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Biosynthesis of Indigo Dye by Newly Isolated Naphthalene-Degrading Strain *Pseudomonas s*p. HOB1 and its Application in Dyeing Cotton Fabric

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Abstract Indigo is one of the oldest dyes manufactured chemically and is mostly used in textile, food, and pharmaceutical industries. However, owing to the environmental hazards posed by the chemical production, the present scenario in the field stipulates a biosynthesis alternative for indigo production. The present study describes an indigenously isolated naphthalene-degrading strain *Pseudomonas* sp. HOB1 producing a blue pigment when indole was added in the growth medium. This blue pigment was analyzed by high-pressure thin-layer chromatography and other spectroscopic techniques which revealed it to be the indigo dye. *Pseudomonas* sp. HOB1 showed ability to produce 246 mg indigo liter^{-1} of the medium. The K_m for the enzyme naphthalene dioxygenase which is involved in indigo formation is 0.3 mM, and V_{max} was as high as 50 nmol min^{-1} mg dry biomass^{-1}. The bacterial indigo dye was further successfully applied for dyeing cotton fabrics. The high indigo productivity of *Pseudomonas* sp. HOB1 using naphthalene as growth substrate and its applicability on cotton fabrics, therefore, stems the probability of using this culture for commercial indigo production.

Keywords Dyeing · Indole · Indigo production · Naphthalene degradation · Naphthalene dioxygenase (NDO) · *Pseudomonas* sp.

Introduction

Indigo (Vat Blue 1; C.I. 73,000) is one of the world's largest-produced dyes and is widely used in the textile industry [1, 2]. The enduring popularity of denim has ensured persistent commercial significance of indigo. It is classified as vat dye since it is insoluble in water and needs to be converted into water-soluble form by reduction to leuco form of the dye.

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Structurally, indigo is an aromatic compound [3]. Besides serving as dyestuff, indigo and indirubin (an indigoid pigment) has established therapeutic importance in a number of diseases including Alzheimer's disease [4], certain forms of cancer [5–7], and delayed hypersensitivity [8]. Indigo was initially extracted from plants of *Indigofera* spp. but is presently manufactured chemically [9]. Merits of chemical synthesis are its higher productivity, simple production process, and high purity. However, the chemical synthesis of indigo provoked not only health problems to workers but also severe environmental pollution due to toxic materials and catalysts used and the toxic by-products and waste water produced and released from nearly 1980s onwards. As the awareness of the importance and urgency to protect the environment is increasing, interest is growing in finding environment-friendly and producer-friendly methods for the production of indigo. Microbiological indigo synthesis process can, therefore, pave way to newer avenues in green chemistry.

Many microbial processes for indigo synthesis have been reported [1, 9–11], but the main concern is the lower productivity in comparison to chemical processes. Hence, there is inclination towards isolation of microbes that are capable of producing indigo at an appreciable rate. Majority of aromatic hydrocarbon-degrading bacteria have the ability to produce indigo dye [10, 11]. The bacterial catabolism of such xenobiotic compounds involves dioxygenases, exemplified by naphthalene dioxygenase (NDO; E.C. 1.14.12.12), which contains a Rieske iron-sulfur cluster and a non-heme iron center [12]. They have potential application in the bioremediation of chemical pollutants as well as for the production of oxygenated value-added biomolecules. NDO, which initiates naphthalene degradation in aerobic bacteria, is a multicomponent enzyme system in which electrons are transferred from NAD(P)H to a terminal Rieske non-heme iron oxygenase component (Oxygenase $_{NAP}$) through Reductase$_{NAP}$ and Ferredoxin$_{NAP}$ [13]. When indole is provided as a substrate to such culture, the dioxygenation of indole by NDO occurs to produce *cis*-2,3-dihydroxy-2,3-dihydroindole. The *cis*-diol intermediate subsequently undergoes spontaneous dehydration to indoxyl followed by spontaneous dimerization, thus forming indigo (Fig. 1) [12, 14]. Besides indole, several derivatives of indole have been used for biosynthesis of different dyestuffs by dioxygenase enzyme [14]. The NDO-based production of indigo from glucose using recombinant *Escherichia coli* cells had been at the stage close to commercialization [14]; however, the process suffered from low stability of dioxygenase enzyme resulting into poor productivity.

