Biocatalytic Production of Commercial Textile Dye - Indigo

THESIS

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DOCTOR OF PHILOSOPHY

By

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Under the Supervision of

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CERTIFICATE

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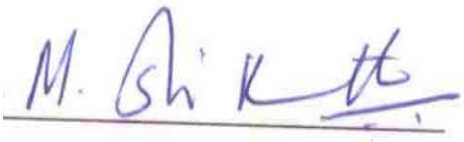

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ABSTRACT

Indigo, is blue of blue jeans, a dye used from ancient times for dyeing of fabrics, now its use is also extended to food and the pharmaceutical industries. From the history of its invention the dye was produced from different variety of plants in Europe and Asia. Indigo is also termed as vat dye, which means that it needs to be reduced to its water soluble *leuco* form before dyeing. 'Vat' is referred to as a vessel used for fermentation of the Indigo leaves. Today the dye is produced chemically on large scale leading to environmental pollution. The 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). Biological route for indigo production could be an alternative for sustainable indigo production. The present study focuses on isolation of bacteria from oil contaminated garage soil for the biological production of indigo. The strain identified as *Pandorea sp.*, was found to be an effective indigo producer in presence of indole as a substrate and silicone oil as a biphasic organic solvent. The production of pigment was confirmed using TLC-thin layer chromatography, UV-visible spectrophotometer and FTIR- analysis. For effective indigo production the following parameters like concentration of substrate-indole, pH, and temperature were optimized under single and two phase study carried out in vial at lab scale. The production of bio-indigo was optimized using response surface methodology with quartic model. The parameters selected for the optimization were – substrate (indole) concentration and temperature of the medium, at fixed pH 7. The maximum indigo concentration obtained under the optimized condition was found to be 0.4 mM. The model prediction and experimental results matched with an increase in bio-indigo production. The kinetics

parameter, V_{max} and K_m found for the indigo formation for single and two phase system revealed the advantage of using second phase and its effect on the affinity of the enzyme for the substrate indole, the result helped to decide the use of silicone oil-medium system for scale up of the work. The lab work was scaled up to bioreactor operation with a capacity of 2.5 liter. The bioreactor under optimized condition was analyzed for single and two phase study. Different bioreactor configurations were studied so as to get the best possible route to be applied for Indigo production at commercial level. To study the effect of different reactor configuration the reactor was operated in Batch, Fed batch and Continuous operation (First and second mode) to compare both single phase and two phase effect on the product formation. The study revealed the increase in the indigo formation as we moved from batch to fed batch and finally to the continuous operation of the reactor. In the continuous second mode operation, the substrate injection was done continuously whereas the product withdrawal was carried out thrice in a day to get the maximum productivity of indigo 0.5 mM, which was maximum as compared to the entire configuration studied in this work.

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VAISHNAVI TUSHAR UNDE

Dedicated

To all my Mentors

BRIEF CONTENT

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LIST OF SYMBOLS AND ABBREVIATIONS

ANNOVA- Analysis of Variance
BLAST – Basic local Alignment Search Tool
C =C – Carbon - Carbon bond
C-H – Carbon -Hydrogen Bond
C-N – Carbon -Nitrogen bond
DOE – Design of Experiment
DNA- Deoxy Ribonucleic acid
3D – Three dimensional
etc – Et cetera
FTIR- Fourier Transform Infra Red
g/l – Gram per liter
h – Hour
HCL – Hydrochloric Acid
IR – Infra Red
mM- Milli Molar
ml – Milli Liter
mg/L – Milli gram per Liter
mm of Hg – Milli meter of mercury
N-H – Nitrogen Hydrogen bond
OD – Optical Density
pH – Negative Logarithm of Hydrogen ion concentration
RPM- Revolution Per Minutes
RSM- Response Surface Methodology
Rf – Retardation factor
TLC – Thin layer chromatography
UV- Ultra Violet
^{o}C – Degree Centigrade
µl - Microliter
$ - Dollars

1. INTRODUCTION

Earth is a beautiful combination of environment and life, thus there is a need from humans to work in the direction to protect their environment for sustainable development. Rapid industrial development in past have made environmental problems more acute in the countries like India, where industrial development is a prime focus for economic growth of the country as well as the root cause for environmental problems. The wastewater released out from the industries carries toxic and hazardous pollutants, the major pollutant being synthetic chemicals from synthetic dye producing industries. (Pereira L. and Alves M, 2012; Ratna and Padhi B.S, 2012; Rita K., 2012). The dyes are used extensively by industries like textiles, pharmaceutical, food, and leather etc. It is reported that in 1994 the worldwide production of dyes was around 1 million ton. (Adedayo O. et.al , 2004). In 2000, Indian and European countries together consumed 6,00,000 tons of dye per year(Ishikawa Y., et.al 2000) and untill 2013 this figure has gone upto 10, 00,000 tons per year (Saravanan P. et.al, 2013). The production and application of synthetic dyes releases vast amount of waste and unfixed colourant causing serious health hazards and disturbing eco-balance of nature (Saravanan P. et.al, 2013).The most significant dyes is defined to be Alizarin and Indigo (Abrahart E. N, Book,1977).

Among the colours available, the blue pigments are rare in nature and hence associated with wealth (Glowachki E. D., 2012). Indigo dye is an organic compound with distinct blue colour and has great demand. It is one of the oldest textile dyes, produced on large scale and used mostly in textile industries (Ensley et al., 1983; Doukyu et al., 2003; Travasso et al., 2003). 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 (Mutnuri S. et.al, 2009).The vat dyes are insoluble in water and needs to be converted into a water soluble leuco form that effectively penetrates and interacts with the fabric.

Indigo or indigotin (Fig.1.1) is a dye originally extracted from the plant of genus *indigofera* (Bhushan et.al., 2000). The natural extraction process is expensive, the concentration and shade of the dye varied widely from plant to plant and also it could not produce the mass quantities required for highly expanding garment industry.

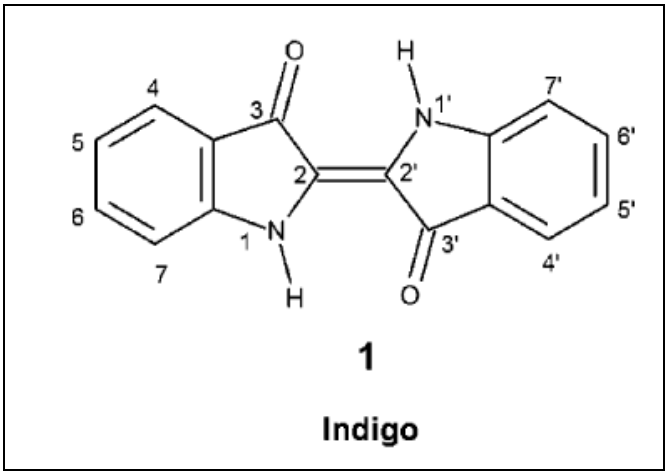

Fig. 1.1: The molecular structure of indigo

(Ref: Indigo and tyran purple, Isreal journal 2012)

Thus in recent years, the synthetic process is used to produce indigo but has come under scrutiny because of the harsh chemicals involved (Laitonjam and Wangkheirakpam, 2011). New and more environmentally responsible methods are being sought by manufacturers. Thus indicating a need for a biotechnological approach for commercial application. Many microbial processes for indigo synthesis have been reported (Madsen and Bollag, 1989, Murdock et al., 1993, Doukyu et al., 2003; Rui et. al., 2005, Pathak and

Madamwar, 2009; Mutnuri S.et. al., 2009) but the main challenge is to overcome the lower productivity of indigo in biotechnological processes as compared to chemical synthesis, which accounts to around several lakhs tons at present. Hence, there is a need to explore isolation of micro-organisms that are capable of producing indigo at higher rate and in large quantity as per today's demand. 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 energy consumption, and being eco-friendly. The microbial biosynthesis of indigo makes use of bacteria to convert a substrate into the highly useful product, Indigo.

Various studies have figured the use of indole as a substrate for the production of indigo (Doukyu et al., 2003; Madsen and Bollag, 1989; Murdock et al., 1993; Rui et al, 2005; Mckay et al., 2005).Indole is an aromatic heterocyclic organic compound that is toxic to microorganism and the toxicity causes microorganism to reduce the production of indigo (Doukyu and Aono, 1997). The microbial conversion of compounds with low water solubility is limited by their solubilization rate (Mutnuri S.et al., 2005), thus low solubility of indole (0.19 g/100 ml @20^{o}C) in water or mineral medium is another limiting parameter for such process. To overcome these limitations the use of water – organic solvent combination can be beneficial. The organic solvent like silicone oil is shown to have higher mass transfer rate for aromatic hydrocarbons (Mutnuri S.et al., 2005).The major objective of this research work is microbial production of indigo dye with special emphasis on control and promotion of bacterial growth and productivity

through control of growth conditions and improving rate of product accumulation through biphasic studies.

2. REVIEW OF LITERATURE

2.1 Colours

Colour is ubiquitous and is a source of information (Singh S., 2006). The whole of the world around us lives in colours. Colours influence our mood and emotions and generally enhance the way in which we enjoy our surrounding. The earlier evidence of the use of colour comes from cave paintings of Cro- Magnon man, which were painted between 10,000 and 30,000 BC, (Kerry K.G. & Cooke D.T., 2001).During ancient times colours were found near their source, and so it shades often differentiated as per the geographical location, as well as class and custom (http://zady.com/features/the history-of-fabric-dye).Colour is either present naturally around us or it is introduced in different materials using dyes. It has the ability to add value to large and variety of products like textiles, leather, paper, food products, cosmetics, plastics, paints , inks, high tech devices like CD's and DVD's, solar cells, medical diagnostics like CT scan and angiography, and thus has great significance. Colours are basically classified into two major groups – Dyes and Pigments.

2.2 Dyes

Dyes may be defined as substances that, when applied to a substrate provides colour by a process that alters, at least temporarily, any crystal structure of the colored substances (Gunay M., 2013). These are the soluble substance that impart colour to the substrate and used majorly in textile industries. It forms a covalent bond or complexes with salts or metals by physical adsorption or by mechanical retention (Othmer K., 2004, Bafana A. et. al, 2011). Dyes are basically aromatic compounds with aryl rings that have delocalized electron systems, which are responsible for the absorption of electromagnetic radiation

that has varying wavelengths, based upon the energy of the electron clouds. Also dyes have presence of a substance called chromophore, which has the ability to make the dye proficient in absorption of the radiation, which makes dyes coloured. The term dye is derived from old English word “daeg” or “daeh” meaning colour (Roy M., 1977). These are any substance, natural or synthetic used to colour various materials and have wide industrial application (NIIR,2004).Industries like plastics, textile, paper and pulp, adhesives, art supplies, beverages, ceramics, construction, cosmetics, food, glass, paints, and polymers uses dyes as a colourant. Whereas the pigment is insoluble substance in granular or a powdered form and used mostly in paints industries (FICCI, 2012). Throughout history dyes and pigments have been major articles of commerce. Manufacture of virtually all commercial products involves colour at some stage, and today some 9,000 colorants with more than 50,000 trade names are used (Abrahart E.N.,1977). There are primarily four sources from which dyes are available- Specialized animal and plant source, Bye – products (Lac dyes), Chemical synthesis and Tissue culture (Vankar P.S.,2000).

It is estimated that over 100,000 different dyes and pigments are used industrially and over 7 x 10^5 tons of synthetic dyes are annually produced worldwide (Gunay M., Zullinger H.,1997, Robinson T. et.al. 2001,Ogugbue C.J. 2011). Different dyestuffs have highly varying chemical characteristics and are selected according to the material to be dyed (Cliona O. et.al,1999). Due to their chemical structure, dyes are resistant to fading on exposure to light, water and many chemicals (Robinson T., 2001). Moreover dyes may be mutagenic or carcinogenic and if contacted, can cause severe damage to

human beings such as malfunction of kidney, reproductive system, liver, brain and central nervous system as well (Dutta M. et.al, 2012).

The making of the dyes and its application on various substrates is an oldest activity by humans, the evidence of which is seen in the archeological history. Natural dyes were used only for coloring of textiles from ancient times till the nineteenth century. A real breakthrough in the history of dyes came when a young scientist, William Perkin, in 1856 accidently discovered a synthetic dye, which is called as Mauveine (Mauve) a basic dye which was the first mass produced synthetic dye (http://dyes-pigments.standardcon.com/what-is-dye.html).

2.3 Natural Dyes

As the name suggest natural dyes are substance that is obtained from natural source such as plants, minerals and insects. These dyes are obtained by extracting it from roots, stems, leaves, flowers, fruits of various plants, dried bodies of certain insects and minerals (Vankar P.S., 2000). From ancient times these dyes were used mainly for colouring fabric (Saxena S., et.al, 2014). Solid evidence, that dyeing methods are more than 4,000 years old has been provided by dyed fabrics found in Egyptian tombs. Ancient hieroglyphs describe extraction and application of natural dyes (Abrahart E. N., 1977). Other than textiles, natural dyes are used in food, pharmaceutical, leather and in handicraft. Natural dyes has advantages like it provided variety of shades, which are, lustrous, soft and soothing for the eyes, the plants can be grown on wasteland. A major advantage of natural dyes is its non toxic effect and hence being environmentally friendly (Gilbert K.G. and Cooke D.T., 2001). Lots of efforts have been taken to extract dyes from different parts of coloured plants; still only few natural dyes have significant use, as most

of these dyes were not stable. Also, it faced limitations like growing and cultivation of colour producing plants, extraction of the colouring component from the source, variation in colour from the same source, low colour value, long dyeing time and poor colour fastness. As a result of these limitations and the invention of synthetic dyes in the second half of the nineteenth century, the use of natural dyes declined (Saxena S. and Raja A., 2014). The natural dyes are also called as mordant dyes as it cannot directly adhere on the fabric hence mordants are required to absorb the natural dyes onto the fabric to improve its colour fastness. A mordant is thus a chemical agent which allows a reaction to occur between the dye and the fabric. In textiles, mordant are used to fix the color in dyeing or fabric printing, especially for fabrics of plant origin –cotton (http://www.nptel.ac.in/courses/116104046/14.pdf).

2.4 Synthetic Dyes

The synthetic dye making industry was set to start, with the invention of " Mauveine"a dye by Henry Perkin in 1856.After which the synthetic dyestuff went into lot of research and invention for production of different colour .The synthetic dyes quickly replaced the traditional natural dyes. As, they cost less, they offered a vast range of new colors, and they imparted better properties to the dyed materials (Garfield S., 2000). There are more than ten thousand dyes available commercially (Ratna and Padhi B.S., 2012). The synthetic dyestuffs now available in several hues are amongst the oldest chemicals produced by man (http://nptel.ac.in/couse/103207082/module9/lecture2/lecture2.pdf). Today all the colours used in textile dyeing are synthetic produced by various ways from byproduct of fossil fuel, e.g. aniline and other aromatic derivatives (Gilbert K.G. and Cooke D.T., 2001).

2.5 Different types of dyes

Most of the synthetic dyes are made from coal tar derivatives. The preparation of synthetic dyes is complex, and tedious operation. Constantly work in improvement of manufacturing technique is going in the industry for better processes (http://www.iiem.com/em/dyes/chapter3.html). The textile dyeing industry consumes around 30,000 tons of different synthetic dyes (Ponraj M. et.al, 2011).The classification of synthetic dyes can be done based on chemical composition, nuclear structure, industrial application or based on source of raw material(http://www.dyespigments.net/types-of-dyes.html). Textile dyes are classified based on their chemical structure as Anthraquinone dyes, Azo dyes, Acridine dyes, Indophenols dyes etc. According to the nuclear structure dyes are classified as – Anionic and cationic dyes. Based on the industrial application technique dyes are classed as Acid, Azoic, Basic, Direct, Disperse, Mordant, Reactive, Solvent, Sulphur, and Vat dyes. Whereas, based on source of raw material dyes are called as natural or synthetic dyes (http://www.dyespigments.net/types-of-dyes. html).

2.6 Global dyestuff industries

Dyestuff sector is one of the major chemical industries in India. It is the second highest export segment in chemical industry. The major industry users of dyes are textiles, paper, plastics, printing ink and foodstuffs. Globally, the textiles sector consumes around 80% of the total production due to high demand for polyester and cotton. The dyestuffs industry has seen an impressive growth all over the world. The world consumption for the dye accounts for printing inks (40%), paints (30%), plastics (20%) and others from segments like textiles. The global market size was estimated around USD 23 billion in

2005, and India's share in the world market is estimated to be 5-6% as compared to China's share of 25% (FICCI, 2006). Overcoming the challenges in production, demand, regulation and changing customers' preference the dyes market has reached great heights and it is expected to grow more in future. Market demand for dye and dye intermediates was expected to grow at a Compounded Annual Growth Rate (CAGR) of 4.7%, from 652,000 tonnes in 2004-05 to 900,000 tonnes in 2010-11(http://www.fiber2fashion.com). The world market for the colorants comprising dyes, pigments and intermediates is presently estimated at $27 Billion. During the last decade, the industry has grown at an average rate of 2-3 per cent per annum. Where other countries in the world market contribute nearly 87.5 per cent of the global production, India accounts for 12.5 per cent (Mehta J., 2012). Whereas today worldwide demand for dyes and organic pigments is still growing at a healthy pace, expected to increase to more than $30 billion by 2019 (Freedonia,2015). Size of the Indian colorants industry was $3.4 billion in FY10 with exports accounting for ~68%. In the international dye market of 800,000 tonnes, one tenth is contributed by indigo, thus the importance of indigo dye is immense (Gilbert K.G. and Cooke D.T.,2001).

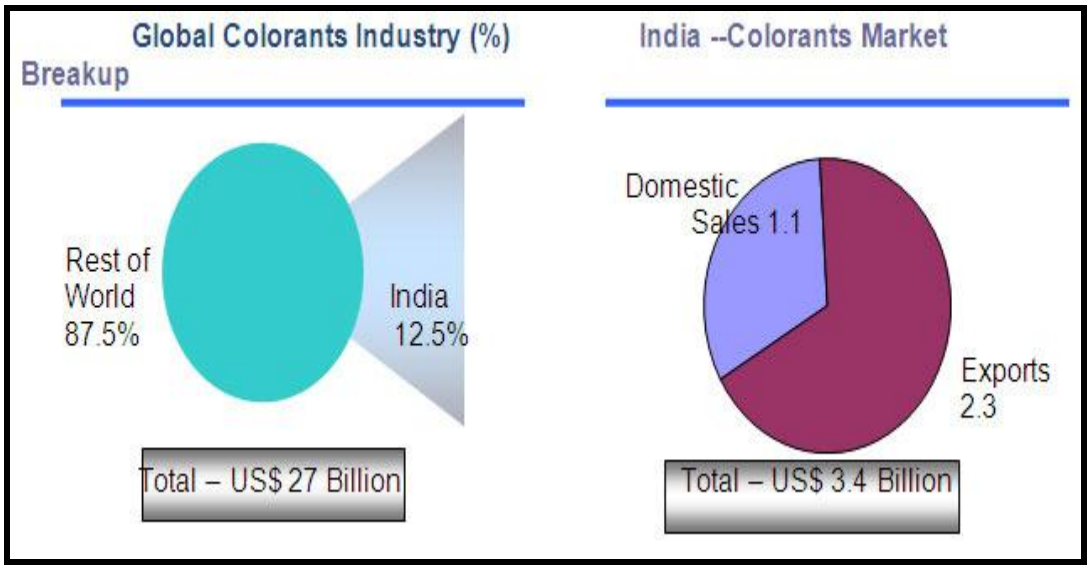

Fig. 2.1: Global and Indian colorant market (Ref .Mehta J., 2012)

2.7 Indian dyestuff industries

India has been a well known producer of textile and has a tradition of making, dyeing, printing and embroidering of cloths since ancient times. In earlier history, Indigo plantations and science in colonial India for the most part appears as a straightforward account of Bengal indigo (indigofera tinctorium)—the natural dye that could colour cloth in intense blue. The beginning of the invention of coal tar derived synthetic indigo in 1897 by the German chemical company - Badische Anilin and Soda Fabrik (BASF), resulted into the end to the production of Bengal Indigo. By the 1920s, the demand for Bengal indigo was dramatically wiped out from most, if not all global markets with synthetics dyes emerging as clear victor (http://www.iiem.com/em/dyes/chapters3.html). India became the global market leader in indigo production early in the 19th century (Engel A., 2007). The following table shows the installed and production of dyes and dyestuff in metric tons (Annual Report, 2011).

