Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 4 (3): 82-88, 2009
ISSN 1816-4935

Biocatalytic Production of a Commercial Textile Dye (Indigo) from a Xenobiont

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Abstract: A Gram negative rod SCV1 was isolated from oil contaminated garage soil. This bacterial strain was used for the production of indigo-a commercial textile dye after induction on xenobiotics like diesel, naphthalene and salicylate. The specific rates of indigo formation are 0.30, 0.38 and 0.35 mg mL^{-1}×h for diesel, salicylate and naphthalene induced bacterial strain SCV1. The bacterial strain SCV1 was hydrophobic in nature as evident from hydrophobicity measurements. Hydrophobic nature gives the advantage to the bacterial strain in adhering to the hydrocarbons. The results of the indigo production by different substrates induced bacterial strain SCV1 suggest that the diesel induced the maximum at 1.75 and 2 mM concentrations. It is also suspected that the uninduced culture i.e., SCV1 enriched on nutrient broth produced other indigoid compounds other than indigo.

Key words: Biocatalysis, xenobionts, indigo, gram negative, hydrophobicity

INTRODUCTION

Biotechnology equips us with the capacity to synthesize materials like pharmaceuticals, dyes, polymers etc., and to biodegrade unwanted materials. The chemical industry is interested in biocatalytic processes for commercial applications as the applications range from the development of microbial processes for specialty and commodity chemicals such as indigo, to the use of bacterial proteins as biochips in future microprocessors.

Pigments extracted from plants, animals and insects have been used as natural dyes. There are primarily four sources from which natural dyes are available namely specialized plant and animal sources by products (especially lac dyes), chemical synthesis and tissue or cell culture by DNA transfer (Lee *et al*., 2008). Blue jeans are an integral part of the youth and at the molecular level, the main components that causes blue colour are carbon and hydrogen (Molecular formula $C_{16}H_{10}N_2O_2$). The organic compound that causes the blue colour to the jeans is called as indigo. Indigo is one of the oldest dyes (Ensley *et al*., 1983) and is still used worldwide for textiles, with an annual production of 22×10^3 tonnes, worth U.S. $ 200×10^6 (Wick, 1995). Indigo is an example of a class of textile dyes known as vat dyes, the term vat referring to the vessel used to ferment the indigo leaves. Vat dyes are typically insoluble in water and must u ndergo a chemical reaction to be converted to a water-soluble form (called the leuco-base form) that effectively penetrates and interacts with the textile fibers.

Indigo can be extracted from many different plant species found all over the world and its use as a natural dye has been recorded since at least 2500 B.C. As is true with many natural dyes, the concentration of dye can vary widely from plant to plant. Due to this natural variation, it is often difficult to obtain reproducible colors from one batch of dye to the next. Thus, for industrial purposes,

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such as the large-scale production of blue denim, synthetic dyes are preferable to natural dyes. Indigo was first synthesized in the laboratory by a German chemist, although this synthesis was not suitable for use on an industrial scale. A number of chemists continued to investigate the synthesis of indigo over the next 20 years with the discovery of an industrial process in the early 1890's. Commercial production of indigo began in 1897. By 1900, synthetic indigo production equaled the yield of farming 250,000 acres of indigo-containing plants. While the production of synthetic indigo contributed to the explosive growth of the German chemical industry at the turn of the century, the fallout was not so favorable for indigo farmers in India and other parts of the world.

More recently, bacterial systems for commercial indigo production have been developed by Murdock *et al*. (1993), which were inspired by the discovery that recombinant *Escherichia coli* expressing naphthalene dioxygenase from *Pseudomonas putida* PpG7 in rich medium resulted in the formation of indigo (Ensley *et al*., 1983). Various monoxygenases and dioxygenases growing on aromatic hydrocarbons have been identified that are capable of indole oxidation to form indigo (Ensley *et al*., 1983; O'Connor and Hartmans, 1997, 1998; Bhushan *et al*., 2000; Gillam *et al*., 2000) and these biological processes are inherently safer than the chemical processes since they do not produce toxins such as aromatic amines (bladder carcinogens) and cyanide (Frost and Lievense, 1994; Wick, 1995).