Microbial biosynthesis of indigo depicts several merits over traditional extraction from indigo-producing plants like higher efficiency, shorter processing period, and no influence of natural environmental factors. Compared with the chemical synthesis of indigo, microbial biosynthesis also deserves distinct advantages, such as lower cost, lower energy consumption, and being eco-friendly.

While much work has been done to improve and optimize the microbial production of indigo, reports are lacking on dyeing of fabrics using microbially synthesized indigo.

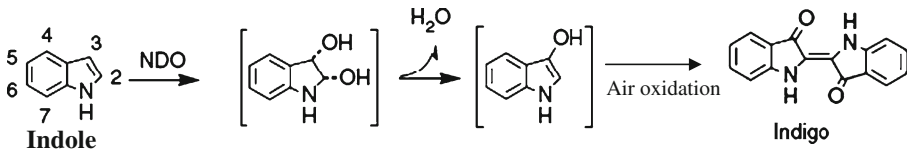

Fig. 1 Naphthalene dioxygenase (*NDO*) catalyzed formation of indigo from indole. Adapted from Kim et al. [14]

Hence, evaluation of the microbially produced indigo for dyeing fabrics is a prime prerequisite to achieve competitive alternative to chemically synthesized indigo dye. In this context, our study was aimed at evaluation of indigo productivity by indigenously isolated naphthalene-degrading strain *Pseudomonas* sp. HOB1, its characterization, and its application for dyeing of cotton fabrics.

Materials and Methods

Microorganism and Medium

Pseudomonas sp. HOB1 capable of degrading naphthalene, a polycyclic aromatic hydrocarbon, was used in the present study. It was isolated from polluted sediments of Amlakhadi canal flowing across the city of Ankleshwar, Gujarat, India. Culture HOB1 was identified as *Pseudomonas* sp. (NCBI accession no. EU849119) by 16S rRNA gene approach. It was routinely grown on 100 mL of Bushnell Haas broth (BHB; Himedia, Mumbai, India) mineral medium supplemented with 200 ppm naphthalene under shaking at 150 rpm at 37 °C. Indigo dye and indole were obtained from Acros Organics (New Jersey, USA, 99.9% purity). All reagents used in the present study were of analytical grade.

Indigenously isolated *Pseudomonas* sp. HOB1 has potential to degrade 97% of 2,000 ppm naphthalene in 24 h and to tolerate initial concentration of naphthalene as high as 60,000 ppm [15].

For inoculation, *Pseudomonas* sp. HOB1 was harvested by centrifugation at 6,000×*g* for 10 min, washed with 0.5 M phosphate buffer (pH 7.0) thrice, and resuspended in appropriate volume of same buffer to have culture density of 1.0 A_{660}. One milliliter of this preparation was used to inoculate the experimental flasks. All experiments in present study were performed in 100 ml BHB with 200 ppm naphthalene, incubated under shaking at 150 rpm at 37 °C and initial medium pH of 7.0 unless specified.

Time Course Study for Indigo Formation

The flasks were harvested at every 2-h intervals. The broth was filtered through Whatman no. 1 filter paper to remove residual naphthalene crystals, and the filtrate containing cells was used to determine the rate of indigo formation by method of O'Connor and Hartmans [16] with certain modifications. One hundred microliters of the filtrate was added to 400 μl of potassium phosphate buffer (pH 7.0) containing 0.3 mM indole, and the mixture was incubated at 30 °C for 45 min for indigo formation. The tube was then centrifuged at 16,000×*g* for 2 min. The supernatant was discarded, and the cell pellet was resuspended in 1 ml of dimethyl formamide (DMF) to solublize the cell-associated indigo. The tube was again centrifuged to remove the cells, and the absorbance of the indigo in the supernatant was determined at 610 nm. Cell growth was determined by measuring absorbance at 660 nm. Flask containing BHB amended with 200 ppm naphthalene along with 0.3 mM indole without culture was used as control.