Table.2.1: Installed and Production capacity of Dyes and Dyestuffs (000' MT)

Major Groups/Products	Installed Capacity (2009-10)	Production (2010-11)
Dyes and dyestuffs		
Azo dyes	8.70	2.80
Disperse dyes	6.50	0.53
Fast colour base	0.6	0.09
Ingrain dyes	0.5	0.7
Optical whitening agents	3.40	3.04
Organic pigment colours	11.00	21.83
Pigment emulsion	6.30	5.63
Reactive dyes	7.90	2.40
Sulphur dyes(sulphur black)	3.30	8.60
Vat dyes (Includes Indigo)	3.00	1.60
Solubilised vat dyes	0.10	0.04
Napthols	3.60	0.07
Total	54.90	47.33

(Sources: Annual Report 2011-2012, DCPC, Govt. of India)

The Indian dyestuff industry is highly fragmented and characterized by a large number of players in the unorganized sector. Today, Indian dyestuffs industry has about 1000 units comprising of 50 large and organized sectors and remaining under Small & Medium Enterprises. Maharashtra and Gujarat account for 90% of dye stuff production in India due to the availability of raw materials and dominance of textile industry in these regions (EIA manual, 2010). The overall production capacity of dyestuffs is 200,000 tonnes per annum (FICCI, 2012). Out of which Indian dyestuff industry meets more than 95% of the domestic requirement (Textile being 60% and 40% for other industries) in spite of the increased standard of quality.

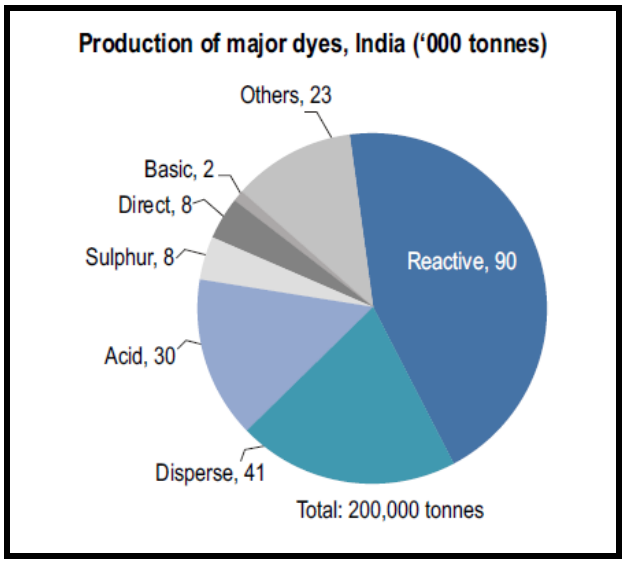

Fig. 2.2: Production of major dyes in India (FICCI 2012**)**

The coexistence of small, large and medium industries in dyestuff production has become a remarkable feature of this industry. The actual production during year 2006-07 until September 2012 is shown in the table below (Table. 2.2) (Envis centre Report).

Table 2. 2 : Production of dyes and dyes intermediate

Sector		Production						
		2006-07	2007-08	2008-09	2009-10	2010-11	2011-12	2012-Sep 12
Dyes & Dye intermediates	**Production(mT)**	90	117	110	149	164	171	86
	Growth rate	29.5	30.5	-6.5	35.8	10.5	4	

In the developing phase India has emerged as a supplier of dyestuff and intermediates particularly in reactive, acid, direct and VAT dyes and some key intermediates. As a result of remarkable growth in export of colorant in last two decade, the industry has achieved a growth of 14.5 % in last decade. The export is further estimated to grow up to

$4.9 billion by 2017(Report-Planning commission 2012). Various action plans are choked for further growth of the dyestuff industry.

2.8 Indigo dye

Indigo (Fig. 2.3) have been known for at least 4000 years (Glowacki E.D., 2012), it is an organic compound with distinctive blue colour. Indigo is one of the oldest dyes that were used for textile dyeing and printing. Asian countries like India, China and Japan had used indigo as a dye for centuries (http //: Fibre2fashion). Throughout history indigo was revered and sought after as a valuable commodity. As blue was rare colour, fabric dyed with indigo was used for fashion as well as for religious rituals, it was considered as a royal colour and hence stood as a symbol of status. Historically, indigo was a natural dye extracted from plants, and this process was important economically. Plants like Dyer's woad (*Isatic tinctoria L*.) from Europe, *Indigofera tinctoria* from India and *polygonum tinctorium* from China and Japan were used for production of indigo. In 1865, the German chemist Johann Friedrich Wilhelm Adolf von Baeyer began working with indigo. In 1880, his work resulted in the first synthesis of indigo and three years later the announcement of its chemical structure. BASF (Badische Anilin and Soda Fabrik) a largest chemical producing company in the world, developed a viable manufacturing process that was in use by 1897, and by 1913 natural indigo had almost been replaced by synthetic indigo. In 2002, 17000 tons of synthetic indigo was produced worldwide (http://en.wikipedia.org/wiki/indigo_dye). Today all of the indigo dye produced, is synthetic, it is the blue of jeans, whereas the production and use of natural indigo has come to extinction.

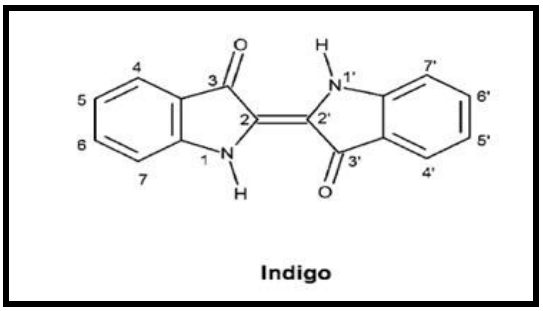

Fig.2.3: Indigo Structure (Glowacki E.D. et.al, 2012)

Indigo which is one of the oldest known natural dyes (Ensley et.al,1983), is a derivative of the colorless glucosides of the enol form of indoxyl, e.g. indican (indoxyl-_-D-glucoside) (Laitonjam W.S.,2011). The indoxyl further dimerises by air oxidation to the blue indigo (Minami Y. et.al , 2000).

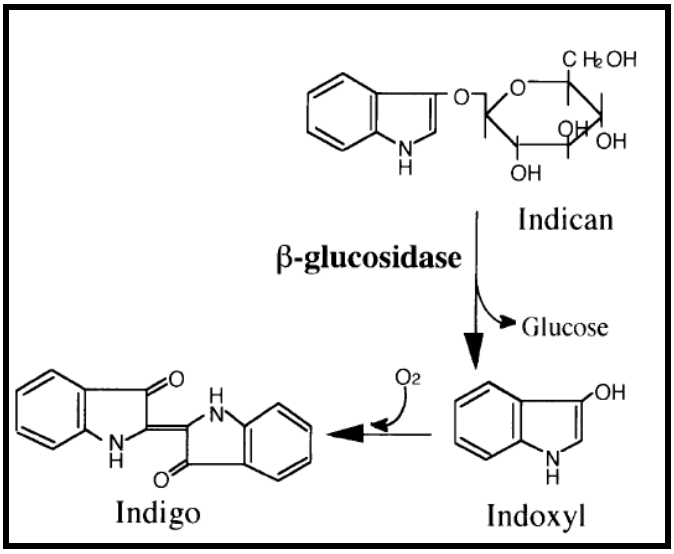

Fig. 2.4: Hydrolysis of indicant to indigo (Minami Y. et.al, 2000)

Indigo is also-called vat dye, which means that it needs to be reduced to its water soluble *leuco*-form before dyeing as shown in the figure below (Buscio V. et.al, 2014). The reduced form is absorbed into the fibers, and when oxidized back to its blue form it stays within the fiber (Vuorema A., 2008). For reduction process, sodium dithionite is used in

most of the industries which cannot be recycled and the disposal of dyeing baths and rinsing water is causing high costs and various problems with the effluent (high salt load, depletion of dissolved oxygen, problems with nasal nuisance, toxicity of sulphide, etc.). Therefore, modern economical and ecological requirements are not fulfilled (Roesslera A. & Crettenanda D., 2004).

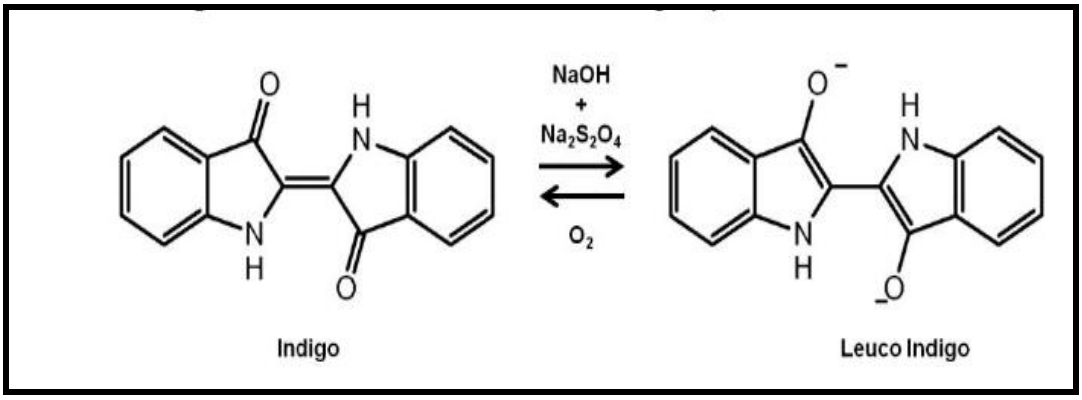

Fig. 2.5: Chemical transformation of indigo to leuco form (Crespi M., Bouzan C.G., 2014)

2.9 Properties of indigo

The chemical structure of indigo, corresponding to the formula $C_{16}H_{10}N_2O_2$, was announced in 1883 by Adolf von Baeyer after eighteen years of study of the dye (Ensley. J, 1985). Bayer received the Nobel prize in chemistry in 1905, Indigo production reaction involved a base-catalyzed aldol condensation of o-nitrobenzaldehyde with acetone, followed by cyclization to 3-indolone and dimerization to indigo (Glowacki E.D. et.al, 2013). Indigo which is also known as indigotin, (CI Vat Blue 1), (2-(1,3-dihydro-3-oxo-2*H*-indol-2-ylidene)-1,2-dihydro-3*H*-indol-3-one) is present at ambient temperature and normal pressure as dark blue-violet needles or prisms with distinct coppery luster (Mojca B. & Kokol V., 2008; Roessler A.,2003). From the electrochemistry it is apparent that indigo

can be both reduced and oxidized at relatively low potentials, with a small electrochemical "bandgap" of 1.7 eV(Glowacki E.D. et.al, 2013).An important optical property of indigo is, it forms hydrogen bonded pigment aggregates , Indigo in the pigment form demonstrates excellent light-fastness and stability, in solution however under the influence of ozone and hydroxyl radicals it will slowly oxidize, into the yellow-colored isatin and anthranilic acid(Glowacki E.D. et.al, 2013). It is practically insoluble in water (Merck Index, 1968) and many other common solvents but it will dissolve to some extent in few of the organic solvents like – chloroform, ethyl acetate, pyridine, tetra hydro furan, and N methyl 2 pyrrolidone (Gilbert K.G. & Cooke D.T.,2001).

Indigo is called as a vat dye i.e. it has to be reduced to its water soluble leuco form before dyeing. The reduced leuco indigo readily would penetrate into the fibers dipped in the vats and after removing the fibre it undergoes re-oxidation in air to produce insoluble blue indigo – now trapped within the fibers. Vat dyes have excellent colour fastness, especially to chlorine and bleach, however if not applied properly it may crock, Vat dyes are applied on cellulosic (Goetz C., 2008).

2.10 Methods of Indigo production

Dyes can be classified as natural, synthetic or biotech dye depending on the method of production. Dyes obtained from natural source like plants, animals or insects are called as natural dyes, those obtained from chemicals and chemical reactions are called synthetic dyes whereas the dyes produced using various species of bacteria are called biotech dyes.

Indigo has been used throughout East Asia since before recorded history but probably originated in India. (Goetz C., 2008). Natural indigo can be derived from a wide range of tropical, sub-tropical and temperate plants from many different species, genera and

families. In tropical and sub-tropical areas, the plants most widely used for indigo production were *Indigofera* spp., of which there are over 350 species. In temperate climates, the most commonly used species was *Isatis tinctoria* or dyers woad. Other species, e.g., *Polygonum tinctorum* (Ai) have also been used in Japan, China and Russia for large-scale indigo production (Gilbert K.G. & Cooke D.T.,2001). Natural indigo is produced by fermenting the leaves of indigo bearing plants. During the fermentation, indican (a colorless, water-soluble derivative of the amino acid tryptophan) in the leaves is hydrolyzed to form indoxyl and glucose by the action of endogenous β-glucosidase and is subsequently oxidized to form indigo by contact with air. The resulting insoluble indigo is then converted to soluble leuco indigo at alkaline conditions, mainly by fermentation Fig 2.6 (Song J. et.al, 2010). Natural indigo when applied to animal fibers like wool and silk shows good colour fastness whereas for vegetable fibers like cotton and linen , it requires metallic oxides or mordant's to fix the colour on the fabric(Sweet D.G., 2012).

Natural indigo dye was used until synthetic indigo was first chemically synthesized at the end of the 19th century (Clark R.J., 1993). Natural dyes have advantages like they are non-allergic, non-carcinogenic and have lower toxicity and better biodegradability than the synthetic dyes (Saravanan P. et.al, 2013). However, since the use of synthetic indigo dye has been preferred to natural indigo it is now widely used to dye cotton fabrics, especially work clothes and blue jeans. Earlier scientist like Baeyer produced indigo from starting material like Isatin, o-nitrocinnamic acid, Baeyer and Drewsen devised a better method in which o-nitrobenzaldehyde was condensed with acetone which also could not enhance the yield of the indigo production, later Heumann developed two methods, in the

first of which aniline, and in next anthranilic acid was used as a starting material which gave improved yield of the dye. This was further improved in laboratory of BASF (Krishnaswamy N.R. and Sundaresan C. N., 2012). In the standard chemical process, N-phenylglycine is fused with sodium amide under anhydrous alkaline conditions at 200 °C to form insoluble indigo via indoxyl formation by oxidation with air. In the chemical indigo dyeing method shown in Fig. 2.6, the insoluble indigo produced is first reduced to water-soluble leuco indigo or indigo white by treatment with sodium dithionite ($Na_2S_2O_4$) and the textile is then dipped in the leuco indigo solution after which it is allowed to come into contact with air to convert leuco indigo on/in the textile to indigo (Song J., 2010).

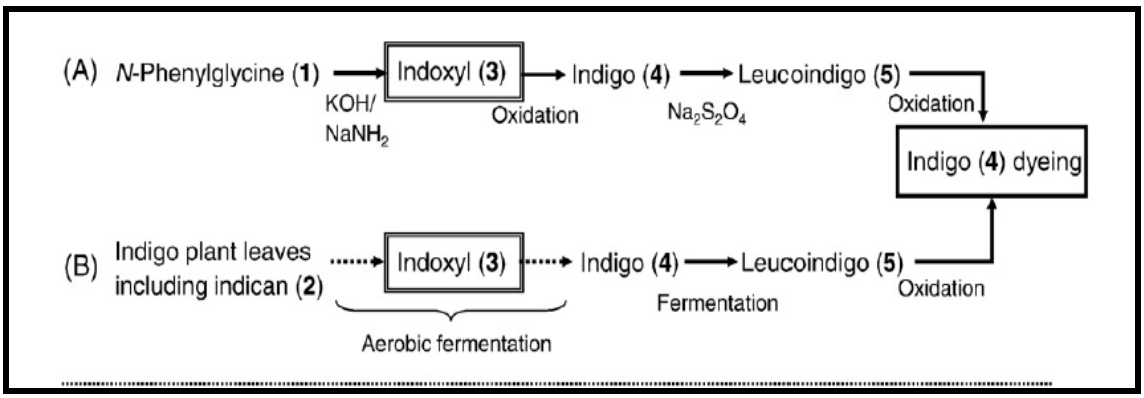

Fig 2.6: Brief scheme for indigo dyeing by the chemical method (A), traditional fermentation method (B).

The synthetic indigo dyes and its use have come under scrutiny because of the chemicals used. Synthetic dyes are of great environmental concern due to their widespread usage and their low removal rate during aerobic waste treatment (Gupta V.K. et.al, 2015). As environmental protection has become a major concern, different eco-friendly alternative are sought for indigo synthesis. As a result, microbial synthesis of indigoid pigments is

commendable over chemical synthesis (Dua A.et. al, 2014). The first microbial indigo production was reported way back in 1920's (Ensley B.D., 1994). After which many microbial processes for indigo synthesis have been reported (Doukyu N. et.al, 2003; Pathak H. and Madamwar D., 2009; Mutnuri S. et.al,2009; Madsen E.L.and Bollag J.M.,1989;D.Murdock et.al, 1993;and Rui et.al,2005) but the main concern is the lower productivity in comparison to chemical processes (Han X. et.al, 2008).

Following table highlights various research works showing Indigo production by the use of different bacteria.

