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Advances in Bioresearch

Optimization of Physical and Nutrition Parameters for Melanin Pigment Production by *Bacillus subtilis* MFKA 7

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ABSTRACT
The present study aims to enhanced the production of melanin by Bacillus subtilis MFKA 7 isolated from soil. Melanin pigment production was carried out in Nutrient broth with pH 5, at a temperature of 40^{o}C, salt concentration of 5%, starch and peptone as carbon and nitrogen sources. For effective pigment production other parameters such as agitation, inoculum size, incubation time were also considered and it was found that 120 hrs. of incubation time along with 120 rpm and 10% inoculum size was effective. Various metal ions which can act as a cofactor in the synthesis of melanin was also studied. $CuSO_4$ at a concentration of 1% was found to be effective. Bacillus subtilis MFKA 7 when grown alone in nutrient broth without any extra supplements of tyrosine and $CuSO_4$, was able to produced only 1.3gm/L of melanin under static condition in 7 days. With all the optimized parameters the bacterial strain was able to produced 3.0gm/L of melanin along with 1% of tyrosine as substrate for melanin production supplemented in the broth medium. The solubility of the obtained melanin was also tested with commercially available melanin i.e., Sepia melanin. Hence, Bacillus subtilis MFKA 7 can be used as potential strain for melanin synthesis.
Keywords: Melanin, Tyrosine, optimization, solubility, *Bacillus subtills* MFKA 7

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INTRODUCTION

Most living organisms contain melanin, which are negatively charged and hydrophobic pigments. It is widely distributed in various taxa's such as bacteria, fungi, streptomyces, plants, insects and even in human being. Melanin is found in most parts of biosphere, ranging from the black pigments found in human skin, hair and eyes to the black epidermis of insects and the browning of fruits caused by oxidation [1]. Melanins are polymers which are synthesized by the oxidative polymerization of chemical such as phenolic or indolic compounds. The key function of melanin synthesis is to protect host cells from harmful ultraviolet (UV) radiations. The functions comprise of defense against physical alterations from the outside environment, defense against UV radiation and energy absorption, and also involving physiological activity-based preservation of intracellular homeostasis [2]. Apart from providing protection to host cell melanins also have various biological properties such as thermoregulation, radical scavenging activities etc. [3]. Melanin nanoparticles have been produced by multiple groups of researchers for a range of applications such as energy storage devices, functional films, protective coating and environmental sensors inspired by the optoelectronics, self-assembling and physio-chemical qualities of natural melanin [3]. Commercially melanins are produced by two methods – chemical synthesis and by extraction of melanin from *sepia officinalis* (common cuttlefish0 [4]. Both the stated methods, present difficulties in producing suitable yield of melanin [5]. Microorganism exhibit a great ability to generate adequate amount of melanin under optimized conditions since they are easily cultured and economically sustainable.

High productivity is needed to produce biomaterials like melanin which are widely used on a large scale. However, the quantity of substrate such as Dopa or L- tyrosine are required to fed into growth cultures and directed towards processing by tyrosinase determines the melanin production yields from different bacteria. Therefore, to improve the efficiency of melanin production, growth optimisation or natural selection of active enzymes have been employed [6]. High synthesis of melanin can be achieved if the organism is subjected into stress environment or even when essential parameters are optimized.

Therefore, present study was aimed to increase the production of melanin from microorganism by employing optimization of one factor at a time. *Bacillus subtilis* MFKA 7 isolated from soil showed melanin production of 1.3gm/L without additional supplement of tyrosine and other nutrients and under static conditions in 7 days of incubation. However, after optimization of parameters like production media, temperature, pH, inoculum size, incubation time, salt concentration, agitation and nitrogen and carbon sources the amount of melanin produced was increased upto 3.0gm/L along with supplemented 1% of tyrosine in 5 days of incubation. Addition of 1% $CuSO_4$ as co-factor can also affect the synthesis of melanin production in bacteria as it activates the enzymes required for melanin synthesis i.e., tyrosinase and laccase enzyme. Melanin pigment was confirmed with an assay and the solubility of the extracted melanin was compared with commercially purchased sepia melanin.

MATERIAL AND METHODS

Various physical, chemical and bioprocess factors which affects melanin production by selected strain of *Bacillus subtilis* MFKA 7 under submerged fermentation condition were optimized to get good yield of melanin. The "one-variable-at-a-time" method was used to examine each parameter's impact on melanin production during submerged fermentation as part of the optimization strategy. Optimized parameters include production media, pH, incubation, agitation, inoculum size, temperature, nitrogen sources, carbon sources, salt concentration and metal ions [7]. All the optimization studies were carried out with 1% tyrosine in the production medium.

Effect of media on melanin synthesis by *Bacillus subtilis* MFKA 7

To maximum melanin production, the impact of different culture media on *Bacillus subtilis* MFKA 7 was studied. In these studies, 100mL of liquid media i.e., nutrient broth, Starch Casein broth, Peptone broth, Tryptone broth and Luria broth were inoculated in 250mL of Erlenmeyer's flask with *Bacillus subtilis* MFKA 7. By incubating all the conical flasks containing liquid media for 5 days at 37°C, melanin was extracted using acid extraction method and the amount of melanin produced was noted and the same liquid media was used for further studies.