Biosynthesis, Extraction, and Characterization of Indigo Dye

Pseudomonas sp. HOB1 was inoculated in BHB broth with 200 ppm naphthalene as mentioned previously. At mid-log phase of the growth, the broth was filtered through Whatman no. 1 filter paper to remove residual naphthalene crystals, and indole was added in the filtrate at a final concentration of 0.3 mM. For indigo formation, the broth was further

incubated under same conditions until blue pigments appeared in the broth. The blue pigment from the whole culture broth was extracted by method of O'Connor and Hartmans [16] with some modifications. The culture broth was mixed with equal volume of chloroform and kept on shaker for 30 min to ensure complete dissolution of the blue pigment in the organic phase. The two layers were separated using separating funnel. The organic layer was collected and centrifuged at 16,000×*g* for 30 min to remove the cell debris. The supernatant was evaporated using Speedvac concentrator dry unit (Thermo Savant SPD111V, USA). The dried pigment was dissolved in dimethyl formamide. The pigment was analyzed by high-pressure thin-layer chromatography (HPTLC) using precoated silica gel 60 F_{254} plates (Merck, Germany). Blue pigment sample (5 μl) and indigo dye standard were spotted on the TLC plate using HPTLC applicator system (Camag, Linomat 5, Switzerland). The solvent system used was chloroform–methanol (15:1). No developing reagent was used as the pigment was colored, and the bands were analyzed visually. The dried pigment was dissolved in DMF and was analyzed spectrophotometrically by UV–visible scan in the range of 200–800 nm. Furthermore, the dried pigment powder was mixed with potassium bromide and analyzed in the infrared (IR) range of 400–4,000 cm^{-1}. Commercial analytical grade indigo was used as standard.

Kinetics of Indigo Formation

The rate of indigo formation from whole cells was determined at different concentration of indole from 0.1 to 1.0 mM. The K_m value for enzyme naphthalene dioxygenase was calculated from the Michaelis–Menten equation. The maximum rates of indigo formation were calculated using Lineweaver–Burk plot.

Indigo Formation Using Other Substrates

Pseudomonas sp. HOB1 was inoculated (as stated previously) into BHB media spiked with 0.02% (*w*/*v*) naphthalene, methylnaphthalene, glucose, salicylate, and succinic acid, respectively, and incubated at 37 °C under shaking condition (150 rpm). Culture suspension was obtained as mentioned earlier (previously). This suspension was used to perform indigo assay to determine indigo formation rate.

Effect of Different In Vitro Conditions on Indigo Formation Rate

Indigo production is cell associated, and the cells produce indigo intracellularly [16, 17]. Therefore, it is necessary to disrupt the cell membrane to release the indigo. This study was conducted to depict the effect of different in vitro conditions on indigo formation rate. Initially, the cells were provided uniform growth conditions. The different in vitro treatments were applied while performing indigo assay to determine indigo formation rate.

Bacterial culture of *Pseudomonas* sp. HOB1 was grown in BHB broth as mentioned earlier. At mid-log phase of growth, cells were harvested and washed with 0.5 M potassium phosphate buffer (pH 7.0). Cell biomass was resuspended in same buffer to obtain a concentration of 1 mg dry biomass ml^{-1}.

Indigo Formation Rate by Resting Cells

The biomass obtained was utilized without any pre-treatment (resting cells) to determine indigo formation rate (as mentioned previously).

Indigo Formation Rate by Permeabilized Cells

The cell suspension was centrifuged at 8,000×*g* for 10 min to obtain cell pellet. Cell pellet was treated with 0.2% (*v*/*v*) Triton X-100 by vortexing for 20 min to achieve cell permeabilization. Permeabilized cell suspension was utilized to determine indigo formation rate (as mentioned previously).

Indigo Formation Rate by Cell-Free Extracts

The cell pellet was resuspended in 5% (*v*/*v*) of the original culture volume in 0.5 M potassium phosphate buffer (pH 7.0). This cell suspension was placed in ice bath and disrupted using an Ultrasonic Probe (Sonics Vibra Cell 500, USA) with amplitude of 35% at 50 W with 9 s pulses and 1 s off mode for 35 min. The sonicate obtained was centrifuged at 32,000×*g* for 35 min at 4 °C. The supernatant (the cytosolic fraction) was filtered through 0.22 μm filters (Millipore, Bradford, USA) to give the cell-free extract. This cell-free extract was used to determine indigo formation rate (as mentioned previously).

