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Short Communication

Indigo production by *Pseudomonas* sp. J26, a marine naphthalene-degrading strain

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A technique developed to determine naphthalene dioxygenase (NDO) activity was optimized and used to study the biotransformation of indole to indigo by *Pseudomonas* sp. J26 whole cells. The maximum production of indigo was achieved at 25 °C using 2.5 mM indole when J26 was grown in the complex medium JPP, while indole concentrations higher than 4 mM proved toxic for cells. The maximum rate of indigo production was 0.56 nmol min^{-1} mg dry $biomass^{-1}$, obtaining 75.5 μM of indigo after 8 h of incubation, while a maximal concentration (138.1 μM) of indigo was obtained after 20 h.

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Introduction

Naphthalene dioxygenase (NDO) catalyzes the first step in the degradation of naphthalene. Cells containing NDO enzymes are also capable of transforming indole to cis-2, 3-dihydroxy-2, 3-dihydroindole, which subsequently undergoes spontaneous dehydration to indoxyl followed by spontaneous dimerization to blue jean dye indigo, that can be measured spectrophotometrically by absorbance at 600 nm. The color formed in the reaction can then be used as an indicator of NDO activity [1]. Attempts at microbial indigo formation for industrial purposes started in 1983, when naphthalene dioxygenase genes from *P. putida* G7 were expressed in a recombinant *E. coli* strain that efficiently converted glucose to indole [1]. After that, several oxidoreductase enzymes like monooxygenases and dioxygenases have been reported to yield indigo from many substrates [2–5].

The degradation of naphthalene, as well as the production of bioindigo mediated by NDO activities, has been studied in *Pseudomonas* species as with the archetype plasmid NAH7 from *P. putida* G7 [6, 7] and the NAH plasmid pWW60-1 from *P. putida* NCIB9816 [8, 9]. *Pseudomonas plecoglossicida* is a fish pathogen bacterium placed in the *P. putida* group that causes hemorrhagic ascites in the ayu fish [10, 11]. Neither studies of PAHs degradation nor indigo production could be found for this bacterium. *Pseudomonas* sp. J26 was isolated from intertidal sediment of Patagonia, Argentina, during a selective enrichment with naphthalene as a sole carbon and energy sources. *Pseudomonas* sp. J26 strain demonstrated to harbor a NDO gene with 99% (866 bp fragment of *nahAc* gene) of similarity to those of *P. stutzeri* AN10 previously described [12] (data not shown). On the basis of producing the largest amount of blue pigment when exposed to indole vapors in agar plates, J26 strain was chosen to measure the capability to produce bioindigo by using indole as substrate in a whole-cell system.

Materials and methods

Culture conditions and dioxygenase test

Bacteria harboring NDOs enzyme activities were selected from 15-day enrichment cultures in the minimal seawater medium MMJP (25 g NaCl/l, 4 g Na_2SO_4/l, 0.7 g KCl/l, 11 g $MgCl_2 \cdot 6\ H_2O$/l, 2 g $CaCl_2 \cdot 2\ H_2O$/l, 0.1 g KBr/l, 0.04 g $SrCl_2 \cdot 6\ H_2O$/l, 0.03 g H_3BO_3/l, 0.003 g NaF/l, 0.002 g $FeCl_3 \cdot 6\ H_2O$/l, 0.002 g NH_4NO_3/l, 0.03 g $NaHCO_3$/l, 0,3 g NH_4Cl/l, 0.05 g $Na_2HPO_4 \cdot 7\ H_2O$/l, pH 7.6) with

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crystals of naphthalene as the sole carbon and energy source. Aliquots of the enrichment (100 μl) were spread on MMJP-agar medium and incubated for 48 h with crystals of naphthalene on the lid of Petri dishes. The selection of colonies expressing NDO was made using the indole test, based on their capability of producing blue color after 24 h when indole crystals were placed instead of naphthalene. This blue color is indicative of indigo formation.