Effect of pH

To study the effect of different initial pH, the 96 hours old culture of *Bacillus subtilis* MFKA 7 was incubated at different pH i.e., 3, 5, 7, 9, 12 in melanin production media at 37° C for 5 days with 100rpm. The pH of the production medium was adjusted using 1N HCL or 1N NaOH. After incubation of 5 days, melanin was extracted using acid extraction assay and the quantity of melanin was weighed.

Effect of incubation

To know the optimum incubation time for melanin production, *Bacillus subtilis* MFKA 7 was incubated at different incubation hours (24 to 168 hrs.) in melanin production media at 37°C with 100 rpm. After the respective incubation time the melanin was extracted and weighed to know the optimum incubation (hrs.).

Effect of agitation

The agitation effect on melanin synthesis was studied by incubating the 100mL of inoculated media in a 250mL conical flask under rotary condition on the rotary shaker (Orbitek, Scigenics, India) at different rotation per minutes, such as 40, 60, 80, 100, 120, 150 and 180 rpm. After being incubated for 5 days at 37°C, the samples were weighed to determine the amount of melanin produced.

Effect of inoculum

Different initial inoculum concentrations (0.5%, 5%, 10%, 15%, and 20%) were used to investigate the optimum inoculum concentration that supports maximum melanin production. The samples were analyzed after 5 days of incubation for melanin production. The studies were conducted at 37°C, pH 5 and agitation of 120 rpm.

Effect of temperature

Different incubation temperature was optimized to maximize the yield of melanin production. Temperatures such as 20°C, 25°C, 30°C, 35°C, 40°C, and 45°C was used to study temperature effect on melanin synthesis. The pigment was harvested after 5 days of incubation.

Effect of nitrogen source

Various nitrogen sources at concentration of 1% in production media was used to investigate the impact of nitrogen source on melanin production. Different nitrogen sources that were used are yeast extract, peptone, urea, beef extract, NH_4Cl, $(NH_4)_2SO_4$, and tryptone. The samples were collected after 5 days of incubation period, and melanin production was examined.

Effect of carbon source

By adding fructose, maltose, lactose, sucrose, starch and glucose separately at a concentration of 1% in the production media, the effect of carbon sources on melanin synthesis was examined. The studies were carried out at pH 5, 120 rpm (Orbitek Scigenics India), 40^0C and 5 days of incubation.

Effect of salt concentration

The ideal concentration of sodium chloride to produce the maximum melanin was optimized. Different sodium chloride concentrations i.e., 0.5%. 1%, 2%, 3%, 4%, 5%, 6%, 8% and 10% were tested for the production of melanin pigment by *Bacillus subtilis* MFKA 7.

Effect of metal ions

The influence of various metals ions on melanin production was studied. Metal ions used were $FeSO_4$, $MgSO_4$, $ZnSO_4$, $CuSO_4$, KH_2PO_4, $FeCl_3$, and $NiCL_3$ (1%).

(2.11) Confirmatory test for melanin

The isolate was cultured for 48 hours at 37^oC after being inoculated into test tube with 10 mL of 0.1% tyrosine as substrate solution along with few drops of chloroform. The bright red color formation indicates the presences of melanin pigment [8].

(2.12) Characterization of solubility of melanin produced from *Bacillus subtilis* MFKA 7

The characterization of melanin produced by *Bacillus subtilis* MFKA 7 was estimated using dis. water, DMSO, 1N KOH, 1N NaOH, 1M NaCl, ethanol, methanol, chloroform, L- butanol, benzene, acetone, ethyl acetate, acetonitrile, 2- Propanol and Petroleum ether. The effect of oxidizing agents such as 1% $FeCl_3$ and 3M/L HCL [9] was also studied. All estimations were compared with standard synthetic melanin Sepia melanin purchased from Sigma Company.

(3) Result and Discussion

(3.1) Effect of media on melanin production

Bacillus subtilis MFKA 7 was cultured in various media like Nutrient broth, Starch casein broth, Peptone broth, Tryptone broth and Luria broth. In nutrient broth with 1% tyrosine, the production of melanin was achieved upto i.e., 2.8gm/L when compared to the other production media. Starch casein broth also supports the melanin production as it was observed that *Bacillus subtilis* MFKA 7 was able to produce 2.2 gm/L of melanin (Figure 1). The least supportive production media was Luria broth where melanin production was less than 1gm/L.

In comparison to other evaluated growth media, nutrient broth contains a good amount of carbon, nitrogen sources, salt along with tyrosine. This could be the cause of melanin production in nutrient broth. Cell development and melanin biosynthesis can also be aided by nutrient broth composition. Chávez-Béjar *et al*., (2013) [10] studied and reported highest melanin pigment synthesis was found in Luria broth of 66mg/L. In the absence of any carbon or nitrogen source in Bushnell broth, *P. stutzeri* produced melanin of 150mg/L.