Table 2.3. : Different bacteria's and substrate used for bio-indigo production by researchers

SR.NO	YEAR	NAME OF BACTERIA	SUBSTRATE USED	REFERENCE	REMARK	EXTRACTION	PRODUCT FORMED (MAX)
1	1965	*Pseudomonas indoloxidans* NCIB 2760	Indole	T Oshima	Conversion of indole to indigotin	Sulphuric acid and Hot Aniline	0.27 µmol/ml
2	1983	Fragment of plasmid NAH7from *Pseudomonas putida PpG7*, cloned and expressed in *Escherichia coli* HB101.	L-arginine hydro-chloride	B D Ensley	-----	Chloroform and ethyl acetate for different test	25 mg/liter
3	1994	Recombinant bacterium-NDO enzymes	Glucose and ammonia salt	B D Ensley	DNA manipulation	Precipitation of solid indigo in the medium	0.5-1 g/h/L of growth medium
4	1995	Bacterial strains *P. putida* F1 and *P. putida* mt-2	indole-2-carboxylate and indole-3-carboxylate	R W Eaton and P J Chapman	-----	Chloroform	Mentioned in terms of OD

5	1997	*Pseudomonas sp.strain* ST-200	Indole	N Doukyu , R Aono	------	----	Isatin and isatic acid production studied
6	1997	Microorganisms expressing styrene monooxygenase (SMO)	Indole	K E Connor,	Styrene, similar to indole	DMF (Dimethyl formamide)	Rate: S-12-10.9 and CA3-12.1 mU/mg dry wt
7	1998	Aromatic hydrocarbon-degrading bacteria expressing different oxygenases	Indole /Styrene	K E Connor	Styrene-grown cells of *Pseudomonas putida* S12 and CA-3 expressing styrene monooxygenase.	DMF (Dimethyl-formamide)	S-12 : Styrene – 8.3 nmol/min mg dry weight.
8	2000	Recombinant *E Coli*	Indole	B. Bhushan et al	------	Chloroform	Sp.rate: 1.40 nmol/min /mg dry biomass
9	2002	Recombinant *E coli*-strain JB102	Tryptophan	A Berry et.al	Tryptophan pathway	DMF/DMS	23 g/L in 42 hours
10	2002	*Acinetobacter* sp. strain ST-550	Indole	N. Doukyu et.al	Water organic –two phase system	Chloroform	292 µg/ml
11	2003	*Rhizobium,Pseudomonas* and Proteus species	Indole	J.Y. Kim, et.al	naphthalene dioxygenase and toluene dioxygenase used	Ethyl acetate	In terms of absorbance for various strains

12	2005	*E coli* strain and plasmids	sodium salicylate and isopropyl -d- galactopir anoside	Jose Luis Royoa, et.al	------	DMF	Strain MPO12 (pMPO3) -45 mg/L
13	2005	*E coli*	_____	He Kyoung Lim	Forest soil Metagen ome Clone	DMF and Ethyl acetate	Absorban ce
14	2007	Co-expression of P450 BM3, glucose dehydrogenas e by *E .coli*	Indole	Yan Lu · Lehe Mei	------	N N Dimethyl formami de	For 5 mM-after 8 hours : 2.9 mM
15	2008	*Rhodococcus sp.* strain T104	Ampicillin and kanamyci n	Na Ra Kwon et.al	-------	DMSO	Wild type strain≈ 50.7 µg/ ml
16	2009	Xenobionts	Indole	SMutnuri et.al	------	Ethyl acetate	0.38 mg/ml h
17	2009	*Pseudomonas sp.*	Napthalen e and Indole	Hilor Pathak & Datta Madamwa r	-----	DMF	246 µg/ml /8 h
18	2010	Recombinant *E. coli* cells harboring a flavin- containing monooxygena se gene	Tryptopha n	Gui Hwan Han et.al	-----	-----	911 mg/L batch process
19	2013	*Escherichia coli* cells expressing phenol hydroxylase	Indole	Shengnan Shi et.al	--------	Ethyl acetate	176.4 mg/L - Dodecane

Thus various bacterial species either as a whole cell or in the recombinant form are used along with substrate like Indole/ tryptophan for indigo production using shake flask method or at lab scale till date. There is a need to produce this environmentally friendly bio-indigo dye by overcoming the limitations in biotech route, scale up and produce the dye to compete the existing technology.

2.11 Bioreactor configuration

Microbial processes for industrial production of commodity chemicals are rapidly gaining practical significance for preparation of high purity products in an environmentally acceptable manner while realizing energy saving (Nagasawa T. and Yamada H., 1995). Production of natural products has become a profitable industry as it carries the benefit of being environmentally friendly; hence attract everyone as the use of such products are safe. Bio-catalytic processes for commercial applications have been the focus of intense research by the chemical industry. It applies to the development of microbial processes for specialty chemicals such as Indigo as well (Springham D. et.al, 1999).

For production of such commodity products bioreactor/ Fermentor design is an important parameter. Greater yield of microbial products at lowest cost demands good design of a reactor which can provide condition like proper supply of nutrients, optimized pH, temperature, cell density, stirrer speed, and oxygen supply. Although many chemicals can be produced by fermentation, the major limitation of such systems is the accumulation of toxic or inhibitory metabolites in the fermentation broth which inhibits cell growth and product formation, resulting into increased cost of the process (Roffler S.R. et.al, 1988). Removal of these metabolites or products can be done within the reactor (in situ) or externally (ex-situ). In situ separation of product has been focused by many researchers

(Daugulis A.J., 1994; Gyamerah M. and Glover J., 1996; Weilnhammer C. and Blass E., 1994).

Thus a two phase bioreactor system can be used for separation of inhibitory products from the Fermentor as well as can help in delivery of toxic substrate. The two phase bio-conversion involves use of two immiscible phases, in which first phase is the medium with nutrients and biomass whereas the second phase is immiscible phase which partitions the substrate in-between the two phases (Januz J., 2001). The use of second phase helps to transfer the toxic substrate from the aqueous phase which enhances the product formation. Also such systems help to control the delivery of inhibitory substrate and have the advantage to overcome low substrate solubility, thus it can be added in higher concentrations. For biotechnological process it is important to obtain economically feasible product concentration (Etschmann M.M.W. and Schrader J., 2006). To maintain high volumetric productivity and to supply high concentration of substrate the second phase helps in delivery of substrate as well as extraction of the product (Lye G.J.and Woodley J.M.,1999).Traditionally in most of the two phase reactors pure solvents has been used as a second phase (Prpich G.P. and Daugulis A.J.,2006). These systems are said to be self regulatory (Prpich G.P.and Daugulis A.J., 2004) and helps to separate the biodegradable products once formed in the Fermentor (Schmid A.et.al, 1998).

The major and crucial step in handling such two phase bioreactor is the solvent selection, wherein the incorrect solvent selection can affect growth of the microorganism, yield of product, uptake of the substrate and removal of the inhibitory components from the system. Thus bioreactor performance can be optimized well by selection of appropriate

solvent, which will be bio-available, safe and has low cost. The two phase study with water –organic solvent has not been studied much for indigo production except some which shows the use of organic- solvents like BEHP - *bis*(2-ethylhexyl) phthalate (Honda K.,2006), oleyl alcohol and BEHP (Shiho Y, 2007), diphenylmethane (Doukyu N.,2002) as suitable solvents for Indigo dye.

Few solvents like Heptane , Octane, Decane , Dodecane has been used as a second phase (Schmid A. et.al, 1998). In this list of solvent, even silicone oil takes it place due to its properties like immiscibility, bioavailability and biocompatibility. Silicone oil is any liquid polymerized siloxane with organic side chains. This oil is shown to have higher mass transfer rate for aromatic hydrocarbons (Mutnuri S. et.al, 2005).

2.12 Gap in the Existing Research

From the literature review it is seen that following routes are available for indigo production

1. Plant extracted Indigo
2. Synthetic Indigo
3. Microbial Indigo

Out of these the production of indigo from plant is an outdated technique and used only by craftsmen these days. Most extensively used, synthetic route has its commercial advantages, but at the cost of environmental pollution. Hence biotechnological method of Indigo production is of current focus.

If Bio-indigo can be produced using the bacterial isolates, then following benefits can be realized,

1. It will benefit the disposal and occupational safety problem due to the reduced use of toxic starting materials.
2. Decentralized production of the dye will result in saving in transportation costs.
3. The biomass accumulated after extraction of the dye can be used for energy generation and the growth media can be recycled preventing the waste generation.

The production of dye with natural characteristics has to satisfy following challenges as compared with the synthetic dyes available today.

1. Produce natural dyes as per the market demand.
2. Produce natural dyes at a comparable price.

3. Produce natural dyes that have properties like high coloured fastness, standard shades, reproducibility.

2.13 Scope of current work

The present research work thus focuses on the production of indigo by bacteria and its increased productivity in a two phase bioreactor.

The first objective of the thesis focuses on the isolation of bacteria from oil contaminated soil sites. The soil samples from different garages were used for the isolation of indigo producing bacterial strain. The purification, enrichment and identification of organism and the indigo producing capability of the organism was tested in this part of work. The organism was confirmed as a producer of bio indigo and hence was used for further study.

The next objective focuses on the optimization of the process parameter to increase the product yield in shake flask experiments. Parameters like temperature and pH were optimized. Comparative study of indigo production was done under both monophase and biphasic substrate releasing conditions. The batch study was validated with Design of Experiment tool. Thus from this part of work optimized parameters were fixed for scale up of indigo production.

The third objective took the shake flask experimental work to a Fermentor (scale up). The comparative experiment with single and two phase study using silicone oil as a second phase is done to estimate the increase yield under prefixed parameters.

The last objective of the work focused on bioreactor configuration which can help to enhance the indigo production and also overcome the operational difficulties in each of

the reactor experiments. A batch, fed batch (single and two phases) and continuous reactor operation were carried out for Indigo production.

To overcome the difficulties encountered in batch and fed batch mode of operation, a continuous operation of Fermentor was employed which can produce indigo on continuous basis as required for commercial application.

Thus the research work carried out results into introduction of a bacterial strain *Pandoreae sp.* capable of producing indigo on continuous basis of operation under the optimized conditions, wherein the cost involved in extraction of dye could be minimized.

The research work is focused on Bio-catalytic production of commercial textile dye Indigo, with the following objectives:

1. To isolate bacteria from oil contaminated soil sites with capability of producing indigo.
2. To experiment various parameters for optimized production of indigo.
3. To produce indigo in a bioreactor under optimized condition.
4. To use best possible bioreactor configuration for continuous production of indigo using biphasic approach.

CHAPTER 3:

Isolation of bacteria from oil contaminated soil sites with indigo dye producing capability.

3.1: ISOLATION OF BACTERIA FROM OIL CONTAMINATED SOIL SITES WITH INDIGO DYE PRODUCING CAPABILITY

Indigo is a blue colored dye used from ancient times for dyeing of fabrics. The dye is produced chemically on large scale leading to environmental pollution and thus a biological route for indigo production could be an alternative for sustainable indigo production. This leads to identification and exploitation of the bacterial species that are capable of producing the indigo dye.

The invention and production of microbial products is primarily dependent on the screening of cultured microbial species for desirable product. Soil is a rich source for microorganisms including bacteria. Bacteria are ubiquitous in nature and constitute a large domain of prokaryotic microorganisms. A number of studies from other parts of the world have been reported on isolation and identification of microbes from oil-contaminated soils (Peter E. K., 2014).

It has been observed that microorganisms that grow on oil contaminated soil are much capable of degrading oil than those microorganisms which are found on non-contaminated site of oil, this can be a very good example of adaptation (Khan A.J. and Rizvi S.H.A., 2011). Oil is a complex mixture of hydrocarbons and other organic compounds, including some organ metallic constituents (Mandri T. and Lin J., 2007). It contains hundreds or thousands of aliphatic, branched and aromatic hydrocarbons (Jain P.K. et.al , 2011)

Since 1980's , many microorganisms expressing mono or di-oxygenase have been reported to possess the ability to transform indole to indoxyl or dihydrodiol, which was then subject to non-enzymatic reaction to form indigo(Yuanyuan Q. et.al,2012). Different

bacterial species like *Pseudomonas Putida* PpG7 (Ensley, B.D., 1983), *Ralstonia eutropha* HF39 (Drewlo S .et. al, 2001), *Pseudomonas* sp. KL28 (Kim.et.al, 2005) etc has been reported to be used for indigo production. Majority of aromatic hydrocarbon-degrading bacteria have the ability to produce indigo dye (Hillor P., 2010).

With this literature background, oil contaminated samples was expected to be a potential source for hydrocarbon degrading bacteria and was used as a sample collection site for isolation and identification of the Indigo producing bacteria. The aim of this part of research work was to isolate bacteria with Indigo producing capability and identify it for further use in this work.

3.1.1 MATERIALS AND METHODS

3.1.1.1 Chemicals

The chemicals used like Bushnell Hass medium, Luria Burteni agar, Indole, along with reagents like acetone and ethyl acetate used in this work were of highest purity available. All the chemicals used are of highest purity grade from Himedia , S D Fine Chemicals and Merck, India.

3.1.1.2 Sample Collection

The sites selected for sample collections were oil contaminated soil samples from garages. The selection of soil samples from garages was based on literature survey and earlier study done by Mutnuri S.et al., 2009. Four different garages from various locations were finalized as sites for soil sample collection. The first garage was from MES College, the second was in Zari, near Zuari Industry, The third garage was from Sancoale and the fourth site was at Queeny area on NH-17 bye-pass highway, all the sites from Vasco (Elevation: 43 m, Coordinates: 15.3981° N, 73.8111° E) Goa, India.

Soil samples from these garages which were seen to be wet with the oil spilled from vehicles were collected. The wet oily soil was collected with the help of spatula, placed into carry bags and brought to the lab for further studies.

3.1.1.3 Microcosm Design

The soil samples were brought to the laboratory and added to different flasks containing distilled water. The washing of soil was done to remove small stones and floating dry grass from the samples. The washing of the soil was done 3 -4 times and the flask carrying the samples in distilled water were kept on orbital shaker (100 rpm) at room temperature for 2-3 hours. The supernatant from each flask carrying microbial community was used for isolating indigo producing bacteria.

3.1.1.4 Enrichment of culture

The supernatant from each flask was inoculated into 100 ml of Bushnell Hass medium in a 250 ml erlenmeyer flask with diesel (1%) as the sole carbon source. The flasks were incubated at room temperature on a rotary shaker at 150 rpm for 48 hours. The growth of the culture was measured by taking optical density at 560 nm using UV visible spectrophotometer (Shimadzu, UV-2450 MODEL) at 24 hr, 48 hr and 72 hr for all the four samples. Samples from all the four flasks were streaked on Luria Bertani (LB) agar plates with 1% diesel on surface. Two different colonies were observed. One colony was seen to be circular, white and slimy whereas the other colony was seen to be circular and yellow in color. Under sterile condition, both the colonies were picked and streaked separately to get a pure culture. The single strain thus isolated was enriched in Bushnell Hass (BH) broth with indole as the sole carbon source.

Indigo formation: Further the enriched pure bacterial strains were tested for indigo production. Indole at a concentration of 2 mM was dissolved in acetone and spread over Luria Bertani (LB) agar plates. The acetone was allowed to evaporate under sterile conditions and the isolated pure cultures were spotted on the above mentioned plate and incubated for 24 hours at room temperature. The white coloured colony resulted in the formation of blue spots after a period of 24 hours whereas the yellow coloured strain was discarded as it did not result in the formation of blue pigment. It was observed that all the soil samples from all the four location contained the bacteria capable of forming the blue colour. Depending on the higher growth of the culture and by using standard microbial technique, pure culture from sample 2 was used for further study.

3.1.1.5 Identification of culture

The DNA extraction of the bacteria was done by using the protocol by Tsai and Rochelle (Tsai and Rochelle, 2001) with some minor modification, 16srDNA amplification: 27F and 1492R primers were used for amplification. The PCR program was used for initial denaturation , which was done at 94^{o}C for 5 minutes, denaturation at 94^{o}C for 30 seconds, annealing at 52 ^{o}C for 30 second, extension at 72 ^{o}C for 1 minute followed by final extension of 10 minutes at 72 ^{o}C (in Biorad mini personal thermal cycler). Further the amplified product was sequenced by an automated DNA sequenser (Applied Biosystems). The applied sequence was used to find out the organism using Basic Local Alignment Search Tool (BLAST) of National center for Biotechnology Information. Mega 5 software was used to construct the phylogenic tree by neighbor joining method. The sequence has been deposited to DNA Data Bank Japan (DDBJ) as -AEBBITSI1 (Accession no - LC149788).

3.1.1.6 Indigo production and Qualitative analysis

For indigo production, two set of indole vial were prepared. To these vials indole dissolved in acetone was added, and acetone was allowed to evaporate. After drying, five ml of medium and 1 ml of inoculum was added and incubated at room temperature for 24 hours. A set of vials were also kept without inoculation to serve as a-biotic control. After 24 hours, product indigo was extracted by adding 5 ml of ethyl acetate to each tube and vortex for five minutes, 1 ml of extracted indigo samples was preserved for further analysis. The analysis of the blue pigments formed was carried out using Thin layer chromatography (TLC) and UV visible spectrophotometer.

3.1.1.7 Standard graph for Indigo

The commercially available indigo ($C_{16}H_{10}N_2O_2$) was dissolved in ethyl acetate to give varying concentrations of indigo solution. The optical density of these solutions was found immediately using U V visible spectrophotometer (Shimadzu, UV-2450 MODEL) at 610 nm. The ethyl acetate was used as blank for this analysis.

3.1.2 Results and Discussion

3.1.2.1 Enrichment of culture and screening for isolate producing indigo

The samples collected from four different garages were studied for the growth of bacteria present. A mixed culture of bacteria capable of growth on diesel as a sole carbon source was enriched by successive sub culturing in Bushnell Hass medium. Optical density measurements of the enrichment culture confirmed the increase in bacterial mass. The sample from the second site (sample two) has shown comparatively higher growth based on the optical density reading at 610 nm (Fig 3.1). Sample two was selected based on higher optical density values as compared to other samples and further treated for isolation of the desired bacteria.

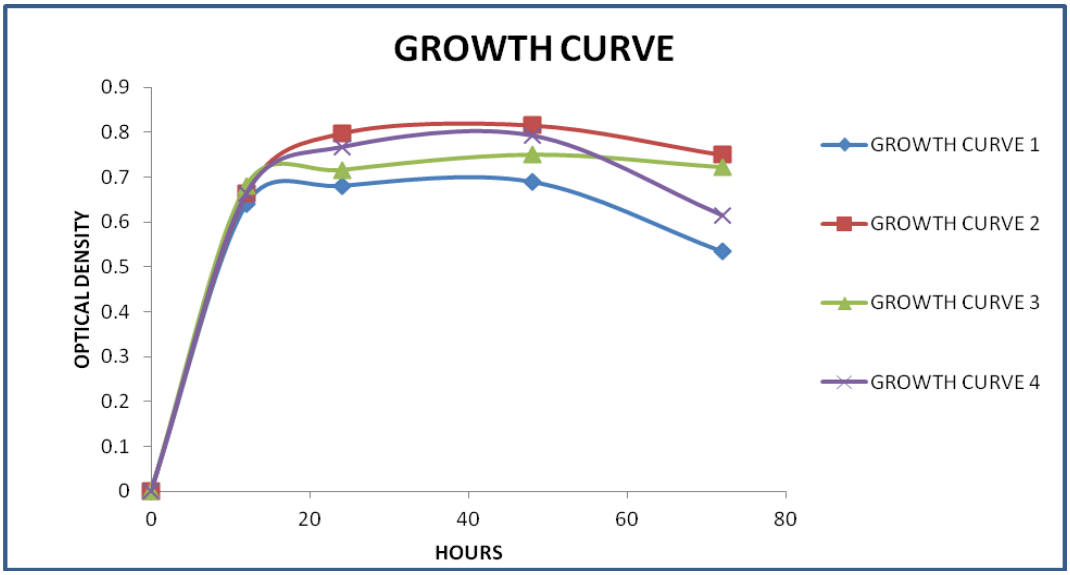

Fig.3.1: Growth of the bacteria from the four locations selected for study

Standard plating technique was used to obtain pure cultures from the consortium. When dilutions of enrichment culture (sample 2) were incubated on agar plates, two different

bacterial colonies became apparent on the enrichment medium agar. The colony morphology is as follows: one of the colonies was yellow in colour and circular in shape. The other one was white in colour with a slimy texture and circular. Both the bacterial strains were subjected to screening using differential medium containing BH agar and Indole. The white colour culture has turned blue in the medium containing indole (Fig 3.2), indicating the production of indigo and was used for further studies.

