



Carbon – Science and Technology

ISSN 0974 – 0546

<http://www.applied-science-innovations.com>

RESEARCH ARTICLE

Received:13/10/2015, Accepted:12/12/2015

Comparative study of production of Bio-Indigo by *Pandoraea sp.* in a two phase - fed batch and continuous bioreactor

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Abstract: Indigo, is blue of blue jeans, a synthetic dye used on large scale all over the world. Chemical production of the dye is taking a new route towards bacterial production to overcome the environmental effects that are posed by the synthetic blue powder (Indigo). In the present work a strain *Pandoraea sp.* isolated from the oil contaminated soil is found to produce blue pigment which is analyzed qualitatively as indigo using UV-visible scan and Thin Layer Chromatography (TLC). The strain is used for indigo production at lab scale in two different bioreactor configurations first the fed batch mode and second continuous mode using two phases. The two phases consisting of medium carrying biomass and the second phase of silicone oil carrying substrate indole. The use of second phase allows higher concentration of substrate injection reducing the inhibition effects of the substrate as well as act as a partitioning agent for removal of the product. In two phase study, the maximum indigo produced was seen to be 0.068 g/L after 22 hours of substrate injection into the Fermentor in a fed batch mode. The maximum yield obtained in this configuration was 19%. For commercial production of bio-indigo a continuous operation is required, which was studied in a bioreactor with 2.5 liter capacity under the optimized conditions. The maximum indigo produced was found to be 0.052 g/L after about 72 hours of operation. The results showed decrease in the production of indigo in continuous mode as compared to fed batch operation, which may be due to the insufficient time available for the bacteria to bio-transform indole into indigo.

Keywords: Continuous reactor, Fed batch reactor, Indigo, Indole.

1. Introduction: Indigo a blue dye has its presence in lives of people for around many centuries, but today its presence and importance in modern fashion has grown and growing toward being one of the oldest and finest dyes still in great demand. A mixed effect of violet and blue, Indigo colour's beauty has been loved by many cultures, royals and artists till date. Even today, it continues to mesmerize and inspire the designers across denim, apparel and interiors. From its origins as a naturally produced dye, most common in India, to its ubiquitous appearance in blue jeans today, indigo has travelled far and wide [1]. Indigo which is one of the oldest known natural dyes [2] is a derivative of the colorless glucosides of the enol form of indoxyl, e.g. indican (indoxyl-D-glucoside) [3]. The indoxyl further dimerises by air oxidation to the blue indigo [4]. Indigo (Figure 1) having molecular formula $C_{16}H_{10}N_2O_2$ was produced from plants but now synthesized chemically, posing environmental hazards [5].

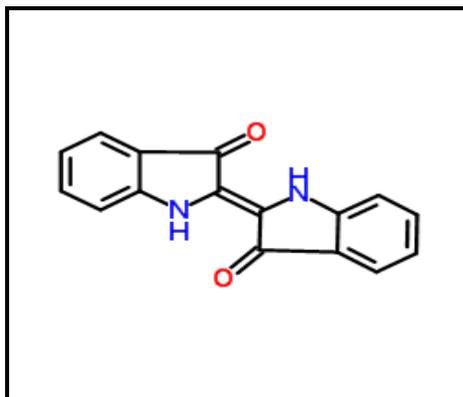


Figure (1): Sketch of the molecular structure of Indigo [6].

