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Production of Indigotin in Submerged Culture Using *Morchella* nov. ES-1

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ABSTRACT

A new strain of the fungus *Morchella* nov. ES-1 (ATCC 20951) that produces blue pigment in submerged culture has been isolated. The blue pigment was extracted by chloroform or ethyl acetate treatment. The crude extract when chromatographed yielded three fractions; a blue one has been identified by mass spectrometry as indigotin. Fermentation studies using enriched media revealed that maximum accumulation of indigotin in culture was approx 24 mg/L in 168 h.

Index Entries: Indigotin; indirubin; fungus; blue pigment; mass spectrometry.

INTRODUCTION

In the course of a study of growing morel mushroom mycelium for food purposes, it was observed that one of our morel mushroom cultures (*Morchella* sp) diffused a blue pigment into the solid medium on which the mycelium was grown. The same blue pigment was observed in liquid media when the fungus was grown in submerged culture. This blue pigment has been identified as indigotin. The structure of indigotin is given in Fig. 1.

Indigotin, a blue-violet pigment long known to man, and which has been used as a dye, is produced mainly by plants, particularly by the leguminous plants of the genus Indigofera. Indigotin itself does not occur as such in plants, but is present as indican, a colorless, water-soluble glucoside. When indican is treated with water, it is hydrolyzed to glucose and indoxyl, which in turn is oxidized by air to indigotin, or, as it is often known, indigo dye.

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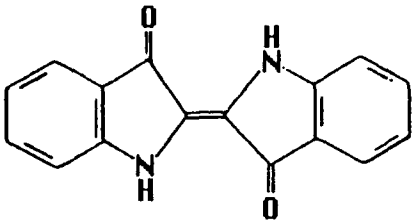

Fig. 1. Structure of indigotin.

Indigotin is also produced by certain bacteria. Several workers (*1–3*) have reported on the microbial production of indigotin in submerged culture by different bacterial strains (e.g., *Pseudomonas indoloxidans, Mycobacterium globarulum,* and *Micrococcus piltonensis*) isolated from soil by enrichment culture techniques, using indole as the sole source of carbon and nitrogen. Eliminating indole from the growth media results in no production of indigotin.

Genetic engineering techniques have been used recently (*4,5*) for the enzymatic and microbial production of indigotin using bacterial strains encoded for tryptophanase enzyme for producing indole in submerged culture in an indole-free media. The indole was then converted to indigotin using dioxygenase enzyme derived from *Pseudomonas putida* (*5*).

The microbial production of indigotin pigment by fungi or higher fungi in solid or submerged culture is a rare phenomenon, and only two other cases of indigotin production by fungal strains, both belonging to Basidiomycetes (mushroom), have been reported. Papazin (*6*) was the first to report on the production of blue pigment in a mutant strain of *Schizophyllum commune* Fr. This blue pigment was later identified by Miles et al. (*7*) as indigotin. Epstein et al. (*8*) and Swack et al. (*9*) also observed a red pigment during the extraction of indigotin from a mutant strain of *Schizophyllum commune* Fr. Strain No. 130, which has been identified as indirubin (a position isomer of indigotin). Average indigotin accumulation by *Schizophyllum commune* strain No. 130 in shake flasks using minimal medium was reported by Swack (*9*) to be in the range of 5–10 mg/L. Falangle and Bobbio (*10*) isolated a single mutant strain from *Agaricus compestris* Fr., which produced red and blue pigments that were tentatively identified as indirubin and indigotin. A brown pigment was not identified. No information on yield or accumulation was disclosed.

MATERIAL AND METHODS

Strain

The organism used in this study was a mutant mushroom strain designed as *Morchella* nov. ES-1 ATTC 20951. This mutant strain was isolated in our laboratory.

Chemicals

Synthetic indigotin (98%) and indole were purchased from Sigma (St. Louis, MO, USA). Ethyl acetate, chloroform, and hexane, all analytical grade, were purchased from J. T. Baker (Phillipsburg, NJ, USA).

Agar Media

The fungal strain was maintained on agar slants composed of 0.5% peptone, 0.5% yeast extract, 0.5% malt extract (all from Difco Laboratories (Detroit, MI, USA), 1.0% glucose, and 1.0% agar. After growth at 25°C, the culture was transferred and maintained at 4°C. Transfer of the culture on agar tubes was repeated every 3 wk in order to obtain viable best growing stock culture.