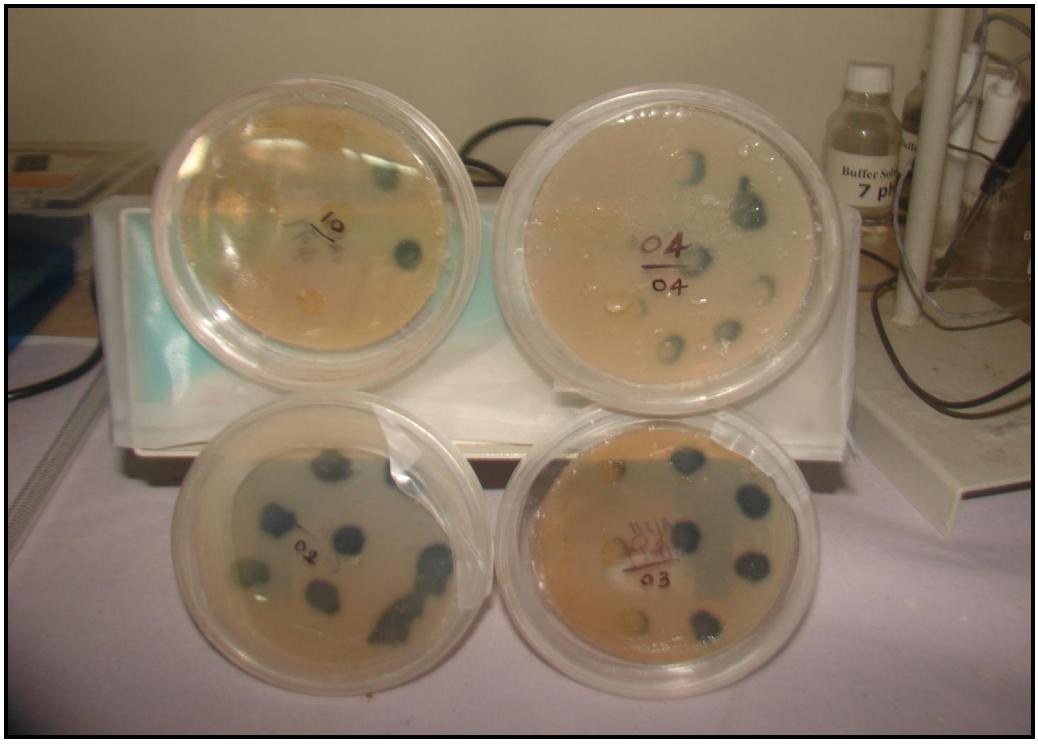

Fig. 3.2: Culture spotted over agar plate shows the blue colour formation in presence of substrate.

3.1.2.2 Identification of culture

Based on the sequencing and BLAST results it was oserved that the isolated organism was *Pandoraea sp.*(Fig.3.3) . *Pandoraea sp.* are gram-negative, non-spore-forming motile bacteria with single polar flagellum, from the family of *Burkholderiaceae* which

belongs to the class of Betaproteobacteria. There are reports on production of indigo using *Burkholderiaceae* family (McClay K. et.al,2005) .However there are no reports, of production of indigo using *Pandoreae* species. This is the first report of use of *Pandoraea* for production of bio-indigo.

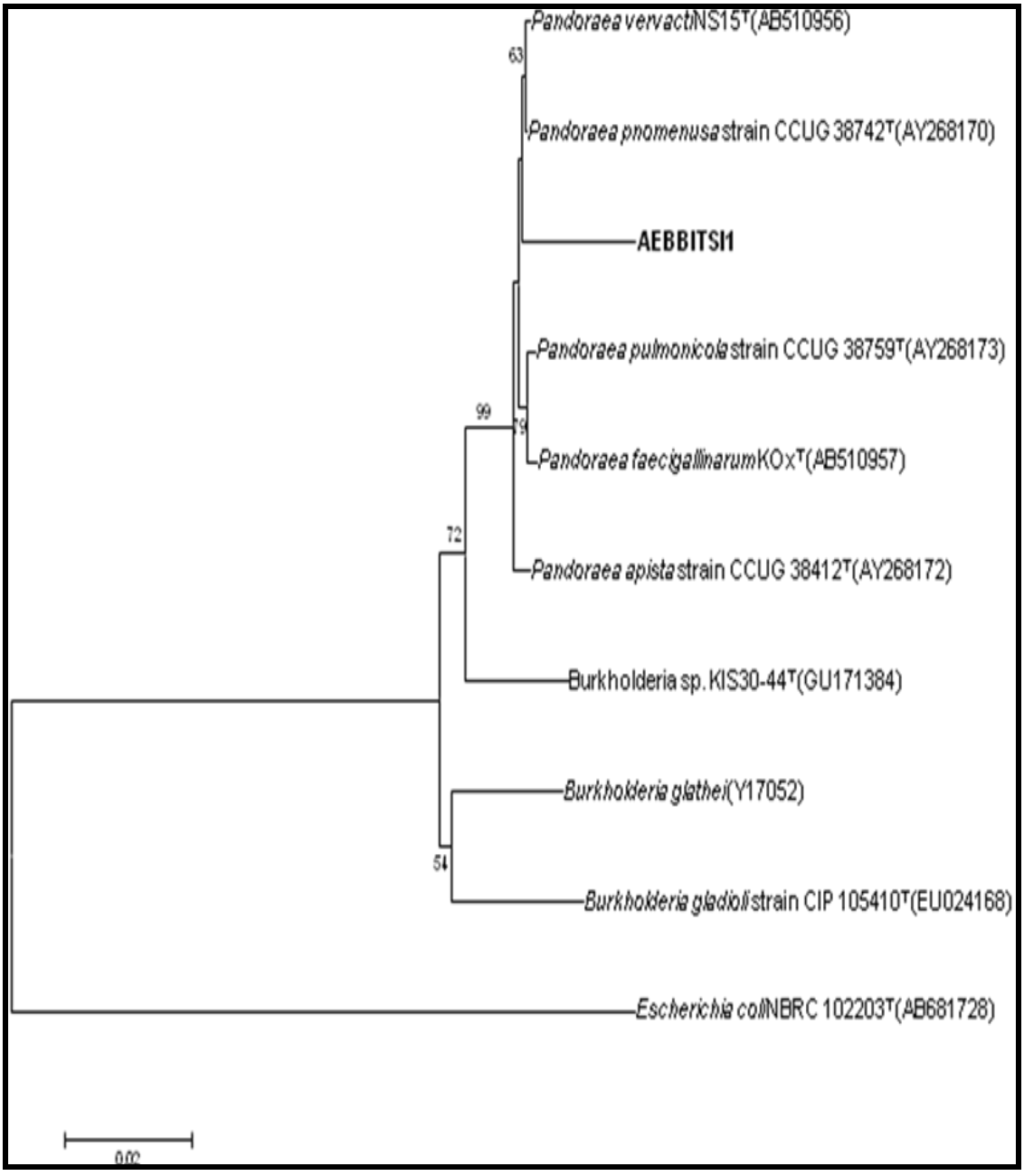

Fig.3.3: Identification of Bacteria, Phylogenic tree

3.1.2.3 Analysis of indigo produced

Verification of the indigo formation was done using Thin Layer Chromatography (Fig. 3.4) and UV visible scan from 200 to 800 nm (Fig. 3.5). The reference used was the synthetic indigo of analytical grade. The Thin layer chromatography result showed the production of two types of pigment, indigo-blue and indirubbin-purple with R_f value 0.86 and 0.82 respectively. The R_f value for synthetic indigo was found to match the synthetic indigo blue pigment value.

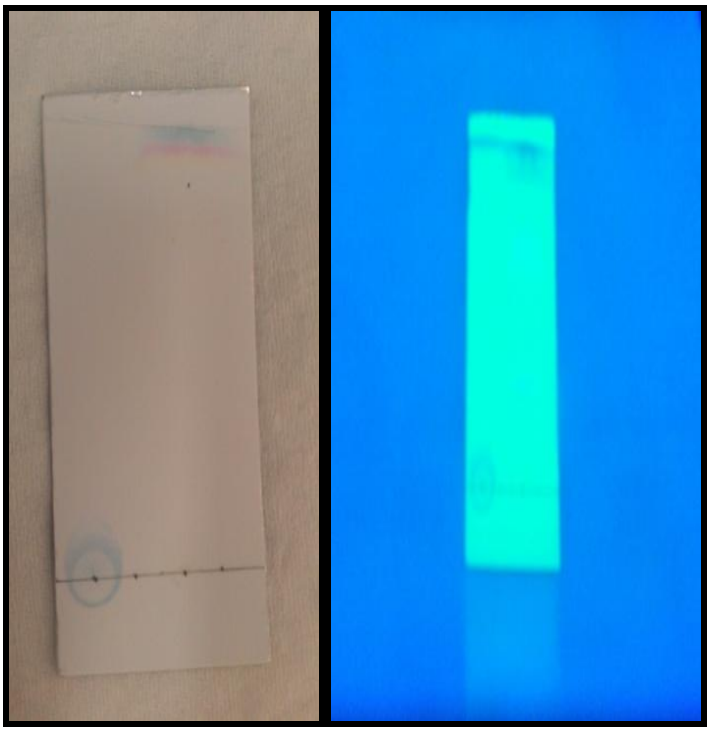

Fig.3.4: Thin layer Chromatography test for blue pigment formed as Bio-indigo

For further confirmation a UV visible scan was carried out for the extracted blue pigment and the synthetic indigo dye, and both was found to match.

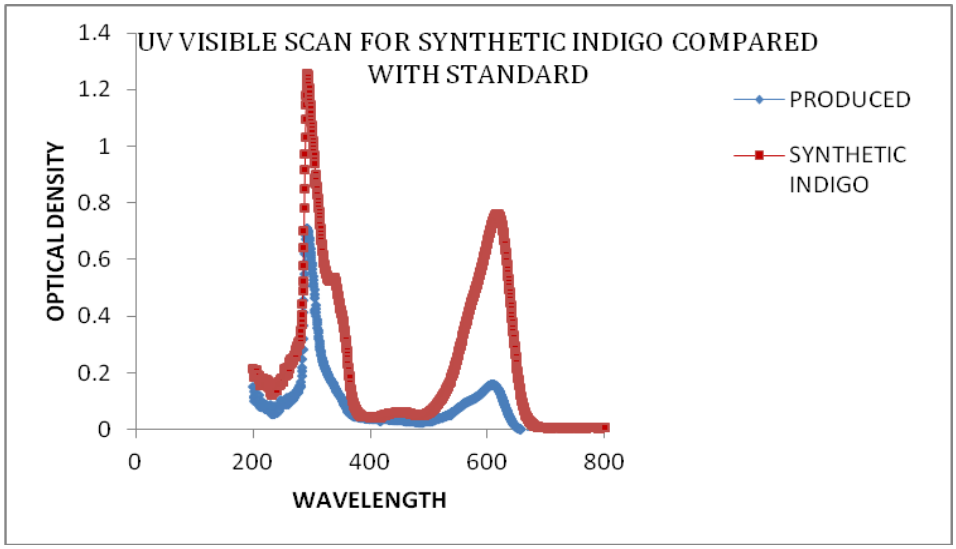

Fig.3.5 : UV Visible scan for Bio-indigo

3.1.2.4 Standard graph

The commercially available indigo was used as a standard to quantify the concentration of indigo produced during various experiments in this study. Thus a plot of optical density on ordinate against concentration of indigo (mM) on Abscissa was plotted as standard graph for further use (Fig. 3.6).

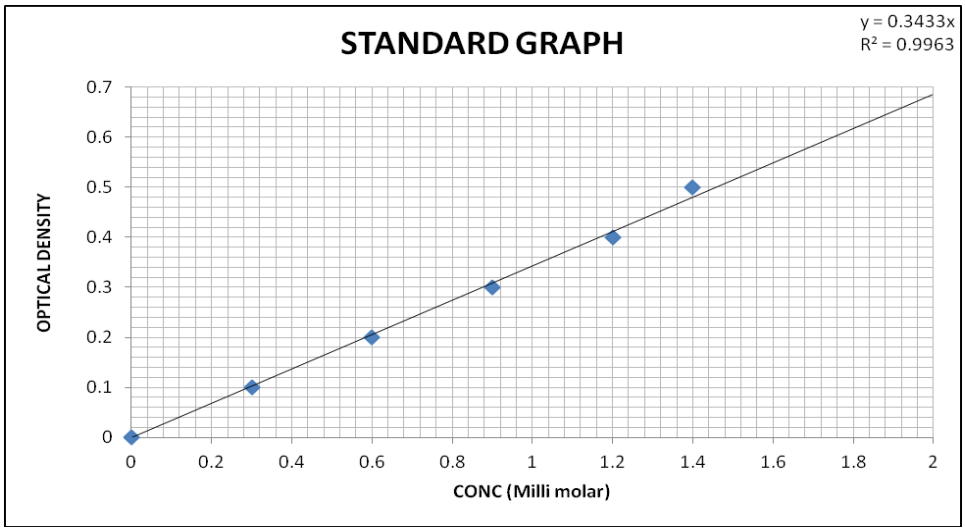

Fig. 3.6: Standard graph plotted using commercial indigo

CHAPTER 4:

Optimization of parameters for indigo production

4.1: OPTIMIZATION OF PARAMETERS FOR INDIGO PRODUCTION

Literature on bacterial indigo production shows different types of hydrocarbon degrading bacteria used for production of this interesting dye. The only limitation in the process is lower productivity and the huge demand in the textile market. Therefore, it is necessary to develop the correct combination of various culture conditions to enhance the growth and the dye production (Palanichamy V., 2011).

For industrial fermentation process, fermentation medium and fermentation process condition plays a critical role because they effect the formation, concentration and yield of a particular fermentation end product. This affects the overall process economics, therefore, it is important to consider the optimization of fermentation medium and process conditions to maximize the profits from fermentation process (Schmidt, 2005). Today different techniques are being used for enhancing the yield of the biocatalysis products. Recombinant DNA technology is one of the techniques applied for Bio-indigo production from E-coli which challenges the chemical synthesis of the dye (Derek G. S. et.al, 1999). Optimization methods are the use of statistical and mathematical tools to analyze the effect of different parameter on the product formation. Response surface methodology (RSM) is one among them. It is a compilation of statistical and mathematical techniques widely used to determine the effects of several process variables on response and to optimize different biotechnological processes (Annapurna K., 2009).

The present work highlights the use of RSM for optimization of indigo production in laboratory. The influence of identified parameters and their individual and interactive

effects is studied for indigo production, by performing a series of planned experiments and analyzing the response.

Another, very interesting and most recent technique of optimization is the use of ATPS (Aqueous Two Phase Systems) for biological products. The ATPS has the advantage like, it provides gentle environmental conditions, the interfacial tension between the two phases is low, it has stabilizing effects on the biological activity, straightforward and relatively simple, easy to scale-up and reliable process, makes it a novel method for recovery of biopharmaceuticals, environmental remediation, proteins purification and for extractive bioconversion (Karnika R.. 2010).Another advantage for two phase system is the controlled delivery of a toxic substrate dissolved in an organic phase to a cell-containing aqueous phase (Januz M. ,2001).

Different solvents were tested on the bacteria by conducting the toxicity test and the selection of appropriate solvent for two phase experiment was done. This was followed by a comparative single phase and a two phase study to understand the effect of second phase on increase in the yield of product formation.

In later part of work the kinetics parameters were also studied to help the optimization of the indigo production process. The formation of indigo dye with time helped to determine the rate of the product formation in single as well as two phase system. Further the kinetics parameters, V_{max} and K_m were calculated based on the Michaelis-Menten and Lineweaver Burk plots for this experiment.

4.1.1 MATERIALS AND METHODS

4.1.1.1 Chemicals

Chemicals used: Commercially available Bushnell Hass medium (BH), Indole, Silicone oil, Ethyl acetate, Acetone, Hexane, Methanol, Chloroform, Dimethyl formamide, Sodium Hydroxide and Hydrochloric acid were used for the present work. All the chemicals used are of highest purity grade from Himedia , S D Fine Chemicals and Merck, India.

4.1.1.2 Methodology

To overcome the challenge for bio-indigo production a comparative, single and two phase study was conducted to produce indigo. Parameters like substrate concentration, temperature, pH were optimized to reduce the cost of bio-indigo production.

4.1.1.2.a Comparative study: single and two phase with variation in substrate concentration

In this study a novel technique of use of two phases was experimented to test the increase in the yield of indigo as compared to a single phase bacterial production. Various solvents like Ethyl Acetate, Hexane, Acetone, Chloroform, Dimethyl formamide, and Silicone oil were tested for growth of *Pandorea sp.* well as for indigo production. The culture was inoculated in the medium with the substrate indole, in presence of the solvents at room temperature on shaker at 150 rpm. The indigo production was observed only in the presence of acetone and silicone oil as a second phase. Indigo produced in presence of silicone oil was more as compared to acetone, thus silicone oil was used as a second phase for further experiments.

To optimize the indole concentration in absence as well as in presence of second phase, the culture was subjected to varying concentrations of substrate indole. The indole concentration was varied from 0 mM to 4 mM, with 0.5 mM interval in-between. Two sets of indole vials were prepared. To these vials, solution of indole dissolved in acetone at varying concentration was added. Acetone was allowed to evaporate under sterile conditions. In first sets of vials, 4 ml of sterile BH medium and 1 ml of silicone oil and for second set of vials 4 ml of sterile BH medium alone was added for comparative study. All the vials were inoculated with 1 ml of inoculum and incubated in orbital shaker at 150 rpm at room temperature for 24 hours and measured for indigo production. A set of vials were also kept without inoculation which served as a biotic control. The indigo produced was studied in two sets, first in absence of silicone oil and another in presence of silicone oil as a second phase.

4.1.1.2.b Design of Experiments (DOE) for indigo production

RSM for Design and analysis study was carried out using variation in substrate concentration and temperature to understand the relationship between the yield and possible interaction between temperature and indole concentration.

A few experiments were conducted to judge the crucial process variables that affect the indigo dye production. It has been found that factors like temperature, indole concentration and pH affect the production of indigo. RSM was used to evaluate extent of two process variables viz. Indole concentration and temperature on Indigo production. pH value of the medium was kept constant for all experiments as too low (pH 4) or too high pH (pH 10) values were giving void results. Two levels of each factor were selected with a center point to account for the non linear nature of the response. Three replicates of

each experiment were performed to account for the variability by chance. The process variables and their level selected is shown in Table 4.1.

Table-4.1 Experiment process variables and levels selected for RSM studies

Factor selected	Designation	Lowest level (-1)	Middle level (0)	Highest level (+1)
Indole concentration	X1	2	3	4
Temperature	X2	30	35	40

For the factors and their levels selected, RSM has generated 9 experiments based on center composite design.To account for the noise factor in the experiment, each experiment was repeated thrice.Thus a scheme of 27 experiments was generated and performed. Table 4.2 shows the plan of experiments along with the responses in terms of amount of Indigo Produced.

Table 4.2 : Experiment design and Indigo produced for RSM studies

	FACTOR 1	FACTOR 2		
SR. NO	**INDOLE CONC (milli molar)**	**Temperature (^oC)**	**INDIGO PRODUCED (milli molar)**	**AVG INDIGO (milli molar)**
1	2	30	0.39	0.39
			0.38	
			0.388	
2	2	35	0.39	0.39
			0.38	
			0.39	
3	2	40	0.4	0.41
			0.42	
			0.42	
4	3	30	0.38	0.38
			0.39	
			0.36	
5	3	35	0.37	0.38
			0.39	
			0.38	
6	3	40	0.46	0.44
			0.43	
			0.42	
7	4	30	0.38	0.36

			0.34	
			0.37	
8	4	35	0.38	0.38
			0.38	
			0.37	
9	4	40	0.3	0.34
			0.35	
			0.36	

4.1.1.2.c. pH variation :

Further to optimize and analyze the production of indigo in acidic and basic environment. The acid and alkali was added in the medium for single phase and two phase experiment. Sodium hydroxide Solution and dilute Hydrochloric acid was added in drops to change the pH of the medium. The pH of the medium was measured using pH meter. The pH was set at different values as 6, 7, 8,9,10 and 11 for both set of experiments. The medium at different pH was inoculated and incubated at room temperature for 24 hours in presence of indole.