The chemical synthesis of indigo has resulted into health problems to workers along with severe environmental pollution due to toxic materials and catalysts used and the toxic by-products and waste water produced and released since past years [7]. To overcome the problems related with the use of chemical indigo on large scale, various studies and research is done worldwide. The study is now focused on production of Bio-indigo, dye which will have natural characteristics, environmental friendly, and can be produced on large scale. Natural extraction of indigo from plant is a tedious, uneconomical and unrealistic process of producing natural indigo, thus an approach to produce indigo dye using bacteria which will pay way to greener chemistry is researched today. Microbial biosynthesis of indigo has been studied since 1928 [2] and from then, reports are posted by researcher all over the world to produce indigo from substrate indole using bacteria [8]. But the main apprehension in microbial indigo production is its lower productivity as compared to chemical synthesis [9]. Also the process of indigo biosynthesis is not economically feasible owing to the very low solubility and the high toxicity of indole, substrate loading needs to be increased, and the toxicity of indole has to be minimized to make the process feasible [10]. Thus, a biphasic system consisting of two immiscible phases has been used to overcome both the limitation in this process [11]. Traditionally, in most of the two phase reactors pure solvents has been used as a second phase [12]. These systems are said to be self regulatory [13] and helps to separate the biodegradable products once formed in the Fermentor [14]. To produce bio-indigo at industrial level different reactor configuration can be used. In addition, metabolic engineering could be successfully applied to solve a problem far downstream of the bioreactor in production of important commodity chemicals [15]. Many industrially important bioreactor operations involving microbial and animal cells are carried out in fed-batch mode [16]. With this literature background, the present studies aimed at isolating the indigo producing bacteria and optimize the indigo production using two phase comparative study between fed batch and continuous bioreactor, to get the best yield of the product.

2. Materials and methods: All chemicals used in the experiments were of analytical grade purchased from Himedia, Mumbai, India. Bio-Reactor of Bio-Ferm was purchased from Zenith Engineers, Agra, India. The solvent – ethyl acetate used was of analytical reagent grade from SD fine chemicals, Mumbai, India. Silicone oil LR from S. D. Fine chemicals, India.

3. Experimental procedure:

a. Isolation of Indigo producing bacteria: The bacterium isolated from oil contaminated soil was found to be *Pandora sp.* using DNA extraction technique and was capable of producing a blue pigment, analyzed to be indigo [17]. The confirmation of indigo production was done using thin layer chromatography and UV visible spectrophotometer.

b. Two phase fed batch bioreactor study: The indigo producing strain was used in bioreactor using two phases. The first phase used was a BH medium carrying the bacteria whereas the second phase was silicone oil an immiscible phase which acted as a carrier for indole as well as a solvent for extraction of product from the broth. For the study, experiments were conducted in a Bioreactor having total volume of 2.5 liters and working volume of 1 liter. The reactor was maintained at 7 pH and temperature at 30 °C with 800 ml of medium. The stirrer speed was maintained at 180 RPM and Dissolved oxygen at 100 %.

Initially the culture was grown separately in an Erlenmeyer flask; and was then inoculated into the reactor for growth in presence of 1 % diesel oil as a carbon source. The culture was allowed to grow into the reactor up to mid log phase and then was injected with premixed solution. The solution consisted of 3 mM of indole into 200 ml of medium and 200 ml of silicone oil in a well mixed condition on a magnetic stirrer. The oil added into the reactor acted as a carrier for indole to the bacteria increasing the bioavailability of the substrate to the bacteria. After about 22 hours of operation culture was seen to turn blue with formation of indigo. Thus to quantify the indigo formed some oil was removed along with 200 ml of culture for analyzing the growth of the bacteria. Using a spectrophotometer the amount of indigo formed and growth was found out.

Further the experiment was continued by adding the solution of indole in medium into the Fermentor, and after specific interval of time the parameters were analyzed. Thus the Fermentor was operated in a fed batch mode with input of feed solution on continuous basis but the product withdrawal (oil phase) was done only after 162 hours of operation.

c. Two phase continuous study: The continuous operation of reactor is required for industrial production of product. Thus the objective of this work was focused on industrial bio- indigo production as well as to overcome the limitation of saturation of indigo into oil phase as in fed batch mode of operation.

The continuous operation experiment was conducted under same condition as the fed batch operation. In this experiment, after the growth of culture in the reactor, the substrate solution was injected carrying 3 mM indole. After 24 hours the indigo formation was observed and from this point a continuous operation was started. For continuous study two peristaltic pumps were used, one pump was used to feed the solution carrying substrate with silicone oil and another pump to withdraw the product (Figure 5). The flow rates of both the streams were maintained at 16 ml per hours. The product was withdrawn from top and the substrate solution was injected at the bottom of the reactor. After every 24 hours the product sample collected in the flask was allowed to stand and analyzed for indigo produced and bacteria growth using spectrophotometer.