The present research work was aimed at isolating xenobiont which could be used for the production of commercial textile dye after inducing with various xenobiotic compounds (Indigo).

MATERIALS AND METHODS

All the chemicals used were of highest purity grade available.

Mineral Medium

Bushnell Haas (BH) medium was used for all the experiments in this work. The composition of BH medium is (g/L) KH_2PO_4-1.0, NH_4NO_3-1.0, $MgSO_4.7H_2O$-0.2, $FeCl_3$-0.05, $CaCl_2.2H_2O$-0.02 and at a pH of 7.2.

Enrichment of Culture

Soils from pristine environment as well as oil contaminated environment were selected for isolation of hydrocarbon degrading bacteria. Soil samples were collected from agriculture field, college campus and nearby motor garage. After collecting soil samples, one gram of soil from each sample was mixed with 10 mL of mineral medium and kept on a rotary shaker in a horizontal position at 150 rpm for 30 min. After 30 min, 5 mL of supernatant from each sample was inoculated into 100 mL of BH medium, respectively with 1% diesel oil as the sole carbon source.

The growth of the different soil samples with diesel as the sole carbon source was monitored (every 24 h) by measuring the optical density at 560 nm. After 4 days of enrichment, 5 mL of different culture were transferred to fresh medium. The enrichment of the cultures was repeated thrice. Based on significant increase in cell growth, the enrichment culture from oil contaminated garage area was selected.

Isolation of Bacteria

The enriched culture on diesel was plated on nutrient agar plates for strain isolation. The dominant colony of the agar plates (one colony named as SCV1 out of three) was used for further studies. The selected bacterial strain SCV1 was subjected to Gram's staining and the morphology was checked under 100X of compound microscope.

Hydrophobicity of the Bacterial Strain SCV1

Five milliliter of each enrichment cultures and 5 mL of diesel were taken in 20 mL test tubes. Initial OD at 560 nm of the culture was measured and the test tubes were vortexed vigorously for 1 min. After vortexing, the diesel and the aqueous phase were allowed to separate for 30 min. The aqueous phase was then carefully removed with micropipette and then OD of the aqueous phase at 560 nm was measured. Hydrophobicity is discribed by Zhang *et al*. (1997) and calculated as follows:

$$\text{Hydrophobicity} = 100 \times \frac{1 - \text{OD of the equation Phase}}{\text{OD of the equation Phase}}$$

Indigo Production

The bacterial strain SCV1 was inoculated into BH medium with either naphthalene (20 mg L^{-1}) or salicylate (20 mg L^{-1}) or diesel (1%) as the sole carbon source and incubated at 30°C. Every 24 h, 2 mL of culture was withdrawn and cells were pelleted by centrifugation at 10000 rpm for 10 min. The pellet was resuspended in 5 mL of BH medium along with 2 mM Indole and 4.3 mM Glucose. The vials were incubated at 30°C and after 24 h the indigo concentration formed was quantified.

Prior to this experiment indole dissolved in acetone were added to dry glass vials. Acetone was allowed to evaporate and the medium was added to carry out indigo production experiment as described above.

The rate of indigo production by the bacterial strain SCV1 were also determined at different concentrations of indole (1, 1.25, 1.50, 1.75, 2, 2.50, 3, 3.5 and 4 mM). This experiment was done individually for naphthalene induced, salicylate induced and diesel induced bacterial strain SCV1.

Indigo Extraction

The whole culture broth (5 mL) was mixed and shaken with equal volume of ethyl acetate in a 20 mL glass vial. The mixture was vortexed vigorously for 2 min and this was repeated thrice. After vortexing the vials were kept in the freezer overnight. After overnight storage, ethyl acetate fractions (which are not freezed due to much lower freezing point than water) for spectrophotometric quantification of indigo concentration. The medium gets freezed and the organic solvent (ethyl acetate) does not freeze as the freezing point is much lower than water. The indigo concentration in the ethyl acetate extracts which is formed when the indole is oxidized in the presence of dioxygenase enzyme was quantified at OD 600 nm by comparing to a standard curve for Indigo. Standard curve is prepared using the commercially available indigo.

