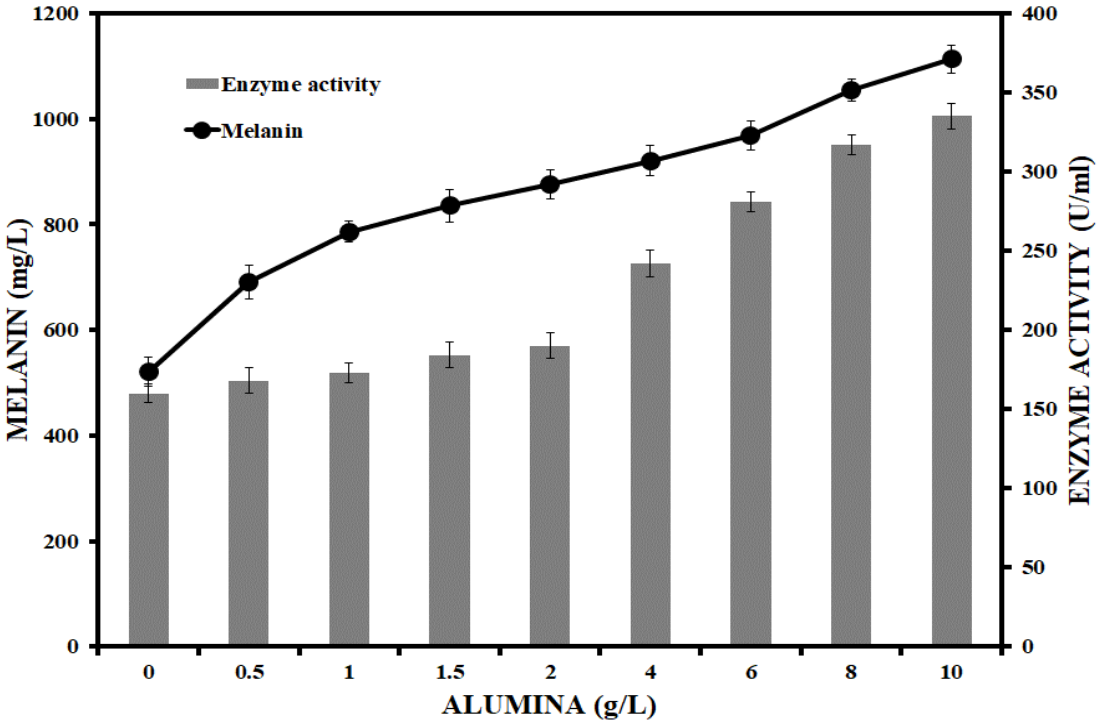

Figure 4-31: Effect of adsorbent (alumina) on melanin production and enzyme activity of cells (APPENDIX Table 7-23).

Figure 4-31 shows that melanin production increased as adsorbent dosage increased. Tyrosinase enzyme activity also improved by increasing the adsorbent dose, which is evident from Figure 4-31. At 10 g/L adsorbent concentration, the reducing sugars, as well as the nitrogen sources, were completely consumed by *P. stutzeri* by 48 hours and therefore, higher adsorbent concentrations were not investigated further.

Figure 4-32 demonstrates the adsorption of melanin onto alumina. As shown in figure 4.32B, the alumina adsorbed the melanin produced in the broth. The growth medium containing free melanin and melanin bound on alumina particles was collected at the end of the bioprocess. A 0.1 M NaOH solution was added to the alumina particles obtained to desorb melanin from alumina particles, which is exhibited in figure 4.32C. The mixture was centrifuged to separate alumina particles. The melanin produced was quantified by measuring the optical density of supernatants at 400 nm using a spectrophotometer.

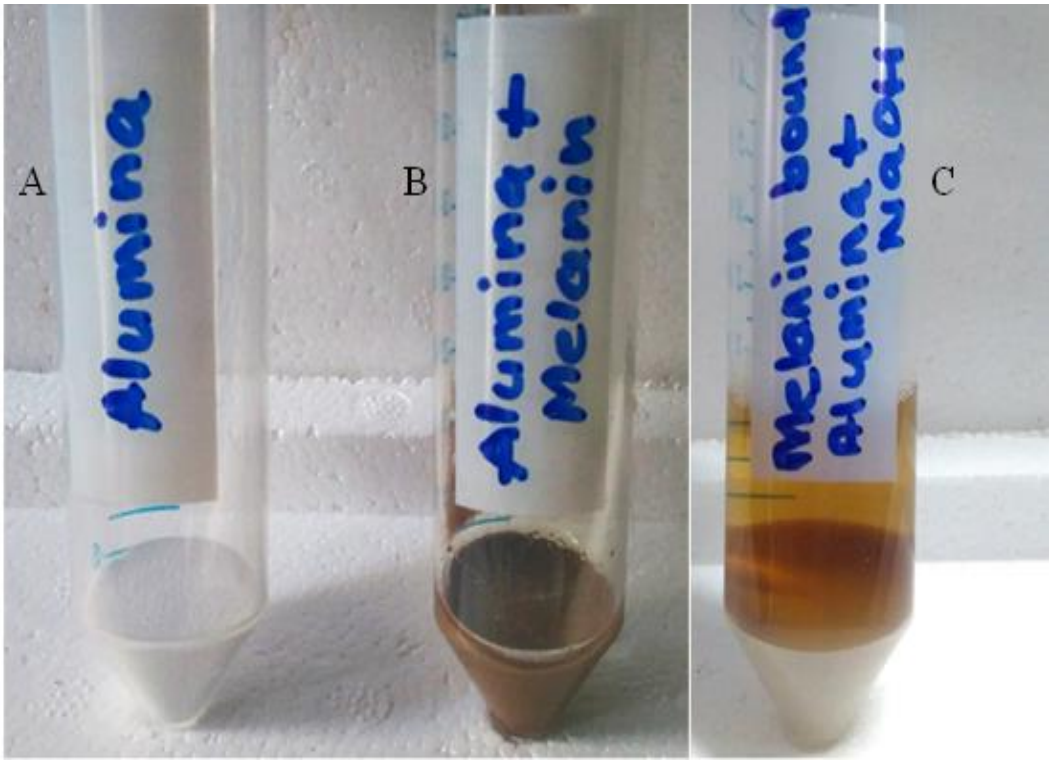

Figure 4-32: Adsorptive bioprocess A) Alumina. B) Melanin bound alumina. C) Desorption of melanin particles from alumina after the second stage of desorption.

Adsorptive bioprocess was carried out in a 3 litre stirred tank bioreactor with the addition of alumina (10 g/L) and the following figure shows results of the bioprocess.

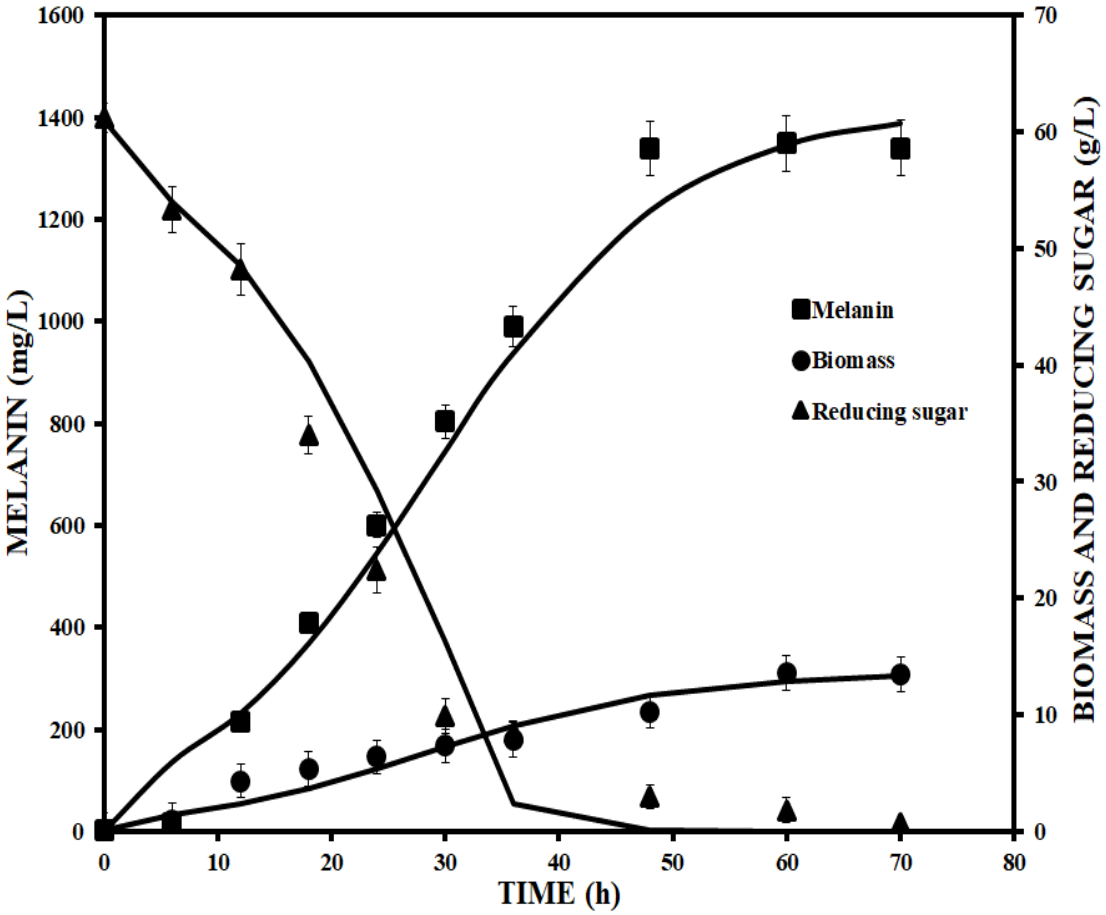

Figure 4-33: Melanin production in stirred tank fermenter with the addition of adsorbent alumina. (— simulation results) (APPENDIX Table 7-24).

The adsorptive bioprocess in a 3L fermenter enhanced melanin production from 520.96 mg/L (Figure 4-20) to 1349.03 mg/L (Figure 4-33), which is about 2.6 fold increase in the same optimized medium. About 1200 mg/L melanin bound to the alumina whereas about 150 mg/L melanin remained in the supernatant. The carbon sources, as well as the nitrogen sources, were completely consumed by *P stutzeri* by 48 hours. The important growth parameters are mentioned in Table 4-5.

Figure 4-34 compares the melanin production at different time intervals in a normal batch bioprocess and adsorptive bioprocess in a stirred tank bioreactor under optimized conditions. It also shows the amount of melanin remaining in the supernatant during the adsorptive bioprocess. The melanin concentration remained nearly constant in the supernatant, in the range of 95 to 110 mg/L in presence of alumina, thus reducing the inhibitory effect.

