Environmental Challenges 4 (2021) 100157

Contents lists available at ScienceDirect

Environmental Challenges

journal homepage: www.elsevier.com/locate/envc

Influence of various oxidation parameter(s) for natural indigo dye formation from *Indigofera tinctoria* L. biomass

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ARTICLE INFO

Keywords:
Natural indigo
Indoxyl
Fermentation
Oxidation
Aeration
Particle size

ABSTRACT

Aeration or oxidation is one of the crucial steps during the plant-derived natural indigo dye production process. However, the lack of scientific information to determine adequate aeration conditions during natural indigo dye production is one of the significant limitations of the process. Therefore, the present study focuses on selecting suitable parameters to assess the optimum oxidation for indigo dye formation. Initially, the effect of direct oxidation parameters, such as dissolved oxygen (DO), oxidation–reduction potential (ORP), and pH on indigo dye formation, were evaluated. However, insignificant (p>0.05) co-relation was observed between the direct parameters on indigo dye formation. Alternatively, due to the agglomeration and water insolubility properties of indigo dye, particle size measurement was considered for real-time monitoring of indigo dye formation. It was established that the minimum indigo particle agglomeration size of ≥100 μm could be considered as a significant parameter to determine the indigo dye formation. It can act as an indirect indicator of adequate oxidation.

1. Introduction

The persisting popularity of blue-dyed jeans in the textile and fashion industries made indigo the most demanded dye. The current commercial production of indigo is carried out via synthetic route, which is derived from the reaction of aniline, formaldehyde, and hydrogen cyanide. These compounds are derivatives of petroleum and are highly toxic in nature. Not only the reactants but the significant amount of wastes derived from this process are also hazardous and draws serious environmental concerns (Rebelo et al., 2014). The replacement of synthetic indigo relies on natural routes, predominantly based on plant biomass (Shahid et al., 2013).

As an alternative to synthetic indigo dye production, the natural indigo dye production process is a green alternative that uses organic raw materials such as plant biomass and water without the addition of any chemical agents (Pattanaik et al., 2020a). Globally, the traditional indigo dye production process has been carried out either by crushed leaf method or water steeping method from various indigo bearing plant biomass available in the world (Angelini et al., 2003; Bechtold et al., 2002; Campeol et al., 2006; Maugard et al., 2002; Teanglum et al., 2012; Pattanaik et al., 2020b). In India, indigo dye extraction has been carried out by the traditional water steeping method using *Indigofera tinctoria* L. biomass (Bechtold and Mussak, 2009).

Indigofera tinctoria is a legume plant naturalized to tropical Asia, as well as parts of Africa. This plant belongs to the kingdom-plantae, family-fabacae, subfamily-faboideae, genus-*Inigofera*, species-*tinctoria*. It is a shrubby herbaceous plant (1–2 m tall), stems erect, copiously branched, leaves compound, leaf-rachis including the petiole 2.5–10 cm long; leaflets 5–13 in number and oval, oblong, obovate in shape of 10–27 mm long, 4–17 mm broad; flowers pink, standard orbicular obovate (4.2 mm long); pods glabrescent, 15–35 mm long and 2 mm wide, reflexed, slightly straight; seeds 3–15 (usually 6–12), much longer than broad. It is widely cultivated in tropical regions, from sea level to 300 m grows well along riverbanks, roadsides, and grassy fields. Continuous rain or too much water may kill plants. The common indigo plant is reported to tolerate annual precipitation of 6.4–41.0 dm, mean yearly temperature of 10.5–40.4 °C, and soil pH of 4.3–8.7 (Duke, 1981).

Unlike other caretonoid (annotto seeds and corolla tubes), anthroquinone (madder and safflower), and naphthoquinone based dyes (henna and walnut shells), where the natural dyes resides in the plant material in terms of pigments and extracted directly through various solvent extraction techniques. However, the natural indigo is a product of secondary metabolite and obtained from plant material through several conversion steps (Saxena and Raja, 2014). The method of indigo dye extraction from *Indigofera tinctoria* plant has been consisting of steeping, beating, and dye recovery stages. In the steeping process, the

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https://doi.org/10.1016/j.envc.2021.100157
Received 23 March 2021; Received in revised form 18 May 2021; Accepted 21 May 2021

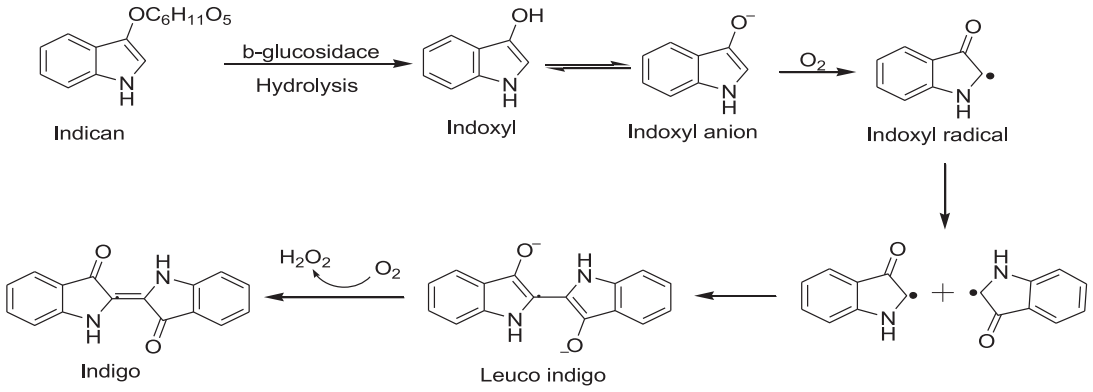

Fig. 1. Mechanism of the conversion of indican into indigo *via* indoxyl (Bechtold and Mussak, 2009).