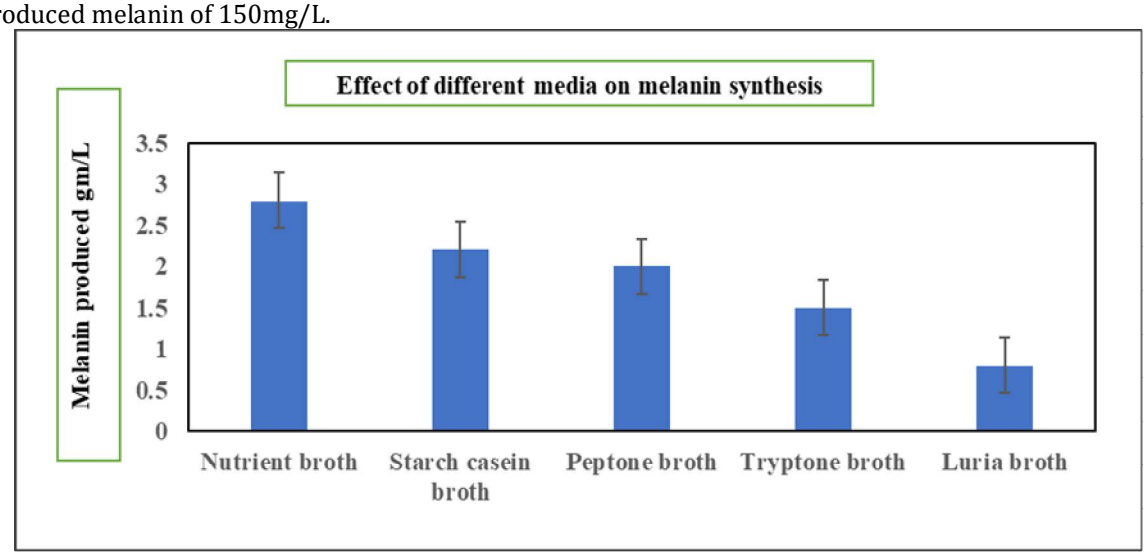

Figure 1: Effect of different media on melanin synthesis by *Bacillus Subtilis* MFKA 7

(3.2) Effect of pH on melanin synthesis

The synthesis of melanin by bacteria is extremely sensitive to the pH of the surrounding environment, and the changes in pH have major impact on the amount and quality of melanin produced by the cell. In bacteria, or other micro-organism melanin synthesis frequently peaks at an ideal pH range, variations from this range either stops the melanin synthesis process or can change the properties of the pigment greatly. The enzymatic activity of important proteins involved in melanin synthesis is influenced by pH, which may alter the yield and molecular structure of the pigment. *Bacillus subtilis* MFKA 7 was cultured at various initial pH ranging from 3 to 12. It was found that maximum melanin production was achieved at pH 5 which yielded 1.8gm/L of melanin. At pH 3 melanin synthesis was found to be low and also beyond pH 7 (Figure 2). In marine *Streptomyces*, melanin production was seen at a pH 7.0 [11].

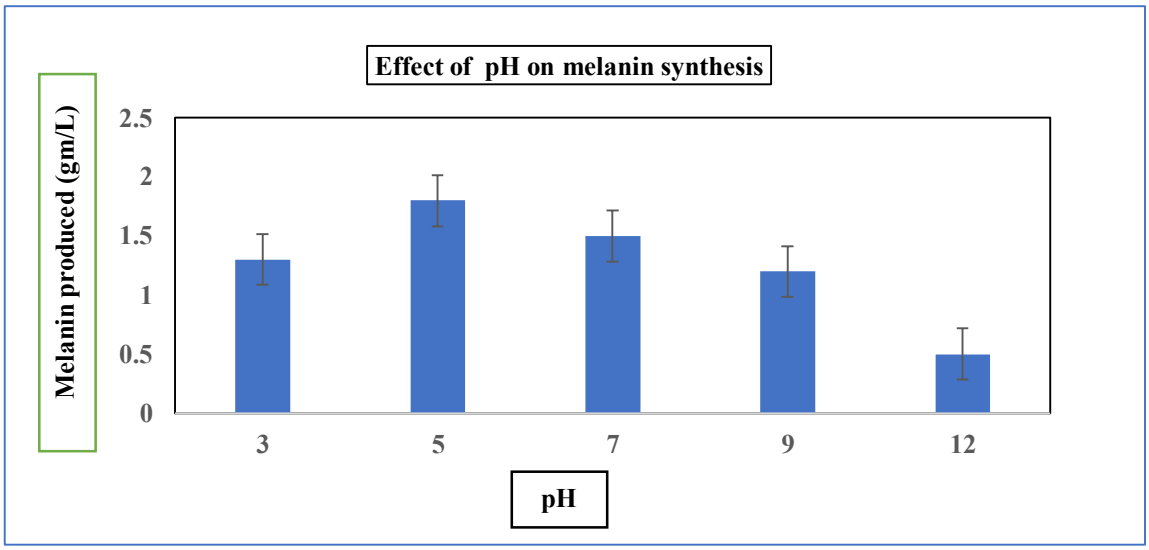

Figure 2: Effect of pH on melanin synthesis by *Bacillus Subtilis* MFKA 7

(3.3) Effect of Incubation

During incubation period, the color of the production medium turned from colourless to light black and then dark black as the day progresses. On 5th day i.e., 120 hours a noticeable change was observed in the color of the medium (Figure 3). Melanin production from *Bacillus subtilis* MFKA 7 was started from the 24 hours and increased by 120 hours. The melanin production on 96 and 120 hours was observed to be same after which it decreased. The melanin production on 120 hours i.e., 5 day was found to be 1.5 gms/L. *B. safensis* isolated from garden soil, was shown to produce 6.96g/L of melanin after 10 hrs. of incubation [12].