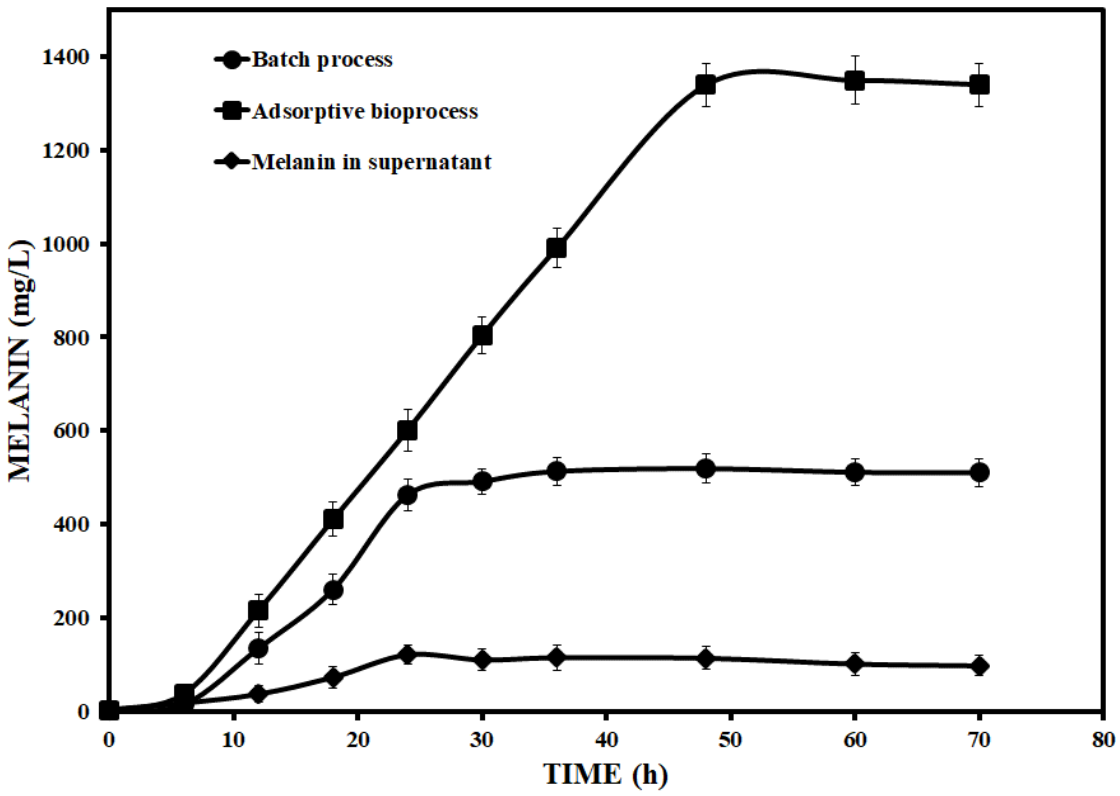

Figure 4-34: Production of melanin in normal batch bioprocess and adsorptive bioprocess (APPENDIX Table 7-25).

From the figure, it is evident that there is an enhancement in melanin production due to the addition of adsorbent in a batch process.

Parameter	**Normal batch process with optimized medium**	**Adsorptive bioprocess with optimized medium**	**% increase**
μ_{max} (1/h)	0.145	0.25	72.4
v_x (g/l/h)	0.15	0.28	86.6
α (g/g)	0.081	0.105	29.6
$Y_{X/S}$ (g/g)	0.14	0.21	50.0
$Y_{P/S}$ (g/g)	0.0083	0.023	177

Table 4-5: Values of growth parameters obtained through parameter estimation.

Table 4.5 compares the growth and product formation parameters for a normal batch bioprocess and adsorptive bioprocess for melanin production. The table is self-explanatory and proves that adsorptive bioprocess increased the specific growth rate, yield coefficients and hence productivity of melanin production. The amount of proteins, sugars, and cells/DNA adsorbed onto alumina was 2.51 mg/g, 1.52 mg/g and 0.18 mg/g, respectively. These values are negligible as compared to the adsorption values of melanin onto alumina.

The following figure explains general principal of adsorptive bioprocess of melanin production.

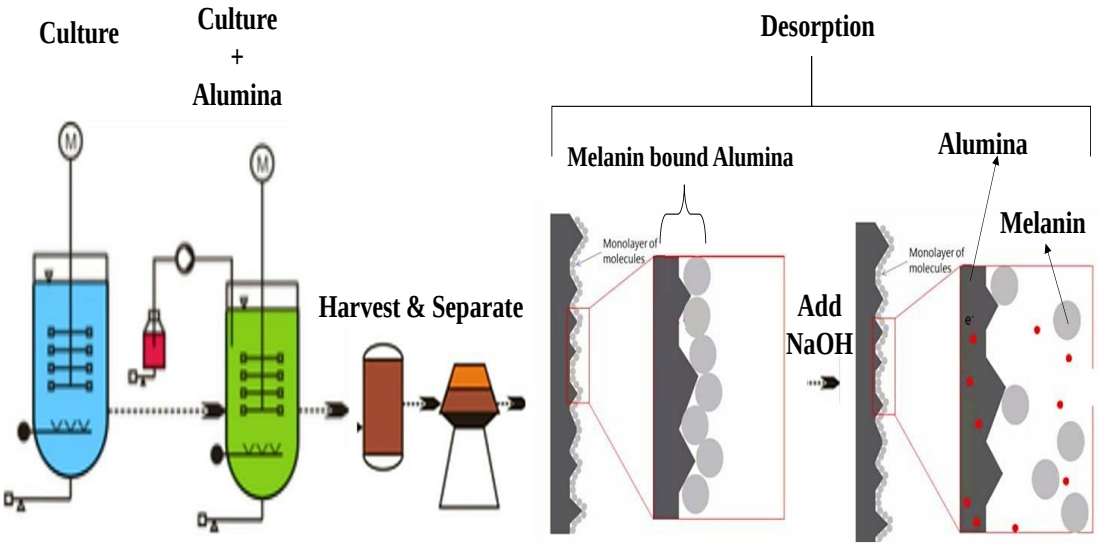

Figure 4-35: Adsorptive bioprocess for melanin production

As mentioned in the above figure, the alumina was added into fermenter when the melanin concentration was about 140 mg/L (8 hours after inoculation) and the bioprocess was continued without changing any parameters. The broth was harvested at 68 hours and alumina was separated by centrifugation at 600 rpm for 5 minutes. The alumina was washed three times with water followed by alcohol wash. The melanin bound onto alumina was desorbed by 0.1N NaOH solution.

Figure 4-36 compares the melanin production at different stages of this research work. The figure confirms that the melanin inhibition was reduced which is visible in increased melanin production by more than 100% in the same medium, i.e., optimized medium.

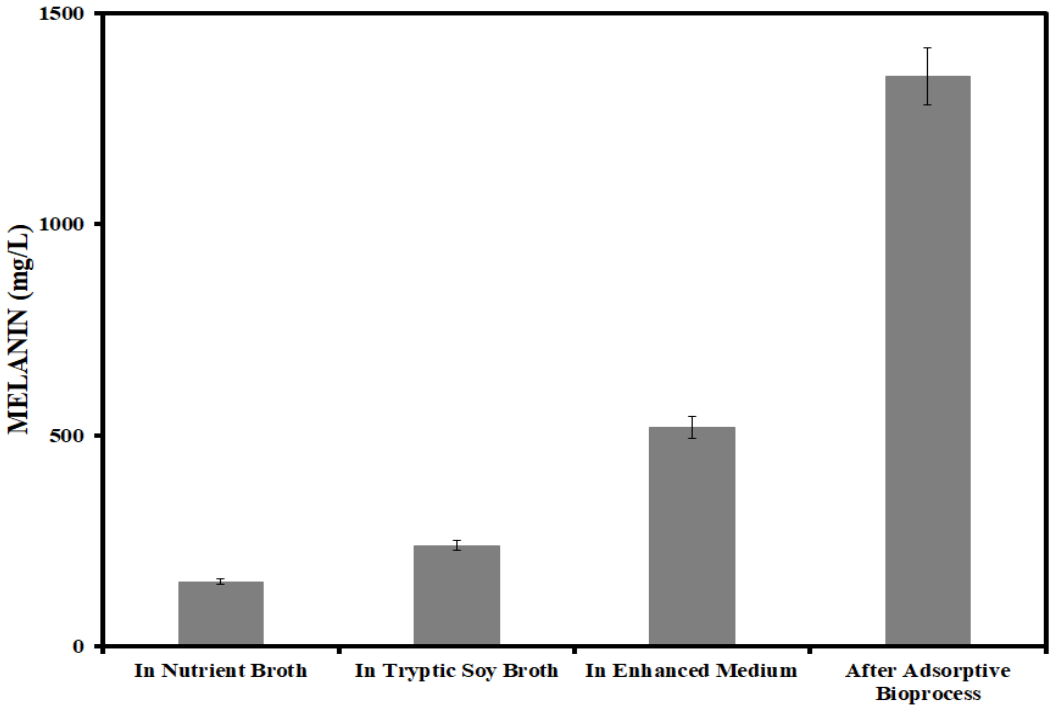

Figure 4-36: Melanin production during different stages of optimization and bioprocess development.

Overall, as above figure depicts, the melanin production enhanced from 153 mg/L at the initiation of the project to 1349.03 mg/L to date. Volumetric productivity of melanin increased from 2.12 mg/L·h at the initiation of the project to 29 mg/L·h to date. Moreover, the adsorptive process described here can be easily incorporated in any bio-production facility without any modification in the existing plant.

4.8.1 Determination of melanin binding capacity of alumina

Adsorption isotherm helps to describe the interaction between the adsorbate and adsorbent. Freundlich adsorption isotherm is applicable to multilayer physical adsorption whereas Langmuir isotherm is based on the assumption of monolayer adsorption, the uniform energy of adsorption in the plane of the adsorbent surface, non-interaction between the adsorbed ions and availability of all identical binding sites for monolayer adsorption. Following form of Langmuir isotherm was used to determine the melanin binding capacity of alumina. The linear form of Langmuir isotherm can be expressed as follows (Dutta et al. 2015):

$$\frac{C_e}{q_e} = \left(\frac{1}{q_{max}}\right) * \left(\frac{1}{\mathrm{b}}\right) + \frac{C_e}{q_{max}} \qquad (4\text{-}12)$$

Where, C_e (mg/L) is the equilibrium concentration of melanin in the solution, q_e (mg/g) is the amount of melanin adsorbed per specific amount of adsorbent at equilibrium, q_{max} is the maximum loading capacity, which is the amount of adsorbate required to form monolayer and b is a constant, which represents the affinity of adsorbent to adsorbate.

The following figure (Figure 4-37) shows data fitting to Langmuir adsorption isotherm. The maximum adsorption capacity of melanin onto alumina from pure melanin solution as well as from the broth at pH 8 was found to be 127.42 mg melanin/g alumina and 113.84 mg melanin/g alumina, respectively. The amount of proteins, sugars, and cells/DNA adsorbed onto alumina were 2.51 mg/g, 1.52 mg/g and 0.18 mg/g, respectively.

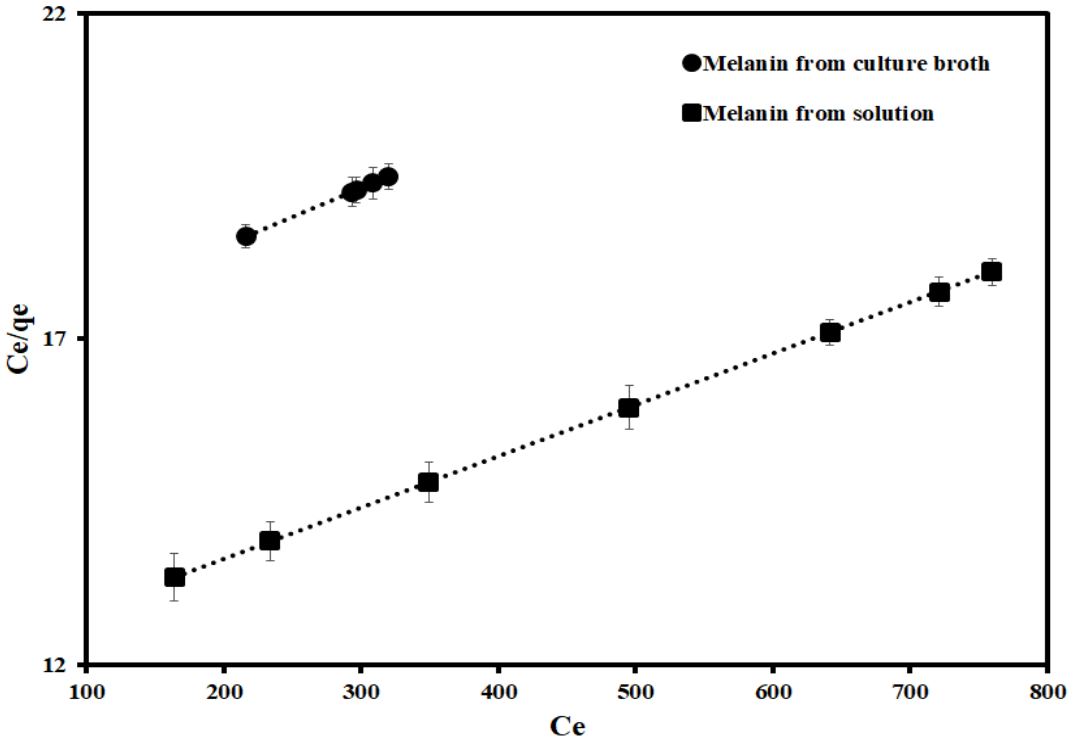

Figure 4-37: Langmuir adsorption isotherm for adsorption from melanin solution (APPENDIX Table 7-26) and from culture broth (APPENDIX Table 7-27).

