Effect of different physico-chemical parameters for natural indigo production during fermentation of *Indigofera* plant biomass

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Abstract Natural indigo production from *Indigofera* plant biomass requires fermentation of biomass, oxidation of fermented broth, settling of oxidized product (indigo), filtration and recovery. In this study, we have investigated roles of physico-chemical parameters during fermentation with respect to product yield. The study showed that water-to-biomass ratio (1:10), fermentation duration (0, 6, 12, 18, 24 h), pH (6–7.5), dissolved oxygen concentration; DO (0.5–3 mg ml⁻¹), oxidation reduction potential ORP (+50 to –300 mV) and temperature (25–40 °C) during fermentation, oxidation and dye recovery from the broth are directly or indirectly related to indigo yield. Biomass fermentation for 12 h at 40 °C incubation temperature yields the highest biogenic indigo (2.84 mg g⁻¹) out of the different experimental conditions.

Keywords Natural indigo · Parameters · Fermentation duration · Process optimization · Production

Introduction

Global awareness for pollution mitigation ensuing from synthetic colorants has resumed interests for utilization and production of biogenic natural dyes. The ingredients for large-scale dye production through chemical synthesis are mostly hazardous. Left over of these ingredients in wastes from dyeing industries have ravaging effects on ecosystem.

On the other hand, natural dye reflects a harmonious, sustainable relationship with the ecosystem. Synthetic dyes have almost replaced their natural counterparts in dyes and pigment sectors due to their persistence as they are recalcitrant to degradation, and commercially developed dyeing methods. Indigo retains its position as one of the world's most important industrial chemicals, with the current annual world consumption of indigo and other vat dyes being ca. 33 million kg (Roessler and Jin 2003). Environmental awareness and health consciousness of the people have renewed the demand of natural dyes in world market. Dyes are indispensable components of the chemicals sector and the environmental protection is now one of the major thrust areas for research and development in this sector, especially in the area of minimizing use of toxic ingredients in dye production, toxicity reduction of the left overs and degraded by-products of dye stuffs in dye and fabric industries (Watson 1991; Anon 1992). Indigo can be derived from plants and animal sources to substitute its synthetic counterpart, which is obtained from hazardous ingredients and by-products of oil and coal tar industries. However, traditional knowledge base for natural indigo production has been lost after advent of synthetic indigo. The traditionally developed conventional method in India and some other countries has become rudimentary as a rural craft and legacy of some families.

Indigo usage dates back to thousands of years, it is one of the oldest natural textile dye used by mankind (Ensley et al. 1983; Fitzhugh 1997; Seldes et al. 1999). There are evidences of indigo being used centuries back in the fabric used for wrapping 'mummy' in ancient Egypt (Gilbert and Cooke 2001). The association of India with indigo is discovered in Latin connotation of the word, "indikon", meaning 'blue dye from India' or more literally 'Indian substance'. The Romans used the term 'indicum', which

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conceded eventually into English as the word indigo (Jain et al. 2010). The use of indigo plant for dyeing fabrics blue was known to Indian civilization from past 3000 years (Kaufman et al. 1999). Its worldwide appeal extends both geographically and demographically and it has recognized itself both as a fashion article and a standard article in leisure and work wear. Natural indigo can be obtained as of a broad assortment of tropical, subtropical and temperate plants from varied species, genera and families (Schunk 1955; Martin-Leake 1975). *Indigofera* species are predominantly used for indigo production in tropical and subtropical areas. The genus *Indigofera* L. is a member of family *Leguminosae* under dicotyledons. It is a large family of herbs and shrubs having antiseptic, purgative and stringent properties (Balamurugan and Selvarajan 2009). *Indigofera* plant biomass is used as raw materials for indigo dye production and leaves contain high concentration of the dye compounds. The indigo dye stuff is not readily available as such in the plant, but as a secondary metabolite known as indican (indoxyl- β -D-glucoside). In most of the indigo producing plants indican is the precursor (Maugard et al. 2001), whereas in *Isatis* (Woad) species an ester isatan-B (indoxyl-5-ketogluconate) is the precursor (Epstein et al. 1967). Indigo extraction from plant biomass entails several steps; indican located within cell vacuole is released from plant biomass through partial fermentation by steeping under water, hydrolyzed to indoxyl and glucose by the native β -glucosidase (Minami et al. 1997). Formation of indigo occurs when two molecules of indoxyl combine spontaneously in the presence of oxygen under alkaline condition (Maugard et al. 2002), which is carried out through aeration of the ferment depicted in Fig. 1. Condensation of indoxyl with isatin and dioxindole leads to formation of indirubin and isoindirubin as by-products of