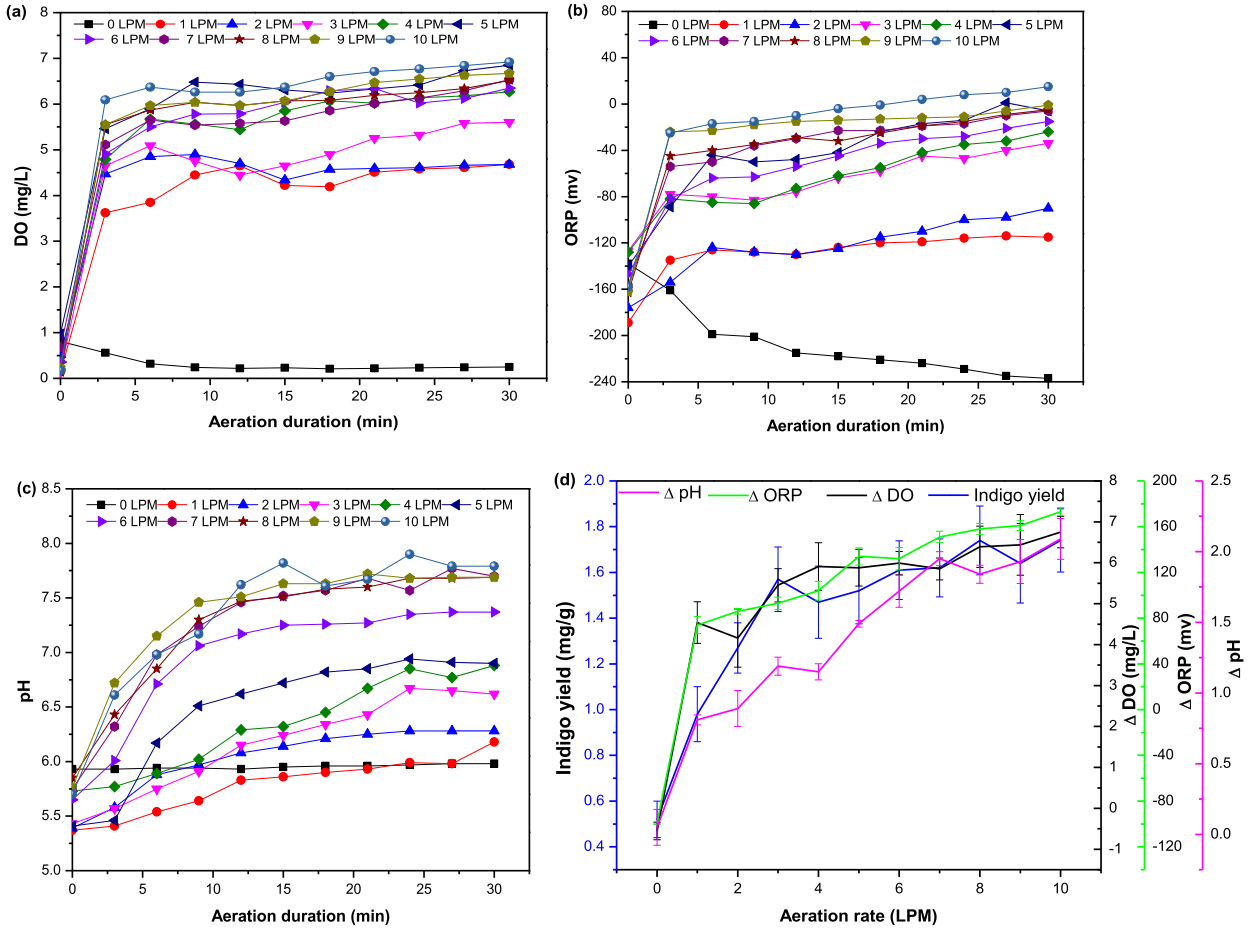

Fig. 2. DO (a), ORP (b), and pH (c) profiles and change in oxidation parameters (DO, ORP, and pH) with indigo yield (d) at different aeration rates.

release of indoxyl molecule and glucose moiety occurs from its precursor (indican), located in the plant vacuole. This hydrolysis reaction is catalyzed by glycosidase (β-glucosidase) enzyme of plant or microbial origin (Minami et al., 1997; Oberthür et al., 2004). In the second step or beating process, the released indoxyl undergoes spontaneous dimerization in atmospheric oxygen to form indigo. As these indigo particles are hydrophobic and insoluble in water, they form aggregates and sediment readily, which is considered as the third step or dye settling stage (Pattanaik et al., 2019).