From the affinity constant b given in the above mentioned equation, a dimensionless constant called separation factor, R_L which helps to define whether the adsorption process is favorable or not was calculated using following equation (Saini and Melo 2013; Wang et al. 2015),

$R_L = 1/(1+b*C_i)$ (4-13)

Where *Ci* (mg/L) is the initial melanin concentration. The value of $R_L > 1$ implies unfavorable adsorption process, $R_L = 1$ is linear, $0<R_L<1$ represents favorable adsorption process and $R_L = 0$ implies that the adsorption process is irreversible. In this scenario, the R_L value for adsorption of melanin from melanin solution was between 0 to 1 at melanin concentrations ranging from 100 mg/L to 2000 mg/L, representing the favorable adsorption process.

4.9 Downstream processing of melanin

Following chart explains the conventional extraction process of melanin (Almeida-Paes et al. 2012; Schmaler-Ripcke et al. 2009). Recovery of melanin is mentioned after each step of the extraction process.

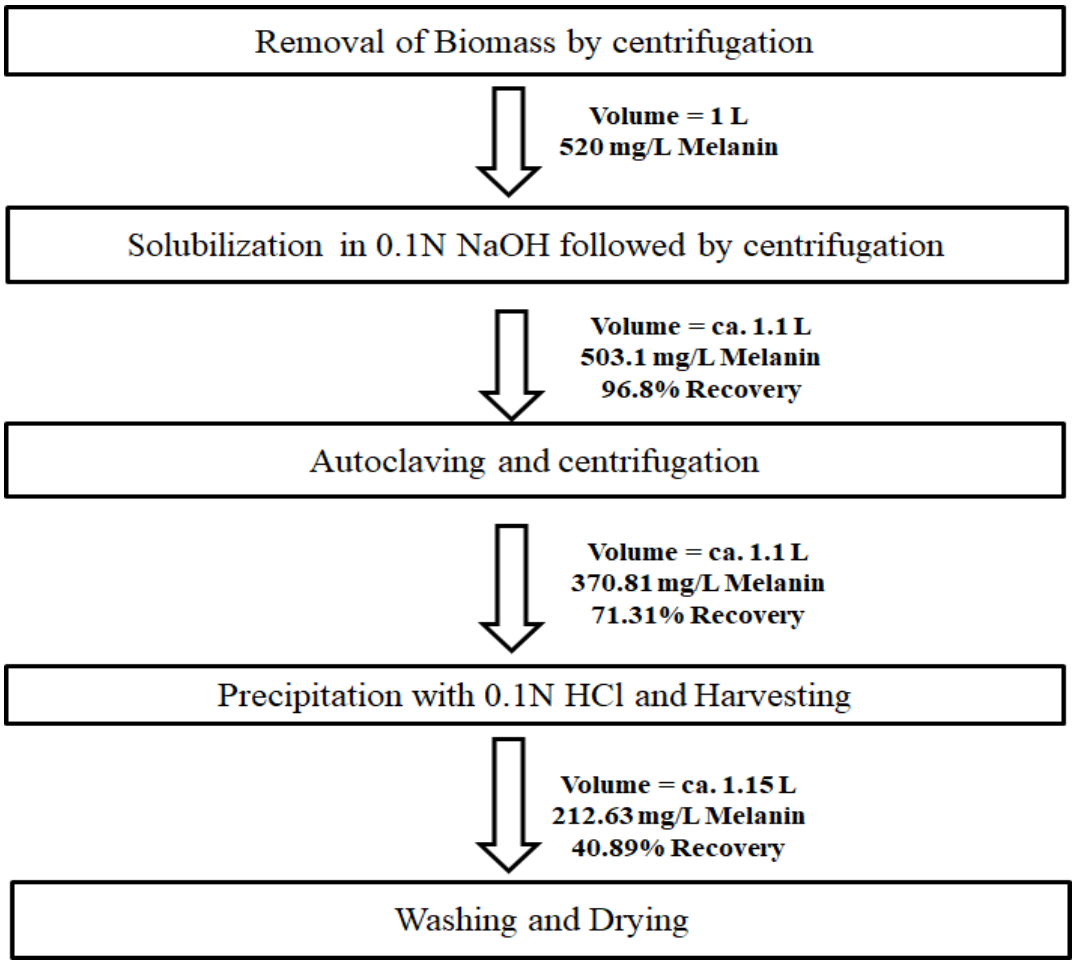

Figure 4-38: Conventional extraction process.

Melanin recovery was about 40.89% using the conventional process, which is very less and major part of melanin produced was still remaining in the acidified solution. The melanin formed a colloidal solution and could not be precipitated out due to very less concentration in the mother liquor. Hence, the process was modified by extracting with different solvents. Usually, organic solvents are good extractants of pigments.

Solvent	**Effect**
Ethyl alcohol	-
Methyl alcohol	-
Ethyl acetate	-
TFA	-
Phenol	+
Toluene	-
Chloroform	-
Glycerine	-

Table 4-6: Solubility of melanin in different solvents

While investigating the solubility of melanin in different solvents (Table 4-6), melanin was readily soluble in phenol. Hence phenol was used for extraction of melanin.

The following figure (Figure 4-39) explains steps involved using phenol extraction. Two changes were made in this process as compared to the conventional extraction process. Autoclaving step was removed and phenol was introduced as an extraction solvent of melanin from final acidified solution.

When phenol was added to the supernatant in the ratio of (2:10), more than 90% of the melanin in the supernatant extracted into the phenol layer and this reduced the volume handling. The melanin dissolved in phenol was recovered by solvent vaporation in vacuum. Unfortunately, phenol is carcinogenic in nature and therefore can not be used at commercial scale for extraction of melanin.

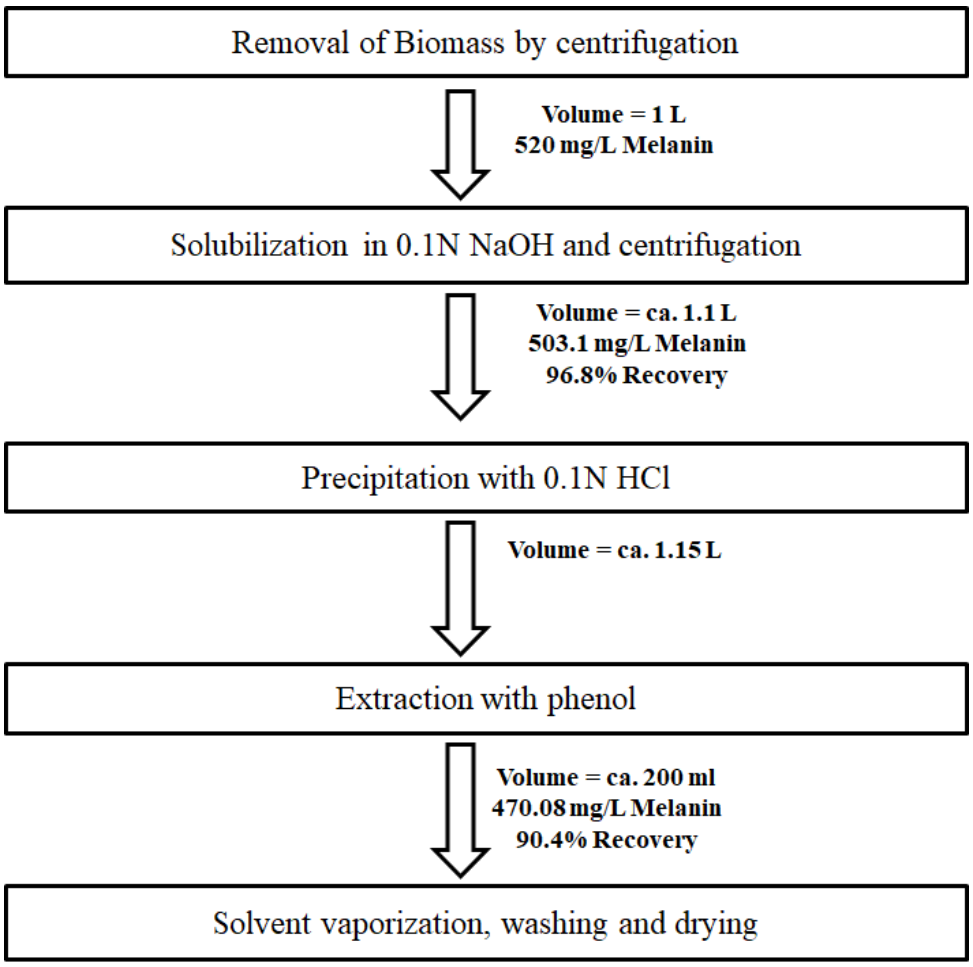

Figure 4-39: Extraction with phenol.

Therefore, the principle of adsorptive bioprocess was applied to the extraction process and an adsorptive extraction process was developed through which the melanin remaining in the solution was extracted. The steps of this extraction process are mentioned in the following figure (Figure 4-40). During the adsorptive bioprocess, most of the melanin produced was adsorbed onto alumina. The alumina was separated from the broth. The supernatant was collected after adsorptive bioprocess and was acidified to pH less than 3 for melanin precipitation. To this, alumina at a

concentration of 0.5 g/L was added and kept for four hours for adsorption. Almost 98% melanin adsorbed onto alumina from the broth.