Inoculum

Inoculum for submerged culture was prepared by homogenizing the contents of a culture tube using a Tekmar® Tissumizer (Cincinnati, OH, USA) and transferring the homogenized seed to a 250-mL Erlenmeyer shake flask containing 100 mL of the following medium: 10 g/L glucose, 1.0 g/L $MgSO_4 \cdot 7H_2O$, 5.0 g/L casamino acid (Difco), 5.0 g/L yeast extract (Difco), 5.0 g/L Soytone (Difco), 6.7 g/L NH_4NO_3, 1.0 g/L KH_2PO_4, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 0.02 g/L thiamine HCl, and minerals 0.025 g/L $CuSO_4$, 0.054 g/L $FeCl_3 \cdot 6H_2O$, 0.085 g/L $MnSO_4 \cdot 4H_2O$, 0.144 g/L $ZnSO_4 \cdot 6H_2O$, and 0.2 g/L $NaO_4 \cdot 2H_2O$. The inoculum was shaken reciprocally at 27°C and 120 rpm, and the medium was adjusted to pH 6.4 before incubation. After 48 h of incubation (before the liquid medium changed color to blue), the inoculum mycelium was homogenized aseptically using the Tekmar® Tissumizer, and the mycelium from a single shake flask was used as seed culture for a fermentor.

Fermentation and Growth Conditions

Fermentation growth curves were obtained either from 500 mL laboratory shake flasks containing 200 mL of media, or from a 15 L fermentor (Biolaffite) containing 10 L of liquid vol. Medium was essentially the same as described above except derivation in glucose or casamino acid amounts added during experiments. Cultivation in a fermentor was performed at an agitation speed of 300–400 rpm, an aeration rate of 1 vvm, and at temperature of 28°C. Dry mycelium weight was determined at regular intervals in the usual way. The change in pH and glucose consumption were also followed side by side during the course of fermentation by removing samples (50 mL) at various time intervals from the fermentor.

Mycelium Extraction Procedure

Pellet mycelium (100 g) was washed twice with 0.5 L distilled water and blended for 30 min in a Waring® blender. The resultant suspension was again filtered and the filtrate was placed for 24 h in a vacuum dryer at 35–40°C for drying. The dry mycelium was ground in a waring blender and the mycelium was extracted three times with ethyl acetate (0.5 L), which removed a dark blue color extract. The blue pigment extract was evaporated almost to dryness, yielding a dense blue extract.

Purification

The pigment extract was purified by dissolving the pigment extract obtained in 100 mL of ethyl acetate and loading it on a silica column (Silica PAE 1000 chromatographic grade silica, Amicon Division (Danvers, MA, USA), using a mobile phase of 50% ethyl acetate and 50% hexane. Four different colored fractions appeared: blue, red, yellow, and green. The blue and red fractions were collected and evaporated under vacuum to yield two 50-mL portions of very intensive concentrated dark blue and red fractions.

Colorimetric Analysis

In order to monitor blue pigment (indigotin) synthesis in the mycelium during cultivation, the following assay for pigment analysis was used. Culture sample was removed at various time intervals from the fermentation broth. Mycelium was removed by filtration and dried overnight in an oven. A 1.0 g sample of dried mycelium was extracted with 20 mL of ethyl acetate for 4 h, centrifuged to remove mycelium, and 1.0 mL of the upper (ethyl acetate) layer was transferred to a quartz cuvet. The optical density of each extract at 605 nm was measured to determine the amount of indigotin produced in the mycelium during cultivation. The readings obtained were compared with a standard reference curve of indigotin in ethyl acetate made from a series of solutions containing known concentrations of indigotin. The same procedure was repeated using 10 mL of filtered fermentation broth in order to determine indigotin produced in the medium during cultivation.

RESULTS AND DISCUSSION

Location of the Blue Pigment in Solid Culture

Microscopy revealed that the pigment is located in the wall of the hypha. More precisely, the pigment is found in two forms: in some filaments, the pigments form large granulations that are located outside the