So far, extensive work has been done on the improvisation of indigo dye yield from various indigo-bearing plant species by implementing different physicochemical methods. Laboratory scale experiments with various combinations of process parameters for steeping or fermentation process, such as a decrease in pH (1–3) and increase in temperature (40–70 °C) have been conducted to enhance indigo yield from *Polygonum tinctorium* and *Indigofera tinctoria* (Bechtold et al., 2002; Bechtold and Mussak, 2009; Stoker et al., et al., 1998). In India, process improvements for indigo dye extraction from *Indigofera tinctoria* plant biomass was carried out by acid extraction and subsequent neutralization using alkali (Sarangdhar and Henriques, 2007a). Recently, the indigo dye has been extracted from *Lonchocarpus Cyanescens* leaves by cold maceration in alkaline $Na_2S_2O_4$ solution (Oduro and Addo-Yobo, 2013). In another study, fresh leaves of *Isatis* species were used for extraction of indigo using hot water (60, 70, and 80 °C) and alkaline pH (9.0 and

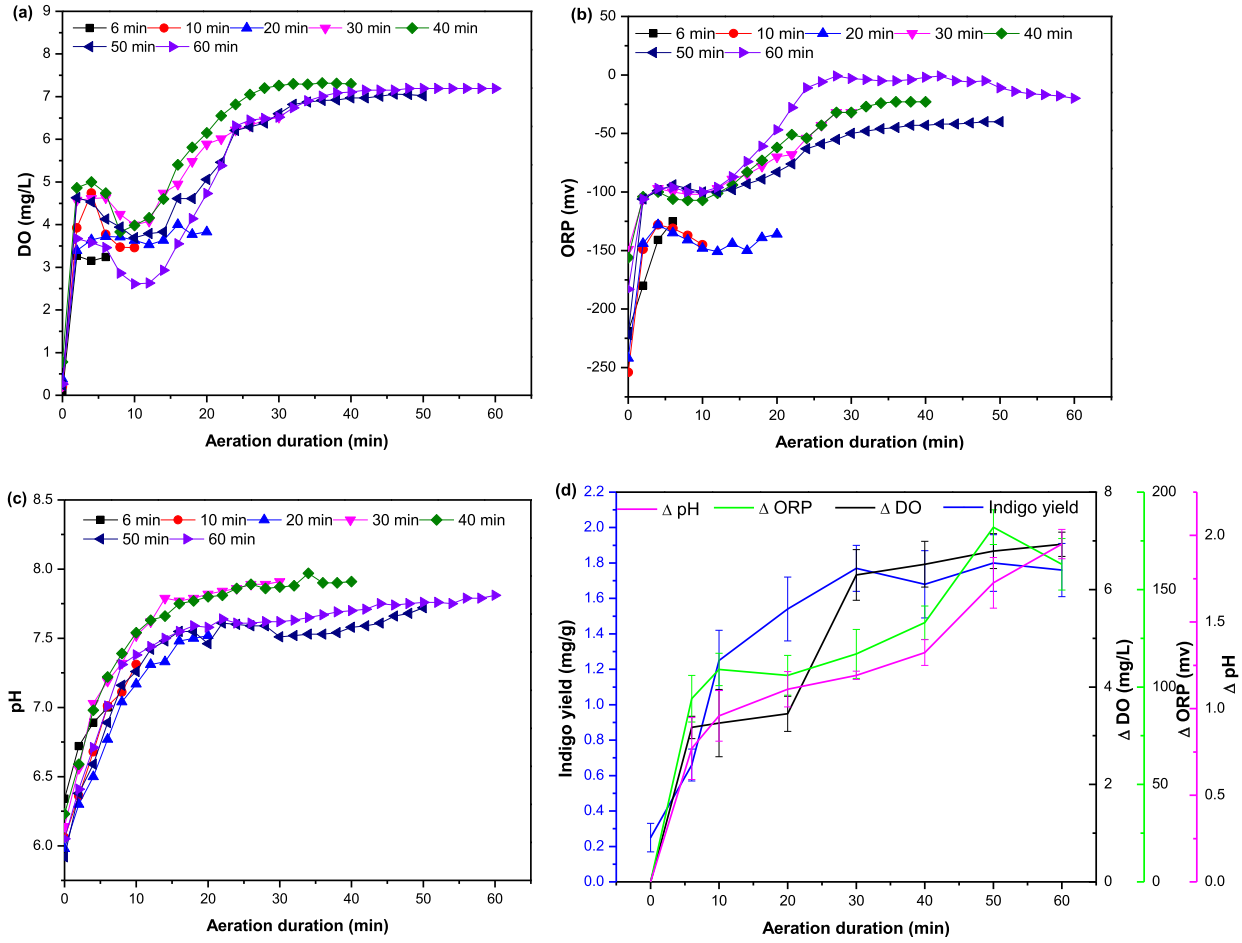

Fig. 3. DO (a), ORP (b), and pH (c) profiles and change in oxidation parameters (DO, ORP, and pH) with indigo yield (d) at different aeration durations.

11.0) (Comlekcioglu et al., 2015). Similarly, for extraction of indigo dye from *Indigofera tinctoria* plant biomass, variation of fermentation time (12, 24, 36, and 48 h), temperature (70, 80, and 90 °C), and alkaline condition (pH: 11 and 13) were tried (Hidayati et al., 2018). The effects of commercial β-glucosidase enzymes was evaluated on hydrolysis of indican and improvement of indigo dye yield using plant *P. tinctorium* (Angelini et al., 2003; Maugard et al., 2002). Likewise, the application of various microbial strains in fermentation or hydrolysis of *Indigofera* leaves has also been investigated (Sarangdhar and Henriques, 2007b). Most of the developments have been carried out on the improvement of the fermentation process. However, concerning environmental sustainability, economic feasibility, and ease of applicability, these improvements have their limitations for large-scale applications.

