Research article
A biotechnological approach to optimization and production of melanin by *Brevibacillus invocatus* strain IBA, under submerged fermentation

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(Received: November 2021 Revised: November 2021 Accepted: December 2021)

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ABSTRACT

Introduction and Aim: Melanin is a macromolecule with many biological activities, found across the animal, plant and microbes. This study focuses on parametric optimization of melanin production using a microbial.

Materials and Methods: The soil samples were diluted to tenfold and spread plate technique was employed. The isolated cultures were subjected for melanin production the potential strain was selected and employed for mass production using nitrate broth supplement with L-tyrosine. The culture identification is by biochemical, microscopic and molecular sequencing and data retrieved through NCBI. Different physical parameters are used for the optimum growth of isolated culture.

Results: The potential microbial culture was identified through 16s rRNA was *Brevibacillus invocatus* strain IBA with accession number 696201.1. This is an effective culture produced tyrosinase activity was 3282 U mg⁻¹ and 0.554 g l⁻¹ cell mass. The incubation duration highest melanin production was 48 hours, with 0.328 g l⁻¹. Melanin production was better with 0.352 g l⁻¹ at 120rpm agitation. In pH 6.5 remained establish to be optimum with 0.47 g l⁻¹. Optimum temperature at 35°C high yield with 0.487 g l⁻¹. Sucrose remained establish to be best carbon source with 0.512 g l⁻¹ of melanin; while Tryptone as nitrogen sources produced the maximum melanin 0.548 g l⁻¹. Thin layer chromatography was one of the useful methods to detect melanin from crude to pure melanin had an Rf value of 0.62.

Conclusion: Chemical synthesis of melanin is expensive and involves multiple steps, animal or plant sources are cumbersome process. There are limitation and challenges to use the animal origin melanin for therapeutics and for food process. This investigation provides knowledge on factors affecting the melanin production using a bacterial culture in submerged fermentation technique.

Keywords: *Brevibacillus invicatus*; tyrosinase activity; molecular sequencing; submerged fermentation; chromatographic technique.

INTRODUCTION

Melanins are macromolecules that are generated when phenolic or indolic chemicals are oxidatively polymerized. The pigments that result are frequently black or brown in colour, although they can also be found in a variety of other hues. Melanin’s are likewise negatively charged and hydrophobic. The enzyme tyrosinase (1, 3) initiates the conversion of melanin from L-tyrosine through a variety of enzymatic and non-enzymatic processes. The initial stage in the synthesis of melanin is the oxidation of L-tyrosine to L-3, 4-dihydroxyphenyl alanine (L-DOPA) by the tyrosinase (monophenolmonooxygenase EC 1.14.18.1) catalyser. The tyrosinase enzyme then converts L-DOPA to dopachrome. Dopachrome is converted to melanin through a series of non-enzymatic oxidoreduction reactions (1, 4). Tyrosinase is an enzyme involved in the synthesis of L-DOPA and melanin (1, 5).

Melanin is found in both the animal and plant domains, and is also produced by microbes (6-8). Innumerable bacteria and fungi have been reported earlier to produce melanin. *Escherichia coli* W3110 (9), *Bacillus cereus* (10), and *Klebsiella sp.* GSK, (11), *Cryptococcus neoforms*, *Aspergillus fumigatus*, and *Pneumocystis carinii* are among the major bacterial and fungal species that produce melanin (6, 7). Microorganism-produced melanin appears to be critical for organisms’ defence against environmental stress. In vitro studies have discovered...
that melanized fungi exist additional resistant to UV light-induced and oxidant-mediated damage, temperature extremes, hydrolytic enzymes, heavy metal toxicity, and antimicrobial drugs than non-melanized fungi (12). Manufacturing of Melanin by Bacterial species in fermentation technology is one of the newest fields of research owing to the situation low cost and simple purification procedures. Bacterial melanin pigments are biodegradable, less poisonous, and more stable than synthetic and plant-based pigments. Microbial melanin pigments are used in a variety of industries as food colorants, dyes, and medicinal agents. Because of its ability to generate colours, Actinobacteria is regarded one of the most important bacterial species (13, 14).