4.1.1.2.d. Rate of indigo formation

To determine the minimum time required for the production of indigo, experiment was done at indole concentration of 3 mM. Two set of indole vials were prepared after evaporation of acetone as discussed earlier. One set was used for single phase and the other was used for two phase (silicone oil). Both the sets were incubated with the pure culture at room temperature. After every 2 hours the culture was withdrawn under sterile condition and mixed with equal volume of ethyl acetate in the vial. The extraction of the indigo was done by vortexing for 3-5 minutes. Ethyl acetate and silicone oil was used as a blank for single phase and two phase study respectively.

4.1.1.2.e. Kinetics study for calculation of Vmax and Km from a batch study of Indigo

Chemical Kinetics is the study of reaction rates of a chemical process whereas for Biological systems the enzyme kinetics studies the chemical reactions catalyzed by the enzymes. Enzymes are highly specific. Typically a particular enzyme catalyzes only a single chemical reaction or a set of closely related chemical reactions. The clarification of the mechanism of an enzyme can only be understood with the help of kinetics of the reaction where the enzyme acts as a catalyst for the biological reaction (Paul C. et.al, 2007). An enzyme-catalyzed reaction of substrate S to product P, can be written, $E+S \xleftrightarrow{k1/k-1} ES \xrightarrow{k2} E+P$, solving and rearranging this equation we get,

$$ES = \frac{V_{\max} S}{S + K_m}; V_{\max} = k_2 E_{tot}, K_m = \frac{k_1^{'} + k_2}{k_1}$$

To characterize the enzyme catalyzed reaction K_m and V_{max} needs to be determined. The V_{max} is the maximum reaction rate attained at higher concentration of the substrate and K_m is the Michaelis constant, which is a substrate concentration which permits the enzyme to achieve half the value of V_{max}.In this part of work, the experiment was done to calculate the rate of the reaction at different concentration of the substrate from 0 mM to 4 mM with 0.5 mM intervals. The indigo formation was quantified by a UV-visible spectrophotometer. The rate of indigo formation was plotted against the substrate concentration based on the Michaelis-Menten kinetic and was further linearised to get the kinetic parameter, V_{max} and K_m using the Line weaver Burk plot.

4.1.2 Results and Discussion

4.1.2.1. Comparative study of indigo production between Single and Two phase

The indigo produced was studied in two sets (Fig.4.1), first in absence of silicone oil and another in presence of silicone oil as a second phase.

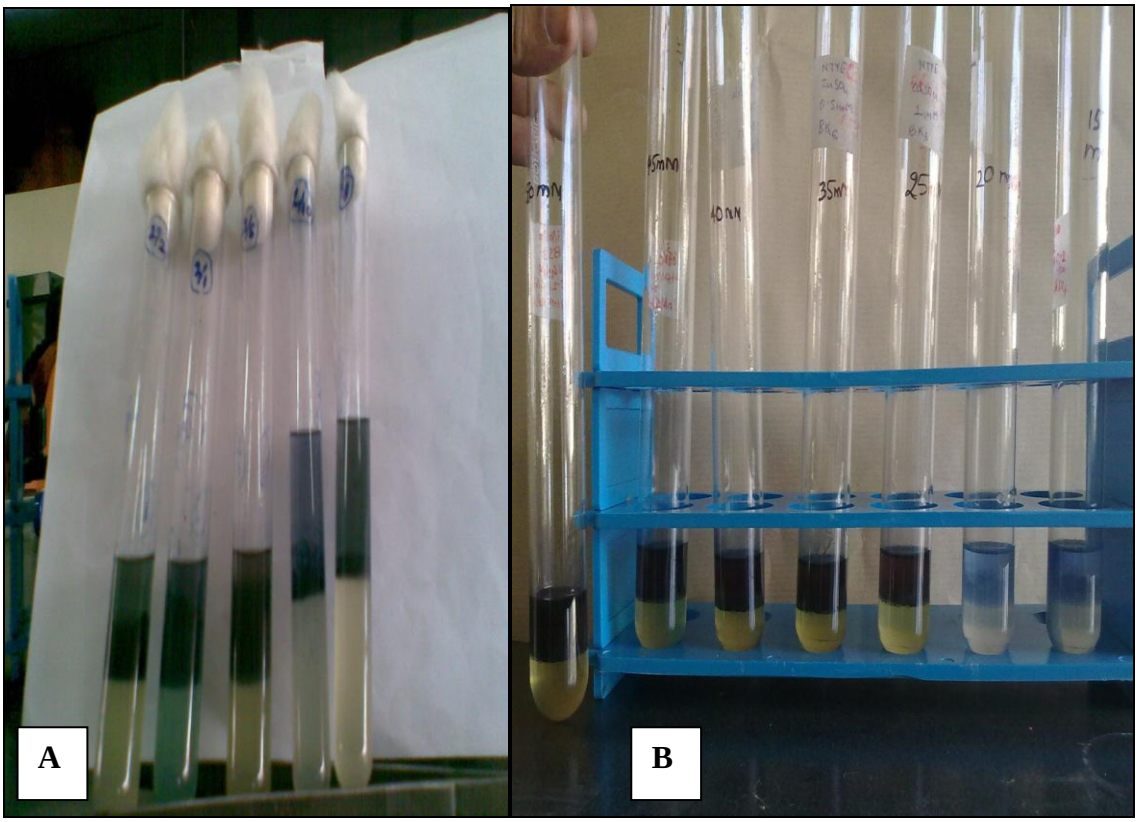

Fig.4.1: Bio-Indigo production at lab scale (A) Single phase and (B) Two phase studies

In such a two-phase aqueous–organic system, the substrate is solubilized in the immiscible organic phase and allowed to transfer into the organic phase. Thus, the substrate concentration in the biotic phase can be maintained below the inhibitory level. Also the organic phase helps the increased bioavailability of the substrate to the bacteria, by increasing the surface area for contact, resulting into an increased production of the product. The highest indigo production of 0.6 mM (0.16 g/L) was obtained at 3.5 mM concentration of indole (Fig.4.2).

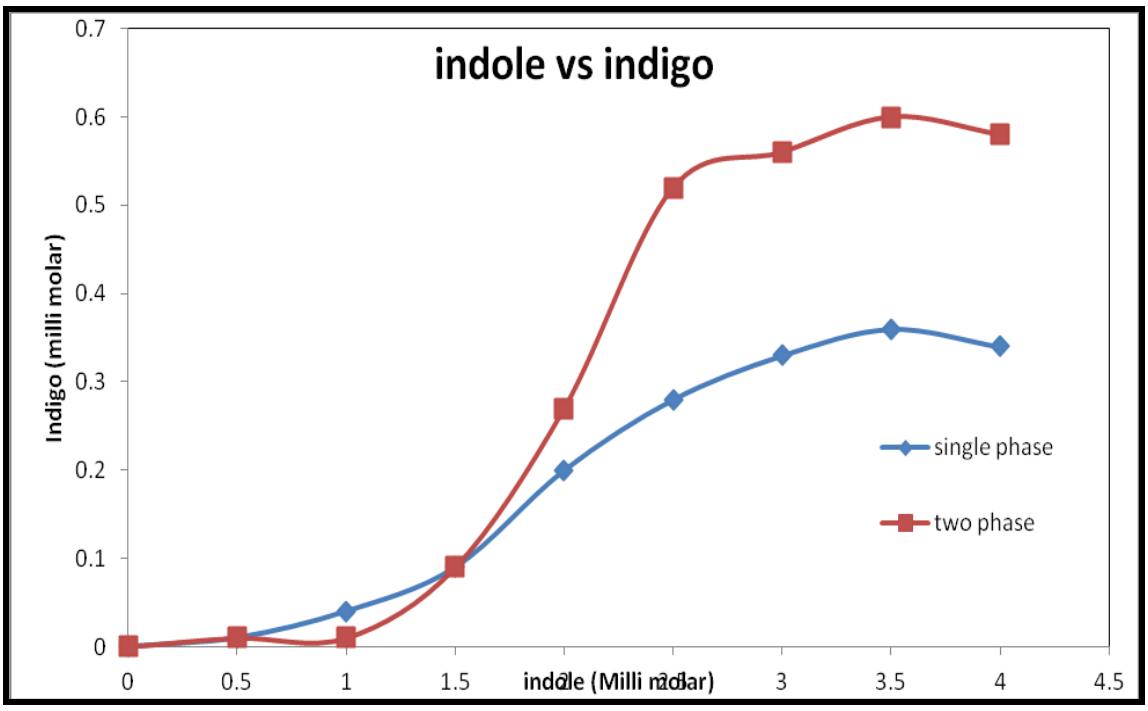

Fig. 4.2: Indole vs Indigo formation in vial

4.1.2.2: Analysis and Experiments

To determine the extent of significance of process variable on the Indigo production, Analysis of Variance (ANOVA) was done at 5 % level of significance as shown in Table-4.3. It is evident from the results that Indole concentration and temperature have strong interaction effect on the Indigo production. This interaction also results in a non linear response as it can be seen that interaction terms A^2B and AB^2 were found to be significant. The quartic model used resulted in the model with F-value of 8.80 and P values of < 0.0001, which implies the model is significant.

Table 4.3: Analysis of Variance (ANOVA Table for RSM studies)

ANOVA for Response Surface Quartic Model (Aliased)						
Analysis of variance table [Partial sum of squares - Type III]						
Source	Sum of Squares	df	Mean Square	F value	p-value Prob > F	
Model	0.019296296	8	0.002412037	8.800676	< 0.0001	significant
A-A	0.00015	1	0.00015	0.547297	0.469	
B-B	0.0054	1	0.0054	19.7027	0.0003	
AB	0.002408333	1	0.002408333	8.787162	0.0083	
A^2	5.56E-06	1	5.56E-06	0.02027	0.8884	
B^2	0.001422222	1	0.001422222	5.189189	0.0352	
A^2B	0.003402778	1	0.003402778	12.41554	0.0024	
AB^2	0.001469444	1	0.001469444	5.361486	0.0326	
A^3	0	0				
B^3	0	0				
A^2B^2	0.001556481	1	0.001556481	5.679054	0.0284	
A^3B	0	0				
AB^3	0	0				
A^4	0	0				
B^4	0	0				
Pure Error	0.004933333	18	0.000274074			
Cor Total	0.024229630	26				
The Model F-value of 8.80 implies the model is significant. There is only						
a 0.01% chance that a "Model F-Value" this large could occur due to noise.						

To account for the simultaneous variation of the Indole concentration and temperature, 3_D surface plots were drawn (Fig. 4.3). The maximum Indigo production of around 0.44 mM was observed at 3mM indole concentration and 40^0C.There is gradual increase in Indigo at 40^0C when Indole changes from 2 to 3 milli molar. However it decreases further as the Indole concentration increases. This could be due to the toxic effect of increased concentration of indole on the bacteria.

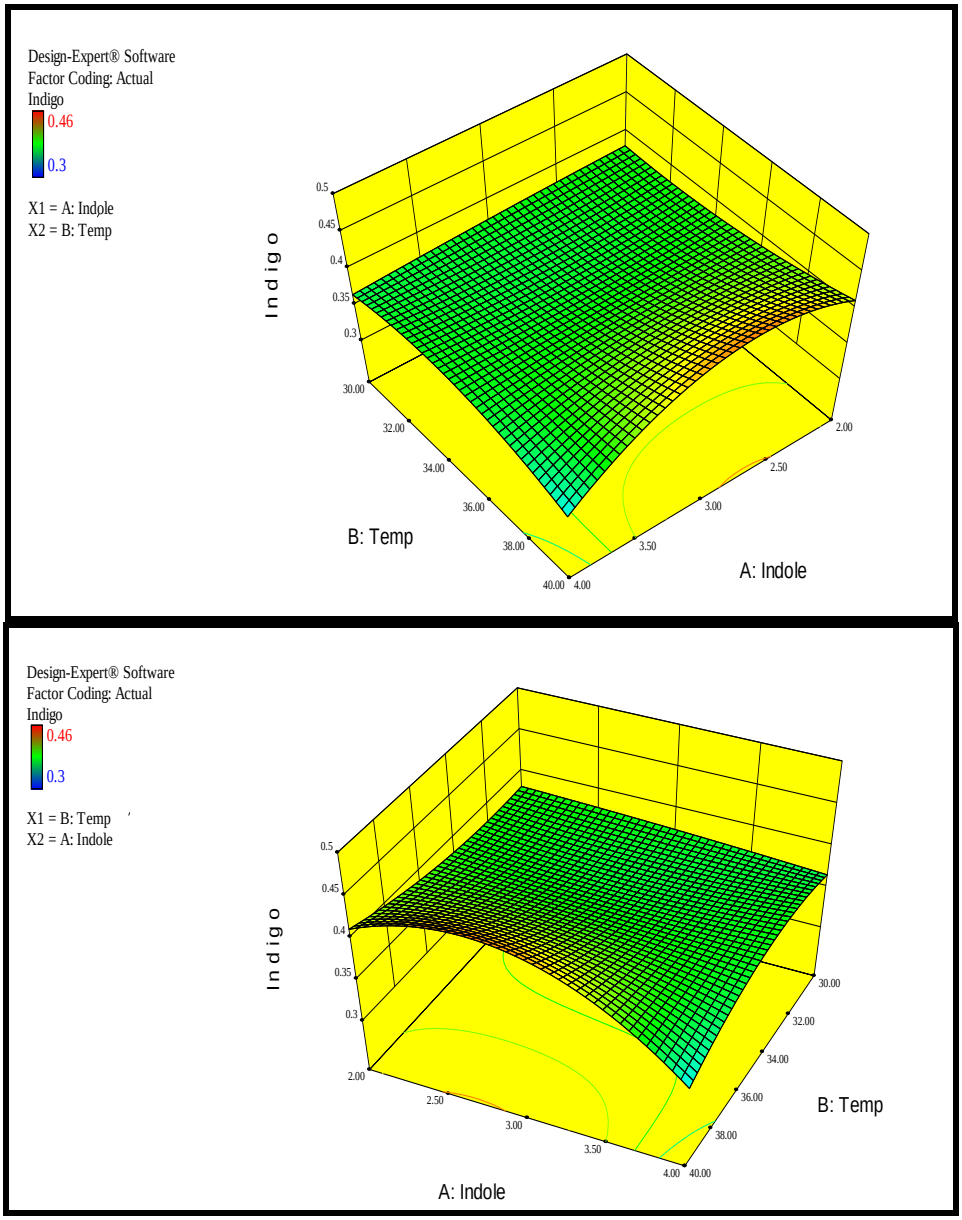

Fig.4.3: Design of Experiment results for optimization of parameters

Empirical Model for the Indigo Production

An empirical model was developed and the experimental results were compared with the model to validate it and 3D plots were generated.

Based on the above findings, a regression equation has been developed by accounting significant factors affecting the response. Using this equation, the Indigo production can be predicted within the regime of experimentation. The regression equation given below explains about the indigo production(Y) as:

$Y= +3.80 – 0.005\ A +0.030B-0.015A*B +0.0017\ A^2 +0.027\ B^2 – 0.030\ A^2B -0.019\ A\ B^2 -0.033\ A^2*B^2$,

Where A=Indole concentration and B= Temperature.

To validate the model, confirmatory experiments were performed; the indole concentration varying from 2 to 4 mM were used. Experiments for studying the effect of temperature on the indigo production was carried out using water bath which was maintained at temperature 10, 20, 30, 40 and 50^oC, (Fig.4.4),at indole concentration of 3 mM. The result showed no indigo production at 10, 20 and 50 ^oC for single as well as for two phase studies. Thus a temperature range of 30^o to 40 ^oC can be used to achieve higher yield of indigo.

Under 3 milli- molar substrate concentration and at 40 ^oC temperature, the bio-indigo produced was found to match the model results.

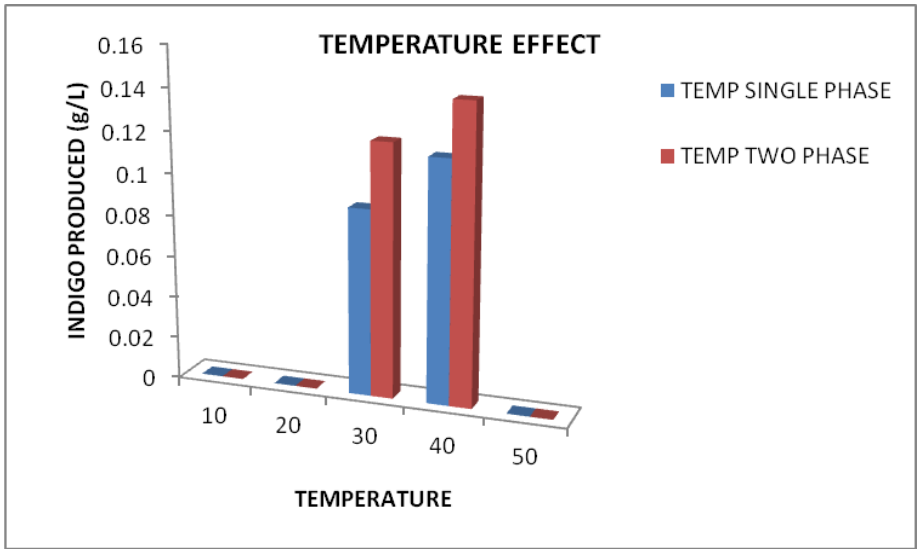

Fig. 4.4: Temperature effect for single and two phase indigo production

4.1.2.3 Variation in pH analysis

The medium at different pH was inoculated and incubated at room temperature for 24 hours in presence of indole. After 24 hours the extraction of indigo was carried out and quantified. The results showed maximum indigo production of 0.12 g/L at pH 7 (Fig.4.5).

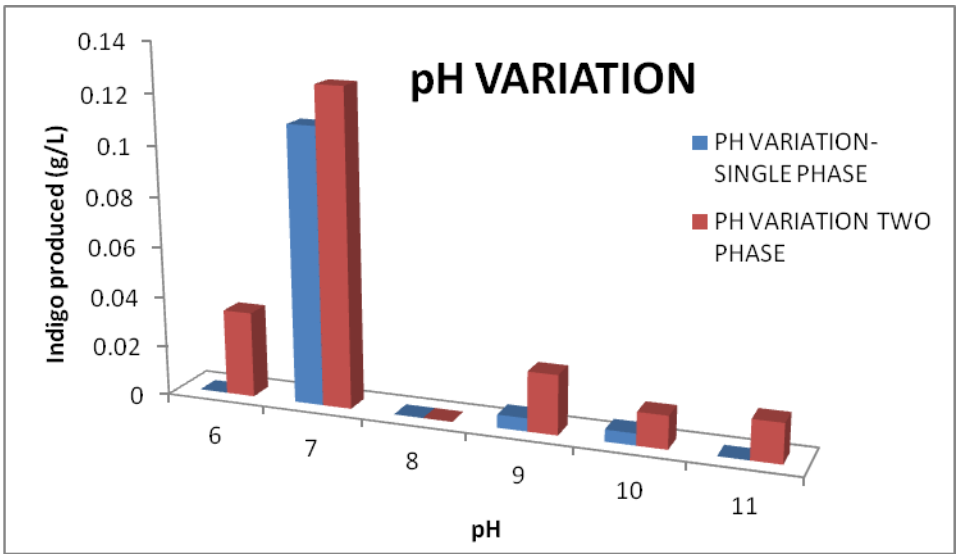

Fig.4.5: pH effect for single and two phase indigo production

4.1.2.4 Rate of Indigo production

Indigo production was found to start only after 4 hours and reached a maximum of 0.16 g/L in presence of silicone oil whereas for single phase it gave maximum indigo production of 0.08 g/L after 24 hours. The two phase study was found to result in increased Indigo formation as compared to single phase .The increase in indigo concentration was as a result of increase in bio-availability of the substrate indole for the bacteria. The oil helped the uptake of the indole supplied and increased the contact surface area for reaction to form indigo from indole. The addition of silicone oil reduced the toxicity of substrate in the biotic phase as well as increased the availability of substrate to the bacteria in the organic phase. The silicone oil was found to help in increase in production of indigo as compared to a single phase. Based on the above results (Fig. 4.6) , silicone oil was used as second phase in a bioreactor for Indigo production.