To enhance indigo yield, attempts have also been made to modify the beating or oxidation stage, where the conversion of soluble indoxyl into insoluble indigo occurs, which is a crucial step as it determines the rate of conversion and dye quantity (Fig. 1). In the traditional indigo dye production process, atmospheric air is primarily used as an oxidizing agent; apart from that, other chemical agents have also been explored for the oxidation process. It is reported that indoxyl to indigo conversion has also been accelerated by high pH (8–11) (Cotson and Holt, 1958). As a result, alkalis like calcium hydroxide (slaked lime), calcium and magnesium carbonate, calcium chloride have been explored for enhancing the oxidation process (Bechtold et al., 2002). However, these chemicals add impurities to the newly formed indigo and are not recommended for commercial use (Gracia-Macia and John, 2004). Further, the application of ammonia (NH_3) gasses as an oxidizing agent resulted in a cleaner indigo dye production as compared to the other alkali agents. However, due to energetically expensiveness, this process also has a limitation with respect to large-scale dye production (Stoker et al., 1998). Therefore, atmospheric air would be preferred as a cost-effective and environmental-friendly oxidizing agent.

In the conventional natural indigo dye production process, oxidation is carried out by the infusion of atmospheric air into the fermented broth through the manual beating process. Further, the optimum oxidation time has been decided by manual observation of indigo dye formation based on the traditional knowledge of the dye producers, which is deprived of any scientific knowledge. Regulating the oxidation process by manual observation might leads to insufficient aeration by affecting the dye yield. Therefore, the selection of suitable parameters to identify the required oxidation condition for indigo dye formation is essential, which is considered as the primary objective of the present study.

2. Materials and methods

2.1. Plant material

Indigofera tinctoria biomass was cultivated at Micromodel complex, IIT Delhi, during the month of April-September 2017. The *Indigofera* seeds with 80% germination were collected from indigo dye manufacturers, Villupuram district, India. The *Indigofera* seeds were sown in 200 m^2 of agricultural land with a line spacing of nearly 50–60 cm in the Micromodel complex, IIT Delhi.

2.2. Experimental basis

The present study has considered two possibilities to determine the suitable oxidation parameters for indigo dye formation.

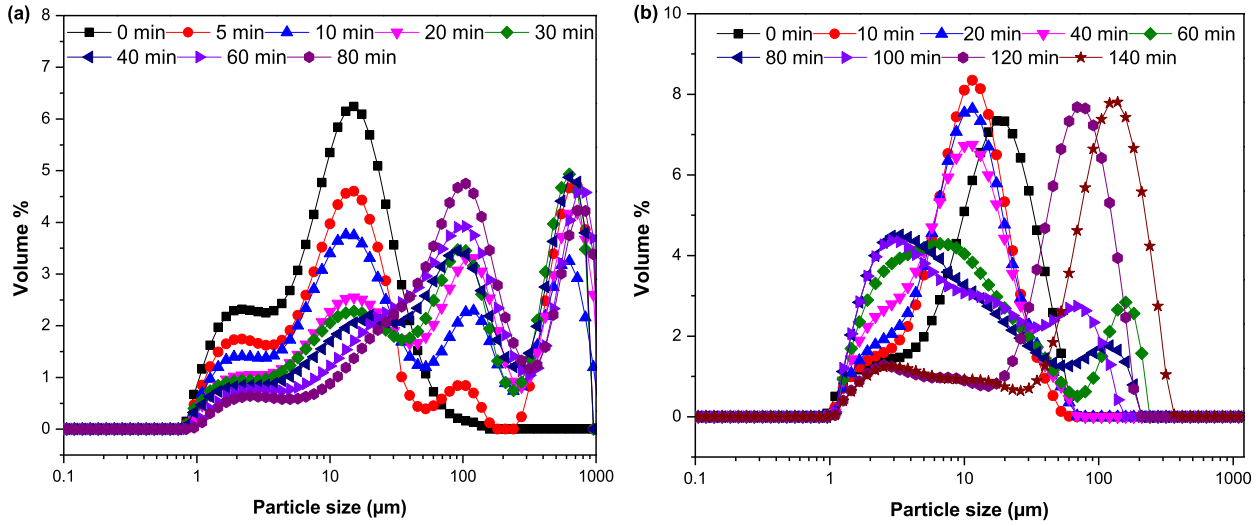

Fig. 4. Indigo dye particles distribution with course of time in D1 (a) and D2 (b) samples.

2.2.1. Direct parameter(s)

A specific aerator system was installed during oxidation to understand its role in the indigo dye formation process. The most crucial parameter during aeration was considered to be dissolved oxygen (DO), as it is the direct measure of the amount of oxygen present in the system. Again, indoxyl to indigo formation is an oxidation reaction (Bechtold and Mussak, 2009), so the measurement of oxidation–reduction potential (ORP) or redox is a crucial parameter to understand the reaction state of the system during aeration. Further, indoxyl oxidizes to its ketonic form during oxidation by releasing the proton (Bechtold and Mussak, 2009). Therefore, pH is also an essential parameter for observation.

2.2.2. Indirect parameter(s)

In the indirect method, the role of indigo particle size was measured by concerning indigo dye formation irrespective of any specific aerator system. Indigo particle size could act as an alternative/ indirect parameter to measure the required amount of aeration during the indigo production process.