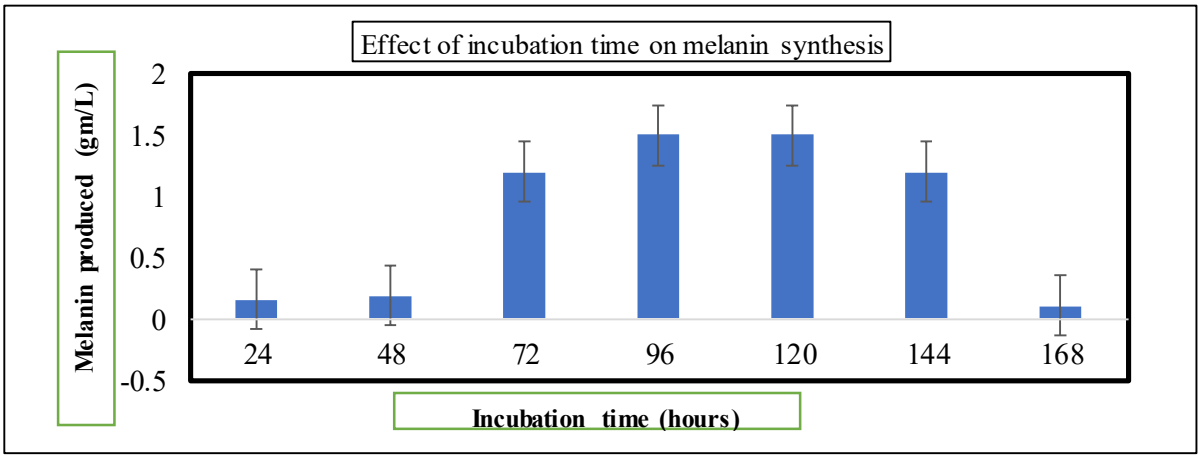

Figure 3: Effect of incubation time on melanin synthesis by *Bacillus Subtilis* MFKA 7

Effect of Agitation

Any microorganism's growth is determined by the oxygen transfer rate. The frequency of shaking has an impact on the rate at which oxygen is transferred from the air into liquid medium. The highest melanin synthesis was studied and found to be 1.8gm/L of melanin, was observed at 120 rpm (Figure 4). Melanin production reduced at lower or higher agitation speed (40 and 180 rpm). *Pseudomonas otitidis* DDB2 with agitation speed of 120rpm for duration of 24 hours was able to produced extracellular melanin [13] in optimized broth.

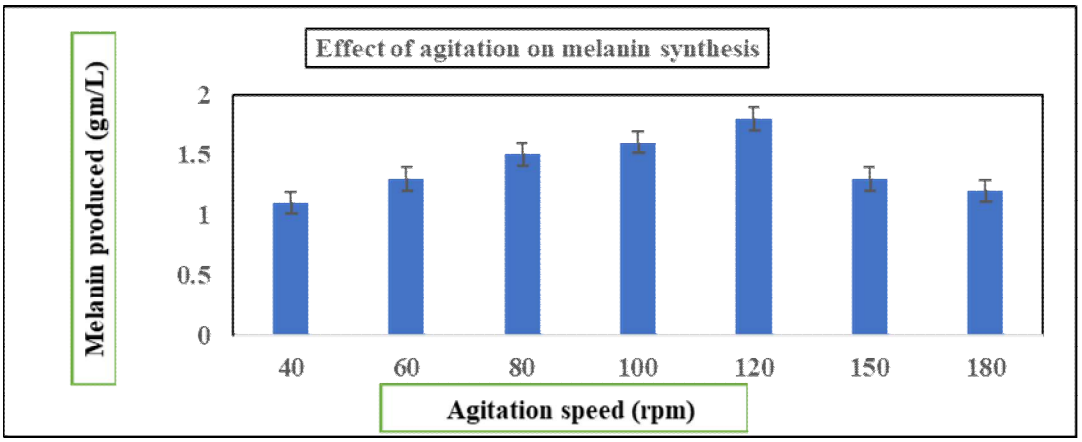

Figure 4: Effect of Agitation on melanin synthesis by *Bacillus subtilis* MFKA 7

Effect of Inoculum size

Inoculum size of microorganism play a critical role in fermentation studies of various products. Smaller inoculum size prolongs the non-synthetic lag phase of fermentation process. Larger the inoculum size rapidly depletes the nutrients and lower the synthetic fermentation process (Mahajan *et al.*, 2024). Mahajan *et al.,* (2024) found that inoculum percentage also influences various growth and synthetic activities [14]. Higher concentration of inoculum depletes medium nutrients faster without boosting productivity, therefore an optimal inoculum percentage is critical for a successful bioprocess. *Bacillus subtilis* MFKA 7 showed highest melanin production i.e., 2.2gm/L with 10% inoculum (Figure 5).