RESULTS AND DISCUSSION

Enrichment of Cultures

Three different bacterial consortia i.e., soil from oil contaminated garage soil, from agricultural field and soil from college campus were isolated and enriched on diesel as the sole carbon source. Based on the increase in optical density (560 nm) of atleast 0.1-absorbance unit, the bacterial strain from oil contaminated garage soil was selected (Fig. 1). The ability of the bacterial strain from oil contaminated garage to grow better on diesel compared to other bacterial strain may be due to the pre exposure of the bacterial strain to the oil in its natural environment. From the bacterial consortium of oil contaminated garage area a gram-negative rod was isolated and named as SCV1. This strain was the dominant strain when the sample from the garage area was plated on nutrient agar. This strain alone was used for further studies.

Growth of SCV1 on Diesel and Naphthalene

The screening procedure had strongly selected for bacterial consortia that was able to grow on diesel. The dominant bacterial strain SCV1 from oil contaminated garage soil was tested for its growth

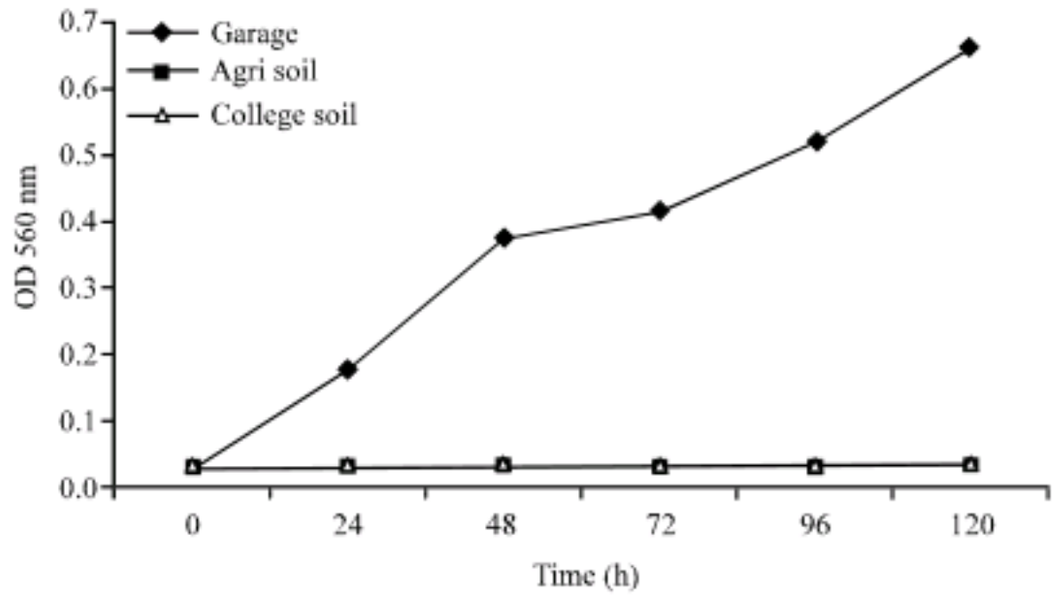

Fig. 1: Enrichment of Different Soil Samples on Diesel

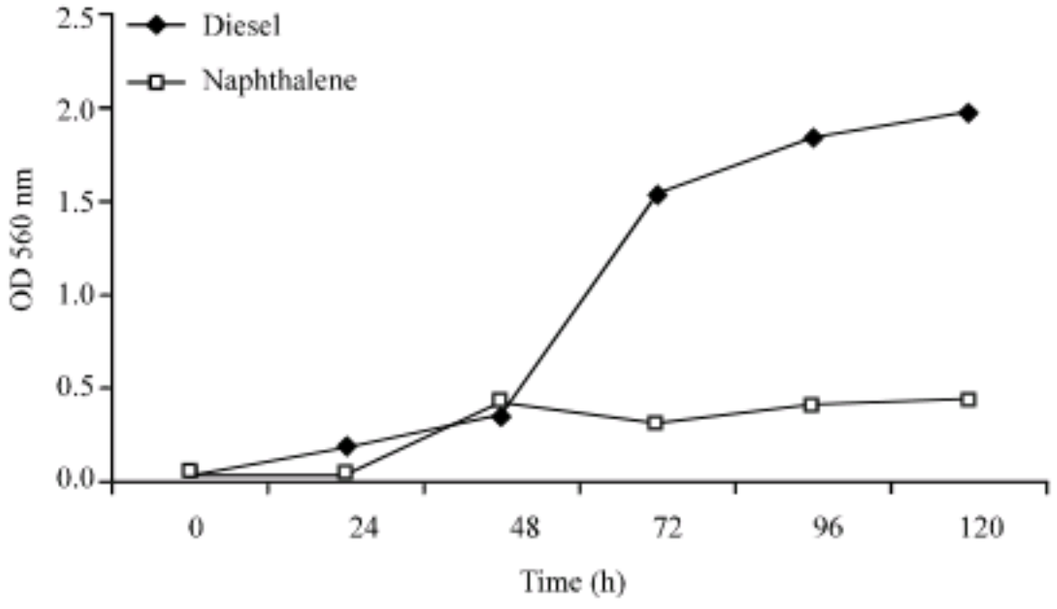

Fig. 2: Growth of bacterial strain SCV1 on diesel and naphthalene