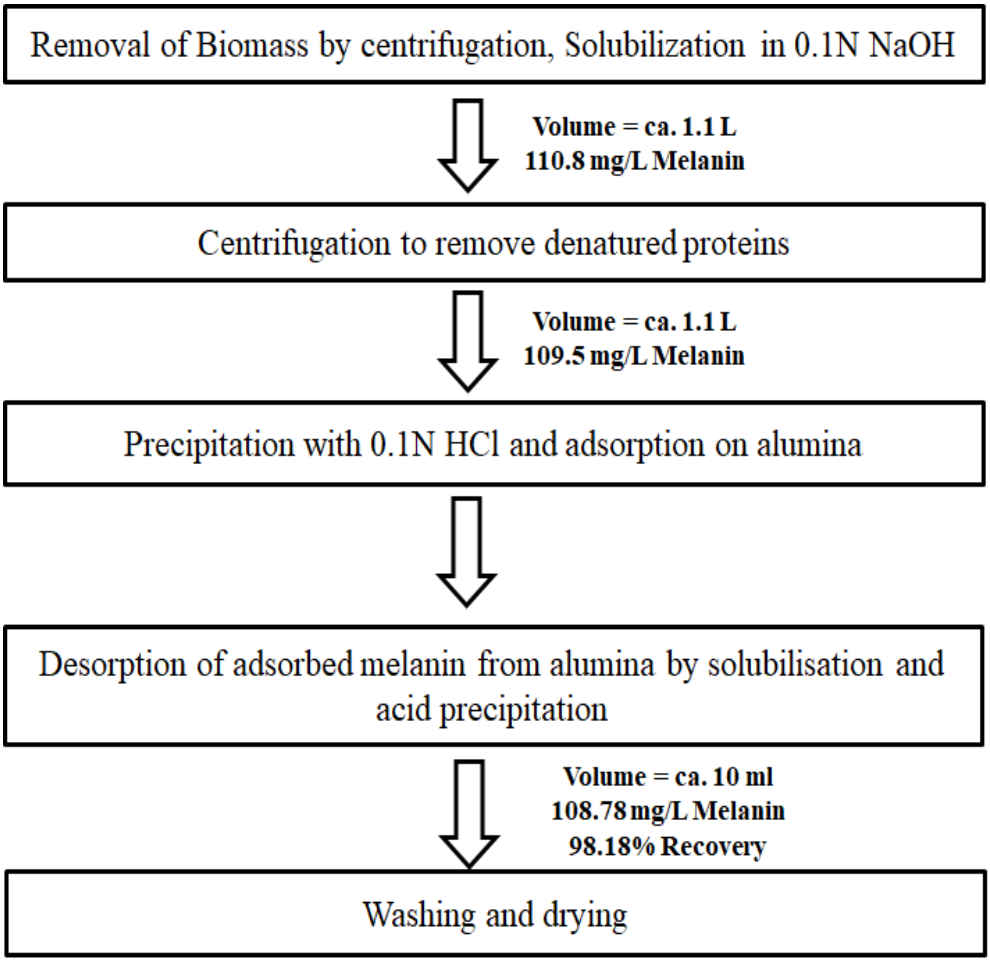

Figure 4-40: Adsorptive extraction process.

The alumina adsorbed melanin was desorbed in 5 mL of 0.1N NaOH solution. Alumina was contacted twice with 0.1N NaOH solution to desorb all the melanin. The working volume decreased from 1.15 L to about 10 to 15 ml. This solution was acidified to pH less than 3 with hydrochloric acid to precipitate melanin. Since the volume of melanin solution decreased drastically, the concentration of melanin increased; hence, the precipitation was much more than the conventional extraction process. The precipitated melanin was washed twice with water followed by washing in alcohol. It was then dried and stored at 4ºC for further use. Around 98% of melanin was recovered by this method.

Method	Recovery
Conventional extraction	40.9%
Phenol (1:10)	90.4%
Adsorptive extraction process (at pH < 3)	> 98%

Table 4-7: Comparison of melanin recovery efficiency of different extraction processes.

Thus as table 4.7 compares the three extraction processes, the findings indicate that the adsorptive extraction process using alumina as an adsorbent can be an efficient (recovery > 98%) alternative for conventional extraction method.

4.10 Characterisation of melanin

4.10.1 UV-Visible spectrophotometric analysis

The spectral property of the melanin was studied to confirm its nature. The UV-Visible absorption spectrum of the biosynthesized melanin was found to be in agreement with the synthetic melanin with an adsorption maxima between 200 nm and 300 nm.

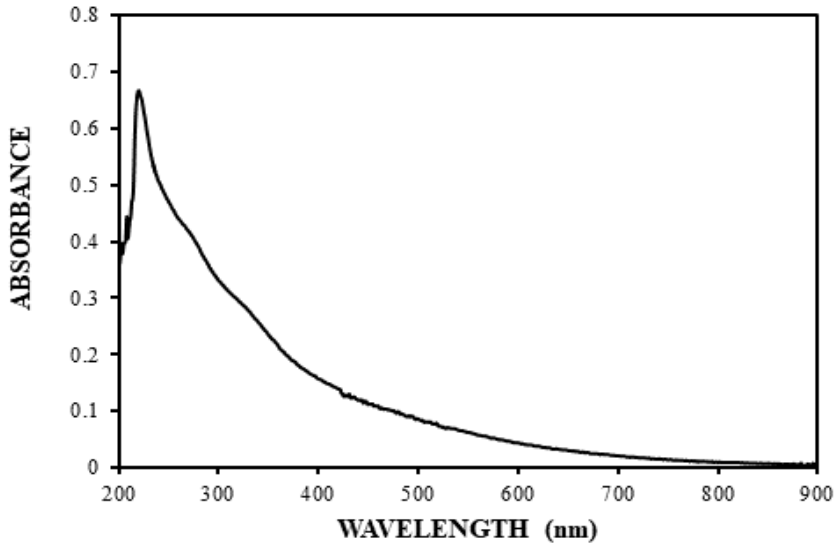

Figure 4-41: The UV-visible absorption spectrum of melanin produced by *Pseudomonas stutzeri*.

4.10.2 SEM and TEM analysis

SEM results showed similarities between biosynthesized melanin and synthetic melanin which appeared as homogenous spherical particles (Figure 4-42).

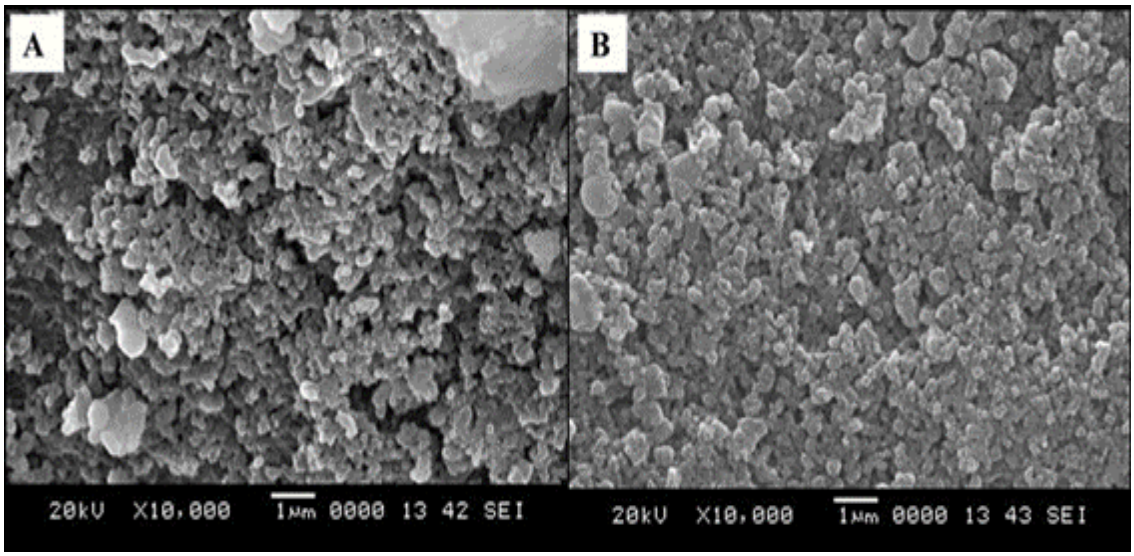

Figure 4-42: SEM image of (A) synthetic melanin and (B) biosynthesized melanin

The TEM imaging was done with TEM, JEM-2100, JEOL make. Figure 4-43 shows an aggregate of melanin molecules of size in the nano range.

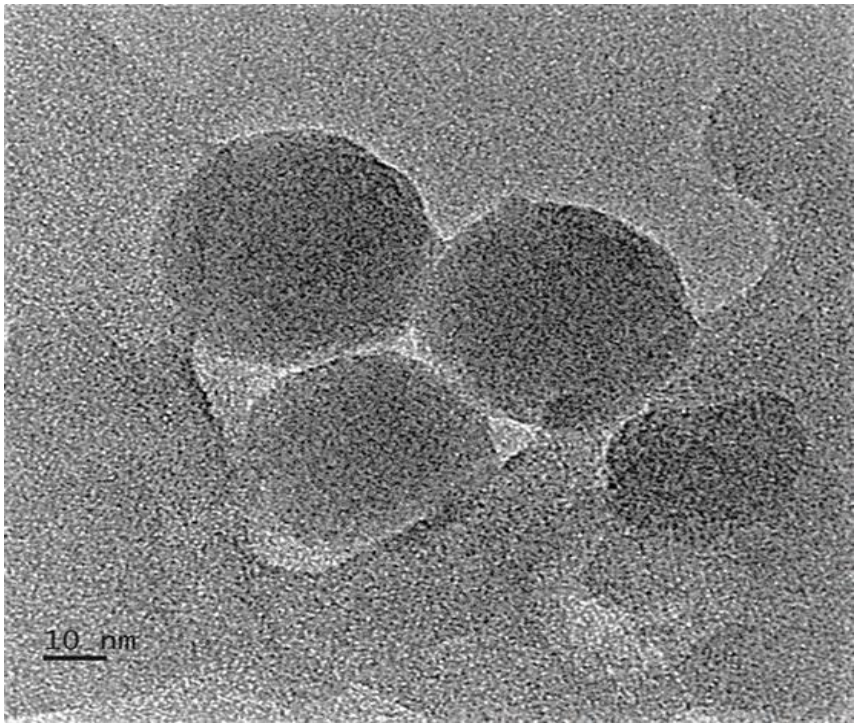

Figure 4-43: An aggregate of melanin particles

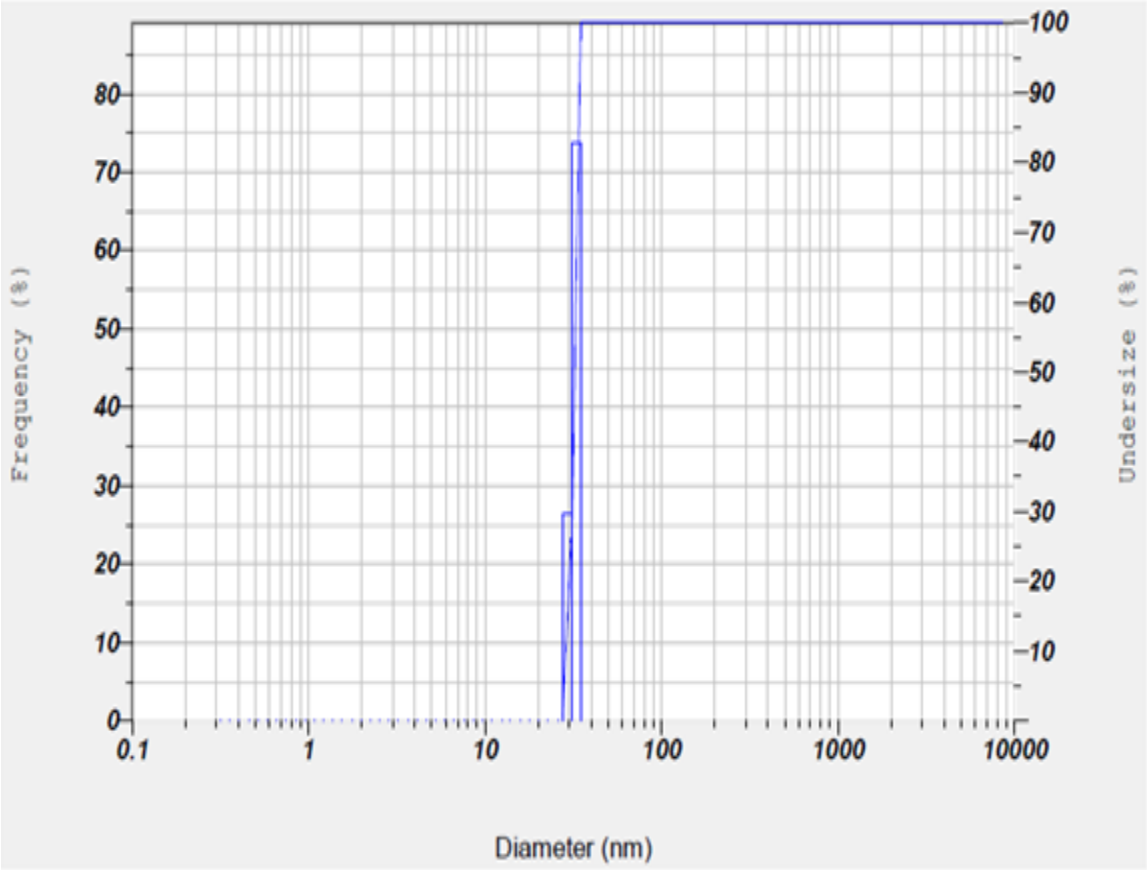

Figure 4-44: Particle size analysis of the biosynthesized melanin.