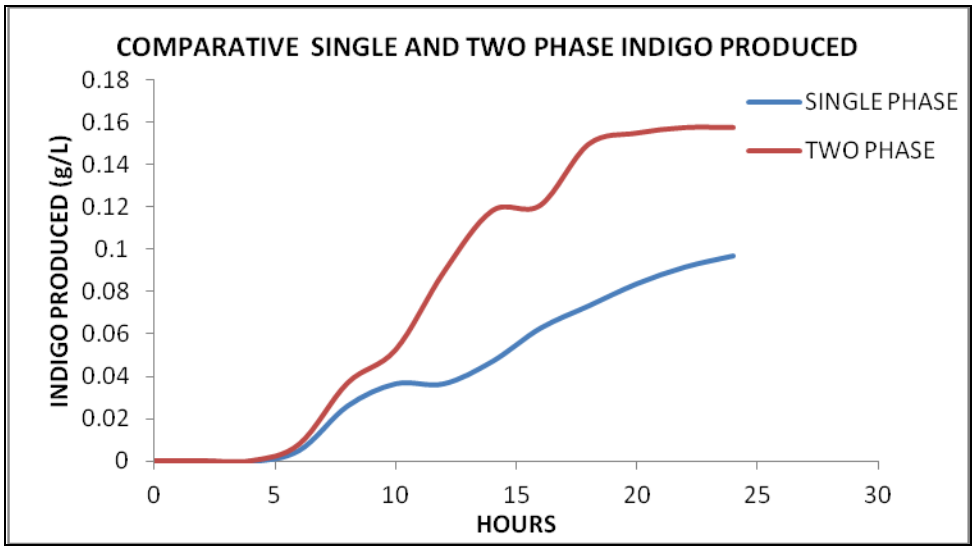

Fig.4.6: Rate of indigo produced in single phase and two phase

4.1.2.5 Kinetics parameter V_{max} and K_m

The rate of indigo formation was recorded with respect to the variation in the substrate concentration (Indole) from 0 mM to 4mM with an interval of 0.5 mM. The kinetics parameters were found using the Michaelis-Menten (Fig. 4.7) and Lineweaver Burk plot (Fig. 4.8) for both the single as well as for two phase experiments. The K_m and V_{max} for the enzyme involved in indigo formation for single phase was found to be 3.33 mM and 0.66 mM/day respectively. Whereas the K_m and V_{max} was found to be 2.22 mM and 0.90 mM/day respectively for two phase system.

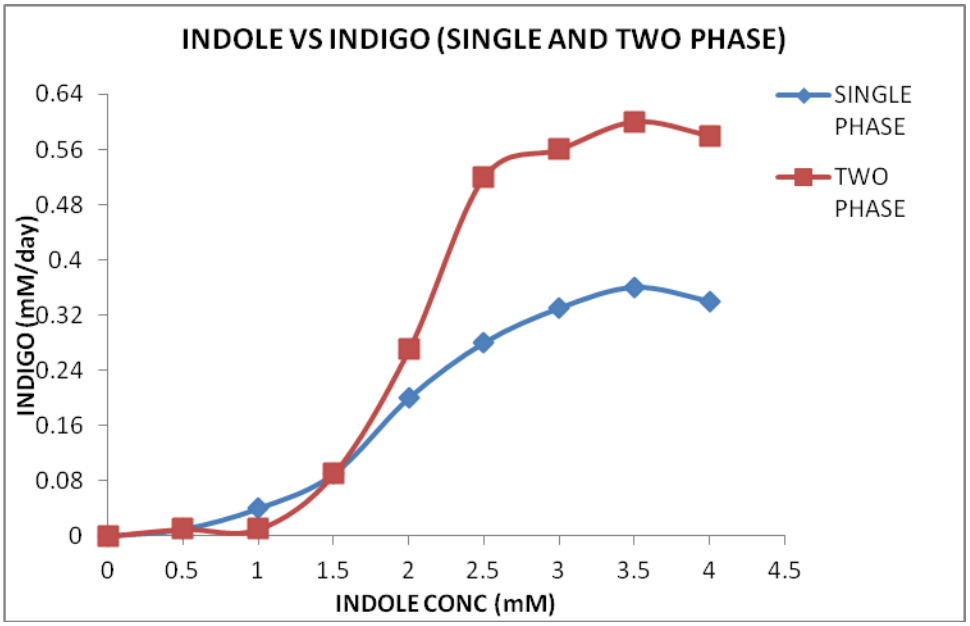

Figure. 4.7 Michaelis- Menten plot for single and two phase system

The K_m value in Lineweaver Burk plot represents the affinity of the enzymes for the substrate, lower value of K_m , better affinity of enzyme to the substrate and V_{max} represents the maximum product formation. The results obtained in the experiment reveals the affinity of the enzyme toward the substrate and the maximum product formation for single and two phase system. It is found that the enzyme shows more

affinity for the substrate in the two phase system (K_m = 2.22) as compared to single phase (K_m = 3.33) resulting into more product formation in silicone oil –medium system. The V_{max} for two phase system is found to be 0.90mM which is greater as compared to single phase. Therefore based on the kinetics a two phase system is found to favor the dye production and can be used for scale up studies.

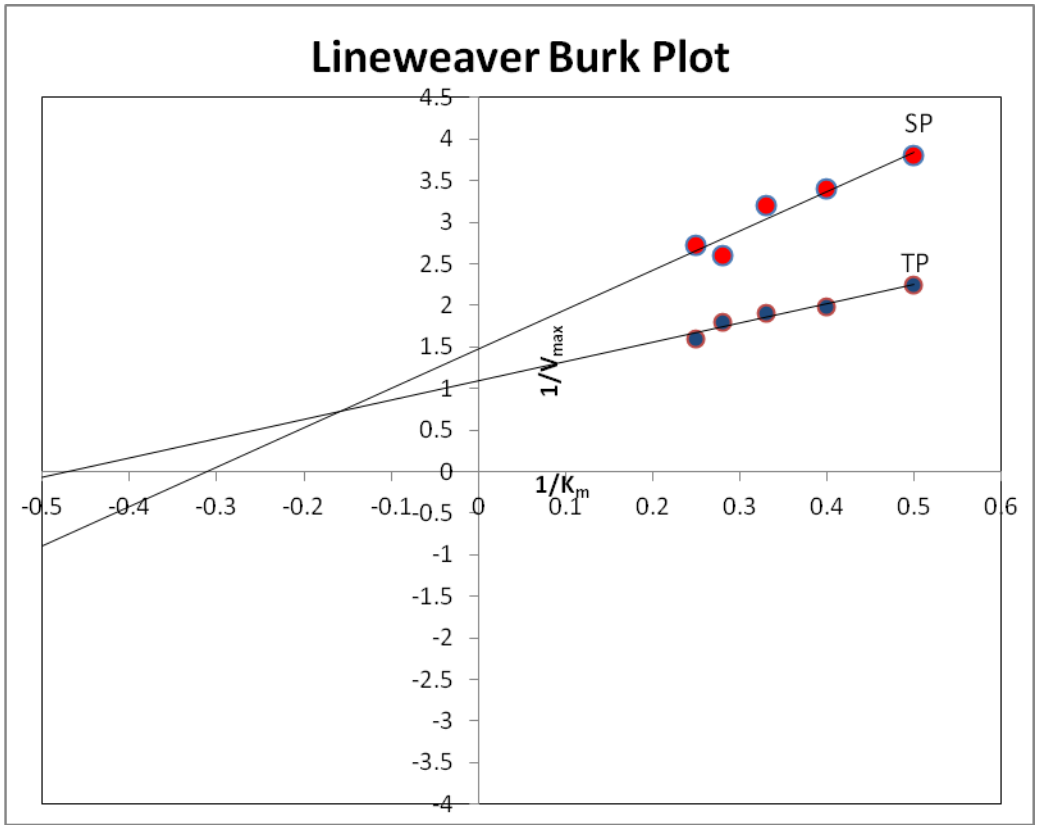

Figure. 4.8 Lineweaver Burk Plot for single phase (SP)and two phase (TP)system

CHAPTER 5:

Scale up of indigo production at lab scales in a bioreactor and optimize it for commercial application.

5.1: SCALE UP OF INDIGO PRODUCTION AT LAB SCALE IN A BIOREACTOR AND OPTIMIZE IT FOR COMMERCIAL APPLICATION

Bioreactors are the mechanical vessels in which organisms are cultivated in a controlled manner and/or materials are converted or transformed via specific reactions to form a particular product. The proper selection and design of the bioreactor will determine the optimal commercial bioprocess and the corresponding capital investment. The bioreactor should not be regarded as an isolated unit, but as part of an integrated unit operation with both upstream (preparation) and downstream (separations) unit operations (John W., 2002).

Bioreactors of different configuration can be used in production of biological products. Based on the mode of operation bioreactors are classified as Batch, Fed-Batch and Continuous bioreactors. Batch reactors have simple construction and are suitable for small production but may lead to increased batch time, due to charging and discharging of the reactor (Mukesh D. et.al ,2004).

Many, if not most, industrially important fermentation and bioreactor operations are carried out in fed-batch mode, producing a wide variety of products (Henry C. Lim et.al, Cambridge University Press, 2013). Fed-batch Fermentation process can be accomplished within a single day with minimal supervision, and can be done on a small (2L) scale that is scalable to 30L or more capacity(Moulton, 2013). On the other hand continuous bioreactors provides high production rate, and better product quality due to constant reaction conditions (Mukesh D. et.al, 2004). Selection of the bioreactor configuration is thus a major task for product formation and its optimization to increase the yield of the product.

This part of work focuses on scale up of shake flask experiments to bioreactor operation for commercial application. A comparative single and two phase study is carried out to study the effect of two phase on the yield of Bio-indigo in a reactor. The experiments are conducted in a 2.5 liter capacity bioreactor vessel in Fed batch mode and Continuous mode to identify the configuration best suited for Indigo production. For each of this configuration a comparative study between single phase and two phases using silicone oil is done.

5.1.1 MATERIALS AND METHODS

5.1.1.1 Chemicals and Material

All chemicals used like indole, silicone oil ,ethyl acetate in the experiments were of analytical grade purchased from Himedia and S.D fine chemicals, Mumbai, India..

Bio-Reactor of Bio-Ferm was purchased from Zenith Engineers, Agra, India.

5.1.1.2 Bioreactor Study

Fermentor from Bio-Ferm was used for scale up experiment in this part of work at optimized condition, The two modes of operation used are - Fed batch (mostly used in industry due to better efficiency) and Continuous. Bioreactor, of 2.5 liters capacity was used with a working volume of 1 liter. The reactor was operated under optimized conditions i.e, temperature at 30 ^{o}C, Initial pH at 7 and substrate concentration of 3 mM . The reactor was operated in single phase and two phases with impeller speed at 200 RPM and DO maintained at greater than 70 %.

A. FED BATCH SINGLE PHASE STUDY (Fig.5.1-A):

The shake flask study carried out in the earlier part of the work was scaled up in a bioreactor. The bioreactor after sterilization was inoculated with the culture. The culture was grown till the mid log phase. The toxicity test for appropriate solvent selection was done to inject substrate indole into the medium. The challenge to supply solid crystalline indole to the bacteria under sterile condition was overcome by supplying indole through acetone. This was done by dissolving 3 mM indole into 2 ml of acetone and fed to the reactor carrying growing culture. To maintain the single phase mode a very low volume i.e 2ml of acetone was used for 1000 ml of culture medium in the reactor. Further the substrate injection was done after specific intervals of time based on growth of bacteria as well as the product formation. The injection was done at 47^{th} ,91^{st} and 145^{th} hour. Growth of bacteria and indigo produced was recorded using spectrophotometer at 560nm, 610 nm and respectively. The blue pigment formed in the reactor was analyzed using FTIR for the identification and confirmation of the dye produced.

B. FED BATCH TWO PHASE STUDY (Fig.5.1-B)**:**

The culture was allowed to grow till it reaches a mid log phase (based on OD) in the fermentor, at this point the pre-dissolved indole, 3 mM into 200 ml of medium was fed into the Fermentor along with 200 ml of silicone oil as a second phase (fed point 1).The OD for culture growth and the indigo produced were carried out, it was found that the indigo production started as early as 9^{th} hour (Fig.5.8) and reached a maximum after 22 hours. The limitation of addition of indole directly into the medium was resolved in this experiment by dissolving 0.35 g of indole into autoclaved medium, on a magnetic stirrer for 45 minutes based on solubility of indole in water (0.19 g/ 100 ml).The addition of 200 ml of silicone oil, helped in increase in the production of indigo due to increase in surface area and it made the substrate availability to the bacteria easy.

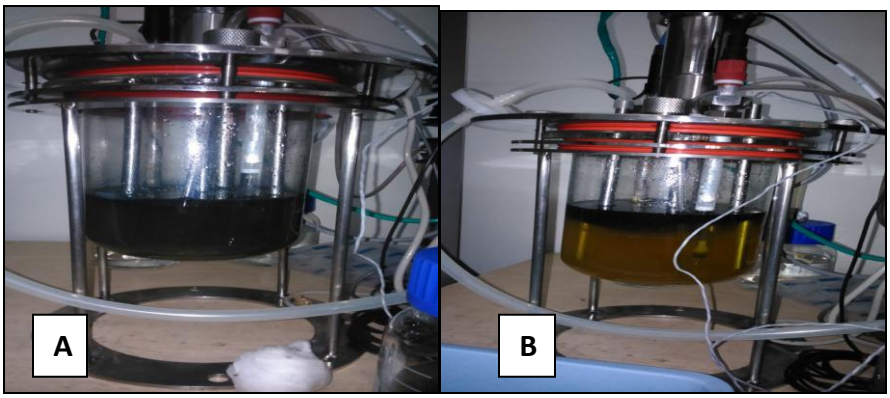

Fig. 5.1: Bioreactor with Single phase (A) and Two phase (B)

C. CONTINOUS FED BATCH OPERATION

The bioreactor operation was carried out on a continuous basis wherein the oil was removed from fermentor after sufficient indigo was produced to prevent saturation and then fresh oil and medium was supplied for further continuous production of indigo in the Fermentor.

In this experiment the reactor was started with 800 ml of medium and inoculated culture was allowed to grow. It was fed with premixed solution which consists of 0.35 g (3 mM for the reactor culture) of indole dissolved into 200 ml of medium and 200 ml of silicone oil in a well mixed condition on a magnetic stirrer. The solution was added to the reactor at a flow rate of approximately 16 ml / hour using a peristaltic pump. After about 24 hours of operation the indigo formation was observed.

Another flask of substrate (indole – medium –silicone oil) solution was prepared and fed to the reactor. Simultaneously another peristaltic pump was used to with draw the product from the reactor at the same flow rate as the feed. During this operation the feed was injected at the bottom of the reactor whereas the product was withdrawn from the top surface as silicone oil is on top. After every 24 hours the sample collected in the flask was allowed to settle and analyzed for indigo produced and for bacterial growth using spectrophotometer. The stirrer was maintained at 200 RPM which allowed premixed silicone oil to partially float and partially mix with the reactor culture.

5.1.2 Results and Discussion

a. Fed batch Single phase:

The initial injection of 3 mM indole with 2 ml of acetone into the culture helped the bacteria to grow and convert indole into the product indigo. Subsequent additions of 3 mM indole with 2 ml acetone did not result in increase in production of indigo. Further addition of indole into the culture in the same amount as earlier decreased the growth of the cells and very little increase in indigo formation as shown in the Fig. 5.2 and Fig 5.3 respectively. After the fourth fed point of substrate injection the indigo production was drastically reduced and also the growth of cells dropped.

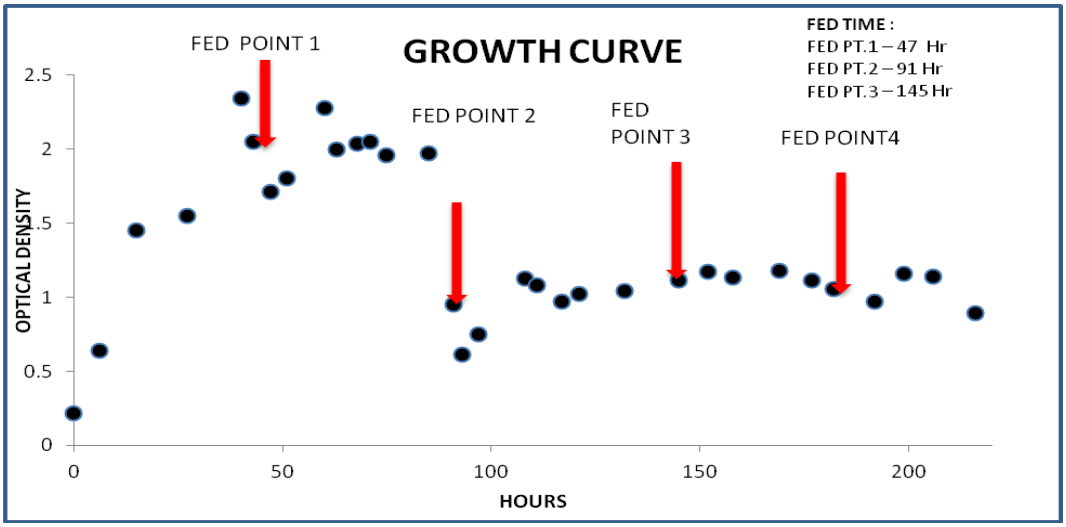

Fig.5.2: Growth of bacteria in bioreactor under fed batch operation (Single phase)

Indigo production was observed initially due to the availability of substrate for the culture, further due to the drop in growth, another dose of substrate was provided, and this resulted in decrease in indigo production though some increase in growth of the cells was recorded. This slight increase in growth may be due to the indole available as the carbon source and reduced indigo production may be due to the non availability of the

substrate as well as cell for conversion to Indigo, as the cells were not sufficient for bio-conversion. Later the effect of addition of acetone was observed as the growth as well as the indigo production dropped. The accumulation of the acetone in the system with every fed of substrate made the system environment toxic for the bacteria which resulted into the decrease. This method thus may not possibly work for bio-indigo production.