on varying hydrophobic substrates like diesel and naphthalene (Fig. 2). The OD of the bacterial strain SCV1 on diesel as the substrate increased from 0.033 to 1.955 in 120 h. The OD increased to 0.424 on naphthalene in 48 h from an initial OD of 0.033. The result showed maximum increase in growth when diesel was used as a substrate than naphthalene. This may be due to the fact that the mixture of hydrocarbons present in diesel will have a better water solubility than a single hydrocarbon like naphthalene. During growth of the bacterial strain SCV1 on hydrophobic biphasic forming substrate - diesel, adherence of the bacterial strain SCV1 to the oil has been observed. Cell attachment to hydrophobic substrates is of great significance and even a prerequisite to microorganisms from enhanced substrates uptake (Deziel *et al*., 1999; Wick *et al*., 2003). The hydrophobic constituents of the bacteria play an important role in adherence to the interfacial area of a second phase.

Screening for Indigo Production

The results obtained from Indigo formation studies, following induction with diesel, salicylate and naphthalene is shown in Fig. 3. The results are for every 24 h till 72 h. The specific rates of indigo formation induced by different substrates are 0.30 mg mL^{-1}×h for diesel induced cells, 0.38mg mL^{-1}×h for salicylate induced cells and 0.35mg mL^{-1}×h for naphthalene induced cells. The amount of Indigo formed at 48 h is 2.39 mg indigo/g dry wt. of cells when induced by diesel. Induction by salicylate and naphthalene formed 3.8 mg indigo/g dry wt. of cells at 48 h. The above results