indigo biosynthesis. Indirubin is generated from indoxyl and/or dioxindole in an oxygen-rich environment as a side reaction and dioxindole is generated from isatin C. In practice, once the molecule has been hydrolyzed, this combination occurs spontaneously in aerobic condition and indigo precipitates from solution. Dependency of textile industries on non-ecofriendly synthetic dyes is a bottle neck and a future threat to this industrial sector. Natural dyes could ease dependency of dye industries on petrochemicals and reduce their environmental footprints (Sewekow 1988). In some parts of Europe and Japan, R&D for indigo dye production from plant sources is in progress (Minami et al. 1997; Maugard et al. 2001, 2002). The research work of Karaman et al. (2015) suggests high indigo yielding variety in *Istias* genotype which could help in selection of plant varieties for breeding in Turkey. Sandoval et al. (2010) reported hypocotyls explants of *Indigofera suffruticosa* Mill. were cultured on MS media supplemented with different plant hormones yield indican concentration 16.67 mg g^{-1} tissue biomass. Indigo yield was also affected by different light intensities, plants grown under greater light regimes when transferred to lesser light conditions, indigo production declined from 400 to $150 \text{ mg plant}^{-1}$ (Stoker et al. 1998a). The aspect of using indigo from *Indigofera* plant as phyto-medicine especially acting as antioxidants were also investigated (Singh et al. 2015).

In view of the current market demand and environmental importance, it is pertinent to reinvent the lost natural indigo dye production process from plant biomass and retrofit it for commercial and economic benefit. This investigation was carried out to understand the effect of different physico-chemical parameters that affects *Indigofera* plant biomass fermentation and identify the important

Fig. 1 Process formation of indigo and its other derivatives from indican

factors that could be utilized to regulate the fermentation process towards enhancement of indigo from plant biomass.

Materials and methods

Plant material

Indigo plants (*Indigofera tinctoria*) were grown at NEERI campus, Nagpur, India. Seeds were germinated in pots, seedlings were raised under glass house and planted in fields under sunlight. Plant biomass (leaves along with rachis) from 4-month-old plants were used in the experiments.

Lab-scale experimental setup

Leaves were collected from plants grown in experimental plot (30 g of leaves), put into the vessel of the prototype reactor and immersed in water with biomass:water ratio of 1:10 (30 g of leaf biomass to 300 ml of water). The leaves were steeped under water using weight blocks to maintain anaerobic condition. Key parameters of the water used for fermentation, i.e., pH, redox potential, dissolved oxygen and temperature were recorded before adding the biomass. All experiments were repeated thrice.

The study of fermentation stage was the main focus of this experiment (Erickson and Fung 1988). The incubation period was set for different hours (0, 6, 12, 18, and 24 h) under different temperature ranges (25, 30, 35 and 40 °C) to determine the effect of change in temperature on indigo dye production with variation in incubation period. Combinations of temperature and incubation period were also carried out. After specified hours of incubation remaining plant biomass was sieved out. The influencing factors such as pH, dissolved oxygen (mg l^{-1}) and redox (mV) of the fermented broth were measured. Soon after this, the fermented broth was taken for oxidation in the next step of indigo production. The fabricated prototype reactor had a port for air inlet. The inlet was connected to an air blower and air flow was regulated through a rotameter for maintaining flow rate at 4 l min^{-1} (liter per minute). The prototype reactor was also fitted with a mechanical agitator unit for agitation up to 900 rpm (rotations per minute). Based on preliminary experiments, oxidation duration through air sparging was fixed 20 min for all experiments. During oxidation the prototype reactor was covered with a tight cap to prevent spillage of broth from the reactor due to agitation. After completion of oxidation, the influencing factors, viz. pH, dissolved oxygen (mg l^{-1}) and redox (mV) of the fermented broth were measured. The oxidized broth was collected in Oak Ridge tubes and centrifuged under

7826g for 20 min at 22 °C for settling dye from the broth. The supernatant was siphoned and the pellet was collected. The tubes were kept in hot air oven at 70 °C for 5–6 h. The settled indigo yield (crude dye) in mg was determined by subtracting weight of identical empty tubes from the weight of oven-dried tubes containing indigo.