In this study, *Brevibacillus invocatus* strain IBA (26) is used to produce extracellular melanin using submerged fermentation technique. The primary goal of this research is to optimise different parameters such as Temperature, pH, agitation rate, incubation length, evaluation of carbon and organic nitrogen sources for successful melanin production. Thin layer chromatography was used to figure out the purity of melanin. This is novel approach on the use of the *B. invocatus* strain IBA (26) for melanin synthesis and optimization investigations. This Information is obtained through research on optimization of melanin production by submerged fermentation technology and how it may be used to produce melanin using a bacterial strain *B. invocatus* (26). These research articles’ findings can be related with previous literature, allowing for futuristic conclusions to be reached.

**MATERIALS AND METHODS**

**Chemicals**

All the chemicals and media used were from Sigma Aldrich (USA), Himedia (India) and SRL (India), and were of the highest purity and analytical grade. The chemicals used are given in the respective sections below where it was used.

**Media Used**

*Nutrient agar (Isolation Medium)*

Peptone 0.5 %, Beef extract 0.3 %, Yeast extract 0.3 %, NaCl 0.5 %, Agar 2 % and pH 6.5 (26).

*Screening media (Nutrient Agar with L-Tyrosine)*

Peptone 0.5 %, Beef extract 0.3 %, Yeast extract 0.3 %, NaCl 0.5 %, CuSO₄ 0.004%, L-tyrosine 0.5% Agar 2 % and pH 6.5.

*Production medium (Nutrient Broth with L-Tyrosine)*

Peptone 0.5 %, Beef extract 0.3 %, Yeast extract 0.3 %, NaCl 0.5 %, CuSO₄ 0.004 %, and L-tyrosine 0.5 %, and pH 6.5.

**Isolation, screening and molecular identification of tyrosinase-producing bacteria**

Soil samples rich in phenolic compounds, from paddy fields enriched with water from Amblikoppa (15.3577230N 74.9437630E) in the Dharwad region of India, was collected. The soil sample was serially diluted (10 fold) in 0.85 % saline up to 10⁸ dilutions. The serially diluted samples from 10⁻¹ to10⁸ were spread on isolation media for bacteria (Nutrient agar). Bacterial colonies obtained from the plates were subculture. Each colony was screened for melanin pigment production on Nutrient agar with L-tyrosine (Screening media). All of the bacterial colonies that had dark brown pigments around them were chosen for further testing. The bacteria that showed high production of melanin and tyrosinase were chosen, and subjected to molecular identification by PCR and sequencing (26).

**Optimization studies**

**Bacteria**

*B. invocatus* strain IBA (26) was isolated from paddy field soil and was chosen for further optimization investigation, since it produced the highest melanin and had the greatest activity among the other isolates during screening.

**Submerged fermentation**

*B. invocatus* strain IBA (26) was used for various optimization parameters. The inoculum generation and incubation periods ranging from 0 to 48 hours was identified initially. The Physical characteristics, such as agitation, temperature, pH, incubation length, and nutritional variables such as carbon sources, and nitrogen sources were studied using submerged fermentation. The factorial design of experiments, known as the "one factor at a time method," was used in this work to improve the reproducibility of the experimental data and to optimise the biosynthetic process as a whole. The experimental factors are changed one at a time, while the other parameters stay constant. Unless otherwise noted, all tests were done in triplicate with a control group. Production of melanin was confirmed by Thin Layer Chromatography (TLC) technique and tyrosinase production was quantified by tyrosinase activity test by dopachrome method (26).