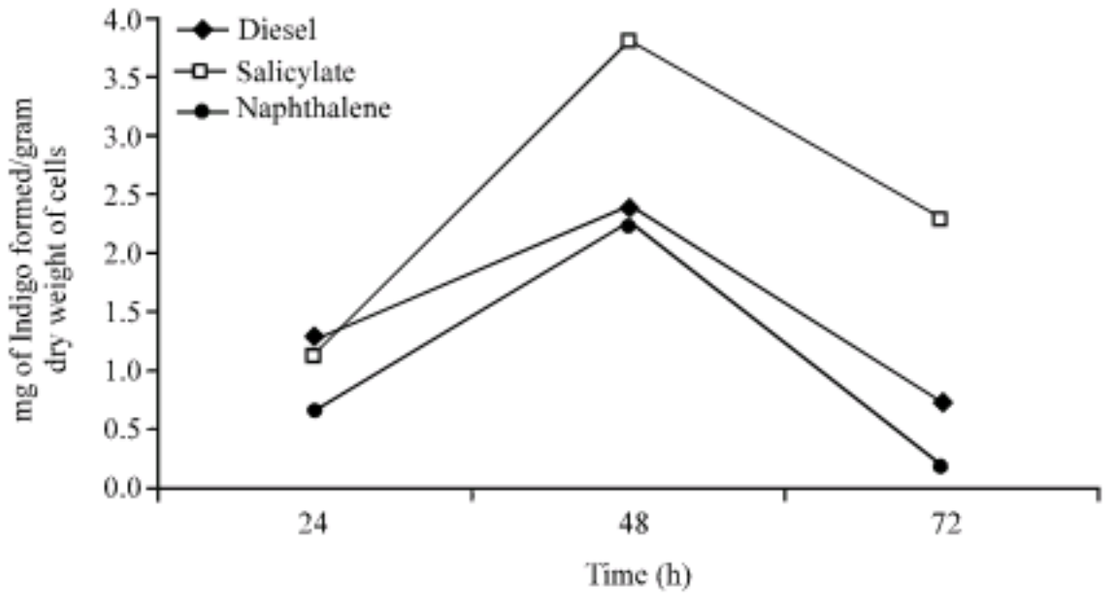

Fig. 3: Indigo production by the bacterial strain SCV1

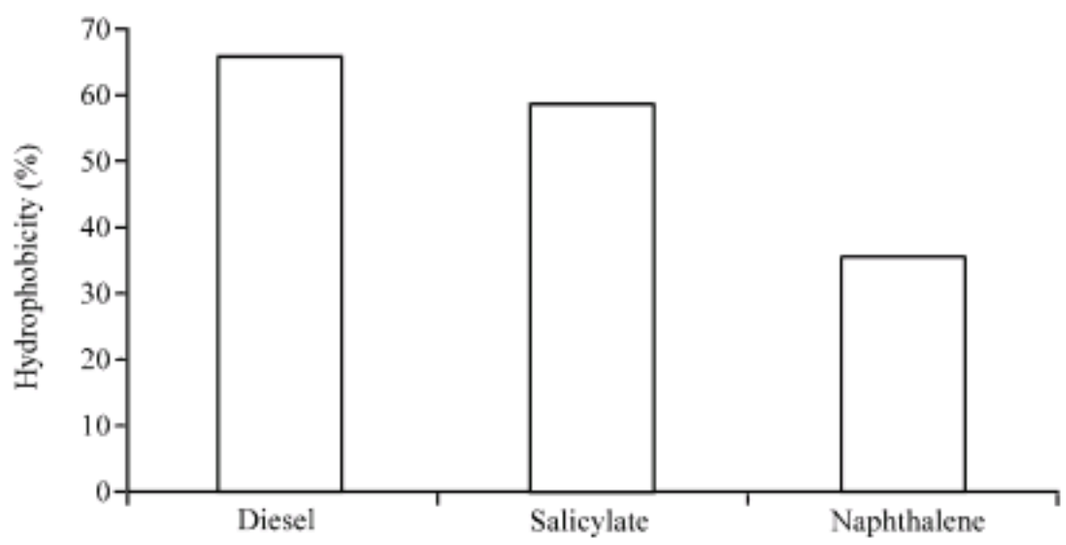

Fig. 4: Hydrophobicity (%) of the bacterial strain scv1 on different substrates

revealed that the hydrocarbons (Diesel or Naphthalene}) or hydrocarbon metabolite (Salicylate) could induce the bacterial strain SCV1 for the production of Indigo and salicylate induced cells showed about two fold increase in Indigo production.

Hydrophobicity of the Bacterial Strain SCV1

The hydrophobicity of the bacterial strain SCV1 on diesel, salicylate and naphthalene was measured at 96 h (Fig. 4). The bacterial strain showed 65% of hydrophobicity on diesel, 58% on salicylate and 35% on naphthalene. The importance of cell surface properties for the utilization of organic compounds has been indicated earlier by Rosenberg and Rosenberg (1981) and Wick *et al*. (2003). They showed that bacteria with high affinity for hydrocarbons utilized hydrophobic substrate like hexadecane more effectively. Based on present hydrophobicity results, it seems that the bacterial strain SCV1 has better access to hydrophobic substrates and the difference in indigo production may not be due to limitation in bioavailability of inducing compounds.