NDO activity assays

J26 strain was selected as a dark blue colony by indole test, and was identified as *Pseudomonas* sp., closely related to *P. plecoglossicida* using biochemical tests and sequencing of 1400 bp of 16S rRNA gene (99.7% similarity). Blue color of J26 was developed after 6–8 h of exposure to indole vapors. For NDO activity assay, J26 strain was grown overnight in MMJP or JPP medium (20 g NaCl/l, 2 g peptone/l, 1 g yeast extract/l, pH 7.6) with naphthalene crystals, at 25 °C with shaking (150 rpm). Three different sources of naphthalene were used in the culture media for growth and NDO induction. Stock solutions of naphthalene (100 mM) in dimethylformamide (DMF) and methanol were made and added to previously inoculated medium to obtain a final naphthalene concentration of 1 mM. The data of NDO activity obtained in these assays were compared with those using naphthalene crystals. When growth reached late exponential phase, cells were harvested by centrifugation (16,000 × *g* for 4 min at 4 °C), washed twice with physiological solution, resuspended in 10 g NaCl/l to a cell density of 0.9–1.0 and kept on ice for NDO activity determinations. Absorbance at 600 nm (A_{600}) was measured as a function of indol oxidation by whole cells of the J26 strain [13].

The oxidation reactions were initiated by adding indole (5 μl 100 mM) in aqueous solution directly to cell suspension (195 μl) and indigo formation was monitored spectrophotometrically at 600 nm over 3 h in a 96-wells microplate reader. The blank consisted of the use of water instead of indole. The NDO activity was determined as the initial rate of indigo formation by plotting the increase in absorbance at the first 2 h of reaction, while the NDO specific activity was defined as the rate of indigo formation normalized to the cell optical density (absorbance units min^{-1} $OD_{600}{}^{-1}$). All the experiments were performed independently in triplicates and the results given here are the mean of the three assays.

Indigo quantification and recovery

To provide an accurate indigo determination, a polypropylene vial scarification method was developed. Indigo formation reaction was started simultaneously in 30 polypropylene vials containing 0.5 ml of washed cells (OD = 1.0) previously grown in JPP medium, by adding indole from a 100 mM indole in DMF stock solution. Each reaction mixture was analyzed following the method of O'Connor [2] with few modifications. Samples were incubated at 25 °C in a water bath, in darkness without shaking. Tubes were collected every 30 min during the first two hours and every 60 min over a 10 h period. Two vials were taken for analysis each time and the complete reaction mixture was analyzed. Reaction mixture was centrifuged at 16,000 × *g* for 4 min and supernatant was carefully pipetted off. After that, the cell pellet was resuspended in 1 ml of DMF and incubated at room temperature with shaking for 12 h in darkness to ensure removal and dissolution of indigo associated to cells. The tubes were then centrifuged and supernatants were pooled with previously obtained. The A_{600} of the supernatants was determined.

The maximal absorbance of indigo was experimentally determined by mass spectrum at different wavelength, using a standard indigo solution dissolved in DMF. The concentration of indigo extracted from J26 whole cells was calculated by using a molar extinction coefficient at 600 nm (maximal absorbance of indigo determined) of 3,530 cm^{-1} M^{-1}. The correspondence between dry biomass and OD_{600} was also estimated.

Results and discussion

Pseudomonas sp. J26 could grow in JPP medium without naphthalene. However, this culture condition resulted in no further transformation of indole to indigo. Therefore, naphthalene produced an induction of NDO activity, necessary for further biotransformation of indole (data not shown).

While J26 did not grow when DMF was used as organic solvent, a specific activity of NDO of 0.0007 absorbance units min^{-1} $DO_{600}{}^{-1}$ was reached when indole was added in methanolic solution. However, this value was almost 60% lower than the NDO activity reached when naphthalene crystals were added into the culture media before inoculating, where the specific activity was 0.0017 absorbance units min^{-1} $OD_{600}{}^{-1}$.