2.3. Experimental condition for indigofera *biomass hydrolysis or fermentation*

After the onset of flowering, 75 g of freshly harvested biomass was chopped into 25–30 cm size, placed in 1 L beaker, and soaked in 750 mL water (biomass to water ratio ~1:10). The beaker was airtight to facilitate anoxic conditions and kept in an incubator for 16 h, at a temperature of 30 ± 2 °C for fermentation of biomass. Subsequently, the fermented broth was filtered and oxidized in a 1 L glass beaker with a working volume of 700 mL. Further, to evaluate the suitable parameters for oxidation, two different sets of batch experiments were performed.

2.4. Batch experiment to determine suitable parameter(s) for oxidation

In the first set of batch experiments, an air compressor with a sparging system was used to provide diffused atmospheric air into the oxidation system. The oxidation of the fermented broth was carried out in a 1 L glass beaker (working volume of 700 mL) at a room temperature of 28 ± 2 °C. To observe the regulating parameter(s) for identification of indigo formation, OFAT (one-factor-at-a-time) method was used. By maintaining the oxidation duration of 30 min, the effect of aeration was investigated by comparing the performance at different aeration rates (0–10 LPM) with an increment of 1 LPM. Similarly, with a constant aeration rate of 3 LPM, oxidation was carried out at 8 different oxidation durations (0, 5, 10, 20, 30, 40, 50, and 60 min). In each batch experiment, the primary process parameter observed during the oxidation process was the DO concentration in the broth. Along with DO, the other process parameters considered for observation were ORP and pH. The change in all the process parameters after the end of each batch experiment has been recorded (Eq. (1), Eq. (2), and Eq. (3)) along with the indigo dye yield.

$$\Delta \text{DO} = \text{final DO of the oxidized broth} - \text{initial DO of the fermented broth} \quad (1)$$

$$\Delta \text{ORP} = \text{final ORP of the oxidized broth} - \text{initial ORP of the fermented broth} \quad (2)$$

$$\Delta \text{pH} = \text{final pH of the oxidized broth} - \text{initial pH of the fermented broth} \quad (3)$$

2.5. Batch experiment for the measurement of particle size of indigo during oxidation

In the second set of batch experiments, the optimum condition for the indigo dye formation process was determined by measuring the indigo particle size by a laser particle size analyzer (Mastersizer 2000, Malvern Instruments, UK). The freshly recovered fermented broth was utilized for particle size analysis in two different dilutions. Due to instrument detection limitation, the highest dilution of the sample was made with 30% fermented broth in 70% distilled water, named dilution 1 (D1). Similarly, dilution 2 (D2) consists of 15% fermented broth and 85% distilled water. A total sample mixture (fermented broth and water) of 1000 mL was homogenously mixed with an inbuilt agitator system (agitation speed of 2400 rpm) for uniform mixing and distribution of indigo particles. Except for the inbuilt agitator system, no external mechanized aeration system was provided for aeration and oxidation of the fermented broth, which was carried out by spontaneous diffusion of atmospheric oxygen. Sampling of oxidized broth was conducted at a regular interval of time by the autosampler attached to the particle size analyzer. The overall particle size of the broth was determined by considering the D_{50} value, which represents 50% of the total particle size distribution. Periodic sampling of oxidized broth was also carried out to measure the indigo dye content.

Other than the particle size analysis, another set of experimentation was conducted to visualize indigo particle agglomerations using a compound microscope (LEICA DM 500) with a spontaneous exposure of atmospheric oxygen to the fermentation broth. Images of indigo particle agglomerations at different time intervals were captured, and the particle size was measured by using ImageJ software (National Institute of Health, Bethesda, USA).

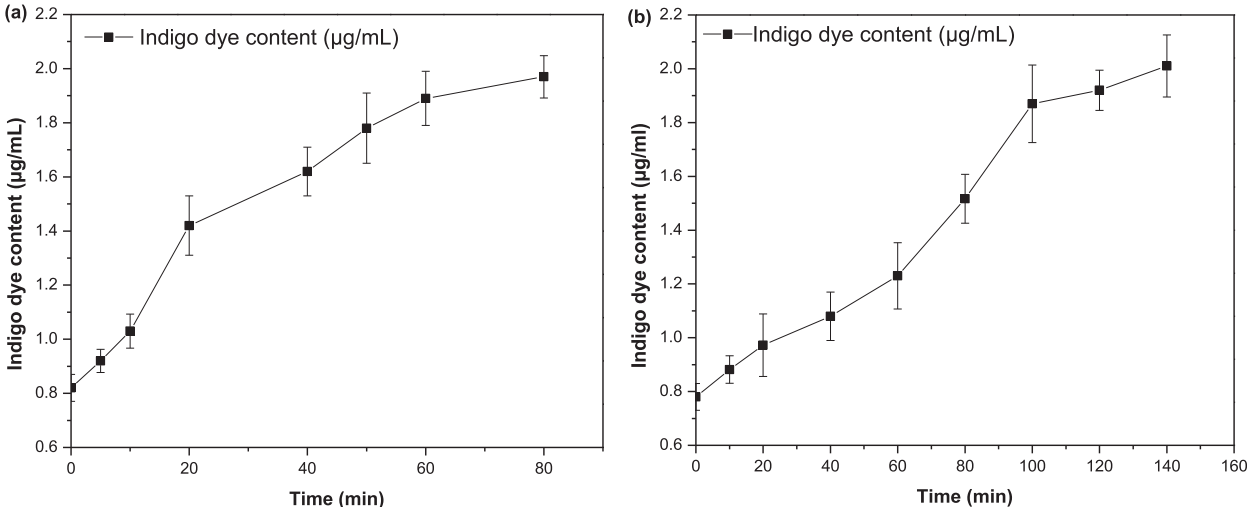

Fig. 5. Indigo dye content in D1 (a) and D2 (b) samples.