**Optimization of incubation period**

Using production medium, the influence of incubation period on melanin production was examined. Ten different flasks with production medium were inoculated and incubated in the shaker incubator at 37°C and 120 rpm. Estimating melanin was used to look into the effect of incubation time. At every 6 hrs interval, one flask is removed and checked for melanin production (26).
Optimization of agitation rate

Different agitation rates were tested for better growth and optimal melanin synthesis. Individual Erlenmeyer flasks were incubated for up to 48 hours in static and at 40, 80, 120, 160, and 200 rpm. The temperature of the incubation chamber was kept at 37°C (26).

Optimization of pH

pH of media ranged from 4-9 was tested to identify optimal pH for production of melanin. Setting the initial pH of the medium to 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, and 9 using 0.2N HCl and 0.2N NaOH was done. The flasks were incubated for up to 48 hours at 37°C with 120 rpm (26).

Optimization of temperature

Seven flasks with production media were inoculated with the culture. Each flask was incubated at 20, 25, 30, 35, 40, 45 and 50°C in shaker incubator to identify the optimum temperature for melanin formation. Each flask was incubated at 37°C and 120 rpm for up to 48 hours (26).

Evaluation of carbon sources

The influence of several carbon sources were investigated by adding 1 g l⁻¹ of each carbon source in the medium. Glucose, sucrose, lactose, fructose, maltose, cellulose, starch, and glycerol were tested as carbon sources. All the flasks were incubated at 37°C and 120 rpm for up to 48 hours (26).

Evaluation of organic nitrogen sources

To test the different nitrogen sources for optimum melanin synthesis, a medium containing sucrose, was supplied with each organic nitrogen source at a concentration of 1 g l⁻¹. Gelatine, casein, soya peptone, tryptone, meat extract, beef extract, yeast extract, and peptone were among the organic nitrogen sources studied. All the flasks were incubated at 37°C and 120 rpm for up to 48 hours (26).

Melanin production

The isolated bacterium was cultured in nutrient broth supplemented with L-Tyrosine (Hi Media, India). In 250-ml Erlenmeyer flasks, two mL (2 %) of a 6-hour culture was inoculated in 100 ml of the production medium for melanin synthesis. The flasks were incubated for 48 hours in a shaker incubator at 37°C and 120 rpm. Melanin synthesis in the broth was measured spectrophotometrically at 475 nm using UV spectrophotometer. Synthetic melanin (Sigma-Aldrich, St. Louis, USA) was used as a standard to generate standard curve (15, 26)

Tyrosinase assay

The dopachrome technique was used to measure tyrosinase activity. The absorbance at 475 nm was monitored continuously for 5 minutes in the UV-VIS spectrophotometer at 30°C, and the standard reaction mixture contained 0.5 ml 8 mM L-dopa, 1.5 ml 0.1 M sodium phosphate buffer (pH 6.8), 0.5 ml 0.5 mM SDS, and enzyme extract (cell free supernatant was obtained by centrifuging at 1000rpm for 10 min.) in a total volume of 3 ml. The quantity of enzyme necessary to oxidise 1 mol of L-dopa per minute under the given conditions is defined as one unit of tyrosinase activity (16, 17, 26), which was estimated using the molar extinction coefficient of dopachrome (3600 M⁻¹ cm⁻¹). Lowry et al. (16, 18) used bovine serum albumin as a reference to evaluate protein content.

Detection of melanin by thin layer chromatography

TLC tests were performed on several samples. L-Tyrosine, L-Dopa, crude (nutrient broth and bacterial culture), crude melanin, purified melanin and melanin. The samples were run on a TLC using a silica gel G (1 mm thick) on a glass plate and a solvent solution of n-butanol: acetic acid: water (70:20:10, v/v). Spraying with ninhydrin reagent detected bands, and Rf values of resolved bands were computed (16, 19, 26).