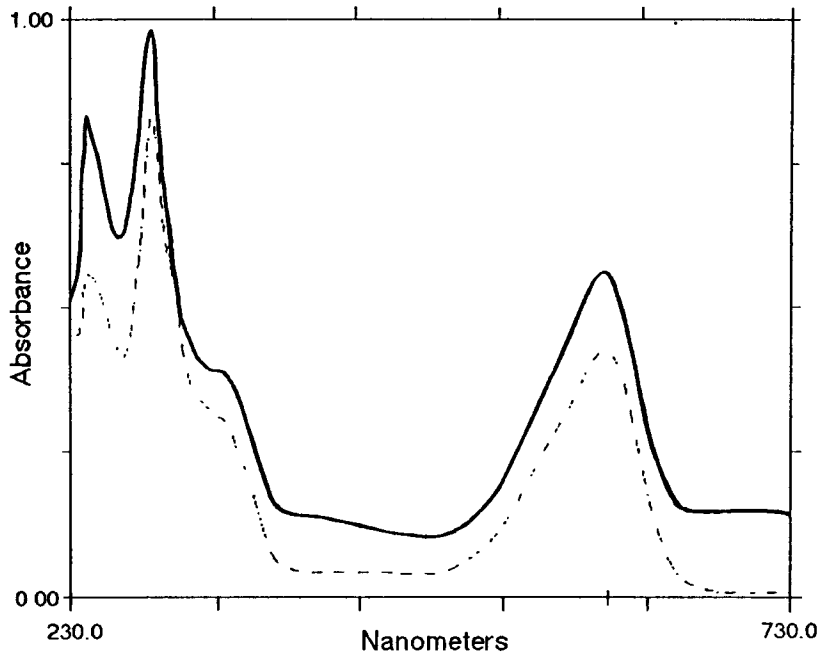

Fig. 2. Ultraviolet adsorption spectrum of indigotin extracted with ethyl acetate from the culture medium of pigmented strain *Morchella* nov. ES-1. Upper spectrum: blue pigment. Lower spectrum: synthetic indigotin.

mycelium, whereas in others, it appears inside the hypha as a continuous layer of granules. The pigment was practically insoluble in water, ethanol, and methanol, soluble slightly in acetone, hexane, and benzene, yielding purple solutions with various degrees of a reddish tint. Extraction with chloroform or ethyl acetate yielded a dark blue solution that was very stable.

Identification

UV-Spectrometric Analysis

The blue fraction that was obtained from the silica column, as described in Materials and Methods, was evaporated and the dry blue pigment obtained was identified as indigotin by comparing its UV spectrum in ethyl acetate with pure indigotin obtained from Sigma (Fig. 2) using a Gilford Response UV-VIS Spectrophotometer. The second red/purple pigment fraction that was purified by chromatography on silica column could be tentatively identified as indirubin, since its absorption maximum (Fig. 3) is at 550 nm, which is characteristic of indirubin (*7,9*).

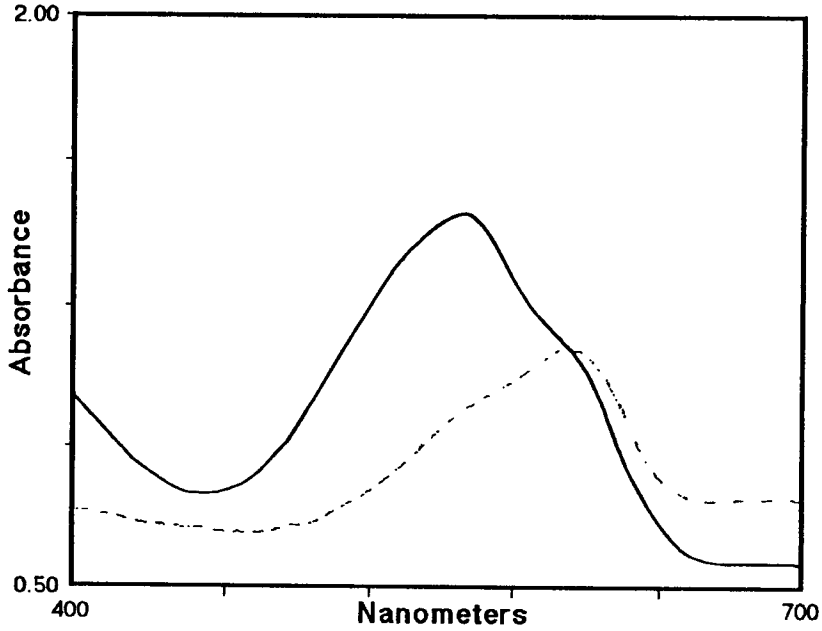

Fig. 3. Ultraviolet absorption spectra of indirubin extracted with ethyl acetate from the culture medium of pigmented strain *Morchella* nov. ES-1. Upper spectrum: indigotin. Lower spectrum: indirubin.