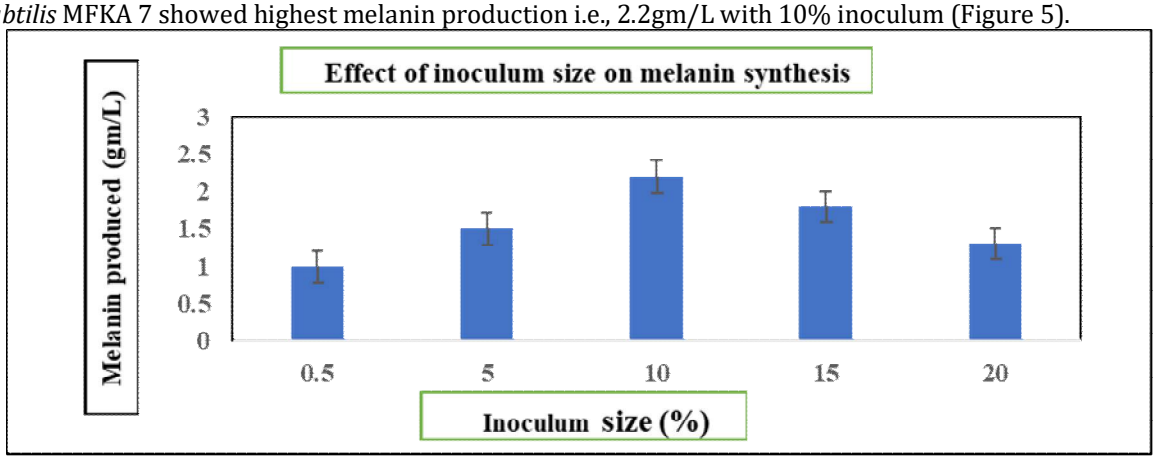

Figure 5: Effect of Inoculum on melanin synthesis by *Bacillus subtilis* MFKA 7

Effect of Temperature

Effect of various temperature was studied by culturing *Bacillus subtilis* MFKA 7 at various temperatures ranging from 20 ^{o}C to 45^{o}C. It was found that maximum melanin production was seen at a temperature of 40^{o}C which resulted in the production of 2.6gm/L (Figure 6). In *Pseudomonas otitidis* DDB2, it was observed that melanin synthesis increased rapidly at a temperature of 30 to 40^{o}C and more slightly at 45^{o}C, which yielded around 0.13gm/L of melanin [15].

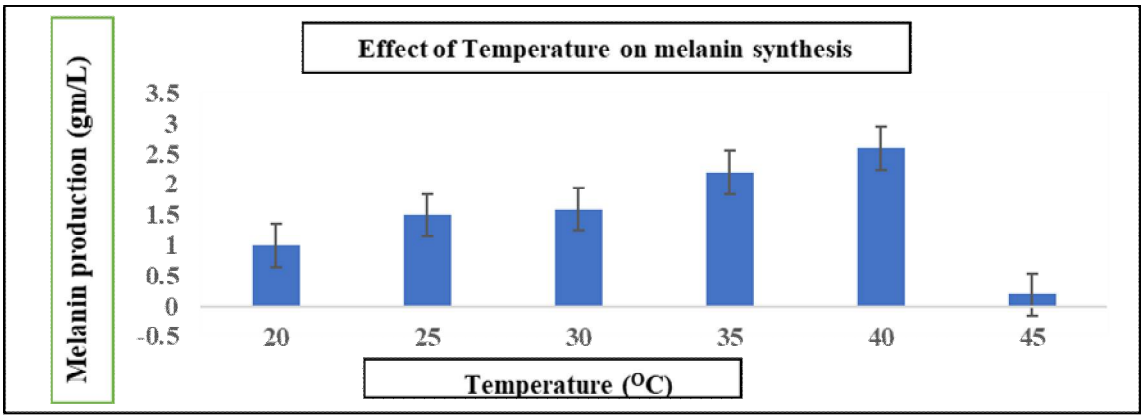

Figure 6: Effect of Temperature on melanin synthesis by *Bacillus subtilis* MFKA 7

Effect of nitrogen source

The synthesis of melanin pigment is generally dependent on expression and regulation of metabolic enzymes like tyrosinase. This in turn is influenced by a variety of dietary and physicochemical variables. Different nitrogen sources such as peptone, yeast extract, urea, beef extract, NH_4Cl, $(NH_4)_2SO_4$ and Tryptone (Figure 7) were studied. All studied nitrogen sources, organic or inorganic, can support melanin synthesis by *Bacillus subtilis* MFKA. The results showed that peptone act as one of the efficient nitrogen sources as it resulted in maximum melanin production of 2.8 gm/L, followed by $(NH_4)_2SO_4$ (1.9 gm/L) and followed by beef extract (1.6 gm/L). The results are in the line with that recorded earlier where yeast extract in combination with peptone served as effective nitrogen source for melanin synthesis in yeast and actinomycetes [15].