Indigo estimation

Dried indigo pellet was dissolved in di-methyl sulphoxide (DMSO) and quantified through spectrophotometric analysis (Haucke and Graness 1995; Puchalska et al. 2004; Jacquemin et al. 2006). Calibration curve was plotted using standard indigo (Sigma-Aldrich, R304949) at the concentration of $0.01\text{--}0.2 \text{ mg l}^{-1}$ with an increment of 0.01 mg l^{-1} . Known weight of the dried biogenic indigo pellet was dissolved in DMSO and absorbance was read at 620 nm (Wu et al. 1999; Christie 2007). Indigo content in the dried pellet of each experiment was calculated using the standard curve.

Apparatus

The pH and oxidation–reduction potential were carried out using pH meter (EUTECH instruments pH 510 cyberscan) and Redox meter (EUTECH instruments pH 510 cyber-scan). The precipitated indigo was obtained through drying of fermented product in Oven (Bio-Techniques India). Settling of indigo precipitate was carried out by centrifugation of the broth (Multifuge-1, Sorvall Heraeus). The lab-scale fermentation was performed in a fabricated reactor prototype. Aeration was done using Pump (PALL, vacuum pressure pump). UV–visible spectrophotometer (UV-1800-SHIMADZU) was used for spectrophotometric estimation of pure indigo.

Results and discussion

Effect of fermentation durations and temperatures on indigo yield

The experiments were carried out under different fermentation periods with a constant value of biomass:water ratio (1:10). The total crude yield during fermentation is depicted in Fig. 2a–d. Each experiment was carried out at four different temperatures, i.e., 25, 30, 35 and 40 °C. These temperature ranges were chosen based on the literature reports on effect of surmounted temperature on secondary metabolite productions in plants due to variation in permeability, metabolic regulation and rate of intracellular reaction that affect growth and production (Morison and Lawlor 1999; Yu et al. 2005; Chan et al. 2010; Narayan

Fig. 2 Graphical representation of indigo crude and pure yield (mg g^{-1}) at different fermentation durations (6, 12, 18 and 24 h) and temperatures: (a) 25 °C, (b) 30 °C, (c) 35 °C, (d) 40 °C

et al. 2005). In literature, nearly >1% indigo yields were reported from *Indigofera* biomass, i.e., approximately 100 kg from 10,000 kg biomass per hectare (Ergashev 2009). Our investigation shows that under 25 °C experimental setup and 18 h fermentation, crude indigo yield (dried mass on weight basis) was highest, i.e., 5.89 mg g^{-1} plant biomass, on other hand after 12 h fermentation pure indigo yield (on spectrometric estimation) was highest, i.e., 1.41 mg g^{-1} plant biomass. Under 30 °C experimental setup after 18 h fermentation duration crude indigo yield was 3.95 mg g^{-1} plant biomass. But, pure indigo yield was highest, i.e., 1.59 mg g^{-1} biomass after 24 h of fermentation. Thus, it indicates that there is no much correlation between fermentation duration and crude yield, but pure indigo yield was more when fermentation duration was extended from 12 to 24 h. However, due to higher fermentation duration more cell debris and impurities were formed in the dye stuff. This lead to reduction in dye quality, and require purification of the yield product for

extraction of indigo dye stuff from the product for quality enhancement.

Similar experiments was performed by Stoker et al. (1998b) on lab scale, where temperature of the woad's broth was maintained at 30 °C for 24 h yielding $38 \text{ mg indigo g}^{-1}$ plant biomass, and as the temperature increased from 60 to 90 °C indigo yield was decreased prominently. In our study, crude indigo yield was highest 6.36 mg g^{-1} plant biomass at 35 °C after 24 h of fermentation, and pure indigo yield was highest 2 mg g^{-1} after 12 h of fermentation under similar temperature. At 40 °C fermentation, highest crude indigo yield was 5.39 mg g^{-1} biomass after 24 h of fermentation, and pure indigo was 2.84 mg g^{-1} biomass after 12 h of fermentation. Teanglum et al. (2012) found that indigo yield from *Marsdenia tinctoria* plant was 0.25 mg g^{-1} after 24 h fermentation at 40 °C. A Korean research team worked on *P. tinctorium* suggested that without pretreatment (washing), the leaf biomass yield $0.03 \text{ mg pure indigo g}^{-1}$ biomass during scale-up