Application of the Indigo Produced for Dyeing Cotton Fabrics

Dyeing Process

The indigo dye was harvested from culture broth as stated previously and dried to powder in Speedvac concentrator unit. This indigo powder was used to dye cotton cloth according to procedure reported by Nizar et al. [18] with certain modifications. Indigo being a vat dye, it is insoluble in aqueous medium. So to make it soluble, indigo was converted into its soluble form leuco-indigo by providing alkaline conditions. Two hundred milligrams of aluminum powder was added to 20 ml of distilled water. The solution was stirred, and 1 ml of 50% (*w*/*v*) sodium hydroxide was added. Two hundred milligrams of indigo powder was added to this solution. The solution was stirred for approximately 90 min. The solution decolorized from the blue color of indigo to the greenish-yellow of the leuco form. The fabrics were dyed at a liquor ratio 60:1 (dyeing bath volume (ml)/fabric weight (g)). The cotton cloth was dipped in dye bath for 20 min and allowed to air dry.

Evaluation of Dyeing Quality

The dyeing of fabrics was evaluated by color measurements using UV–Vis spectrophotometer (Lambda 19, Perkin-Elmer, USA) with UV-Win Lab 2.3 software (Perkin-Elmer, USA). To calculate the color yield (*K*/*S*) value, the reflectance of the dyed sample was measured at 660 nm and transferred to (*K*/*S*) according to the Kubleka–Munk function [18].

Results and Discussion

Time Course Assay for Indigo Formation

Periodic monitoring of indigo formation and cell growth was conducted to determine the time for maximum indigo formation by *Pseudomonas* sp. HOB1. Indigo formation increased linearly during initial 8 h of the growth and reached maximum to 246 μg ml^{-1} and decreased thereafter (Fig. 2). Indigo formation was superior when compared to other

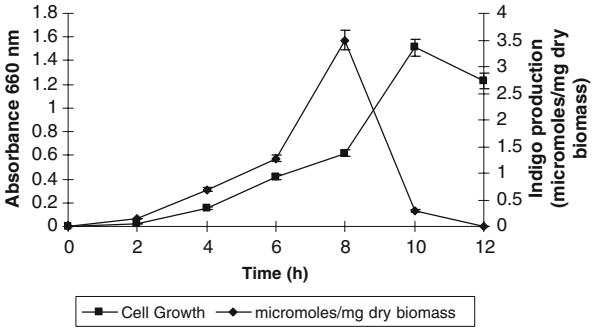

Fig. 2 Time course assay for indigo production at 37 °C with initial medium pH of 7.0

studies (Table 1). No indigo formation was observed in the control flask suggesting the role of *Pseudomonas* sp. HOB1 in indigo biosynthesis.

Characterization of Indigo Dye

Naphthalene degrading *Pseudomonas* sp. HOB1 used in the present study produced two types of pigments, viz: blue pigment and purple pigment when analyzed by HPTLC (Fig. 3). The R_f values of blue and purple pigments were 0.85 and 0.8, respectively. The R_f value for blue pigment matched exactly with that of authentic indigo dye, i.e., R_f value of 0.85.

For further confirmation of the blue pigment as indigo dye, it was analyzed using UV–visible scan from 200 to 800 nm and by Fourier transform IR (FT-IR) of blue pigment. The UV–visible spectrum of the extracted blue pigment was identical to that of the authentic indigo dye (Fig. 4).

The infrared spectrum is specific to any compound and can be used as its "fingerprint" for identification purposes. Figure 5a, b depicts the infrared spectrum of the extracted blue pigment and the commercial indigo dye, respectively. The FT-IR spectrum of the purified blue pigment shows a peak at 3,398 cm^{-1} that is attributed to the N–H stretching in secondary amines. A peak at 1,617 cm^{-1} may be attributed to C=O stretching coupled with C=C stretching. Similar peak has been observed in the IR spectrum of authentic indigo dye (Fig. 5b). Further, weak bands in the range of 1,350–1,000 cm^{-1} have contributions from C–N stretching of the amine group. The peak around 735–770 cm^{-1} may be assigned to C–H out of the plane bending in aromatic group. Thus, from the IR spectrum of the microbial synthesized compound, it appears that it has indigo moiety which is blue in color.