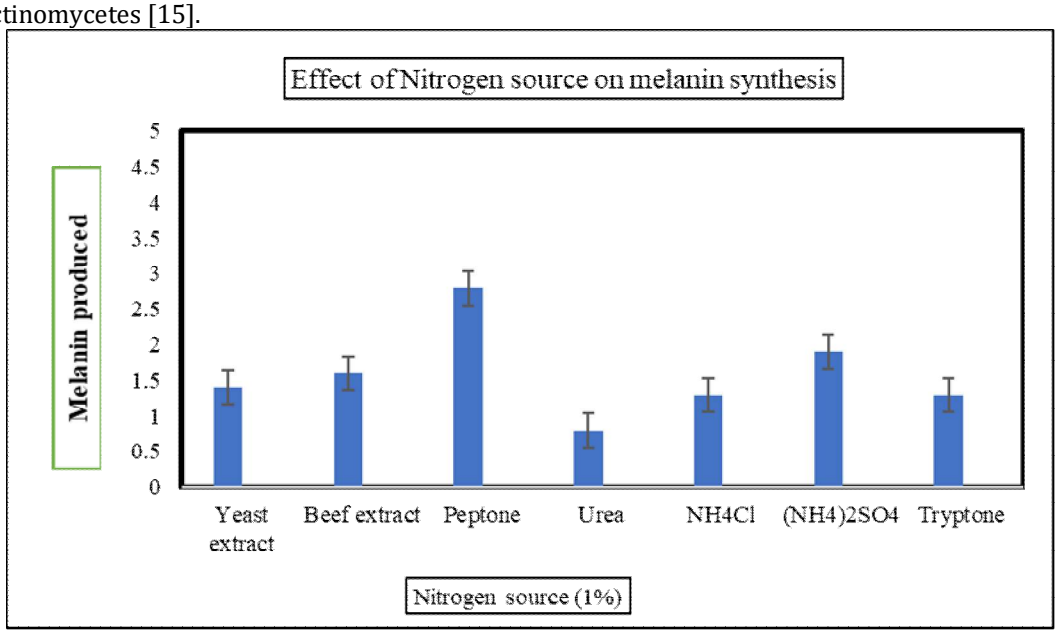

Figure 7: Effect of nitrogen sources on melanin synthesis by *Bacillus subtilis* MFKA 7

Effect of carbon source

The influence of several carbon sources such as maltose, lactose, sucrose, fructose, starch, glucose in 1% concentrations in melanin synthesis were studied. According to the findings, starch was found to be the best carbon source for melanin production which yielded 2.5gm/L, while maltose as a carbon source could result in low melanin production (Figure 8). The culture produced a significant amount of melanin while growing on starch as the only carbon source. According to studies, bacteria that produce melanin are thought to have a role in the recycling or reusing of organic compounds and the breakdown of complex carbohydrates [16].

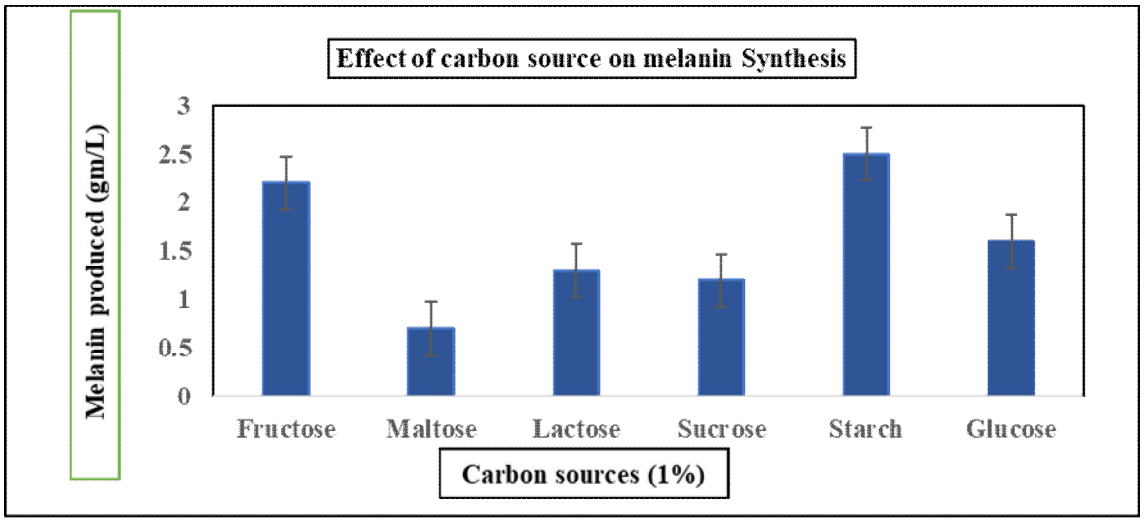

Figure 8: Effect of carbon sources on melanin synthesis by *Bacillus subtilis* MFKA 7