Mass Spectrometric Analysis

A chloroform extract of the blue pigment obtained from *Morchella* ES-1 mycelium was chromatographed on a silica gel column as described in Materials and Methods, and the blue band was collected. The collected sample was analyzed by Probe-MS in the electron impact mode on a Finnigan TSQ-70 mass spectrometer. The sample was heated from 50°C to 350°C at the rate of 30°C/min during the experiment. Mass spectrometric analysis of the blue pigment revealed that the blue pigment is indigotin. For further identification, pure indigotin was compared to the blue pigment (Fig. 4). The remarkable similarity between the two mass spectra clearly indicates that the blue pigment extracted from *Morchella* ES-1 is indeed indigotin.

Indigotin Production

Figure 5 presents the kinetics of indigotin production in a fermentor using enriched media. Curve A presents the amount of indigotin released in the culture broth and curve B presents the amount of indigotin accumulated in the fungal mycelium. Pigment production in the fermentor began at about 48 hours. At 72 h of cultivation, the broth medium begins

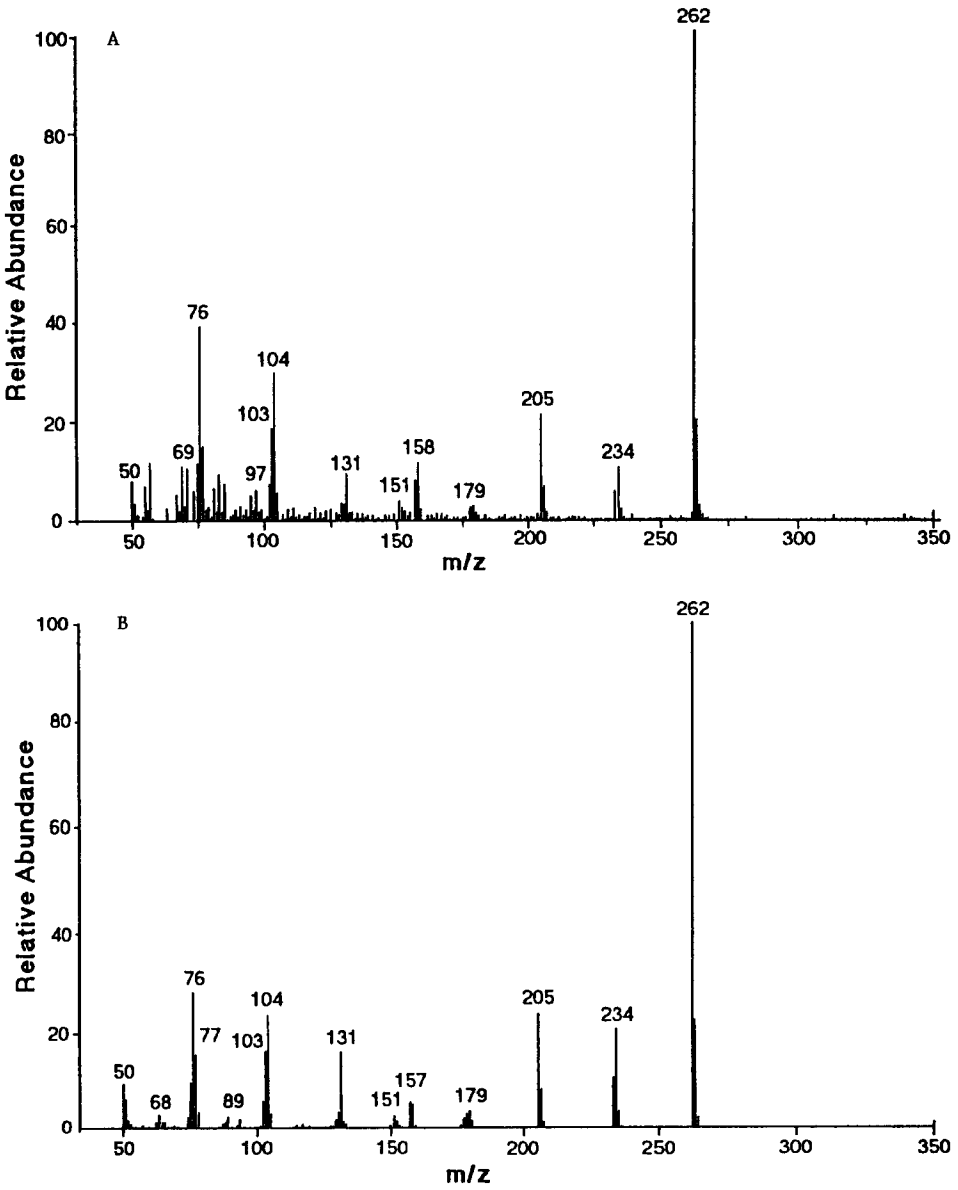

Fig. 4. Comparison of mass spectra (Probe-MS) of (A) blue pigment extracted from *Morchella* nov. ES-1 and (B) pure indigotin.