Table 1 Indigo production by different bacterial cultures.

Indigo produced (μg ml^{-1})	Time (h)	Culture	Reference
246	8	*Pseudomonas* sp. HOB1	Present study
52	24	*E. coli*	Doukyu et al. [1]
292	24	*Aceinaobacter* ST 550	Doukyu et al. [1]
662	24	*E. coli*	Han et al. [22]
160	12	*E. coli*	Choi et al. [23]

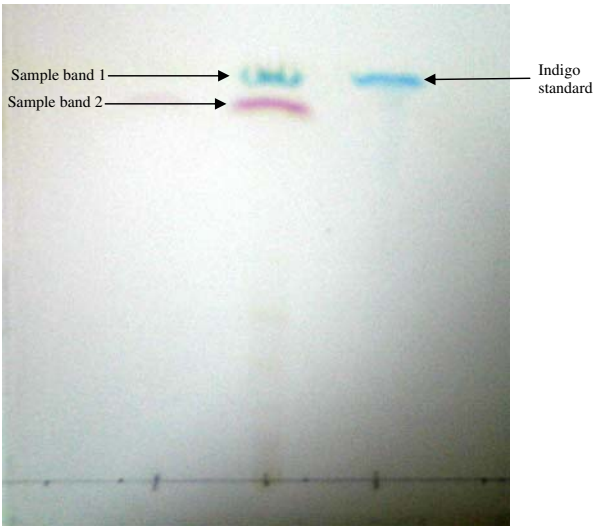

Fig. 3 TLC plate showing separation of indigo dye

The results of HPTLC, UV–visible scan and FT-IR confirmed that the blue pigment produced as a consequence of biotransformation of indole is the indigo dye.

Kinetics of Indigo Formation

Naphthalene dioxygenase is the enzyme present in *Pseudomonas* sp. which is responsible for the biotrasformation of indole to indigo [12, 14]. The specific rate of indigo formation by *Pseudomonas* sp. HOB1 was found to be 77.8 nmol min^{-1} mg dry $biomass^{-1}$. This rate of indigo formation is 50–100 times higher as compared to the indigo formation rates (0.60, 0.80, 1.2, and 1.5 nmol min^{-1} mg dry $biomass^{-1}$) reported by Bhushan et al. [19] under different inducing conditions with wild-type *Pseudomonas putida* strain as well as

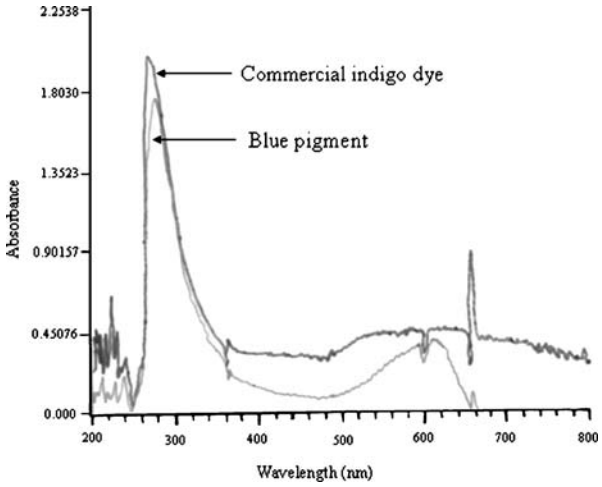

Fig. 4 The overlay of UV–visible scan from 200 to 800 nm of blue pigment and commercial indigo dye