2.6. Indigo dye yield

The oxidized broth with indigo particles was collected by centrifuging at 19,419 × *g* for 15 min. The collected particles were dried, and the weight of crude dye was estimated using Eq. 4.

$$\text{Crude dye yield}\left(\frac{\text{mg}}{\text{g}}\right) = \frac{\text{Final weight (mg)} - \text{Initial weight (mg)}}{\text{Weight of biomass (g)}} \quad (4)$$

The pure yield of indigo was estimated by dissolving the crude indigo (~1 mg/10 mL of solvent) into a mixture of N-methyl pyrrolidone (NMP) with 0.5% of BHT (Butylated hydroxytoluene) (Gracia-Macias and John, 2004). The absorbance of the dissolved dye was measured at λ_{max} of 613 nm. The various indigo standard was prepared by the procedure mentioned above using the synthetic indigo powder of 95% purity (Sigma-Aldrich). The concentrations of the samples were determined from the calibration curve. Experiments were conducted in triplicates, and the mean data with the standard deviation have been reported. The relationship between each process parameter(s) on indigo dye yield has been evaluated by regression analysis using MINITAB 16.

3. Results and discussions

Aeration is the major contributor to oxidation, which dimerizes the indoxyl molecules to indigo (Bechtold and Mussak, 2009). For optimization of aeration during indigo dye formation, the effect of both aeration rate and aeration duration needs to be evaluated concerning indigo dye yield.

3.1. Effect of aeration rate on indigo dye formation

With a constant oxidation duration of 30 min, the effects of different aeration rates were investigated, and the profiles of oxidation parameters with respect to indigo dye yields have been represented in Fig. 2. The oxidation parameters (DO, ORP, and pH) are observed to be steadily increasing with respect to the increase in the flow rate of air. The maximum DO concentration in the fermented broth remained within 5 mg/L in the aeration rate of 1 LPM and 2 LPM. Whereas at 3 LPM, it reached up to 5.6 mg/L. The DO concentration remained within 6–7 mg/L at the aeration rate from 4 to 10 LPM (Fig. 2a). The DO concentrations hit within the initial 3 min of aeration for all the flow rates, followed by a relatively slower rise. A similar observation was also found in the case of ORP profiles during the different aeration rates (Fig. 2b). The ORP increased drastically from −237 to +15 mv at an aeration rate from 0 LPM (no aeration) to 10 LPM, signifying the improvement in oxidation condition in the fermented broth.

Similarly, in the case of pH, which is increasing continuously with the increase in aeration rate until it reaches a steady state (Fig. 2c). It might be due to the oxidation of indoxyl, and leaching of other phenolic groups out from the plant biomass. Initially, the fermented broth was slightly acidic in nature (pH: 5.43–5.93) moved towards neutral, followed by nearly alkaline conditions with the increases in aeration rate, which reached a maximum up to 7.79 at 10 LPM (Fig. 2c).

From the DO and ORP profile at different aeration rates, slight curvatures of different skewness have been observed. The possible reason for the curve-like shape could be due to the consumption of dissolved oxygen (electron acceptor) majorly by the indoxyl molecules (electron donor) present in the fermented broth for the dimerization and indigo dye formation (Fig. 1). These results are in agreement with the observation during microbial growth in ethanol fermentation, where oxygen acts as an electron acceptor, and the microbial cells act as an electron donor (Lin et al., 2011; Liu et al., 2016). Conversely, in the control sample with no external air supply, the free indoxyl molecule started consuming the available oxygen present in the fermented broth and liberated electron to dimerize, which leads to the reduction in DO and ORP.

Co-relating the change in oxidation parameters (DO, ORP, and pH) with the indigo yield, it can be observed that the increment in air flow rate leads to an increment in indigo dye content. The dye content showed a threefold increase in the yield from 0.52 mg/g to 1.57 mg/g at an aeration rate from 0 to 3 LPM, respectively. Later, a relatively slower increase in dye productivity up to a maximum of 1.74 mg/g was observed at 10 LPM (Fig. 2d). Hence, the increase in dye content could be due to the enhanced supply of atmospheric oxygen into the system at higher aeration rates, which helps in indoxyl to indigo conversion or indigo agglomeration. However, this concept does not hold proportionately with aeration rates beyond 3 LPM. Therefore, aeration rate of 3 LPM was considered the optimum aeration rate for the current experiments. From the regression analysis between the oxidation parameters and indigo dye yield, it was observed that there is insignificant (p>0.05) correlations exist between them (Table 1). The regression equation (Eq. (5)) is shown below.

$$\text{Indigo yield (mg/g)} = 0.386 + (0.349 \times \text{change in DO} - 0.00203 \times \text{change in ORP}) + (0.150 \times \text{change in pH}) \quad (5)$$

3.2. Effect of aeration duration on indigo dye formation

Similar to the aeration rates, the influence of oxidation parameters on indigo dye yield has been evaluated at different aeration durations and has been demonstrated in Fig. 3. Significant changes have been