Particle size analysis of the dispersed solution of biosynthesized melanin in water shows that particles are nanoparticles with a size of 32 ± 0.98 nm (Figure 4-44).

Thus, Scanning electron microscopy, transmission electron microscopy and particle size analysis shows that the melanin molecules are of nano size.

4.10.3 Nuclear Magnetic Resonance spectroscopy (NMR) studies

The ^{1}H NMR spectrum of synthetic melanin and biosynthesized melanin shows signals aliphatic region (Figure 4-45). The nuclear magnetic resonance spectra of these materials in the aliphatic region are almost identical, well resolved signals are observed in both cases. Peaks in the absorption region from 3.0 to 4.0 ppm can be assigned to protons on carbons attached to nitrogen and/ or oxygen atoms in the aliphatic region of the ^{1}H NMR.

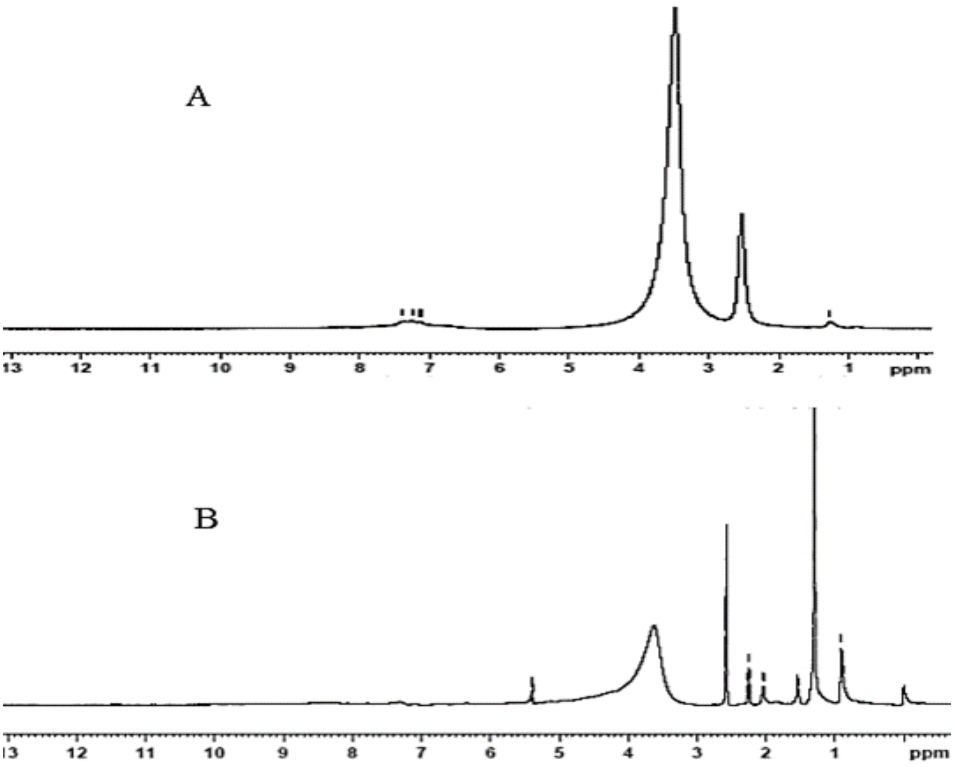

Figure 4-45: (A) The ^{1}H NMR spectrum of synthetic melanin and (B) biosynthesized melanin.

4.10.4 FTIR & SEM of natural melanin and melanin bound alumina

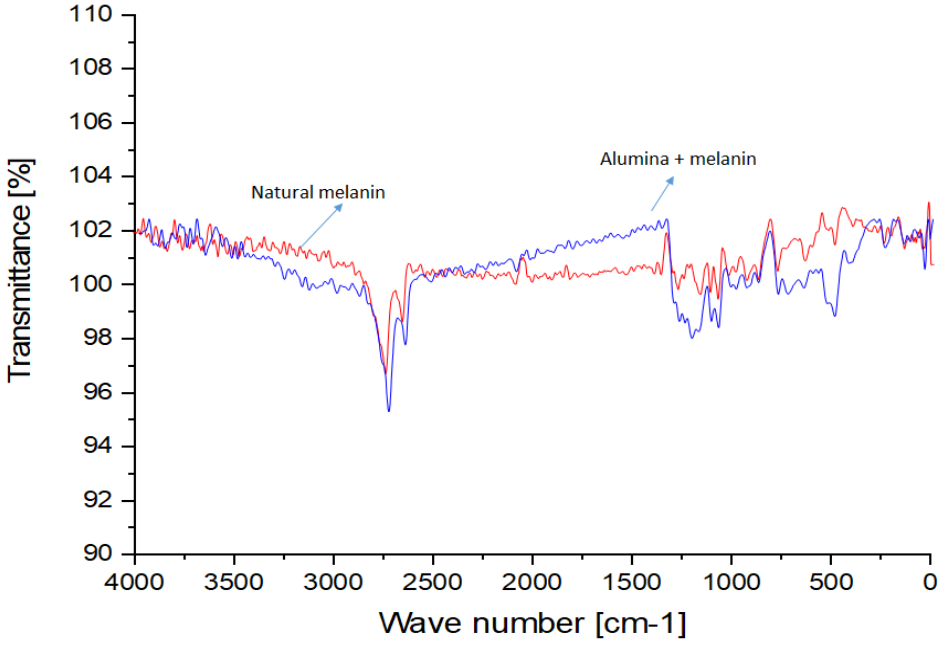

Figure 4-46: FTIR spectra of natural melanin and melanin bound alumina.

From the FTIR spectrum of melanin and alumina bound melanin, the spectrum of alumina bound melanin has shown a change in % transmittance indicating that the alumina is bound to the functional groups.

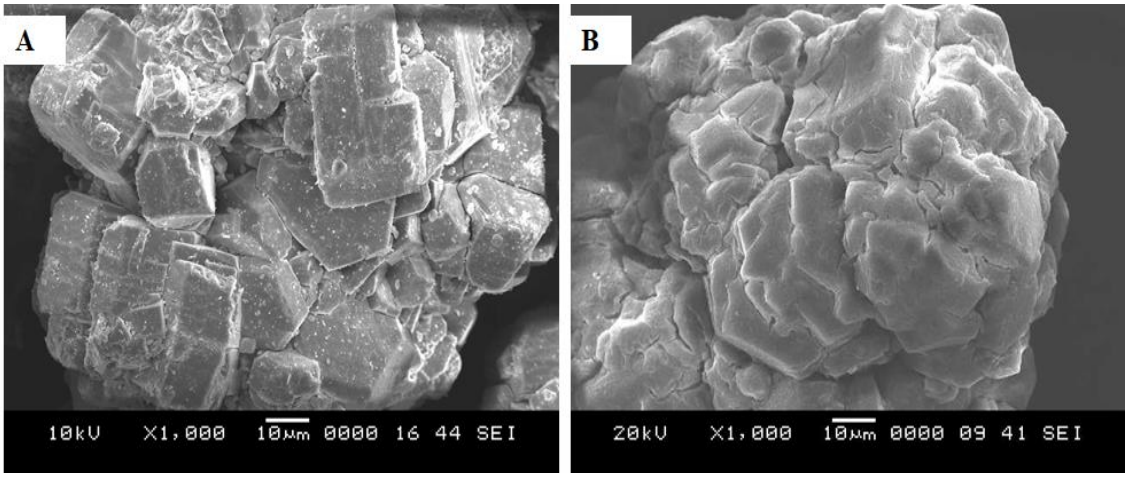

Figure 4-47: SEM image of (A) alumina particle and (B) melanin bound alumina particle.

SEM results showed a change in morphology of alumina particles after melanin adsorption. The binding of the melanin particles resulted in the conversion of sharp edges of alumina particles into smooth edges.

4.10.5 CHN analysis data of melanin and alumina bound melanin

Sample No	Sample Name	N%	C%	H%
1	Biosynthesized Melanin	8.53	45.48	7.09
2	Alumina	Not Detected	Not Detected	Not Detected
3	Alumina bound Melanin	0.26	1.27	0.31

Table 4-8: CHNS analysis data of natural melanin, alumina and melanin bound alumina.

The CHN analysis (Table 4-8) confirms the adsorption of melanin onto the alumina particles where the melanin bound alumina particles show the presence of N, C and O that are present in melanin.

4.10.6 Comparison of FTIR spectrum of natural and synthetic melanin

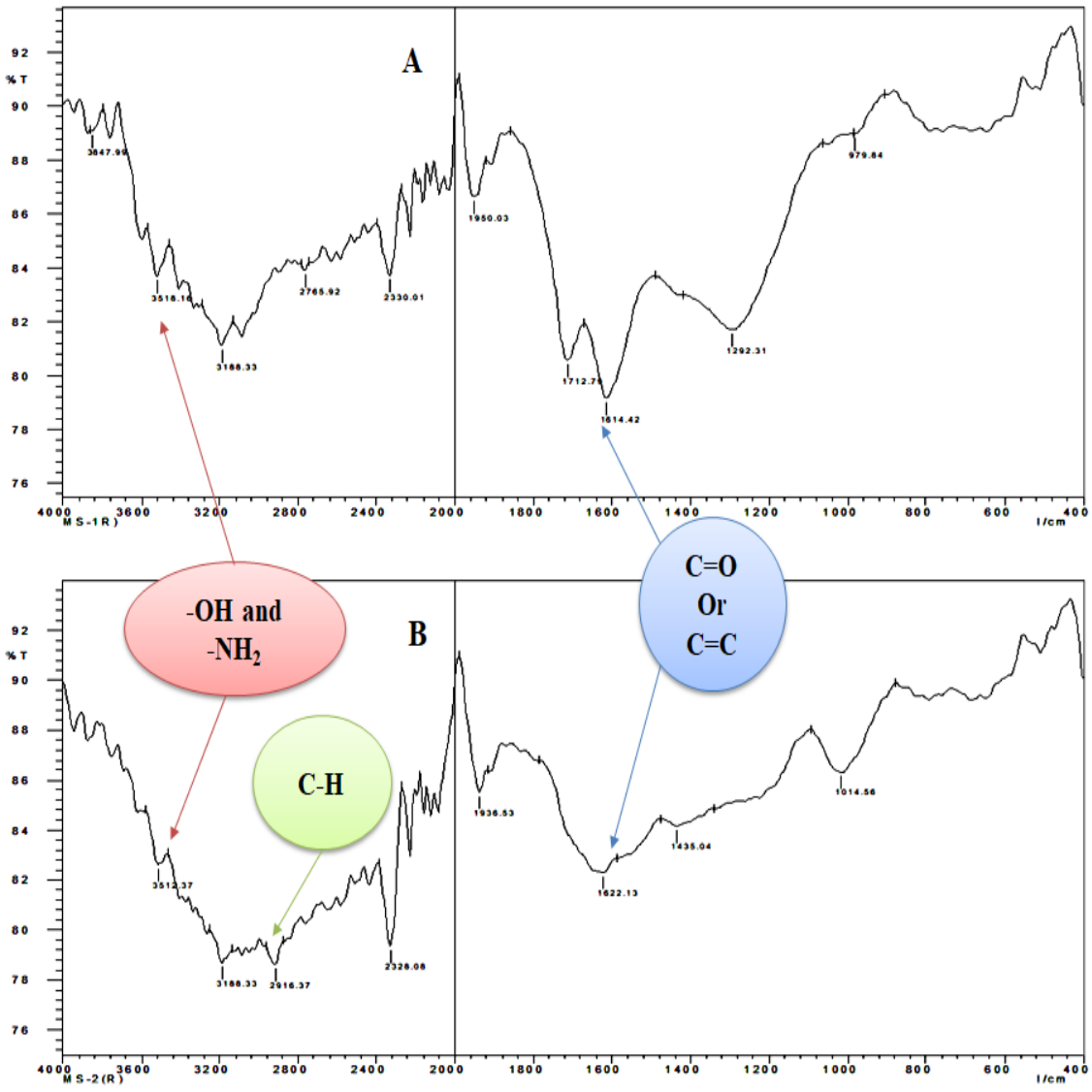

Figure 4-48: FTIR spectra of (A) synthetic melanin and (B) melanin obtained from *Pseudomonas stutzeri*