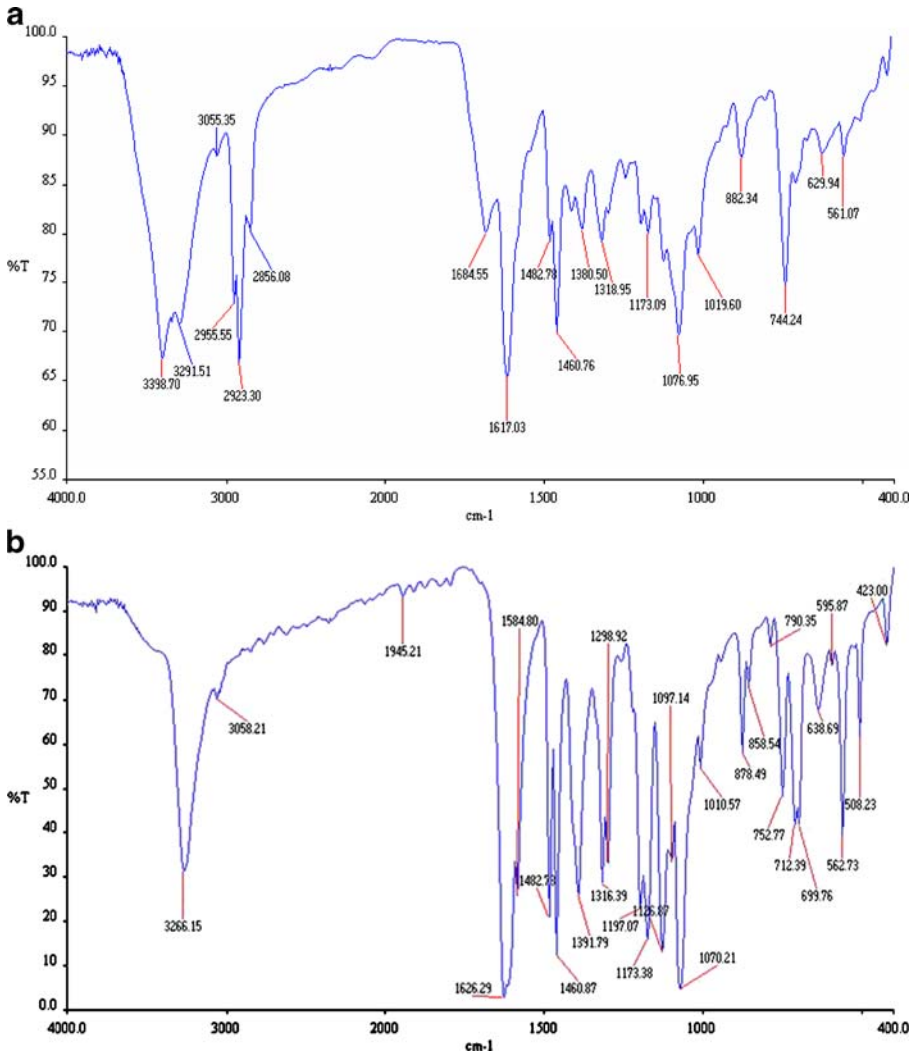

Fig. 5 Infrared spectrum. **a** Infrared spectrum of the purified blue pigment obtained from *Pseudomonas* sp. HOB1. **b** Infrared spectrum of the commercial indigo dye

recombinant *E. coli* strains. O'Connor et al. [16, 20] have reported indigo formation rate of 8.3 and 12 nmol min^{-1} mg dry $biomass^{-1}$, respectively, which is again much less than indigo formation rate by *Pseudomonas* sp. HOB1.

The K_m and V_{max} values for NDO were determined to be 0.3 mM and 50 nmol min^{-1} mg dry $biomass^{-1}$, respectively. This indicates high indigo productivity of *Pseudomonas* sp. HOB1 using naphthalene as growth substrate. This stems the probability of using this culture for commercial indigo production.

Indole, at a concentration beyond 0.3 mM, was found to be deleterious for the culture. A similar result is reported by Bhushan et al. [19] stating that indole concentration higher than 0.5 mM is toxic to the bacteria thus hindering the biotransformation of indole to indigo. Yun and Lehe [21] also reported a sharp decrease in indigo yield by both immobilized and free recombinant *E. coli* cells in a reaction system having indole concentration higher than 0.5 mM.