RESULTS

Isolation, screening and molecular identification of bacteria

The paddy field in Amblikoppa village, Dharwad, India is rich in phenolic components. Hence, the soil sample was collected from this region and subjected to serial dilution and plating on basal media for bacteria (Nutrient agar). Several colonies were appeared on basal media. These colonies were subculture in screening media to identify melanin synthesis. Total of 9 bacterial species were obtained with dark brown to black pigmentation around colonies. Out of 9 bacteria, 1 pure culture which shown higher production of melanin and better tyrosinase activity was subjected to molecular identification by PCR followed by sequencing and identified as B. invocatus (26) and strain renamed as B. invocatus IBA (26). The partial gene sequence of 16S rRNA (678 bp) was submitted to the NCBI gene bank, which is already been published. The accession number for the sequence is 696201.

Optimization studies

Submerged fermentation for the production of melanin was used. For optimization studies, B. invocatus strain IBA (26) was used. Inoculum preparation, Incubation time, Physical parameter such as Agitation rate, pH, temperature, and nutritional parameters such as carbon and organic nitrogen sources are all variables that can be optimised were checked.
Inoculum development and tyrosinase activity

After 6 hours, the crude tyrosinase activity in broth was 1498 U mg\(^{-1}\), with a dry cell weight of 0.192 g l\(^{-1}\). At the 18th hour of incubation, the tyrosinase activity was steadily increased to 2192 U mg\(^{-1}\) with a dry cell weight of 0.456 g l\(^{-1}\). The tyrosinase activity was reduced to 1992 U mg\(^{-1}\) in the 24th hour, while the dry cell weight was increased to 0.459 g l\(^{-1}\). Tyrosinase activity increased steadily after 24 hours of incubation, reaching a maximum of 3280 U mg\(^{-1}\) and a dry cell weight of 0.55 g l\(^{-1}\). After 30 hours, the tyrosinase activity was reduced to 1992 U mg\(^{-1}\) in the 24th hour, while the dry cell weight was increased to 0.56 g l\(^{-1}\). This indicates that the increase in cell mass directly proportional to the tyrosinase activity until 18 hours (Fig 1a).

Fig. 1 (a) Cell mass and crude enzyme activity

The log phase of growth was reached after 6 hours of growth. For melanin synthesis, 1ml of \(B.\ invocatus\) strain IBA (26) cell suspension with an absorbance of 0.32 at 660nm after 6 hours of incubation was inoculated. The activity of tyrosinase increased up to 18 hours and then dropped after 24 hours. This is due to the conversion of L-tyrosine to L-DOPA, which uses tyrosinase produced during the first 18 hours, lowering tyrosinase levels. Whereas an increase in tyrosinase activity was found at 30 hours, this could be due to the conversion of L-DOPA to dopaquinone (7), and tyrosinase synthesis could require a certain concentration of L-DOPA for this process. The nutrient medium inoculated with bacteria was studied during a 6-hour interval period, revealing that no melanin formation occurred until the 12th hour. After 18 hours, a yield of 0.056 g l\(^{-1}\) was observed. The melanin yield gradually increased up to 0.328 g l\(^{-1}\) at 48th hour, whereas a longer incubation period resulted in a modest increase of 0.348 g l\(^{-1}\) in the 54th hour (Fig 1b). This indicates that, the melanin production is higher at 48 hours and further incubation resulted in negligible level of increase in melanin.

Optimization of agitation rate

The highest melanin production (0.352 g l\(^{-1}\)) was attained at 120 rpm, while lower 40 rpm and higher 200 rpm agitation rates resulted in (0.152 g l\(^{-1}\)) and (0.322 g l\(^{-1}\)) melanin production, respectively. Low or no melanin production was detected in static incubation (0 rpm) (Fig. 2). This is due to slow growth due to the oxygen saturation in the medium as well as tyrosinase catalyses the oxidation reactions, which requires a specified concentration of oxygen, which could be maintained with an agitation rate of 120rpm.