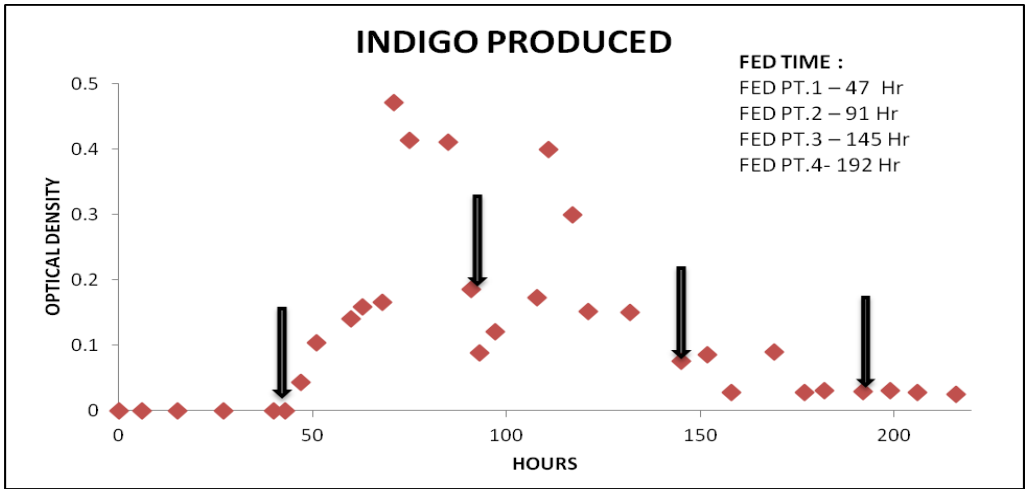

Fig.5.3: Production of indigo under fed batch bioreactor operation (Single phase)

5.1.2. a. (i) FTIR analysis for the pigment formed in Bioreactor:

The samples from single phase experiments were extracted in ethyl acetate and was collected and vaporized using hot water bath leaving behind the dried blue pigment. The dried pigment was mixed with potassium bromide (KBr) and analyzed in the infrared (IR) range of 400 – 4000 cm^{-1}. The qualitative analysis of the blue pigment obtained in the experiment mentioned earlier was done using Fourier Transform IR (FT-IR).The FT-IR set up used is make of Shimadzu, model number IRAffinity1. The FT-IR spectrum is very specific to any compound and hence is the best tool for identification of the compound. The dried blue pigment was mixed with potassium bromide and formed into pellet (Fig. 5.4) for the analysis.

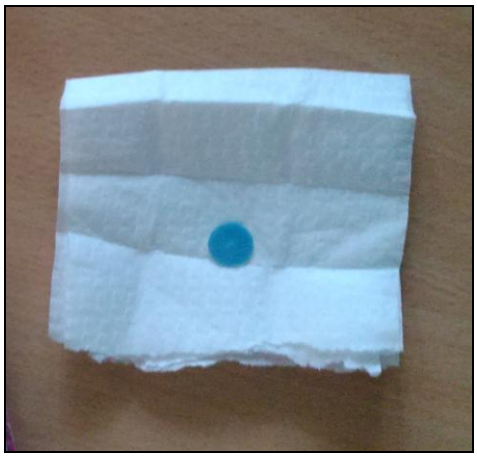

Fig.5.4: Pellet of blue pigment Indigo along with KBr

The FT-IR spectrum of the dried blue pigment shows peak between 3100 cm^{-1} and 3500 cm^{-1} which is due to N-H primary and secondary amines. A peak at around 1600 is due to the C=C stretching. Similarly a peak in the range of 1300 cm^{-1} and 1000 cm^{-1} is due to the C-N amine and lastly the small peak between 600 cm^{-1} and 900 cm^{-1} may be due to C-H aromatic (out of plane bending). Thus the IR spectrum result (Fig 5.5) for the dried blue pigment defines the major functional group present in the commercial indigo dye (Nittaya C, 2002). Hence it indicates the blue pigment produced by bacteria is indigo.

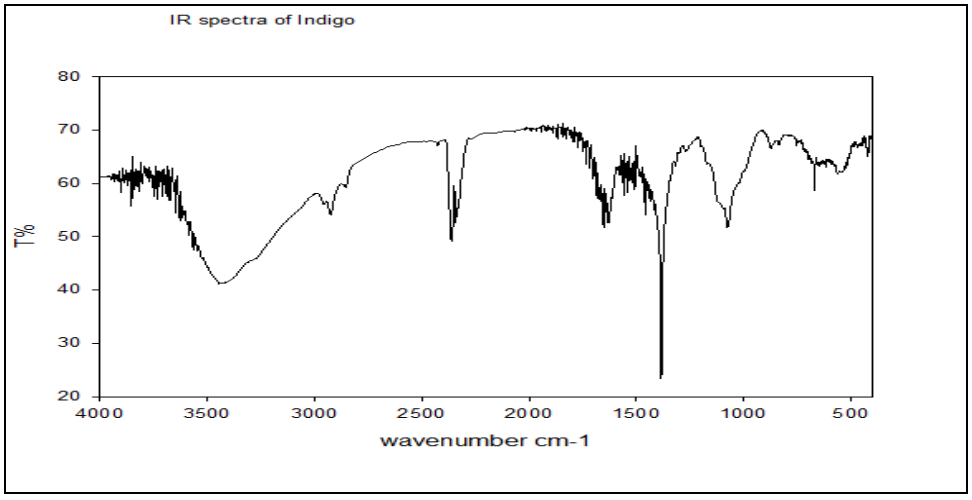

Fig. 5.5 : The FTIR analyses for pigment formed in bioreactor and found to be Indigo

b. Two phase:

As per the single phase results obtained it was concluded that the production of indigo using acetone as a transfer medium of substrate was not effective, thus to overcome the problem of indole addition into the system ,0.35 g of indole was added and mixed to 200 ml of medium (sterile). The mixing was carried out on a magnetic stirrer for 45 minutes. This premixed solution was added along with silicone oil to the reactor ,which helped in increase in the growth of the cells (Fig.5.6) as well as production of indigo due to increase in surface area and it made the substrate easily available to the bacteria.

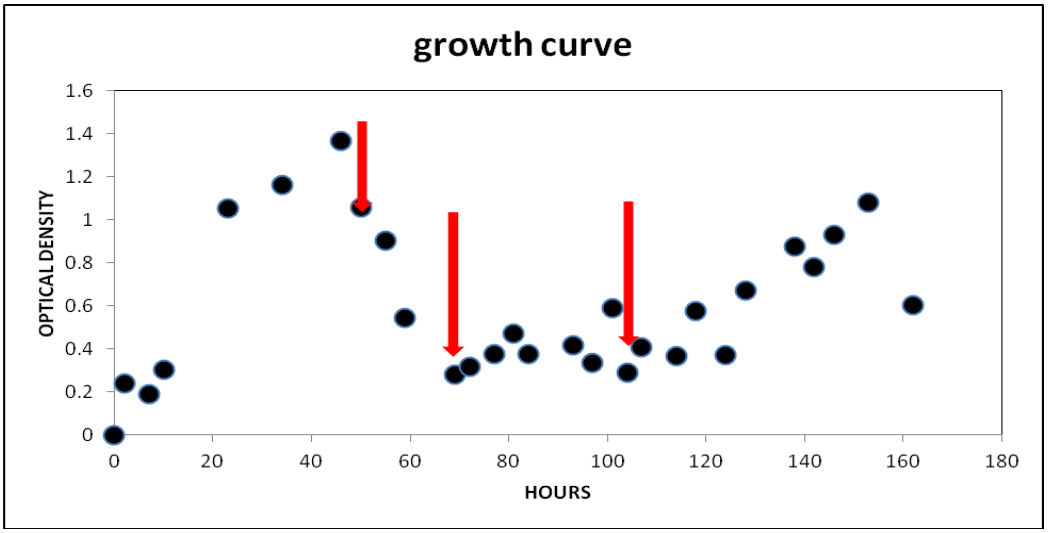

Fig.5.6: Growth of bacteria in bioreactor under fed batch operation (Two phase)

It was found that there was increase in indigo production up to 2 fed point (Fig.5.7) which was quantified only from the top oil layer. Further it was found that indigo was partitioning into the medium which changed the colour of the medium to greenish-blue. This resulted into reduction in the OD for indigo in the oil phase. Thus the silicone oil can be used as second phase to increase the yield, but the oil part can be removed on indigo production to prevent saturation.

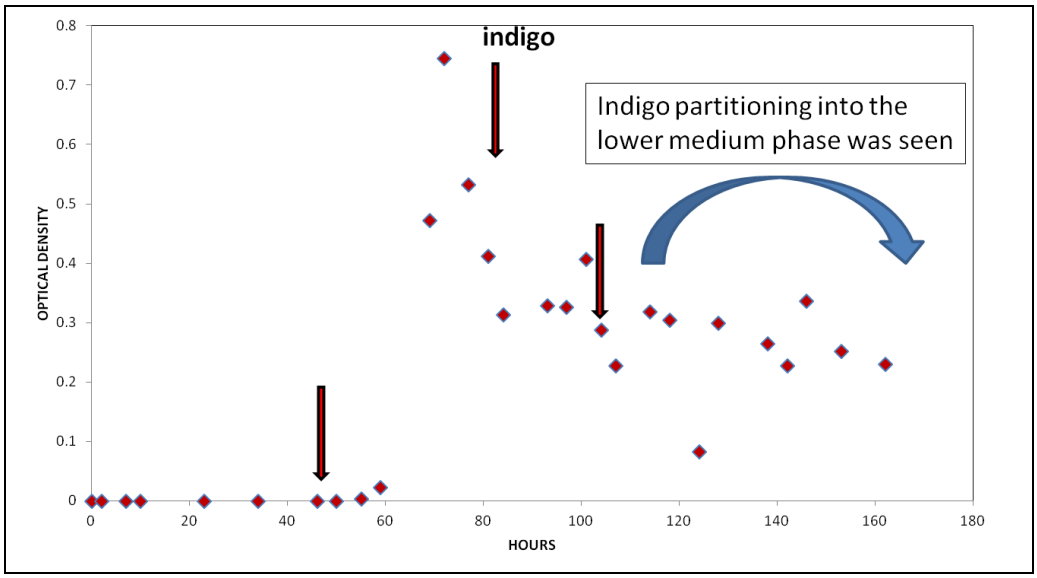

Fig.5.7: Production of indigo under fed batch bioreactor operation (Two phase)

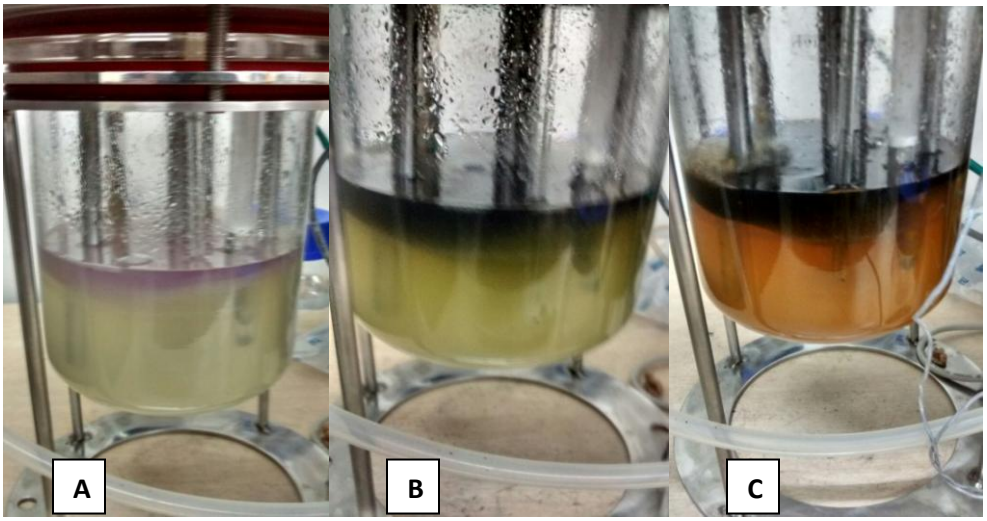

Fig. 5.8:Two phase indigo production after (A) 9 hours, (B) 19 hours and (C) 22 hours

c. Continuous operation:

The experiment was carried out on a continuous basis (Fig.5.9) wherein 400 ml of oil along with 400 ml of culture was removed from Fermentor after 24 hours of operation and formation of indigo. This prevented saturation of indigo in the oil layer and ensured continuous production of indigo in silicone oil where continuous supply of substrate through fresh medium (with indole) was done.

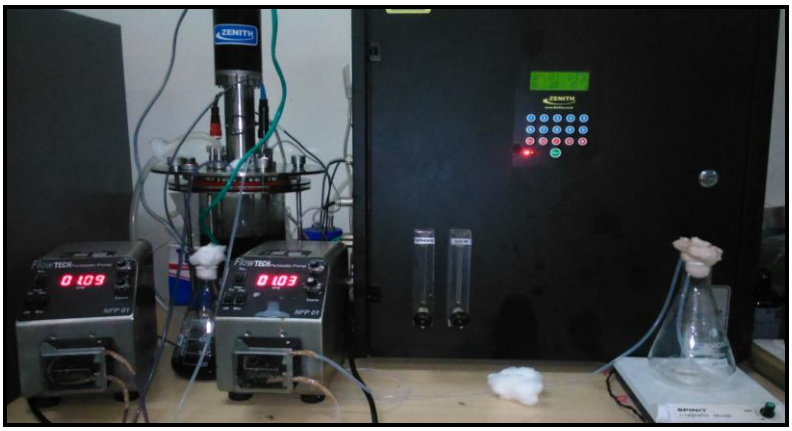

Fig. 5.9: Continuous production of indigo in two phase bioreactor

The flask carrying product was allowed to stand for an hour without disturbing, for proper separation of oil and culture phase. The top layer and bottom layer was analyzed for indigo and bacterial growth using spectrophotometer (Fig.5.10). The blank for the indigo in oil phase was taken as plain silicone oil whereas medium was taken as blank for bottom phase. The experiment resulted into formation of indigo after 22 hours of operation, but it was found that the concentration of maximum indigo formed in this study was less (0.19 mM) as compared to two phase fed batch process (0.26 mM).

This decrease in production was predicted to be due to unavailability of sufficient time for the bacteria to convert substrate into product indigo as the reactor was operated on a continuous basis. When the feed was fed to the reactor, at same time and at same flow rate the product along with culture was withdrawn from the reactor. This might have resulted into removal of some part of un-reacted indole.

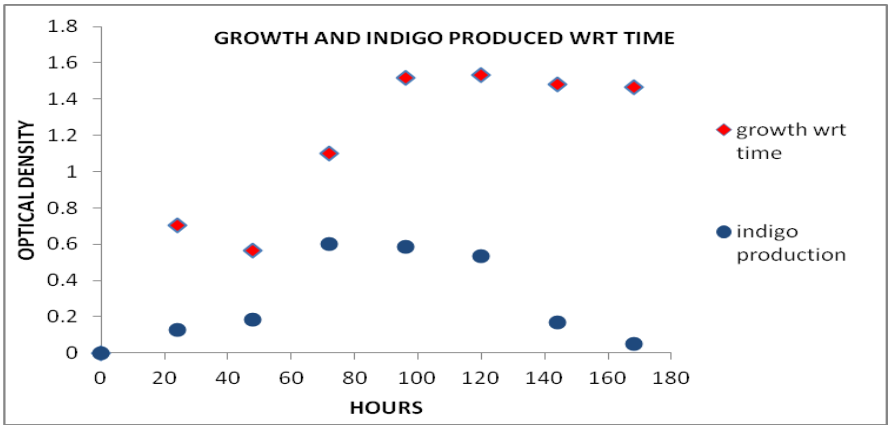

Fig. 5.10: Production of Indigo and growth curve of bacteria in a continuous bioreactor

CHAPTER 6:

Experiment for optimized production of indigo in bioreactor on continuous basis.

6.1: EXPERIMENT FOR OPTIMISED PRODUCTION OF INDIGO IN BIOREACTOR ON CONTINOUS BASIS.

The Indigo dye has been, in demand for years and in future the world demand for dyes and organic pigments is expected to grow 6.0 percent per year to $19.5 billion in 2019(Freedonasia, 2015). To commercialize and to compete with the increasing demand and also to satisfy the productivity requirement it is necessary to produce the bio-indigo on continuous basis. Biotechnology products must compete in economics terms; it's not enough to be environmentally preferable (OECD 2001). A bacterial strain capable of producing higher yield of indigo, modification in the metabolic pathway, optimization of parameters and continuous operation are the avenues which can bring the bio-indigo to commercial grade. The bio indigo produced can support the existing commercial technology. There is no doubt, that in the future, members of colourful family of indigoids to have a promising future application for the benefit of mankind (_Kinghorn D. ,2014).

This part of the work used a continuous production approach for bio-indigo production under optimized condition using two phases. The aim was to produce indigo using bacteria in a continuous bioreactor so as to get higher yield and to develop a process so that it can compete the chemical continuous production of the dye at commercial scale. Separation technique of the indigo produced in a two phase system like filtration, adsorption etc, are experiments carried out in this work along with the application of the dye over a cloth.

6.1.1 MATERIALS AND METHODS

6.1.1.1 Chemicals

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.

6.1.1.2 Bioreactor operation

A bioreactor with 2.5 liter capacity with working volume of 1.5 liter was operated at a temperature of 30 ^{o} C, pH 7, a substrate concentration of 3 mM was used in this experiment . The mode of operation was in two phase with silicone oil as a second phase. The reactor contents were mixed by the impeller which was operated at a speed of 200 RPM. An additional aeration was provided for better mixing and the dissolved oxygen was maintained above 70%.

Experiment:

The continuous operation was carried out in two different modes,

First mode of continuous operation: During this experiment a continuous operation of reactor was carried out in presence of silicone oil as a second phase. Initially Fermentor was inoculated with the culture and allowed to grow, until mid log phase. A substrate solution carrying 3 mM of indole dissolved in 200 ml of medium on a continuous shaker was added to the Fermentor on a continuous basis through a peristaltic pump at a rate of 400 ml/day. Along with the start of the substrate injection, 200 ml of silicone oil was also added to the Fermentor. This complete solution was allowed to mix at 200 rpm at 30^{o}C and at 7 pH for 24 hours. The maximum working volume of the Fermentor was maintained at 1.5 liters. The continuous substrate injection and the silicone oil added initially brought a continuous contact of substrate with bacteria and also helped in

picking up of the product from the medium as produced thus reducing the toxic effect of product saturation in the medium.

After 24 hours the system was switched off, and the silicone oil phase was allowed to stand still and then extracted using a peristaltic pump, during this period of time the substrate feed line was kept on standby mode. After removal of 200 ml of silicone oil and 400 ml of culture from the reactor, the system was switched on. The removal of culture was done to maintain the level of working volume of the reactor. The reactor substrate was switched to run mode on continuous operation. This process was found to result in decrease in the indigo concentration in the oil phase; hence the experiment was further modified to a second mode of operation.

Second mode of operation: Due to reduction in concentration of indigo in the oil phase as well as the decrease in the growth of the bacterial culture. The experiment was further planned, wherein continuous addition of substrate indole and extraction of oil was carried out thrice in 24 hours. In this operation indole (3 mM) dissolved in medium was fed continuously into the reactor at a rate of 400 ml/day using peristaltic pump .The silicone oil was added along with the substrate addition and was started and allowed to mix. The oil allowed the availability of the substrate to the bacteria as well as extracted the indigo produced in the medium. The removal of the oil from the reactor was carried out thrice to prevent saturation of oil phase and also to avoid accumulation of indigo in the medium. The reactor working volume was maintained by removal of the medium once in 24 hours of reactor operation. This allowed the continuous production of Indigo as required to commercialize the procedure.

6.1.2 Results and Discussion

In the first mode of continuous bioreactor operation the substrate was injected into the reactor in the dissolved form in the medium at a rate of 400 ml per day. At the same time, 200 ml of silicone oil was added and allowed to mix with the culture for 24 hours. This resulted into more availability of indole to the bacteria in the medium. After 24 hours of mixing of silicone oil with the culture the reactor substrate feed was kept on stand by mode and the oil phase was extracted using peristaltic pump along with 400 ml of culture medium. The oil was analyzed for indigo formed and the medium was analyzed for growth. The extraction process took 30-45 minutes after which the continuous feed of substrate dissolved in medium along with single addition of 200 ml of silicone oil was done and process continued.