to change color from yellow to dark green blue. Indigotin concentration increased significantly only after glucose was completely exhausted, approximately after 7 d of incubation. The pH of the culture also decreased slowly and the maximum quantity of indigotin was produced when the pH of the culture medium reached pH 5.3.

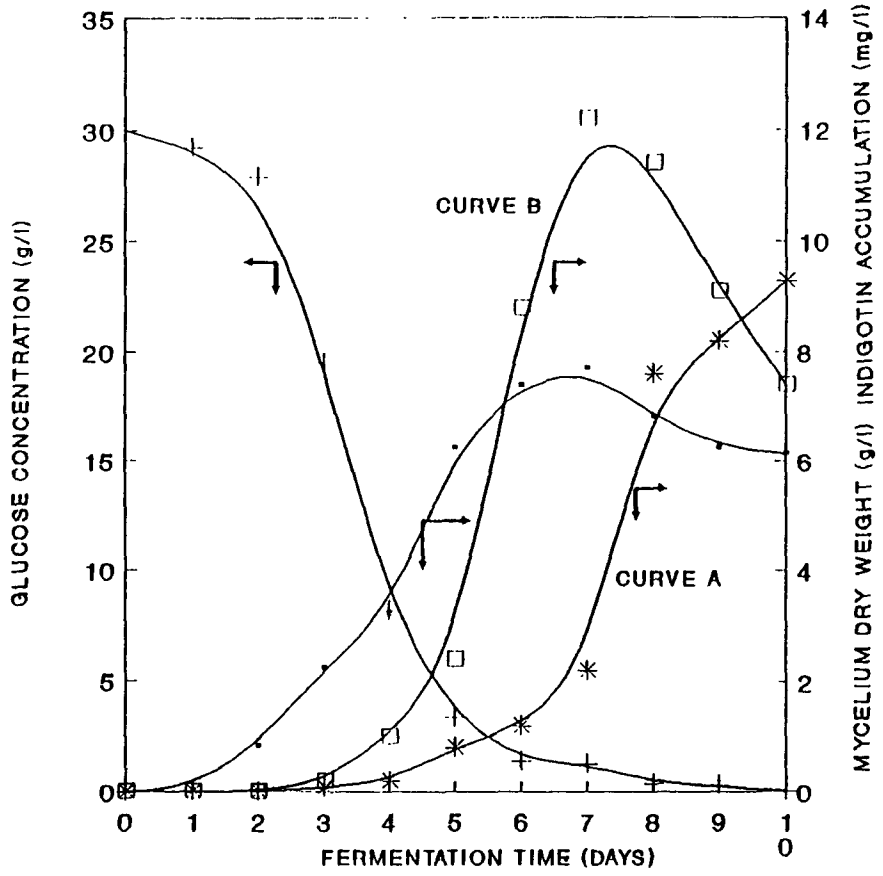

Fig. 5. Growth curve of *Morchella* nov. ES-1 in 15 L fermentor. Initial glucose concentration 30 g/L, initial pH 6.4, cultivation temperature 28°C. Curve A represents indigotin accumulation (mg/L) in fermentation broth and curve B represents indigotin accumulation (mg/L) in mycelium.