The FTIR analysis shows bond vibrations in the peaks corresponding to the functional groups –COOH, -CO, -NH and –OH groups present in melanin. Comparison of the main absorption peaks in the FTIR spectra of the synthetic melanin (Sigma-Aldrich) and melanin obtained from *Pseudomonas stutzeri* showed a high degree of similarity.

The FTIR spectrum showed a broad absorption peak at 1,622 cm^{-1}, which is due to the vibrations of the aromatic groups (C=O or C=C). The peak at 3,512 cm^{-1} was attributed to stretching vibrations of the –OH and–NH_2 groups. While the peak observed at 2,916 cm^{-1} suggested the presence of –CH groups.

4.11 Application of biosynthesized melanin for heavy metal removal

The melanin has numerous functional groups such as =O, -OH, -NH, and –COOH. These functional groups make melanin an ideal choice for heavy metal remediation. Studies on removal of heavy metals were conducted for 12 hours with 25 mg melanin and 10 mg/L heavy metal solution in a batch adsorption in 100 ml conical shake flasks with 25 ml filing volume and 150 rpm.

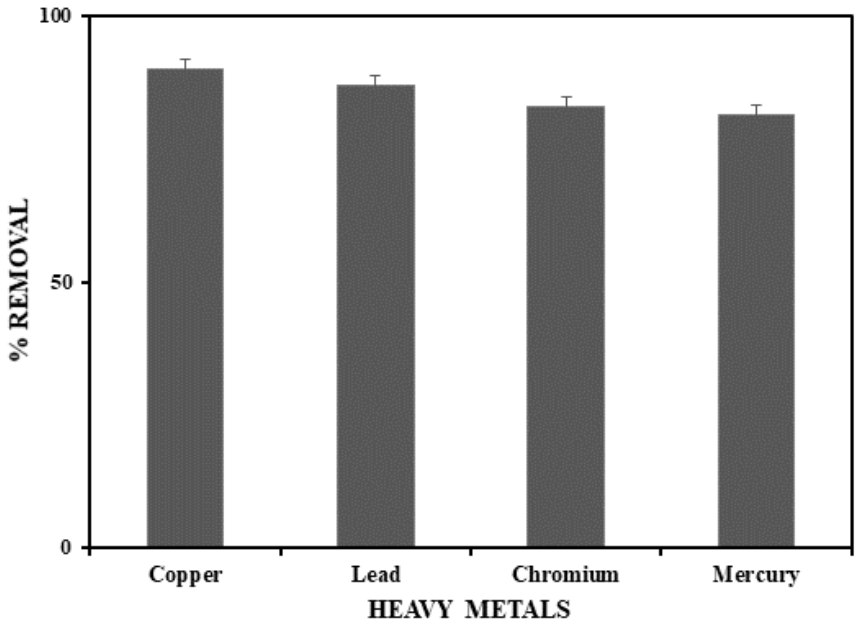

Figure 4-49: The adsorption efficiency of different heavy metals on melanin (APPENDIX Table 7-29).

As mentioned in the above figure, the removal efficiency of copper was 90%, lead was 87%, chromium was 83% and mercury was 81.5%. This study revealed that biosynthesized melanin is an excellent adsorbent, which could remove heavy metals from groundwater.

CHAPTER 5

SUMMARY AND CONCLUSIONS

CHAPTER 5

5 SUMMARY AND CONCLUSIONS

A seawater based sustainable bioprocess for the production of melanin nanopigment is developed and optimized. Moreover, the bioprocess used locally available raw materials, which are not competing for the human food chain, which makes it an excellent choice for commercialization

Optimization of operating conditions reduced bioprocess time from 72 hours to 48 hours. The Optimization of biological parameters and media components resulted in 3.4 fold enhancement in the production of melanin (from 153 mg/L to 520 mg/L) nanoparticles by the marine bacteria *Pseudomonas stutzeri.*

The melanin produced demonstrated feedback inhibition as well as cell growth inhibition. The enzyme tyrosinase responsible for producing melanin was inhibited by melanin through noncompetitive inhibition.

An adsorptive bioprocess reduced melanin inhibition. This technique provides better operational flexibility. The adsorptive bioprocess in a 3L fermenter, increased melanin production from 520.96 mg/L to 1349.03 mg/L, using the same optimized medium. Thus, adsorptive bioprocess increased the productivity of this bioprocess by 2.6 times. The process described here can be easily incorporated in any bio-production facility without further modification in the existing plant.

Melanin production enhanced from 153 mg/L at the initiation of the project to 1349.03 mg/L to date. Volumetric Productivity of melanin increased from 2.12 mg/L h at the initiation of the project to 29 mg/L h to date.

Scanning electron microscopy, transmission electron microscopy and particle size analysis shows that the melanin molecules are of nano size

The biosynthesized melanin exhibited heavy metal binding property. The removal efficiency of copper was 90%, lead was 87%, chromium was 83% and mercury was 81.5%. However, further investigation is required to study adsorption mechanism, kinetics and metal binding capacity of melanin.

With the adsorptive bioprocess, the yield coefficient of melanin over glucose has been increased by 177% (from 0.0083 g/g to 0.023 g/g). However, this yield needs improvement to further reduce the cost of production and channelize glucose for product formation.

Investigations on development of a continuous adsorptive bioprocess and development of an adsorbent, which can specifically bind melanin will be considered as the future aspects of this project.

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6 REFERENCES

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APPENDICES

7 APPENDIX

Time (h)	Biomass (g/L)	Melanin (mg/L)
0	0.01	0.002
6	0.49	49.57
12	1.71	75.89
18	1.76	106.53
24	1.78	110.33
30	1.82	119.01
36	1.94	122.24
42	2.09	131.85
48	2.16	136.59
54	2.18	140.88
60	2.30	144.91
66	2.28	148.42
72	2.21	153.013
78	2.14	152.80
84	1.99	153.52

Table 7-1: Growth of the microorganism and melanin production in shake flask (Figure 4-2).

NB			LBB		
Time (h)	**Biomass (g/L)**	**Melanin (mg/L)**	**Time (h)**	**Biomass g/L**	**Melanin (mg/L)**
0	0.11	10.61	0	0.36	21.74
12	1.56	92.11	12	2.46	148.35
24	2.13	128.84	24	2.57	154.95
36	2.17	136.87	36	2.43	146.63
48	2.15	148.06	48	2.53	152.37
60	2.22	153.23	60	2.71	163.56
72	2.28	154.95	72	2.78	167.58
BHB			**TSB**		
Time (h)	**Biomass (g/L)**	**Melanin (mg/L)**	**Time (h)**	**Biomass (g/L)**	**Melanin (mg/L)**
0	0.099	2.30	0	0.18	10.98
12	0.57	37.95	12	0.74	44.48
24	0.74	43.33	24	1.32	79.77
36	0.94	70.30	36	2.12	127.98
48	1.57	92.40	48	3.66	220.37
60	1.62	91.82	60	3.96	238.45
72	1.67	94.98	72	3.99	241.03

Table 7-2: Growth and melanin production in different growth media (Figure 4-4).

Inoculum Percentage	Biomass (g/L)	Melanin (mg/L)
0.5	2.34	198.99
2	2.41	231.21
5	2.47	266.64
10	2.44	281.42
20	2.58	234.08
30	2.77	203.16
40	2.80	194.84
50	2.71	192.61
60	2.58	177.76

Table 7-3: Growth and melanin production at different inoculum percentage (Figure 4-10).

Inoculum Age	Biomass (g/L)	Melanin (mg/L)
6 Hours	2.05	241.67
12 hours	2.23	261.90
18 hours	2.29	243.62
24 hours	2.25	222.10

Table 7-4: Growth and melanin production at different inoculum age (Figure 4-11).

Temperature	Biomass (g/L)	Melanin (mg/L)
30°C	2.09	232.24
37°C	2.32	252.61
40°C	2.18	245.43
45°C	2.08	198.09

Table 7-5: Growth and melanin production at different temperature (Figure 4-6).

PH	Biomass (g/L)	Melanin (mg/L)
pH 5	1.72	198.28
pH 6	1.92	218.36
pH 7	2.20	245.62
pH 8	2.27	243.04
pH 9	2.16	230.45

Table 7-6: Growth and melanin production at different pH (Figure 4-7).

Shaking frequency	Biomass (g/L)	Melanin (mg/L)
100 rpm	2.24	230.13
150 rpm	2.64	243.90
200 rpm	2.67	248.49
250 rpm	2.50	235.58

Table 7-7: Growth and melanin production at different shaking frequencies (Figure 4-8).

Filling Volume (ml)	Biomass (g/L)	Melanin (mg/L)
10	2.52	262.94
20	2.38	255.78
50	2.29	247.19
100	1.06	243.30

Table 7-8: Growth and melanin production at different filling volume (Figure 4-9).

Trace elements	Concentration g/L)	Biomass (g/L)	Melanin (mg/L)
Cu SO_4	0.05	4.18	277.62
Fe SO_4	0.015	4.14	263.99
$MgSO_4$	0.015	4.17	255.38
$MnSO_4$	0.015	4.11	255.00
$ZnSO_4$	0.05	4.01506	240.67

Table 7-9: Effect of different trace elements on growth and melanin production (Figure 4-14).

Nitrigen sources	Biomass (g/l)	Melanin (mg/L)
control	4.19	291.39
Corn Steep Liquor	3.97	276.33
Soyabean Meal	4.01	285.37
Coconut Meal	4.16	300.00
Cottonseed meal	4.08	292.68
Oat meal	3.83	283.64

Table 7-10: Effect of different nitrogen sources on growth and melanin production (Figure 4-15).

Carbon sources	Biomass (g/L)	Melanin (mg/L)
Control	3.96	261.12
Glucose	4.13	386.66
Starch	3.16	168.58
Glycerol	3.53	204.69
Sucrose	3.61	269.49

Table 7-11: Effect of different carbon sources on growth and melanin production (Figure 4-16).