Effect of salt concentration

Salt concentration is one of the important parameters effecting pigment production. *Bacillus subtilis* MFKA 7 was grown at various NaCl concentration ranging from 0.5% to 10%. Melanin was produced in *Bacillus subtilis* MFKA 7 at an optimal NaCl concentration of 5%, yielding 2.2gm/L of melanin (Figure 8). According to studies, a higher concentration of NaCl resulted in less melanin formation. Although marine bacteria such as *Pseudoalteromonas lipolytica* grew best at 3% NaCl [17], a considerably lower salinity (0.7%) of the medium prevented melanin synthesis, which could be owing to the permanent inhibition of tyrosinase enzyme by salt Cl^- ions [18].

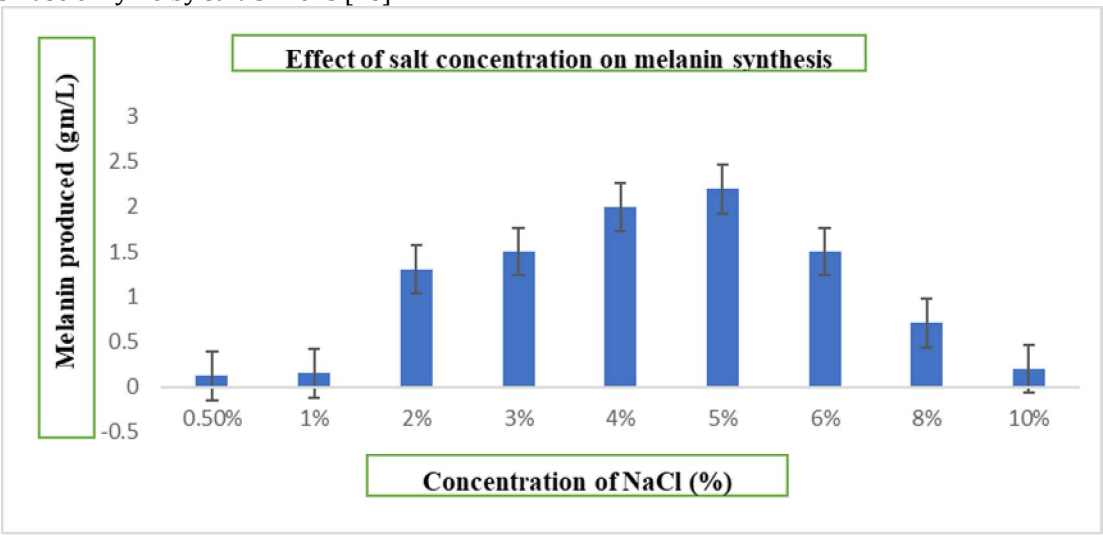

Figure 9: Effect of salt concentration on melanin synthesis by *Bacillus subtilis* MFKA 7

Effect of metal ions as cofactor

Metal ions and their concentration is one of the important factors which influence pigment production. Effect of various metal ions like $FeSO_4$, $MgSO_4$, $ZnSO_4$, $CuSO_4$, KH_2PO_4, $FeCl_3$ and $NiCl_3$ was studied for pigment production using *Bacillus subtilis* MFKA 7. Effect of metal ions showed that 1% $CuSo_4$ is the good metal ions which aids in the production of 2.4 gm/L of melanin (figure 10). After $CuSo_4$, $NiCl_3$ showed highest melanin production followed by $Fecl_3$. Copper plays a key role in the production of melanin as it acts as a cofactor for laccases and tyrosinases enzyme [19]. In many bacterial and fungal species, variations in the quantity of added copper results in uneven coloration [20]. Apart from copper other metals are also known to increase melanin production in lab conditions. Wang *et al.*, (2019) demonstrated enhanced melanin production along with tyrosinase activity by addition of nickel and iron in the production media [21]. According to Gowri and Srivastava (1996), presences of metal ions in the production medium can stimulate stress responses in microorganism, thereby which results in high melanin production [22].