Analyzing the specific rate of indigo production (NDO specific activity), we noted that the maximal value was achieved when J26 was previously grown in minimal MMJP medium, where 100% higher absorbance than those reached with cells grown in JPP medium were obtained (data not shown). Indigo formation was not detectable when indole concentrations lower

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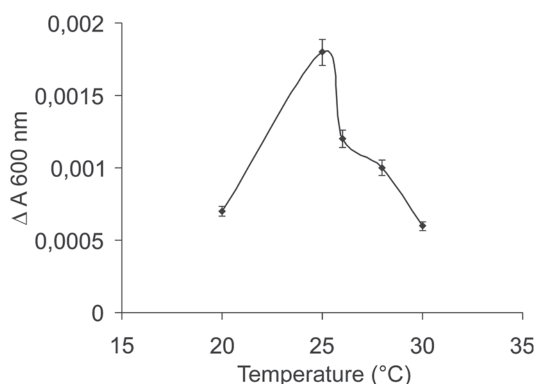

Figure 1. Specific activity of indigo formation was measured after adding indole and incubating at several temperatures within the mesophilic range.

than 1 mM were used, reaching a maximun at 2.5 mM. Activity dropped rapidly at higher concentrations, and was not detectable at 5 mM. This phenomenon could be due to the toxicity of the substrate, as previously reported [14]. Our results are comparable with those reported for *P. aeruginosa* Gs, that could tolerate and degrade up to 3 mM indole and *Pseudomonas* sp. strain ST-200 for which the minimal inhibitory concentration was 2.6 mM. [14, 15]. The highest NDO activity was achieved at 25 °C (Fig. 1), within the mesophilic range of optimal temperature of *Pseudomonas* growth and in accord with previous data obtained for indigo production by different *Pseudomonas* strains [16].

Although the highest specific activity (0.0035 absorbance units min^{-1} $OD_{600}{}^{-1}$) obtained by *Pseudomonas* sp. J26 NDO was achieved using minimal medium MMJP (Fig. 2), it was necessary to concentrate almost 10 ml of culture broth in late exponential-phase due to the low biomass reached in this medium. As a consequence, JPP medium was chosen for further assays of indigo production.

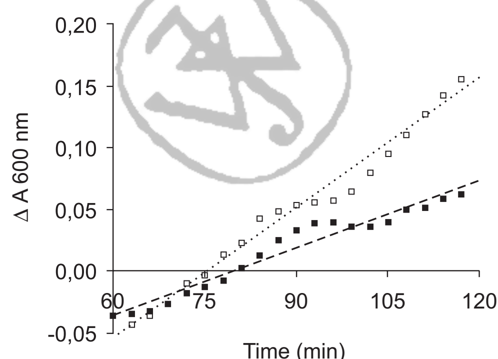

Figure 2. Rate of indigo formation by *Pseudomonas* sp. J26 whole cells grown previously in MMJP and JPP culture media. Filled symbols belong to JPP medium (R^2 = 0.9576) and hollow squares belong to MMJP (R^2 = 0.9837).

When the produced indigo was extracted from the cells using DMF in the vial scarification method, a maximum rate of indigo production (0.56 nmol min^{-1} dry mg $biomass^{-1}$) was achieved at 60 min from the beginning of the reaction and 75.5 μM indigo was produced in the next 8 h. However, the maximal indole concentration (138.1 μM) was reached after 20 h of incubation.

The specific activity of indigo formation reported in this work is lower when compared with recombinant *E. coli* expressing mono or dioxygenase genes [3]. However, specific production of indigo by *Pseudomonas* sp. J26 resulted to be almost 450% higher than those obtained by *Corynebacterium renale,* with 0.125 nmol min^{-1} mg dry cell $weight^{-1}$ [17] and comparable to the indigo production by *Pseudomonas putida* KT2442 [18].

Concluding remarks

In this work, we used a high-throughput and easy technique to determine NDO activity, which consisted in the oxidation of indole to yield indigo. Indigo formation was monitored spectrophotometrically at 600 nm in a 96-wells microplate reader and the formation rate was associated directly with NDO activity. In addition, indigo production was performed in a micro-batch culture from a whole cell system. To our knowledge, this is the first report of NDO activity measures using indole biotransformation and indigo production by a *P. plecoglossicida* related strain.

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