Fig. 3 Relation between changes in pH and indigo pure yield (mg g^{-1}) at different fermentation durations (6, 12, 18, 24 h) and temperatures : (a) 25 °C, (b) 30 °C, (c) 35 °C, (d) 40 °C

production (Shin et al. 2014). Among the four specified temperatures, the setup under 40 °C temperature produced best response for crude and pure indigo yield. In the line of these observations, with notable elevation in temperature and steady increase of fermentation duration crude indigo yield was high, although pure indigo showed a dissimilar attribute. In case of pure indigo, only 30 °C gave a static rise in indigo yield with increase in fermentation duration. In the other three temperatures, pure indigo increased till 12 h of fermentation duration, and showed a gradual fall thereafter. In the other three temperatures, pure indigo increased till 12 h of fermentation duration and subsequently showed a gradual fall.

Effect of pH on pure indigo yield

During biomass fermentation, pH plays an important role for complete or partial fermentation of the samples. pH indicates the acidic or alkaline state of a solution, which represents the dynamic nature of reaction kinetics of

biochemical reactions whether acidic, alkaline, amphoteric or neutral. This will affect indican release from plant biomass and thereby affect dye yield. In the experimental setups, at the end of each fermentation under different incubation period and temperature, pH of the broth was determined to understand the nature of reaction and role of microbes in the biochemical reactions, which regulates the leaf decomposition during the course of fermentation. It was reported that under abiotic stress changes in pH initiates apoplastic oxidative burst which leads to extracellular reactive oxygen species (ROS) production that can regulate reaction dynamics (Chung et al. 2008). Laitonjam and Wangkheirakpam (2011) reported that after 3 days of incubation pH of fermented solution of two indigo producing plants was 4.8. Similarly, Chanayath et al. (2002) reported pH 4.8 from another indigo producing plant *Baphicacanthus cusia* Berm. after 24 h fermentation with indigo yield 5 mg g^{-1} of plant biomass. It was suggested that, during indigo dye production microbes act upon plant cell that facilitate rupture of organelles leading to release of

Fig. 4 Relation between changes in dissolved oxygen (mg l^{-1}) and indigo pure yield (mg g^{-1}) at different fermentation durations (6, 12, 18, 24 h) and temperatures: (a) 25 °C, (b) 30 °C, (c) 35 °C, (d) 40 °C

indican from vacuole and β -glucosidase enzyme from the chloroplast (Minami et al. 1997; Maugard et al. 2001, 2002). The β -glucosidase enzyme acts upon indican cleaving it into indoxyl and glucose. Minami et al. (1996) has reported that activity of this enzyme prompts at a pH of 6.5, which is similar to our observations. We observed pH of the broth at the end of fermentation process was in the range of 6.2–6.8, thus it indicates that microbes involved in indigo production also acts at this pH. There are reports referring optimum enzyme activity for β -glucosidase at different pH ranges 4.5–7 (Kim et al. 2010; Bhatia et al. 2002; Toonkool et al. 2006; Barrera-Islas et al. 2007). Figure 3a–d shows that pH was optimum at 12 h of fermentation incubation duration for all different temperature ranges tried in this study. Among the four different incubation temperatures, incubation at 40 °C yields maximum pure indigo (2.84 mg g^{-1}) at a pH range 6–5.5 after 12 h of fermentation presumably in this incubation time interval microbial activity for the indigo dye production was optimum. During fermentation and oxidation stage of indigo

dye production, 12 h fermentation yields maximum dye corresponding to pH 6–6.8, thus the 12-h fermentation duration was nearly optimum for indigo dye production.

Effect of dissolved oxygen (DO) on pure indigo yield

The dissolved oxygen has important role during the course of fermentation and in other of stages of indigo dye production. It is a relative measure of the amount of oxygen that is dissolved or carried in a given medium. Oxygen was induced to dissolve in medium by diffusion from air through sparging air into the system. It is generally observed that DO levels after fermentation showed marked fall in comparison to the initial value. This DO was assimilated by the microflora present in broth which mediate the biomass fermentation. The microbes take part in lysis of the plant biomass for release of indican to the broth, as well as conversion of indigo precursor 'indican' to 'indoxyl'. Therefore, optimum DO level in the broth is important to support metabolic activity and changing of