Indigo Production at Varying Concentrations of Indole

In the present experiments, indigo production at varying concentrations of indole was determined when the bacterial strain SCV1 is induced with diesel or salicylate or naphthalene. Apart from the above mentioned induction substrates, an uninduced bacterial strain SCV1 (from Nutrient broth}) was also used to study indigo production. The results (mg of Indigo/g dry wt. of cells) are shown in

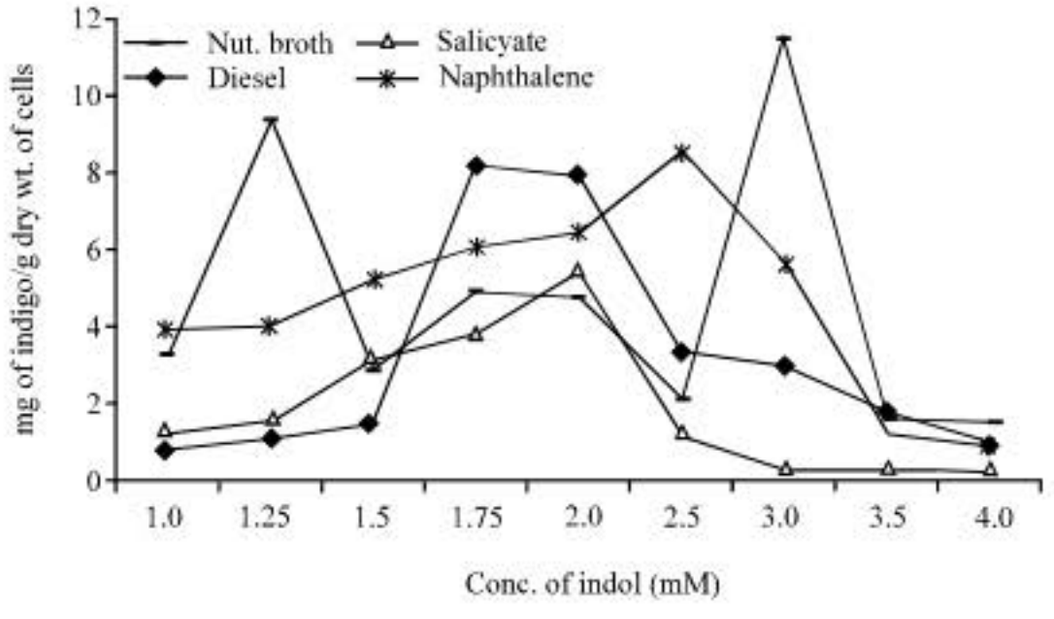

Fig. 5: Production of indigo when Induced by different substrates

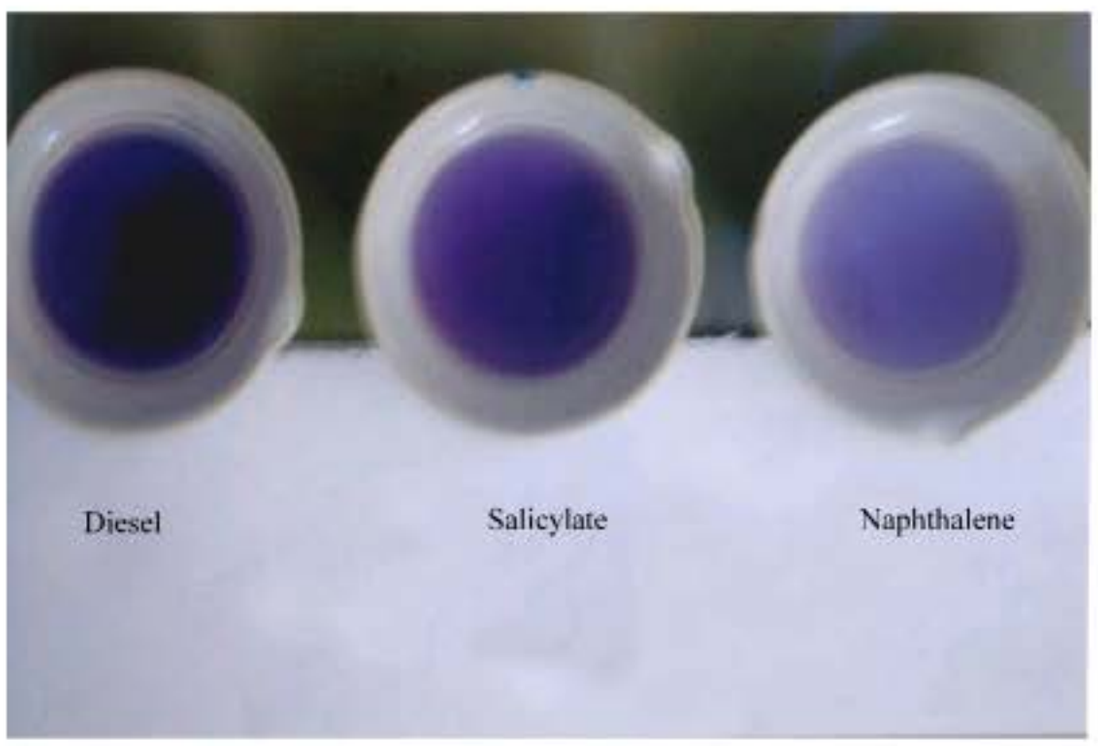

Fig. 6: Ethyl acetate fractions of indigo when induced by different substrates

Fig. 5. The results obtained from indigo production at varying concentrations of indole, following induction with diesel revealed that the indigo production increased till 1.75 mM of Indole. The Indigo produced at 1, 1.75, 2.5 and 4 mM are 0.79 mg Indigo/g dry wt. of cells, 8.18 mg Indigo/g dry. wt. of cells, 3.33 mg Indigo/g dry wt of cells and 0.98 mg Indigo/g dry wt. of cells, respectively. The results obtained from indigo production at varying concentrations of indole following induction with salicylate revealed that the indigo production increased till 2 mM. The Indigo produced at 1, 2, 3 and 4 mM are 1.08, 5.44, 0.28 and 0.22 mg Indigo/g dry wt. of cells, respectively. The results obtained from indigo production at varying concentrations of indole following induction with naphthalene revealed that the Indigo production increased till 2.5 mM. The Indigo produced at 1, 2.5, 3 and 4 mM of Indole are 3.9, 8.57, 5.65 and 0.95 mg Indigo/g dry wt. of cells.