4. Results and Discussion

a. Isolation of indigo producing bacteria : As per reference [17], it is found that the bacterium isolated from oil contaminated garage soil is *Pandoraea sp.* which is capable of producing indigo dye. The blue colour (Figure 2) was analyzed and found to be Indigo using thin layer chromatography and UV- visible spectrophotometer.



Figure (2): Tubes showing formation of bio- indigo in the medium by *Pandoraea sp.*

b. **Two phase fed batch bioreactor study:** The scale up of indigo production was done from shake flask to bio-reactor (Figure. 3) under optimized condition (temperature 30 °C, pH 7). In this experiment the fed batch mode of operation was used to produce indigo.



Figure (3): Bioreactor showing the production of indigo in the oil phase.

The first trace of indigo was found 19 hours after the first injection point of substrate and oil. Further it was found that there was increase in indigo production up to 2 fed point which was quantified only from the top oil layer. But later there was overall drop in indigo in the oil phase (Figure 4).

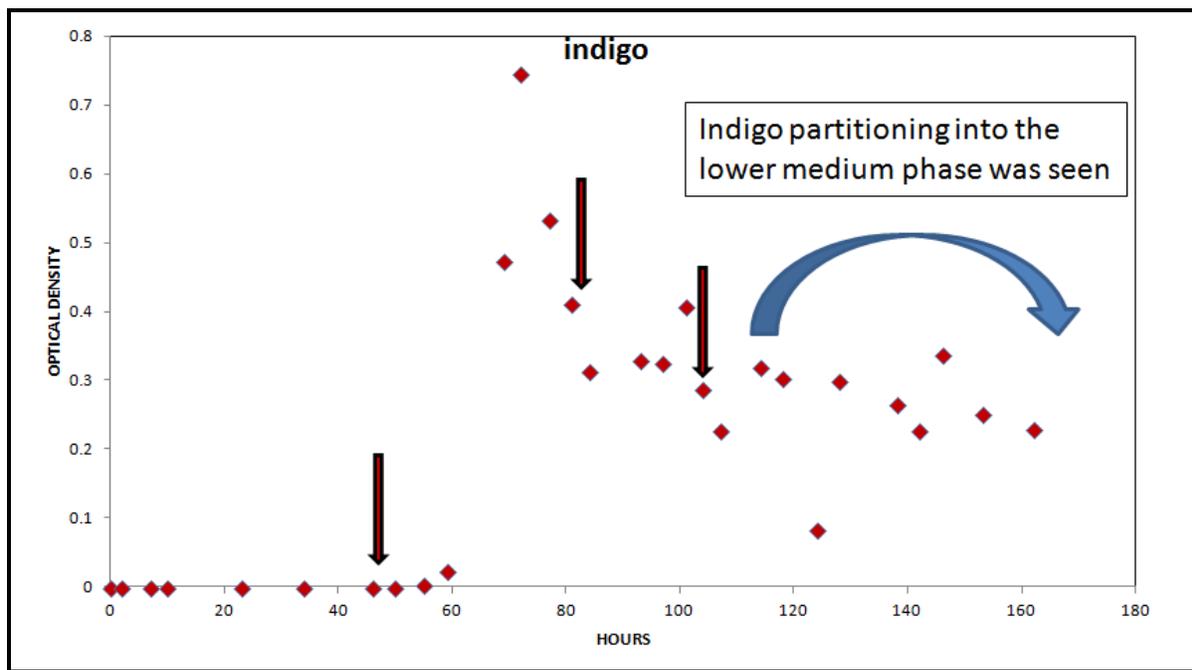


Figure (4): Indigo production in two phase bioreactor using fed batch operation.