Effect of Various Concentrations of Carbon Source

The effect of various concentrations of glucose was studied using shake flasks with different concentrations of glucose. As can be seen from Fig. 6, indigotin production did not increase as expected in the regime of 3–6% (w/v) of glucose in the medium. However, there was a direct relationship between glucose and growth since the mycelium dry wt continued to increase while indigotin production was constant. Above 4% glucose concentration, no further significant increase was observed in mycelium production. Higher dry mycelium weight was obtained in the fermentor than in shake flasks. It seems that the formation of pellet mycelium (4–6 mm) in shake flasks limits the diffusion of nutrients and oxygen from the

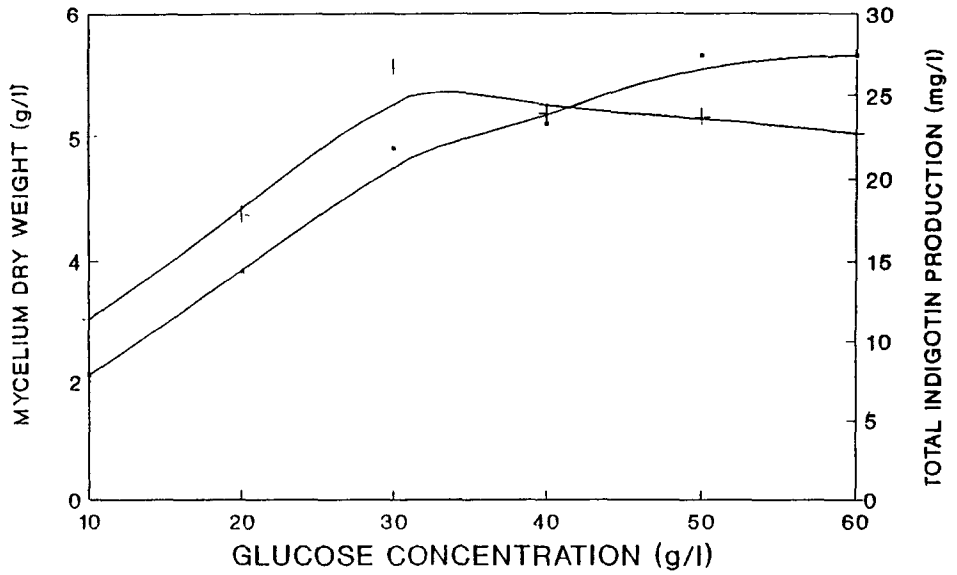

Fig. 6. Effect of initial glucose concentration on indigotin production. *Morchella* nov. ES-1 culture was grown on shake flasks (250 mL) at 30°C, at initial pH 6.0. Total indigotin production in a single shake flask and mycelium dry wt were analyzed after 168 h of incubation. —●— dry wt (g/L); —+— indigotin (mg/L).

medium into the pellets, which results in lower dry wt. Interestingly, no difference in indigotin accumulation (at 168 h) was observed in the cultivation between shake flasks and the fermentor.

Effect of Casamino Acid

When various concentrations of casamino acid ranging from 0.2 to 2.0% (w/v) were added to the growth medium in the presence of 30 g/L glucose, then indigotin production increased almost linearly until 1% (w/v) of casamino acid was added; above this concentration, it was constant. The mycelium dry wt increased linearly over the whole range of casamino acid levels, from 1.2 g/L with no casamino acid to 6.9 g/L with 2% (w/v) casamino acid.

Effect of Indole

Indole was of interest in this study because it was thought that one of the precursors for indigotin synthesis in this particular fungus can be the pathway, indole→tryptophan→indigotin, which is found in bacteria. Shake-flask experiments using enriched medium with addition of indole in the range of 0.01–1 g/L results in the inhibition of the blue pigment production and dry wt of the mycelium. At 0.2 g/L of indole, no growth of mycelium and pigmentation occur.

Economic Factors

To date, indigotin is produced mainly by chemical synthesis. Attempts to use bacteria for microbial synthesis of indigotin in large scale have failed owing to unfavorable economic factors involved in providing indole as a substrate (*5*). Using fungi can therefore be very attractive because, unlike indigotin-producing bacteria, fungi do not require an exogenous supply of indole for indigotin synthesis. In fact, indole is toxic to them in low concentrations as is shown in this work. However, practical application of a fungi, such as *Morchella* nov., probably will not be economically feasible because it takes the fungus at least two days to produce detectable amounts of indigotin, whereas bacteriam require only about three hours (*3*), and the maximum production and yield is very low. It is therefore important to obtain information about the metabolic pathway of synthesis of indigotin in indole free media using fungal strains. Although this study did not produce any information that would, of itself, suggest a plausible biosynthetic pathway for the production of indigotin, it is postulated that the pigment production is dependent on the amino acids present in the medium (such as tryptophan), which leads to the accumulation of indigotin. Further investigations of the metabolic pathway of indigotin using tryptophan and other amino acids for this particular fungus are planned.

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