On the other hand, the cells of the bacterial strain SCV1 under all induction conditions, produced Indigo compared with uninduced cells i.e., from nutrient broth enrichment culture. The uninduced cell culture showed a different colour rather than the characteristic indigo colour when induced with different substrates. The uninduced culture SCV1 from nutrient broth is resuspended in BH medium after washing and change of the colour (results not shown) of the medium was noticed. Figure 6 shows

the ethyl acetate fractions containing indigo when induced by different substrates. Rui *et al*. (2005) showed that their strain *Burkholderia cepacia* G4 changed the colour of the colonies expressing altered monoxygenase enzymes ranged in colour from blue through green and purple to orange indicating the production of isoindigo, indigo, indirubin, isatin etc., due to various hydroxylation products of indole. The brown colour of the cell suspension from uninduced culture in our case might also be due to the production of indigoid compounds other than indigo. However this will be confirmed in our future studies.

The biosynthesis of indigo is not economical compared to chemical synthesis. The costs could be brought down by selecting cheaper substrates as substrate costs constitute more than production costs of any biotechnological product. Our future studies are aimed towards cloning indigo producing gene from SCV1 into *E. coli* so as to produce indigo from tryptophan rich cheaper substrates so as to cut down the production costs. Tryptophan could be used for the biosynthetic production of indole which is the substrate for indigo production.

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