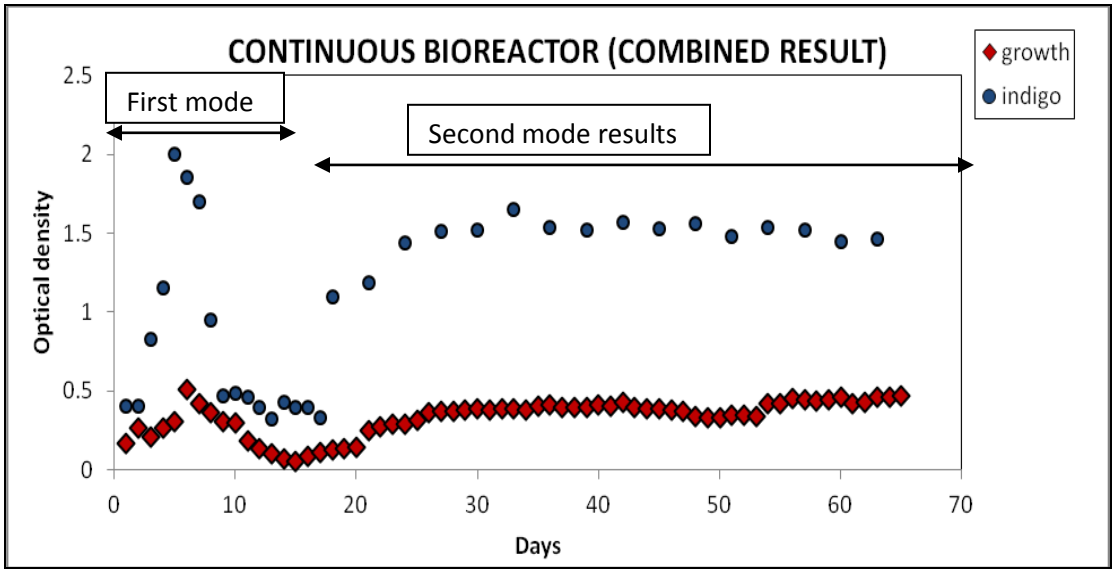

Fig.6.1 : Indigo produced and growth of bacteria in First and second mode of reactor

In the first mode of operation the continuous reactor was started at the optimised condition and the substrate was provided continuous at a rate of 16 ml /h. The system took 4 days to stabilize. On extraction of the product from the oil phase, the increase in the production of indigo was observed,to get the maximum indigo production of 0.68 mM which further reduced due to continuous supply of indole into the reactor, the oil was

found to saturate before the 24 hours, as prefixed extraction hour. Thus continuous supply of substrate and saturation of oil phase resulted into shift of the experiment to second mode of operation.

In the second mode of continuous operation of bioreactor the experiment was modified by reduction in the extraction time. The substrate injection and the bioreactor parameters were maintained at the same conditions. The indole was fed continuosly into the reactor wheras the silicon oil was extracted at 9 am, 2 pm and 7 pm for removal of continuous forming indigo. During this (every 24 h) cycle the culture was withdrawn from the reactor to maintain the working volume. The addition of oil after frequent interval resulted into continuous and stabilised production of indigo along with stabilised growth of the culture. This resulted in stabilised and continuous production of indigo at a concentration of 0.49 mM. The combined result for First mode and second mode of bioreactor operation is depicted in the results shown in Fig.6.1.

Along with indigo extraction , some volume of medium carrying culture was also removed to identifiy the growth of the culture which is shown in Fig 6.1. Thus maintaining the concentration of the bacterial culture in the reactor gave the defined indigo concentration under optimised conditions in a continuous reactor. Based on the concentration of product formed and the biomass available , the yield was calculated as shown in Fig.6.2, which average out as 35% yield under stabilised condition of the bioreactor.

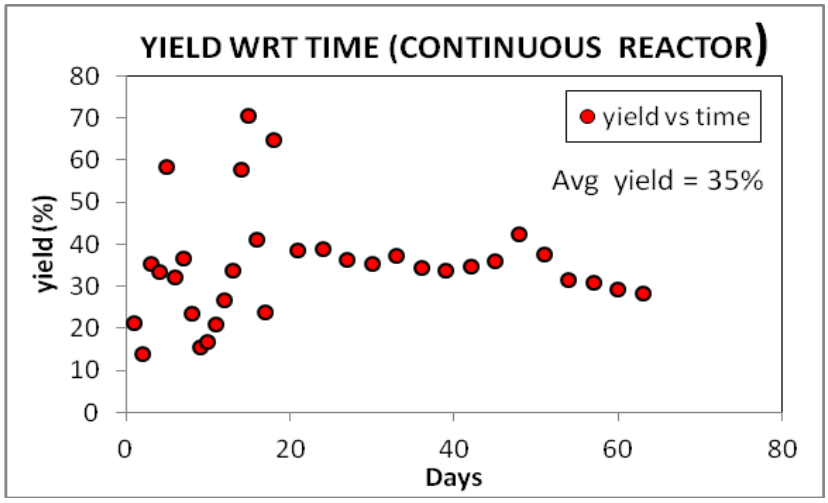

Fig.6.2: Yield (Indigo to Biomass) with respect to time

6.1.3 METHODS USED FOR SEPARATION OF INDIGO FROM OIL PHASE

The indigo dye produced by the *Pandoraea.sp* bacterium in a two phase study was available in a mixed form with silicone oil and to supply the dye it was required to obtain a dried form of indigo. Therefore following separation techniques were experimented to get the dried indigo powder. A qualitative approach was used for carrying out the dye separation from the oil phase so as to select the best method that can be used in future for two phase bio-indigo production.

1. Separation using Centrifugation:

For separation of indigo from the oil layer, the oil layer carrying indigo was centrifuged at 1000 rpm for 30 minutes. After centrifugation, the tubes were found to form a pellet of indigo along with a separate oil layer (Fig.6.3). In this process, complete removal of indigo was not possible as oil also carried indigo in it even after centrifugation .Thus complete separation of oil and indigo to obtain indigo powder by this process will be is a challenge. However the advantage of this process is that a major portion of oil could be recovered and indigo in its oily form could be used for dyeing.

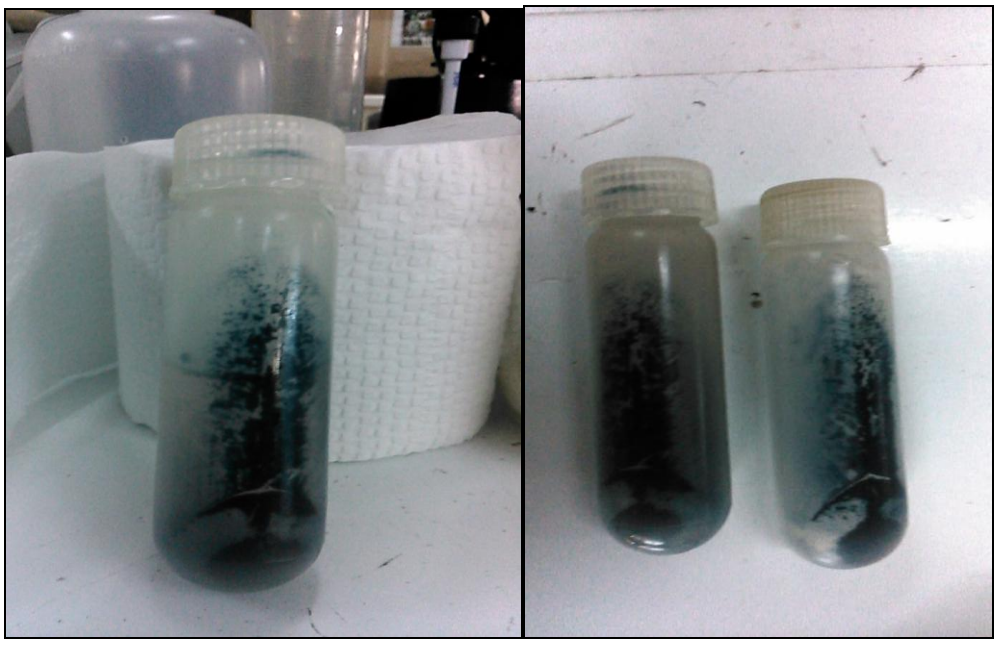

Fig.6.3: Formation of indigo pellet from oil – indigo mixture using centrifuge

2. Separation using Adsorption

In this process a ceramic tile was used for adsorption of indigo pigment from silicone oil. The oil carrying indigo was poured over the tile and allowed to get adsorbed onto the surface. Ceramic being a porous material absorbed the oil and the indigo particle was found to adsorb onto the surface of the solid (Fig. 6.4). The indigo particle was scraped with knife from the surface and found to be little sticky as the particles were oily. Some additional impurities of the ceramic rock were also scrapped into indigo during the process. This technique did not allow any recovery of oil which was found to be a drawback of this method and was not considered for further study.

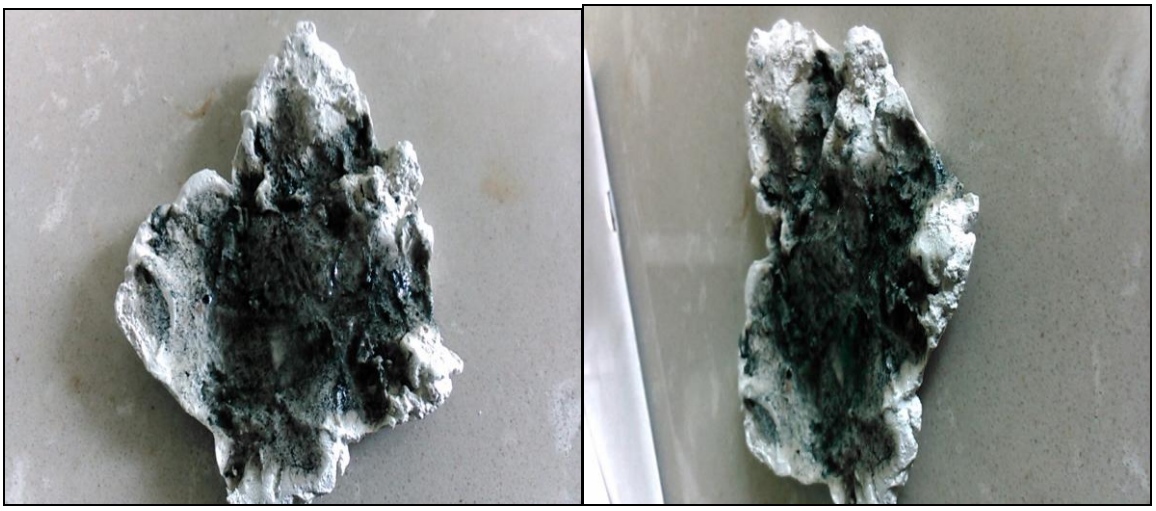

Fig.6.4: Separation of indigo by adsorption on the surface of ceramic tile

3. Separation using Vacuum filtration

The third process used for separation of the indigo pigment from oil was vacuum filtration where in a vacuum pump was used at 700 mm of Hg. The Whatman paper filter paper (No-1) in a Bushnell funnel was used as membrane for separating solid indigo particle from oil under vacuum(Fig.6.5-A). This technique was found to give the best separation as compared to the earlier three methods as it separated maximum oil available in a mixture and that too in less time. However dry indigo powder could not be obtained

as oil stick to the particles (Fig.6.5-B), hence separation of blue pigments from oil completely was not possible.

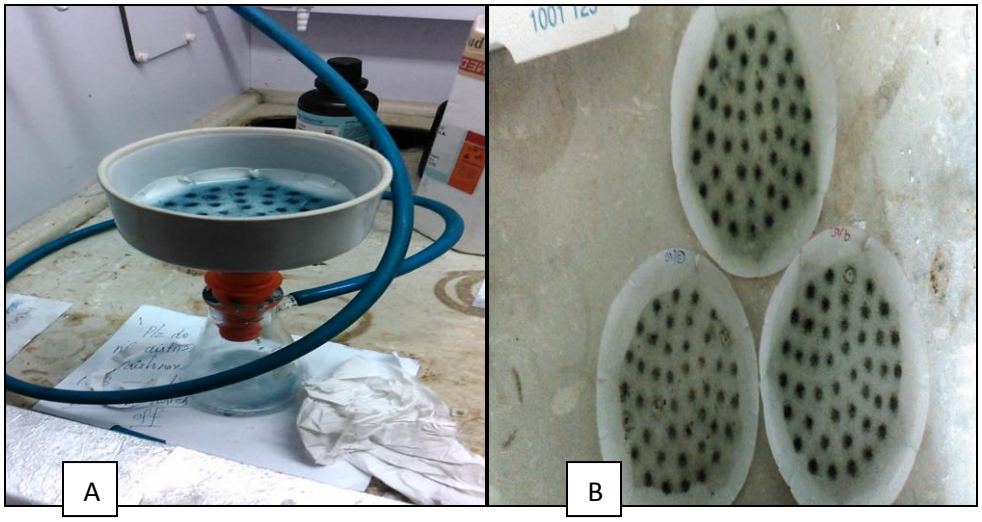

Fig 6.5: Separation of indigo using vacuum filtration

4. Direct spraying of the dye with oil on the cloth for dyeing

As it was observed that the pigment was not separable from oil by the above stated techniques it was decided to dye the cloth directly using oil and pigment mixture. This process worked (Fig.6.6) as the dye when sprayed with a sprayer on the cloth got fastened to the nylon cloth, giving different shades as per the spraying done. As per the literature (Shin E.,2011) silicone oil is used in the textile and dyeing industry as filler to enhance the property of the cloth like - water repellency, softness, durability, heat resistance etc, therefore this technique wherein silicone oil with indigo can be used directly for dyeing.

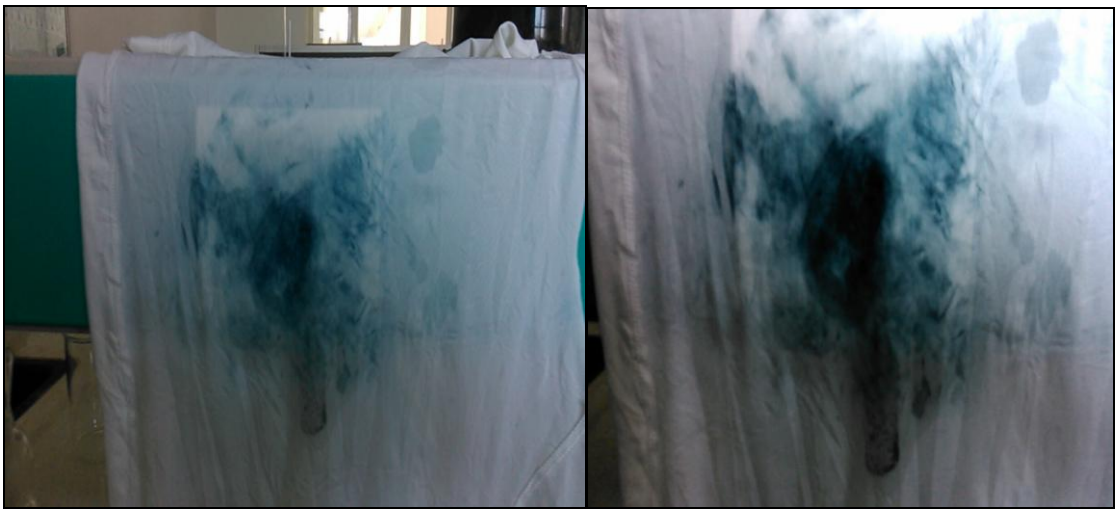

Fig.6.6: Cloth dyed by direct spraying of bio- indigo with silicon oil

Thus the bio indigo produced by *Pandoraea.sp* in oil phase can be directly used to dye the cloth. This will help in major amount of oil recovery. The remaining dye in the silicone oil could also be used.

7. CONCLUSION

The textile industry or the dye producing industry is a core chemical industry which has its global effect on the economy of a country. The environmental effect of this huge industry has diverted the researcher toward the biotechnological route of Indigo dye production. Biological route for the dye production by use of different bacterial strain, different substrate, DNA manipulation techniques are being used to enhance the indigo production till date. However none of the results has actually scaled up to direct industrial application and production of the product on large scale.

The bacterial strain isolated from oil contaminated garage soil, from Zuari nagar, Goa, grown in culture medium with diesel as a sole carbon source showed the capability to produce the blue pigment. It was selected based on its increased growth as compared to the samples selected from other garages. The strain on isolation and purification was identified using DNA sequencing and BLAST technique and was identified as *Pandoraea sp.* from the family of *Burkholderiaceae* which belongs to the class of Betaproteobacteria. The strain effectively produced the blue pigment, in presence of the substrate indole when streaked on the agar plate. The blue pigment further was qualitatively identified as Indigo using TLC and UV scan techniques, thus the strain was used in further studies. Thus from this part of study a bacterial strain capable of producing indigo was identified.

The commercial production of Indigo needs the optimization of the parameters so as to enhance the yield of Bio-indigo. The Design of experiment (DOE) technique used in the study was a helpful tool in increasing the production of indigo and the 3D plots

helped to identify the regime for the experiments. Further these statistical techniques can be applied to evaluate more factors, their interaction, the noise variables and elimination of the noise variables, which helps in increasing the yield of Bio-Indigo. Thus optimization of the process parameter was identified and was used further in scale up of the indigo production. Along with the process optimization, the experiment for analyzing the kinetic parameters like V_{max} and K_m were found out which helped to identify the maximum indigo production rate and the affinity of the enzyme for the substrate indole which will definitely help in scale up study as well as in further use of the enzymes in modification of the indigo production process.

Scale up of the lab result to bioreactor study has brought in the pilot results for industrial production of the dye. During the bioreactor work the fed batch configuration using single phase and two phase study was carried out. This study resulted in better results for two phase operation of the reactor. The continuous operation of reactor was also studied during this part of work to compare its results with fed batch study and also to satisfy the industrial demand of continuous product formation. However from this work it was concluded that fed batch operation dominated over the continuous operation (Continuous supply of substrate and continuous withdrawal of product at same rate) on the basis of indigo production for two phases.

The last part of the research work focused on two aspects of continuous indigo production for commercial application and separation of indigo pigments from silicone oil. A continuous bioreactor operation with two different mode was studied which

resulted into a novel technique of maintaining the substrate indole below toxicity levels and also prevent product saturation in the medium.

From the various extraction techniques used, the vacuum filtration technique was found to be best suited for separation of indigo from oil and also for oil recovery. Direct spraying of indigo in presence of small amount of oil was also found to be effective in cloth dyeing.

The overall conclusions can be drawn from the PhD thesis are:

- The strain *Pandoraea sp.* can be used in future for industrial production of Indigo as it has the potential for the biological Indigo production at industrial level under the optimized conditions of pH-7, substrate- 3mM, and temperature- 30^{o}C.
- To enhance the productivity of the dye, a two phase study done in this research work with Silicone oil as the second phase is surely a way over the single phase indigo production. The major advantage of the two phase results, like the increase in the yield of product at lower substrate inputs and extraction challenge can be used viably in industrial application of the process developed.
- The comparative bioreactor configuration studied in this work has proven the applicability of this process to produce indigo continuously as demanded by the commercial world.

Research Contribution of the work:

- The research work done for the PhD, has identified and isolated a bacteria which has not been reported earlier for Indigo production.
- This work has used a biphasic study for production of indigo which is a novel technique in this area.

The future extensions from the PhD thesis are:

- To study the Chemical Kinetics parameters of the Bio-indigo production in detail to identify the rate limiting step, this can further help to increase the yield of Indigo production.
- To elucidate the surface morphology and the effect of surface properties of silicone oil on the substrate uptake and product extraction in this work.

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