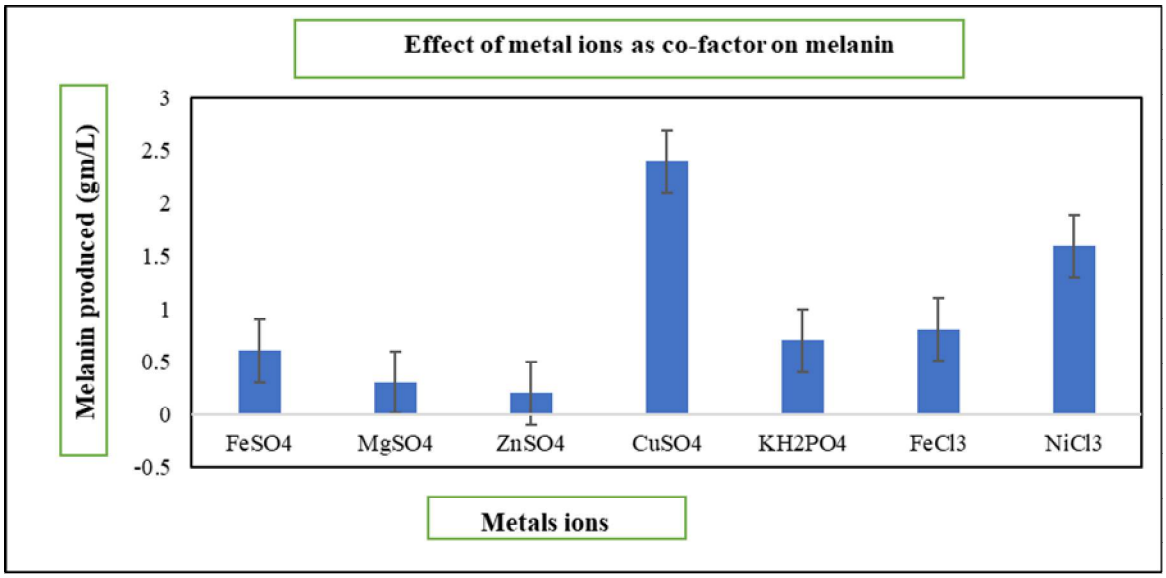

Figure 10: Effect of metal ions on melanin synthesis by *Bacillus subtilis* MFKA 7

Optimized parameters for melanin production

In initial melanin production, *Bacillus subtilis* MFKA 7 was able to produce 1.3gm/L of melanin in nutrient broth without supplementing L-Tyrosine or any other trace metals in 7 days under static condition. After optimizing all the mentioned nutritional and physical parameters, there was a steady increase in the melanin production (Table 1). With all the optimized parameters, the melanin production by *Bacillus subtilis* MFKA 7 can be increased from 1.3 gms/L to 3.0 gms/L in 5 days of incubation.

Table 1: Optimized parameters for melanin production by *Bacillus subtilis* MFKA 7

Process parameters	Optimized parameters
Media	Nutrient broth
Temperature	40^{o}C
Initial pH	5
Agitation	120 rpm
Inoculum percentage	10%
Salt concentration	5%
Carbon source	Starch (1%)
Nitrogen source	Peptone (1%)
Metal ion	$CuSo_4$ (1%)
Incubation time (hrs)	120 (5 days)
Melanin pigment produced	3.0gm/L

Confirmatory test for melanin

The production of a bright red colour indicates the presence of melanin (Figure 11). Bacillus subtilis MFKA 7 was inoculated into 10 mL of 0.1% tyrosine as substrate solution with a few drops of chloroform and incubated at 37^{o}C for 48 hours. The strong red colour indicated melanin production.

Figure 11: Confirmatory test for melanin

Characterization of solubility of melanin produced from *Bacillus subtilis* MFKA 7

The results of solubility characters are summarised in Table 2. The pigment was found to be insoluble in dis. water and organic solvents such as methanol, ethanol, acetonitrile, chloroform, benzene, HCL, and others. Melanin was poorly soluble in DMSO. Some pigment particles remained insoluble even after 15 minutes of vortex in DMSO. In Alkali, like sodium hydroxide and potassium hydroxide the pigment was found to be completely soluble. These chemical properties of *Bacillus subtilis* MFKA 7 melanin were found to be identical with the Sepia melanin purchased from Sigma Company (data not shown). Moreover, melanin of *Bacillus subtilis* MFKA 7 was precipitated with 1% $FeCL_3$ and 3 mol/L HCL.

Table 2: Solubility of melanin extracted from *Bacillus Subtilis* MFKA7

S.NO.	Test	Pigment
1	Colour	Black
Solubility test		
2	Dis. Water	Insoluble
3	DMSO	Soluble
4	1 mol/KOH	Soluble
5	1N/L NaOH	Soluble
6	1M/NaCl	Insoluble
7	Absolute ethanol	Insoluble
8	Methanol	Insoluble
9	Chloroform	Insoluble
10	L- butanol	Insoluble
11	Acetone	Insoluble
12	Benzene	Insoluble
13	Acetonitrile	Insoluble
14	Ethyl acetate	Insoluble
15	2- propanol	Insoluble
16	Petroleum ether	Insoluble
Precipitation reactions		
17	1% $FeCl_3$	Precipitated
18	3 M/LHCL	Precipitated

CONCLUSION

With the recent advancement, melanin has become more important for the use in green technology and material science as an additive that can significantly improve the performance of other materials. In the present study, we describe the synthesis of melanin by promising strain of *Bacillus subtilis* MFKA 7 in response to the growing market demand for melanin. Melanin production was successfully optimized using various physical and chemical parameters. The properties and characters of the extracted melanin was compared with commercially available Sepia melanin. Following optimization, 3.0gm/L of melanin was produced with 1% tyrosine supplemented. Therefore, *Bacillus subtilis* MFKA 7 can be considered as a good option for the production of melanin.

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CONFLICT OF INTEREST: The authors declare no conflict of interest.

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