Time (h)	Biomass (g/L)	Melanin (mg/L)	Sugar (g/l)
0	0.071	0.96	72.68
8	0.84	14.32	62.02
16	2.31	134.32	59.56
28	3.42	259.97	45.08
36	4.84	338.21	37.43
42	5.99	462.18	34.51
48	6.15	491.06	24.78
52	6.20	512.73	21.31
56	6.26	518.74	17.57
64	6.28	510.92	13.96
72	5.94	510.32	12.84

Table 7-12: Growth and melanin production in 3L bioreactor under optimized conditions (Figure 4-20).

Buffer	Biomass (g/L)	Melanin (mg/L)
Control	4.43	284.07
0.01M Tris-HCl	4.30	272.60
0.01 M Citrate buffer	1.33	66.00
0.01 M Phosphate buffer	2.18	137.80

Table 7-13: Effect of different buffers on melanin and biomass production (Figure 4-12).

Buffer concentration (M)	Biomass (g/L)	Melanin (mg/L)
Control	4.43	284.07
0.01M Tris-HCl	4.30	266.14
0.05M Tris-HCl	4.13	228.12
0.1M Tris-HCl	1.70	98.28

Table 7-14: Melanin and biomass production at different buffer concentrations (Figure 4-13).

	Biomass (g/L)	Melanin (mg/L)
Control	3.53	217.84
Arginine (1 mM)	2.95	58.03
Sodium Azide (1 mM)	3.48	203.97

Table 7-15: Inhibition of melanin synthesis pathway (Figure 4-21).

Control (Melanin = 0 mg/L)				Melanin = 100 mg/L			
Time (h)	**Biomass (g/L)**	**melanin (mg/L)**	**Enzyme Activity of cells (U/ml)**	**Time (h)**	**Biomass (g/L)**	**melanin (mg/L)**	**Enzyme Activity of cells (U/ml)**
0	0.11	6.87	0	0	0.07	5.07	0
6	0.44	43.94	11	6	0.14	15.61	5
12	1.08	80.00	34	12	0.38	51.32	23
18	1.29	97.46	50	18	0.69	80.00	41
24	1.67	143.66	71	24	1.04	112.17	54
30	2.06	191.55	92	30	1.26	150.42	73
36	2.34	208.45	107	36	1.60	173.52	97
42	2.92	258.59	120	42	2.40	216.90	110
48	3.21	268.73	134	48	2.76	240.56	122
54	3.25	269.86	137	54	2.81	241.69	127
Melanin = 500 mg/L				**Melanin = 1000 mg/L**			
Time (h)	**Biomass (g/L)**	**melanin (mg/L)**	**Enzyme Activity of cells (U/ml)**	**Time (h)**	**Biomass (g/L)**	**melanin (mg/L)**	**Enzyme Activity of cells (U/ml)**
0	0.05	4.62	0	0	0.01	0.11	0
6	0.11	10.31	2	6	0.045	5.07	0
12	0.19	36.17	9	12	0.13	7.49	4
18	0.43	73.24	17	18	0.34	11.32	12
24	0.55	105.92	24	24	0.44	16.50	20
30	0.74	129.58	39	30	0.59	27.04	25
36	1.21	161.69	55	36	0.94	37.75	29
42	1.56	186.48	68	42	1.31	49.13	31
48	1.88	196.06	74	48	1.48	51.83	34
54	1.90	197.75	77	54	1.55	53.52	35

Table 7-16: Effect of melanin on growth, melanin production and enzyme activity (Figure 4-22, Figure 4-23 and Figure 4-24).

Inhibitor (Melanin) concentration (mg/L)	Tyrosinase (Mushroom) enzyme activity (U/ml)	Tyrosinase (*P.stutzeri*) enzyme activity (U/ml)
0	11700	134
100	9000	122
500	7800	74
1000	6000	34

Table 7-17: Decrease in the enzyme activity with increase in the inhibitor concentration (Figure 4-25).

1/S	1/V (I0)	1/V (I1)	1/V (I2)	1/V (I3)
0.1	161.29	333.33	350.00	461.54
0.2	272.73	375.00	388.00	466.67
1	416.67	545.00	560.00	636.36

Table 7-18: The Lineweaver-Burke plot for tyrosinase enzyme from mushroom (Figure 4-26).

1/S	1/V (I0)	1/V (I1)	1/V (I2)	1/V (I3)
0.1	638	952	1400	2173.9
0.2	766	1105	1503	2299
1	1250	1504	1900	2700

Table 7-19: The Lineweaver-Burke plot for tyrosinase enzyme from *Pseudomonas stutzeri* (Figure 4-27).

Inhibitor (melanin) Concentration (mg/L)	Growth rate in TSB	Growth rate in Optimized media
0	0.125	0.25
100	0.11	0.22
500	0.070	0.14
1000	0.008	0.016

Table 7-20: Maximum specific growth rate at different melanin concentrations in TSB media and optimized media (Figure 4-28).

Adsorbents	Melanin (mg/L)	Zeta potential (mV)
Fuller's earth	72.5928	-1.19
Zeolite	101.7532	-1.34
Celite	104.302	-4.42
Activated Carbon	129.5556	-15.7
Alumina	281.3899	-21.4

Table 7-21: Adsorption of melanin by different adsorbents and their zeta potential values (Figure 4-29).

Adsorbent dosage at different time intervals	Melanin (mg/L)
Control	520.9302
Alumina added at the beginning	527.377
Alumina added after 12 hours	786.7934
Alumina added after 24 hours	712.2535
Alumina added after 36 hours	671.2517
Alumina added after 48 hours	660.6943

Table 7-22: Effect of time of adsorbent dosage (Figure 4-30).

Alumina (g/L)	Melanin (mg/L)	Enzyme Activity of cells (U/ml)
0	520.96	160
0.5	690.72	168
1	786.20	173
1.5	835.28	184
2	875.91	190
4	920.46	242
6	968.58	281
8	1054.83	317
10	1112.99	335

Table 7-23: Effect of adsorbent on melanin production and enzyme activity (Figure 4-31).

Time (h)	Biomass (g/L)	Melanin (mg/L)	Melanin (mg/L) Simulation data
0	0.09	1.51	2.27
6	0.98	17.16	26.30
12	1.71	215.18	231.49
18	2.73	410.00	368.63
24	4.95	601.04	545.80
30	6.92	803.40	745.35
36	9.03	1017.74	938.17
48	8.98	1319.00	1216.84
60	13.02	1349.03	1345.53
70	12.92	1340.10	1375.49

Table 7-24: Growth and melanin production in 3L bioreactor after adding alumina under optimized conditions (Figure 4-33).

Time (h)	Batch process	Adsorptive bioprocess	Melanin in the supernatant
0	0.96	1.51	1.5
8	14.32	17.16	17.2
16	134.32	215.18	36.44
28	259.97	410	72.37
42	462.18	601.04	120.6
48	491.06	803.4	109.6
52	512.73	1017.74	114.2
56	518.74	1319	113.1
64	510.92	1349.03	100.7
70	510.32	1340.1	96.9

Table 7-25: Production of melanin in normal batch bioprocess and adsorptive bioprocess (Figure 4-34).

qe	Ce	Ce/qe
79.92	760.04	18.02
78.7	721.30	17.72
79.08	641.85	17.10
76.12	495.52	15.95
75.14	349.19	14.80
70.77	233.81	13.89
63.60	164.02	13.34

Table 7-26: Langmuir adsorption isotherm data for adsorption from melanin solution (Figure 4-37).

qe	Ce	Ce/qe
18.31	216.37	18.58
20.70	293.50	19.25
21.17	296.78	19.28
23.08	308.76	19.39
27.62	319.83	19.48

Table 7-27: Langmuir adsorption isotherm data for adsorption from culture broth (Figure 4-37).

Media	Melanin (mg/L)
In Nutrient Broth	153.10
In Tryptic Soy Broth	238.00
In Enhanced Medium	518.14
After Adsorptive Bioprocess	1349.03

Table 7-28: Melanin production at different conditions.

Heavy metal	Percentage removal (%)
Copper	90.05
Lead	87.10
Chromium	83.24
Mercury	81.50

Table 7-29: The adsorption efficiency of different heavy metals on melanin (Figure 4-49).

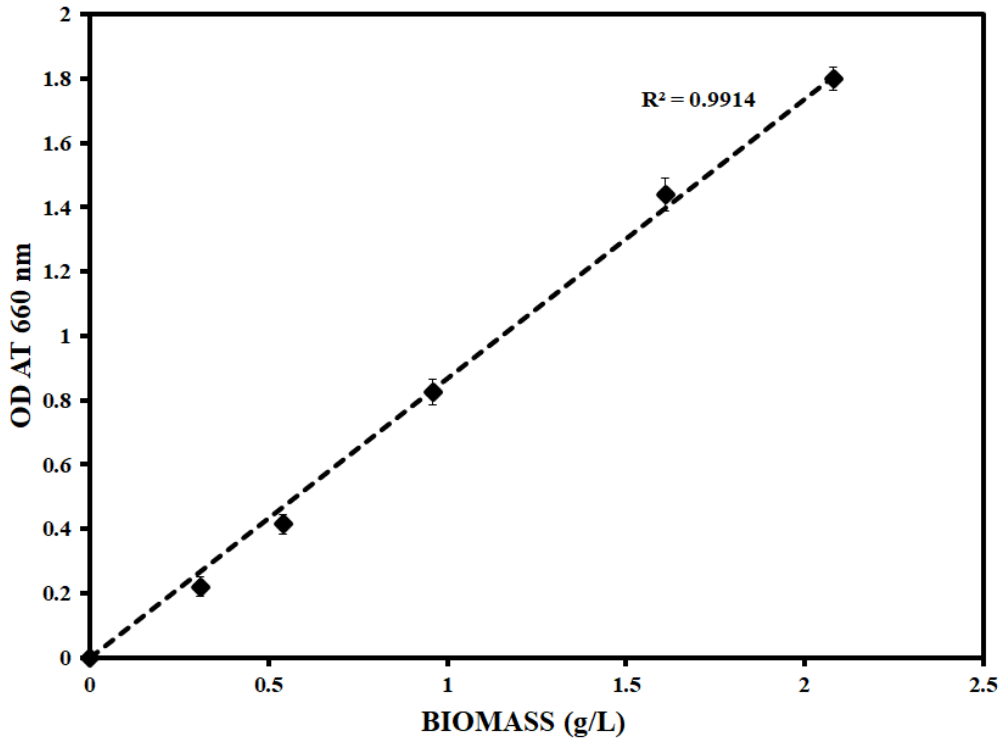

Figure 7-1: Standard graph for biomass estimation.