Fig. 5 Relation between pure indigo yield (mg g^{-1}) and corresponding changes with redox values (mV) at different fermentations (6, 12, 18, 24 h) durations and temperatures: (a) 25 °C, (b) 30 °C, (c) 35 °C, (d) 40 °C

metabolic flux of microbes present in a broth (Calik et al. 2004; Fredlund et al. 2004; Martin et al. 2005). The in-depth study of DO, and its effects on curdlan production through downscale fermentation process and intracellular metabolic changes ensured highest 42.8 g l^{-1} (42.8 mg g^{-1}) curdlan yield at 60% DO concentration (Zhang et al. 2012). Similarly, in fumaric acid production the effect of DO regulation was studied. It was reported that fumaric acid productivity of $0.7 \text{ mg g}^{-1} \text{ h}^{-1}$ was enhanced by 37% when DO concentrations held at 80% (Xu et al. 2012).

The illustration of our study in Fig. 4a–d shows that at 12 h fermentation indigo yield was maximum under the four temperatures investigated. Concurrently, at this duration DO value showed maximum drop indicating its assimilation for microbial activity, and utilization for dimerization of indoxyl molecules to indigo. Reduction of dissolved oxygen after both fermentation and oxidation, justifies the principle that with the advent of a reaction, the DO of a solution decreased complying with the presence and action of microbes in the solution. Slight decrease in DO of a broth indicates that the organism is not able to

consume that much oxygen. As the microbial population in the broth increased in course of time more and more oxygen was depleted, hence the DO value decreased. Therefore, it is presumed that as 12 h fermentation produced maximum indigo yield, it is in agreement to maximum DO reduction at that particular time interval for variable temperature ranges.

Effect of redox potential on pure indigo yield

The redox potential is a function of pH, DO level and redox states of the compounds dissolved in the medium (Hong and Lee 2002). By governing the DO level, the redox potential of the medium can be controlled through reducing agents (Wu et al. 1999). Literature review indicated that oxidation redox potential studies help to scale-up and optimize the fermentation process, like in the cases of xylitol and citric acid production (Kastner et al. 2003; Berovic 1999). In succinic acid production, highest yield of 1.18 mg g^{-1} of substrate was obtained when the redox level reached to -350 mV produced. Our results depicted in Fig. 5a–d shows the changes in redox values during the course of fermentation and its

effect on indigo pure yield. Biological energy is stored and released frequently by means of redox reactions (Martin et al. 2013) which help to understand the metabolic flux of the system. Figure 5a–d represents the experimental setup, where the vector value of spontaneous reaction lies on the Y axis (negative) of the graph, indicating the consumption of oxygen in fermented liquor with high anaerobic metabolic activity. Our findings indicate active participation of microflora, consequently breaking down cell lines releasing indican, which gets cleaved of the sugar moiety to indoxyl. The later gets dimerized with oxygen molecule and finally converted to the indigo dye stuff. Figure 5a–d illustrates that 12 h fermentation at 40 °C gives a redox value of −258 mV, concurrently at this condition indigo yield was the highest, i.e., 2.84 mg g^{−1}. This infers that 12 h fermentation duration is optimum that favors activity of all factors towards indigo dye production.

Conclusion

Bench and laboratory-scale setups were assembled and used for *Indigofera* plant biomass fermentation to analyze key parameters affecting indigo yield to understand the process and identify key process parameters to optimize indigo dye production from biogenic sources. It was observed that physico-chemical parameters during fermentation, viz. temperature, fermentation duration, pH, DO and redox potential of the fermented broth played major role in indigo dye yield. Experiments were conducted under varying incubation temperatures and fermentation durations of the broth, results showed notable changes in dye yield with changes in the latter. With increase in temperature, yield increased and maximum at 40 °C. Similarly, out of four incubation durations, 12 h fermentation incubation showed maximum indigo yield. Our studies have indicated the effects of pH, DO and redox potential upon biomass fermentation. These parameters could be utilized to influence or optimize the process towards enhancing indigo yield. These factors can be further augmented using physical, chemical and biological agents to up or down regulate the indigo production process. The efficacy of this approach at the pilot scale needs to be investigated, and the kinetic relationship between the lab-scale and pilot-scale processes right away needs to be determined.

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Compliance with ethical standards

Conflict of interest The authors state no conflict of interest for this work.

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