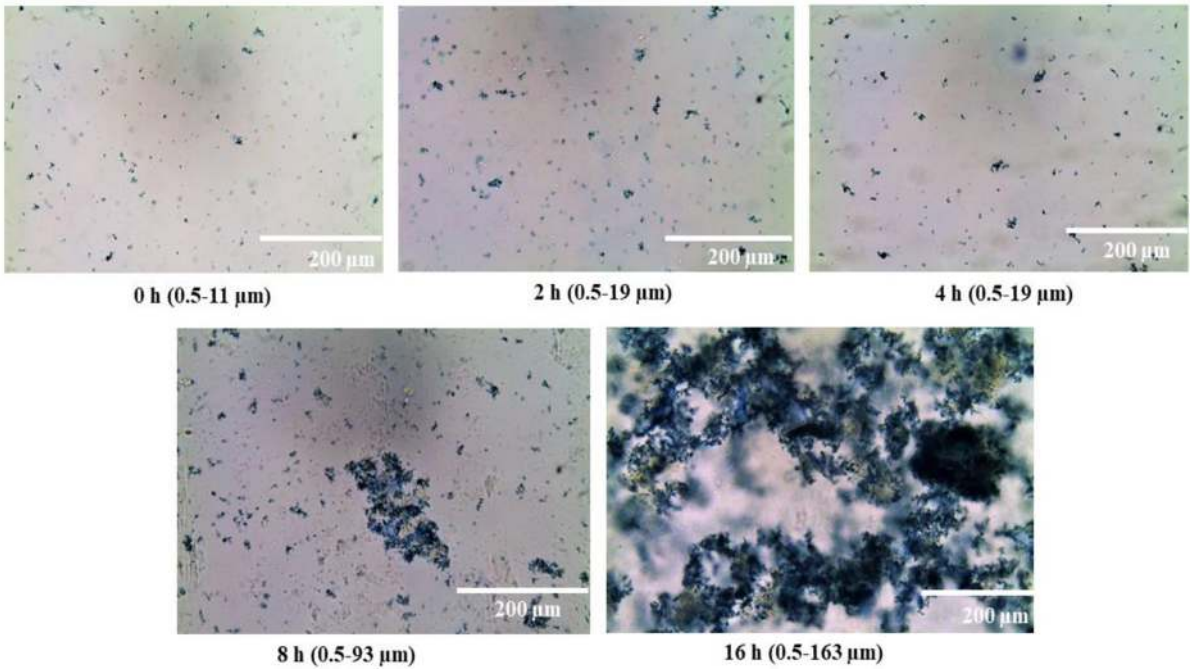

Fig. 6. Microscopic observation of indigo dye particles agglomeration.

Table 1
Regression analysis of oxidation parameters with respect to aeration rates.

Predictor	Coef	SE coefficient	T	p
Constant	0.3864	0.2893	1.34	0.223
Change in DO (mg/L)	0.3486	0.1851	1.88	0.102
Change in ORP (mv)	−0.002030	0.003419	−0.59	0.571
Change in pH	0.15019	0.09562	1.57	0.160

S = 0.115777, R-Sq = 93.2%, R-Sq (adj) = 90.4%.

Table 2
Regression analysis of oxidation parameters with respect to aeration durations.

Predictor	Coef	SE coefficient	T	p
Constant	0.3288	0.2615	1.26	0.277
Change in DO (mg/L)	0.1325	0.1260	1.05	0.352
Change in ORP (mv)	−0.000595	0.008567	−0.07	0.948
Change in pH	0.4198	0.8268	0.51	0.638

S = 0.298056, R-Sq = 85.1%, R-Sq(adj) = 73.9%.

observed in DO, ORP, and pH profiles with respect to variation in the aeration durations. From an initial DO concentration of <1 mg/L in the fermented broth, the DO profiles for all the oxidation duration have been drastically increased to a range of 3.27–4.86 mg/L within 2 min (Fig. 3a). Like the DO profiles, the ORP profiles have also been increased from −245 to 180 (Fig. 3b). Distinct curvatures have been observed in both DO and ORP profiles due to their initial rise and subsequent gradual fall. These observations are similar to the trends obtained previously for different aeration rates. The declining portion of the curve signified that the conversion of indoxyl to indigo occurred much faster than the oxygen dissolution process from air to liquid phase, which made the availability of electron donor (indoxyl) in the fermented broth in much higher concentration than the electron acceptor (oxygen), leads to a negative ORP potential. The basin structure of the curvature could be due to compensation of electron donors by the electron acceptors (oxygen), followed by an uprising of ORP potential and DO concentration, which might be due to higher oxidizing potential because of the availability of more oxygen concentration as compared to the free indoxyl molecule (Lin et al., 2011; Liu et al., 2016). The pH trends of the fermented broth have been observed to be continuously increasing with the increase in aeration duration (Fig. 3c). As indoxyl oxidizes to its ketonic form releases proton and the reaction moved from slightly acidic to neutral pH. Hence the formation of indigo takes place with rising in pH. Like the aeration rate, the indigo dye content also increased with respect to longer aeration duration. However, there is no significant co-relation observed among the oxidation parameters and the dye yield with respect to aeration duration (Table 2).