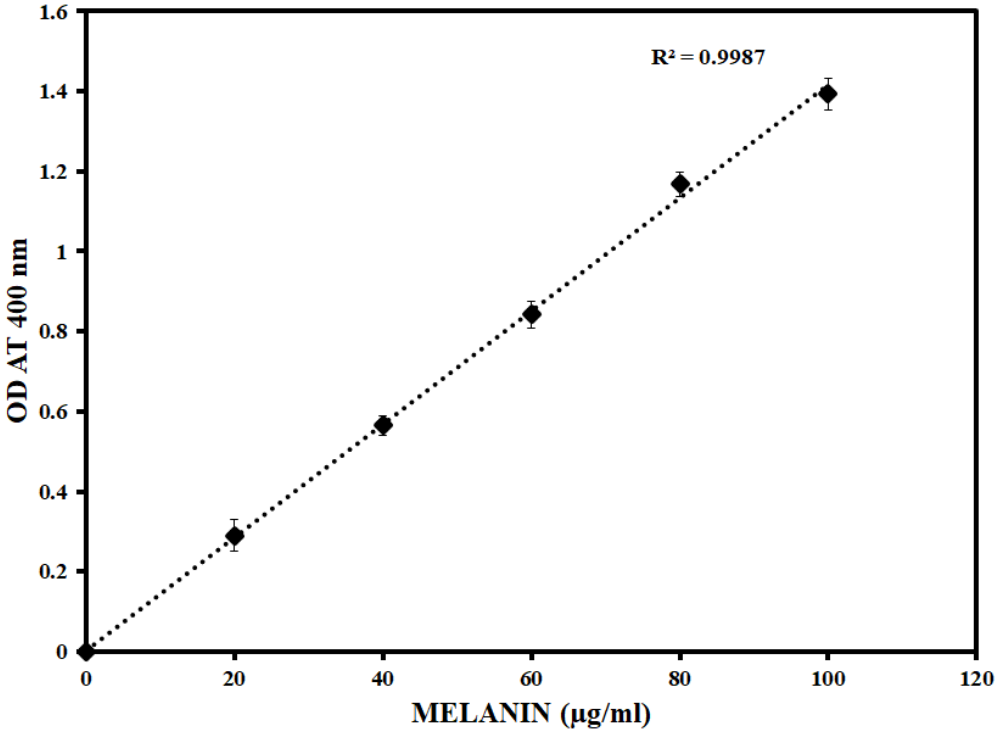

Figure 7-2: Standard graph for melanin estimation.

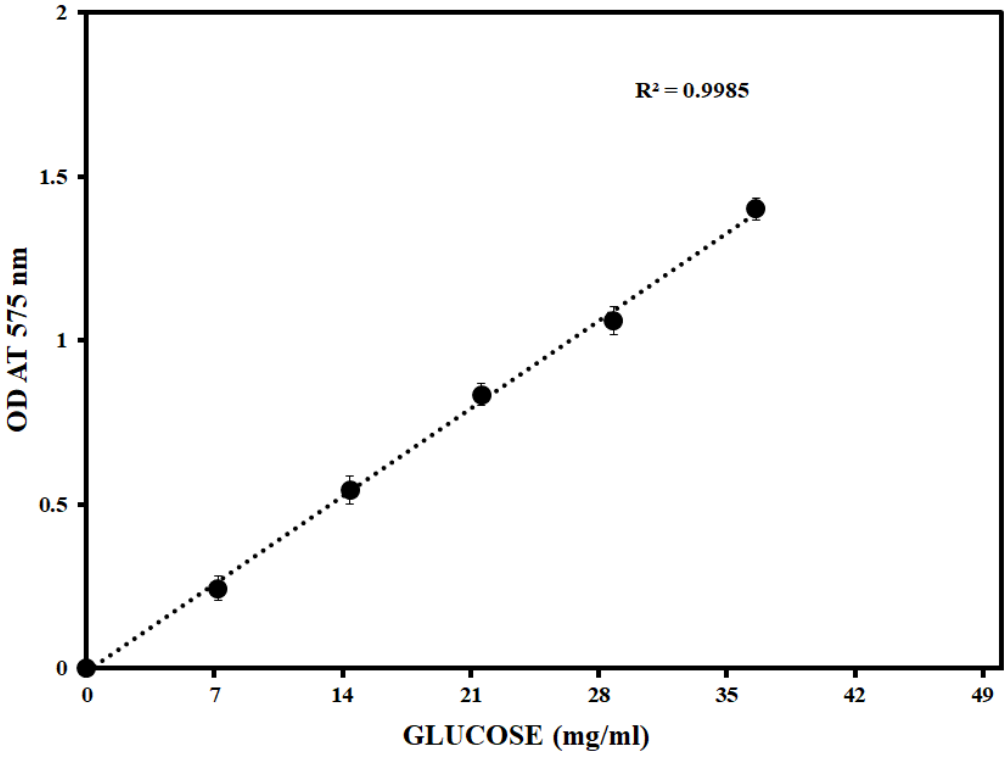

Figure 7-3: Standard graph for estimation of reducing sugars.

7.1 Calibration of pH and DO probes

The pH and DO probes (Mettler Toledo) were calibrated and kept activated for steady value for six hours before fermentation.

The following procedure was followed for pH probe calibration

- Press **"Cal"** button in the control and select pH calibration.
- Now keep the electrode in buffer 1 of pH 4.0 and press enter. Wait till the proper pH value is shown.
- Now keep the probe in buffer 2 of pH 7.0 and press enter.
- **"Calibration successful"** message will appear and save the calibration.

The following procedure was followed for DO probe calibration

- DO probe has a single point calibration at 0% saturation (zero point) or 100% saturation (slope).

- 100% saturation (slope) calibration can be done by dipping the DO probe in distilled water with continuous air supply for 6 hours.
- Press “Cal” button in control when DO probe shows stable value and then press enter two times. **“Calibration successful”** message will appear and save the calibration.

LIST OF PUBLICATIONS BASED ON THIS RESEARCH WORK

Research Journals

- Thaira, H., Manirethan, V., Raval, K., and Balakrishnan, R. M. (2018). "Melanin nano-pigments for heavy metal remediation from water." *Separation Science and Technology*. Taylor and Francis. DOI: 10.1080/01496395.2018.1443132
- Thaira, H., Raval, K., and Balakrishnan, R. M. (2016). "Optimizing the Biosynthesis of Melanin Nanoparticles Used for Heavy Metal Removal." *Research Journal of Chemical and Environmental Sciences*, 4(4S).

Manuscripts under processing:

- Adsorptive bioprocess for melanin nanopigment production from *Pseudomonas stutzeri.* (Communicated).
- Multiproduct bioprocessing using marine *Pseudomonas stutzeri*. (To be communicated).

Book chapter:

- Thaira, H., Bhosle, S. S., Balakrishnan, R., and Raval, K. (2016). "Selection of Medium and Optimization of Process Parameters for Melanin Biosynthesis from Pseudomonas stutzeri." *Biotechnol. Biochem. Eng.*, Springer Singapore, 1–10.

Conferences:

- Harsha Thaira, Keyur Raval, Raj Mohan Balakrishnan. (2016). "Optimizing the Biosynthesis of Melanin Nanoparticles Used for Heavy Metal Removal". National Conference on Recent Advances Chemical, Biochemical and Environmental Engineering (RACBEE).
- Harsha Thaira, Vishnu Manirethan, Keyur Raval and Raj Mohan Balakrishnan. (2017). "Optimizing the biosynthesis of melanin nanoparticles and their application in heavy metal contaminated groundwater remediation". Nanotechnology Applications: Chemical, Energy and Environment (NACEE).
- Harsha Thaira, Naveen Kumar, Keyur Raval and Raj Mohan Balakrishnan. (2017). "Studies On Feasibility Of Organic Solvents For Melanin Extraction".

International Conference On Crystal Ball Vision On Science & Engineering For Societal Upliftment, Goa, Indian JSPA Alumni Association (IJAA)

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EDUCATIONAL QUALIFICATION

COURSES	SCHOOL/COLLEGE	UNIVERSITY	YEAR	SCORE
Ph.D	National Institute of Technology Karnataka	National Institute of Technology Karnataka	Ongoing	
M.Sc (Biotechnology)	Mangalore University	Mangalore University	2013	70.92%
B.Sc (Biotechnology)	Alva's College, Moodbidri	Mangalore University	2010	79.16%
P.U.C	B.A.R.H.S.S Bovikan	Board of Higher Secondary Education Kerala	2007	75%
S.S.L.C	H.S.S Chattanchal	Kerala Secondary Education Board	2005	85%

PROJECTS UNDERTAKEN

1) Agrobacterium mediated plant transfection and PCR.

2) Study on point mutation of topoisomerase-I gene in *Nothaphodytes nimmoniana* producing Camptothecin, an anticancer alkaloid.

SKILLS

Upstream and Downstream Processing, Chromatographic analysis techniques, Microbial & cell culture techniques, Plant tissue culture techniques, Protein isolation techniques, Electrophoresis techniques, PCR, Primer designing, Phylogenetic analysis.

WORK EXPERIENCE

Worked as Jr.Executive in Research & Development at BIOCON from July 2010 to July 2011.

TRAINING, SEMINAR, SYMPOSIUM AND CONFERENCES ATTENDED

- Participated in TEQUIP sponsored one week hands on training and workshop on Analytical Techniques at NMAM Institute of Technology, Karnataka.
- Participated in DBT sponsored five day national workshop on Modeling, Simulation and Optimization of Bioprocesses at National Institute of Technology, Warangal.
- Participated in one day workshop on Multiphase Flow.
- Participated in four day workshop on Recent Challenges in Atmospheric and Earth Sciences at National Institute of Technology, Surathkal.
- Participated in TEQUIP sponsored three day workshop on Skill Development and Stress Management at National Institute of Technology, Surathkal.

PRESENTATIONS IN CONFERENCES

Title of the paper presented	Symposium/Conference	Place and Date
Selection of Medium and Optimization of Process Parameters for Melanin Biosynthesis from *Pseudomonas Stutzeri*.	International Conference on Advances in Chemical Engineering (ICACE-2015)	National Institute of Technology Karnataka, Surathkal Dec20-22, 2015
Optimizing the Biosynthesis of Melanin Nanoparticles Used for Heavy Metal Removal.	National Conference on Recent Advances in Chemical, Biochemical and Environmental Engineering (RACBEE-2016)	Shri Dharmasthala Manjunatheshwara College of Engineering and Technology. Dharwad, Karnataka October 1st, 2016
Optimizing the Biosynthesis of Melanin Nano Particles and their Application in Heavy Metal Contaminated Ground Water Remediation.	International Conference on Nanotechnology Applications: Chemical, Energy and Environment (NACEE-2017)	Sardar Vallabhbhai National Institute of Technology (SVNIT) Surat, India March 22-23, 2017
Studies on Feasibility of Organic Solvents for Melanin Extraction & Heavy metal remediation from groundwater using bacterial melanin.	International Conference on Crystal Ball Vision on Science and Engineering For Societal Upliftment (IJAA-2017)	National Institute of Oceanography, Goa August 7-8, 2017

JOURNAL PUBLICATIONS

Title of the paper	Journal	Authors
Melanin nano-pigments for heavy metal remediation from water.	Separation Science and Technology. Taylor and Francis. DOI: 10.1080/01496395.2018.1443132	Thaira, H., Raval, K., Manirethan, V., and Balakrishnan, R. M.
Selection of Medium and Optimization of Process Parameters for Melanin Biosynthesis from Pseudomonas Stutzeri. (Book chapter)	Springer volume titled "Biotechnology and Biochemical Engineering" ISBN 978-981-10-1920-3	Thaira, H, Bhosle, S, Balakrishnan, R. M. and Raval, K
Optimizing the Biosynthesis of Melanin Nanoparticles Used for Heavy Metal Removal.	Research Journal of Chemical and Environmental science (RJCES). ISSN 2321-1040	Thaira, H, Balakrishnan, R. M. and Raval, K
Kinetic and thermodynamic studies on the adsorption of heavy metals from aqueous solution by melanin nanopigment obtained from marine source: Pseudomonas stutzeri.	Journal of Environtal Management, 214, 315–324.	Manirethan, V., Raval, K., Rajan, R., Thaira, H., and Balakrishnan, R. M.
Effect of Electrodeposited Copper Thin Film on the Morphology and Cell Death of *E. Coli*; an Electron Microscopic Study	Springer volume titled "Biotechnology and Biochemical Engineering" ISBN 978-981-10-1920-3	Augustin, A., Thaira, H., Bhat, KU., and Udupa., KR.

DECLARATION;

I hereby declare that the details furnished above are perfectly true to the best of my knowledge.

Yours Sincerely

Date: 15/10/2018

Place: Mangalore

Harsha Thaira