Fig. 2 Optimization of agitation rate

Optimization of pH

The optimum pH was 6.5, with maximum 0.47 g l\(^{-1}\) melanin production (Fig.3), 0.02 g l\(^{-1}\) at pH 9, and no or less melanin production at extreme acidic at pH 4. The higher yield was obtained at slightly acidic pH. In extremes of pH, the growth of the bacteria was hindered due to acidity or alkalinity, resulting in reduced melanin production.

Fig. 3 Optimization of pH
Optimization of temperature

The *B. invocatus* strain IBA grew at temperatures ranging from 20 to 50°C, producing a maximum of 0.48 g l⁻¹ of melanin at 35°C (Fig 4). At lower temperature (20°C) and higher temperature (50°C), less melanin was produced, with a production of 0.23 g l⁻¹ and 0.045 g l⁻¹, respectively. Because bacterial growth and tyrosinase activity were inhibited at lower and higher temperatures, the melanin output was reduced.

Optimization of carbon sources

Sucrose was found to be an excellent carbon source for melanin formation in this study (Fig. 5), producing 0.512 g l⁻¹ of melanin. Fructose and glucose limits the melanin formation, resulting in lower melanin production of 0.122 g l⁻¹ and 0.212 g l⁻¹, respectively.

Effect of organic nitrogen sources

After testing several organic nitrogen sources (Fig. 6), the medium supplemented with tryptone produced the highest output of melanin (0.548 g l⁻¹). Other nitrogen sources in the medium produced less melanin than tryptone. Beef extract and peptone have been the second and third top in the list yielding 0.501 g l⁻¹ and 0.438 g l⁻¹, respectively. The addition of gelatine in the medium resulted in a substantially lower level of melanin (0.062g l⁻¹).

Thin layer chromatography

Samples were subjected to thin layer chromatography (Fig 7) for detection of melanin. L-tyrosine and L-DOPA were used as a reference standard. Direct broth with culture, (Nutrient broth with bacterial cultures), Cell free supernatant (Crude melanin), Purified melanin, and melanin (reference standard were run on TLC. The Rf value for L-tyrosine and L-DOPA were determined to be 0.47 and 0.50 respectively. The crude melanin had an Rf of 0.58, purified melanin had an Rf of 0.62, and Pure melanin had an Rf of 0.67. Due to presence of other impurities, the crude samples had lower Rf values.

DISCUSSION

Based on enzyme catalysis, researchers investigated the factors that determine the pace of melanin formation. The study's major goal is to produce melanin using enzymes; in this case, submerged fermentation was used since it can eliminate errors throughout the production process. In optimization studies, several parameters like incubation period, agitation rate, pH, temperature, carbon sources, and nitrogen sources are investigated. The enzyme activity (Fig. 1a) was evaluated during the incubation duration (Fig. 1b), and the incubation was studied at 6-hour intervals, demonstrating that no melanin was produced for up to 12 hours. After 18 hours, the yield was 0.056 g l⁻¹, and after 48 hours, it had grown to 0.328 g l⁻¹. At 54 hours, a longer incubation period (Fig. 1b) produced a moderate rise of 0.348 g l⁻¹,
whereas a shorter incubation period produced a minor increase of 0.348 g l⁻¹. This means that at 48 hours, melanin synthesis was higher and that incubation time had no influence on melanin production. Similarly, the agitation rate (Fig. 2) on a shaker incubator was tuned, and the highest melanin synthesis was recorded at 120rpm, around 0.352g l⁻¹. From 0 to 200rpm, the agitation rate was monitored in increments of 40rpm. A pH of 6.5 was determined to be optimal for pH optimization (Fig. 3), with a maximum yield of 0.47g l⁻¹. In both acidic and alkaline pH, melanin production was found to be decreased or non-existent. Acidity or alkalinity inhibited bacterial organisms, resulting in a decrease in melanin synthesis. *Brevundimonas* sp. at pH 7.5 SGJ (6) and recombinant *E. coli* melanin synthesis (10) yielded results that are comparable to and compatible with those of the current investigation. Similar results were obtained with *Bacillus cereus* and *Shewanella* algae (11, 20), as well as *B. thuringiensis* (21, 22).