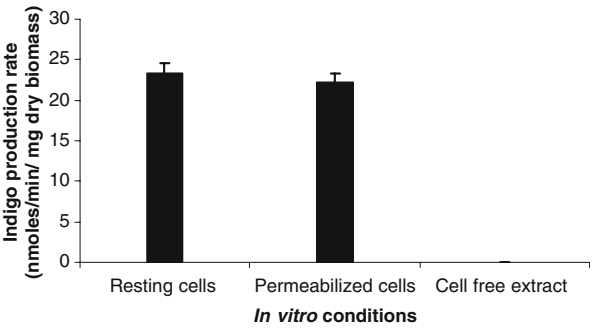

Fig. 6 Indigo production rate under different in vitro conditions at 37 °C with initial medium pH of 7.0

Effect of Growth Substrates on Indigo Formation

Pseudomonas sp. HOB1 was grown on substrates other than naphthalene (methylnaphthalene, glucose, salicylate, and succinic acid) to study the effect on indigo biotransformation. Indigo formation by the cells when grown on these substrates was found to be below detectable levels. These results indicate that indigo-forming activity was strongly induced by naphthalene. This implies that the NDO enzyme depicts specificity for naphthalene as a growth substrate for biotransformation of indigo, and hence, this enzyme is inducible in nature. However, further detailed study is required to support this observation. No similar data are available in the literature for comparison.

Indigo Formation by Resting Cells, Cell-Free Extract, and Permeabilized Cells

Indigo formation rates by resting cells and permeabilized cells were found to be comparable, whereas no indigo formation was observed with cell-free extract (Fig. 6). NDO is a multi-component intracellular enzyme [12]. Therefore, in cell-free extracts, NDO might be getting denatured leading to no indigo formation upon addition of indole. In case of permeabilized cells, treatment with anionic surfactants aided in extraction of intracellular indigo into the supernatant. Therefore, the cells were not required to give dimethylformamide wash while performing indigo assay since indigo was instantly extracted in the supernatant after centrifugation. This is a novel modification in the indigo assay procedure of O'Connor and Hartmans [16]. This modification is significant as it can aid in the downstream processing of microbially produced indigo dye. Moreover, this modified process will be more economic since it eliminated the use of DMF, thereby also being eco-friendly and less time consuming.

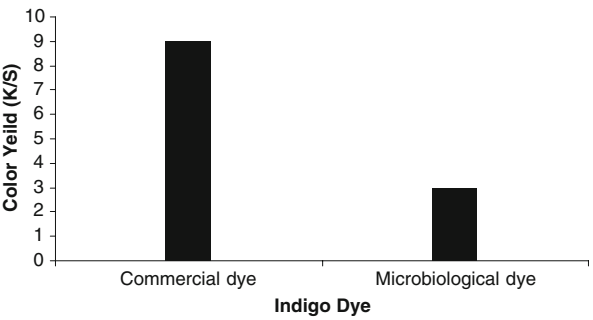

Fig. 7 Dyeing quality measurement of commercial and microbiological indigo dye

Application of the Biosynthesized Indigo Dye for Dyeing Cotton Fabrics

Indigo dye powder and commercial indigo dye were used to dye piece of cotton fabric, and the dyeing quality was evaluated by measuring the color yield (*K*/*S*) at 660 nm. The color yield for the commercial indigo dye was 9, whereas for microbiological indigo dyed fabric it was 3 (Fig. 7). It shows that microbiologically produced indigo dye can be used for dyeing cotton fabrics; however, the color yield is low when compared with the commercial dye. This can be improved by improving the dyeing process. No similar data are available in the literature for comparison of the color yield of cloth dyed from bacterial produced indigo dye. To the best of our knowledge, this is the first report of application of bacterial indigo dye for dyeing cotton fabrics.

Conclusion

The rate of indigo formation by *Pseudomonas* sp. HOB1 is much higher when compared to the reported rates of microbial indigo formation. The addition of anionic surfactants to the cell pellet permeabilizes the cells and leads to release of intracellular indigo pigment into the supernatant. This can aid in the downstream processing of the indigo pigment. Moreover, the bacterial indigo dye thus produced was further successfully applied for dyeing cotton fabrics. These results imply that the culture *Pseudomonas* sp. HOB1 can be a suitable contender for large-scale microbial production of indigo dye.

The system of microbial biosynthesis of indigo is not as complete as that of chemical synthesis, and there still remains a huge amount of work to be carried out in order to reduce production cost and increase production efficiency significantly. Microbial synthesis of indigo, with increasing concern about environment protection, will become, if not replace the chemical synthesis completely, a major breakthrough in the pigment industrial production in near future.

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