In this study, the maximum indigo produced was seen to be 0.068 g/L after 22 hours of substrate injection into the Fermentor, resulting into maximum yield of 19 % in this configuration. Later, dye was found to partition into the medium which changed the colour of the medium to greenish-blue (Figure 5). This resulted into reduction in the optical density (OD) for indigo in the oil phase. Thus, from the experiment it was concluded that silicone oil can be used as second phase to increase the yield, but the oil part needs to be removed on indigo production to prevent saturation and for continuous production of the product.



Figure (5): Bioreactor medium seen to turn bluish green.

c. Two phase continuous bioreactor study: To overcome the limitations of oil phase saturation and to produce indigo on continuous basis, a continuous operation of reactor was done after the first batch production of indigo in the reactor under optimized condition (Figure 6).



Figure (6): Continuous production of indigo in two phase bioreactor.

In this study there was continuous growth of the bacteria due to continuous availability of substrate indole but the indigo production was found to be less as compared to fed batch mode of operation. The maximum indigo produced was found to be 0.052 g/L after about 72 hours of operation (Figure 7). The results showed decrease in the production of indigo in continuous mode as compared to fed batch operation, may be due to the insufficient time available for the bacteria to bio-transform indole into indigo.

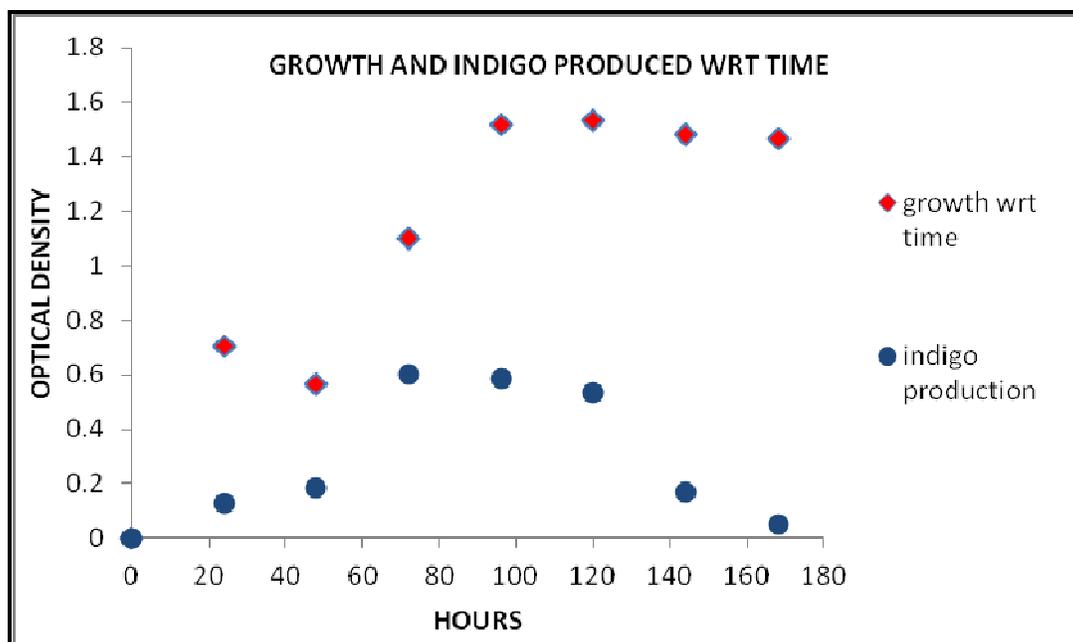


Figure (7): Bacterial growth and indigo produced in continuous two phase bioreactor.

5. Conclusions: In the present study *Pandoraea sp.* was used for indigo production in bioreactor using two different configurations. The work resulted into production of more amount of indigo in a fed batch mode than obtained in a continuous operation of bioreactor. Thus a fed batch mode can be used further for bio-indigo production for industrial basis. The bacteria and other results from this work can be

further exploited for large scale production of dye, which in great demand. Though the microbial bio-indigo production is not as competent as the present chemical synthesis but surely it is an alternate environmentally safe route of production of such commodity chemical.

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