Temperature optimization is utilized (Fig. 4), with temperatures ranging from 20 to 50°C yielding a maximum of 0.48 g l⁻¹ of melanin at 35°C. At lower temperatures (20°C) and higher temperatures (50°C), less melanin was produced. In previous investigations, the temperature employed to produce melanin in *Bacillus thuringiensis*, *Galleria* strain K1, and *B. thuringiensis* sub sp. *Galleria* strain H-14 was 30°C (15, 21). For *Burkholderia cenocepacia* and *Klebsiella* sp., GSJ used 37°C (11). The outcomes of this study were found to be consistent with prior studies indicating melanin development was best around 35°C. In the current study, sucrose was revealed to be an excellent carbon source for melanin production (Fig. 5), giving 0.512g l⁻¹. Fructose and glucose inhibit the synthesis of melanin. Several investigations (9-11, 23) have suggested that in *Bacillus cereus* 58, *Klebsiella* sp. GSK, *E. coli* W3110, and *Aeromonas*, glucose are employed in the manufacture of melanin. Various nitrogen sources were added to the medium to see what effect they had. Tryptone, which produced the most melanin (0.548 g l⁻¹), produced the most. Other nitrogen sources (Fig. 6) resulted in lower or moderate levels of melanin production. In the past, many nitrogen sources have been used, with casein being the best for *Bacillus thuringiensis* H-14 (15). Tryptone was discovered to be the best source of melanin by *Aeromonas* (23). Previously, *B. thuringiensis* sub sp., *Galleria* strain K1 was used in the medium to produce melanin (22). *B. thuringiensis* sub sp., (21), *Galleria* strain K1 was previously used in the media for melanin formation using wheat flour and yeast. Bactotyptone and casein from *Bacillus cereus* 58 (10). Thin-layer chromatography (Fig. 7) revealed the separation of melanin, with an Rf value of 0.62. Similar results were obtained from the isolation, purification, and characterization of physiologically active melanin from marine Streptomyces p. (24, 25). They also discovered an Rf value of 0.74 for the purple patches, which they related to the quick creation of silver nanostructures using a melanin template.

**CONCLUSION**

Chemical synthesis of melanin is expensive and involves multiple steps. Extraction of melanin from animal or plant sources is cumbersome process. There are limitation and challenges to use the animal origin melanin for therapeutics and for food process. The product must be free from animal and / or plant-based contaminations. This could be cost effective production to the purified form of bacteria, fungi and actinomycetes isolated from extreme conditions can produce melanin as a defense mechanism. Exploiting the bacterial or fungal species using biotechnological approach will lower the production cost. In this study, we established optimum fermentation condition for the submerged fermentation in lab scale using a wild strain *B. invocatus* IBA (26) isolated from Dharwad region. The optimized condition provides enhanced yield. This strain could be one of the best sources for melanin. Tweaking the fermentation process and scaling up could yield best quality melanin. To our knowledge, this is the first study to utilize *B. invocatus* (26) for production of melanin using submerged fermentation. This study lays the foundation for the exploitation of bacterial strain *B. invocatus* (26) for production of melanin for utilizing as a therapeutic purpose and as a part of edible substance. However, development of accurate analytical tools to characterize the structure, quality, impurity, and functional relationship is required to understand the melanin production by *B. invocatus* (26).

**ACKNOWLEDGEMENT**

We are thankful to Karnatak University Dharwad, for providing University Researcher Fellowship. We are grateful to P.G. Department of Biotechnology and Microbiology for providing necessary laboratory facilities to carry out the research work. We extend our deepest gratitude to Plant health care and Diagnostics and Dr. Sudhish Jogya for providing facilities.

**CONFLICT OF INTEREST**

Authors declare that there is no conflict of interest.

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