The indigo dye formation (0.25 mg/g) was also observed even at 0th min (sample collected immediately after fermentation with no external aeration), owing to non-maintenance of anaerobic conditions (Fig. 3d). This is due to the spontaneous reaction of indoxyl into indigo with the exposure of atmospheric oxygen during the processing and dye collection stage. Further, with respect to aeration for a longer duration at a constant rate (3 LPM), the dye yield was observed to be increasing. However, at the initial stage, the dye content was increased to a maximum of 1.77 mg/g for aeration of 30 min, followed by nearly a constant dye yield (Fig. 3d). From these observations, it could be inferred that 30 min aeration with an aeration rate of 3 LPM is sufficient to convert all the indoxyl molecules present in the fermented broth to indigo in the current experimental condition. However, further optimization of the aeration parameters such as aeration rates and aeration durations together with respect to the indigo dye yield would lead us to the exact necessary condition required for indigo dye formation in the given experimental condition. Nevertheless, in an open and large-scale indigo dye production process with variability in indoxyl concentration (due to change in indican content in the plant based on age and climatic condition), the optimum aeration condition can be changed (Campeol et al., 2006; Minami et al., 1997).

Further, from regression analysis, an insignificant (p>0.05) relationship was observed between the oxidation parameters (DO, ORP, and pH) and indigo dye yield concerning different aeration durations. Therefore, identifying the optimum aeration condition for indigo dye formation by assessing the direct parameters such as DO, ORP, and pH values is not suitable in the present experimental condition. The regression equation

is as follows:

$$\text{Indigo yield (mg/g)} = 0.329 + (0.132 \times \text{change in DO}) - (0.00059 \times \text{change in ORP}) + (0.420 \times \text{change in pH}) \quad (6)$$

3.3. Measurement of indigo particle size

The two unique characteristics of indigo dye during its development are its particle formation nature and water insolubility. The insoluble indigo tends to form agglomeration due to inorganic or organic impurities in the steeping water, leading to the formation of large-sized indigo particles. The larger indigo particle leads to effective sedimentation, hence enhancing product quality and quantity (Campos et al., 2000; Garcia-Macias and John, 2004). Many previous studies have been reported on indigo particle size measurement, majorly focusing on the indigo dye reduction for dyeing of textiles (Blackburn et al., 2009; Campos et al., 2001; Nicholson and John, 2005; Vuorema et al., 2009). While Garcia-Macias and John (2004) enlightened about the indigo particle size formation by conducting model experimentation using synthetic indoxyl acetate. However, in the present study, the real-time monitoring of the plant-derived indigo particle size has been focused, and experiments were conducted aiming its implementation in the conventional dye production process.

In Fig. 4a and b, the indigo particle size at different intervals of time was measured by exposure of atmospheric oxygen to the fermented broth. In both D1 and D2, it was observed that at 0th min, the formation of indigo particles has already been started with a distribution in the range of 1 to 100 μm. The D_{50} value of 10.62 μm and 11.14 μm was observed for D1 and D2, respectively. The initial observation of the indigo particles could be due to the spontaneous reaction of indoxyl into indigo in the presence of atmospheric oxygen in a low volume of fermented broth. Gradually, with the exposure of atmospheric oxygen, these indigo particles slowly agglomerate and shift to a higher range of particle sizes. A comparatively uniform particle distribution of size >100 μm was observed at an aeration time period of 60 min in D1 and 120 min in D2. The doubling of time for achieving similar particle size could be due to less number of particles present in D2, which justifies that the more the number of particles present in the fermented broth, the less time it takes to agglomerate. Further, the particle size of ≥100 μm is considered to be the minimum particle size for eye observation and sedimentation, so the observation of particle size during aeration could be fixed up to 100 μm. The indigo particle formation during these above experiments was also verified by quantifying the dye content. In Fig. 5a and b, the indigo dye contents have been seen to be increasing with the due course of time; verifying the increase in particle size leads to the collection of more dye particles and simultaneously increases the quantification of indigo dye. Further, the indigo particle agglomeration has been captured by microscopic observations (Fig. 6). According to the previous literature (Garcia-Macias and John, 2004; Bechtold and Mussak, 2009) on indigo particle agglomeration, the dye particles were observed to be agglomerated around the particulate matter originated from soil or plant, which is also found in this study (Fig. 6) having a size of indigo dye ~100 μm.

4. Conclusion

The present study showed some essential aspects of direct and indirect oxidation parameters on indigo dye formation. The effects of direct oxidation parameters (DO, ORP, and pH) and indigo dye yield have been influenced by variation of aeration rate and aeration duration. However, an insignificant correlation was established among them, possibly due to uncontrolled reaction conditions and variabilities in the indican concentration in the *Indigofera* plant biomass, which influenced the indoxyl concentration during indigo dye formation. Hence, in the large-scale natural indigo dye production process, real-time monitoring of indigo dye formation based on these direct oxidation parameters is quite challenging. In contrast, devoid of any specific aeration system, indigo particle size can help in the exact monitoring of indigo dye formation. Further, based on the desired particle size, the aeration rate and duration can be fixed in the indigo dye production process, which enables indigo particle size as an indirect oxidation parameter. By implementing the principle of particle size measurement the uncertainty of manual observation could be avoided during the oxidation process.

Declaration of Competing Interest

Authors declare no conflict of interest.

Acknowledgement

Authors are thankful to MHRDT, Govt. of India and IIT Delhi for providing the necessary infrastructure and resources for this research. Authors also acknowledge Dr. Pritam Kumar Dikshit for his valuable suggestion for improving the manuscript.

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