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Enhancement of indigo dye yield from *Indigofera* plant biomass by using fungal elicitors during fermentation

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Natural indigo dye from biogenic materials is an alternative to synthetic indigo that pollutes the environment. Indigo production from the *Indigofera tinctoria* plant is in practice since ancient time through biomass fermentation in water. Maximum indigo yield depends on complete release of indigo precursor indican from plant biomass during fermentation. In this study, we assessed the role of fungal elicitors on indigo dye yield during biomass fermentation using three strains namely; *Aspergillus niger*, *Trichoderma reesei* and *Sclerotium rolfsii*. Fungal extracts were prepared from fungal mycelia, raised separately on Potato Dextrose agar medium and extracted in a buffer solution containing 1N HNO_3 plus 1N NaOH. The extracts were added to fermentation water in 1, 5 and 10% doses (v/v) separately during biomass fermentation keeping other parameters for fermentation and further processing for dye production fixed. Indigo yield with 10% *A. niger* extract was 1.56% (w/w), which was higher in comparison to control 0.45% (w/w). *A. niger* extract at 10% enhanced indigo yield up to 3.5 times compared to control, and 2.7 and 2.1 times, respectively to other two cultures [*T. reesei* (0.58% w/w) and *S. rolfsii* (0.93% w/w)] used at the same concentration.

Keywords: Dye industry, Elicitation, Indican release, Indigo production, Natural dye, *Neel*

Pollution management in dye and fabric industry is a priority area of research in view of the environmental pollution of the recalcitrant chemical ingredients used for dye synthesis, residual dyes and their degraded intermediates. These negatively affect the physico-chemical properties of water bodies[1,2] eco-systems and some of them are even toxic to organisms[3]. In human civilization, Indigo is the oldest natural dye as come to be known used for dyeing fabric, toy and as an intermediate for synthesis. It is gained from a variety of plant species comes from different regions and landscapes around the globe[4]. *Indogofera tinctoria* of Fabaceae is the commercial variety used for dye yield popularly known as '*Neel*' in India[5].

Commercial indigo dye is predominantly produced through chemical synthesis and the ingredients are hazardous and toxic[6]. Due to its high productivity and purity, synthesis of indigo has replaced extraction from plants albeit their hazardous effects on the environment which has become alarming now[7,8]. Research works have reported the presence of reducing agents, recalcitrant residues and toxic reminiscent in wastewater from dye industry which pose a serious threat to the environment and human health[7-9]. Hence, a cleaner, greener and eco-friendly approach for indigo production is much needed. Supplementing synthetic dyes with their natural counterpart is an alternate strategy to ameliorate the environmental pollution as most of the byproducts during production and application of natural dyes are biodegradable[10]. In the plant derived natural indigo, presence of *cis*-indirubin (isoindirubin, red), *trans*-indirubin (isoindigotin, red) along with *trans*-indigo (indigotin, blue), indigo gluten and traces of flavonoids, makes biogenic indigo more eye-catching due to vivid hue and unique colourations in the fabric[11]. Therefore, natural indigo dyed fabrics have high demand in the European, East Asian and American fashion markets[12].

India had the legacy of indigo production from the *Indigofera* plant[13]. In *Indigofera*; indigo is present as precursor indican as secondary metabolite in vacuole of plant cell[14]. Indican is released from the cell vacuole through fermentation of plant biomass which is accelerated by microbial action. Released indican is

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acted upon by β-glucosidase enzyme that cleaves its glucose moiety to and converts to indoxyl. Indoxyl which upon further oxidation gets dimerized to form indigo[15].

The production of biogenic-indigo is an alternative to chemical processes, being an eco-friendly and sustainable process; however, has the drawback of extreme low productivity. In this context, elicitation is one of the most pertinent techniques to enhance natural indigo production thereby improving the limitation in high yield[16]. On a broader aspect, elicitors are of two types, biotic and abiotic, as shown in Fig. 1[17]. The biotic elicitors are generally derived from microbes or plants whereas the abiotic elicitors are basically chemical compounds. A wide range of abiotic and biotic additives, such as chitosan, β-glucan, sodium acetate, methyl jasmonate and fungal filtrates have been effectively used, direct to exaggerate and stimulated productivity in cell culture regimes[18]. An 'elicitor' is defined as a substance which when introduced in small concentration to living system, enhances or improves the biosynthesis of valuable plant compounds[19]. Fungal elicitation has been reported in recent researches; as one of the prominent methods to increase the yield of metabolic compounds by hastening the biosynthesis pathway of biologically important compounds[20]. Low energy requirements and mild reaction conditions have provided new avenues for microbial production of indigo keeping with benign environment[21]. Here, we have investigated the effect of three different fungal strains as elicitors, namely *Aspergillus niger* (513), *Trichoderma reesei* (1052) and *Sclerotium rolfsii* (1084) on natural indigo production from *I. tinctoria* plant.

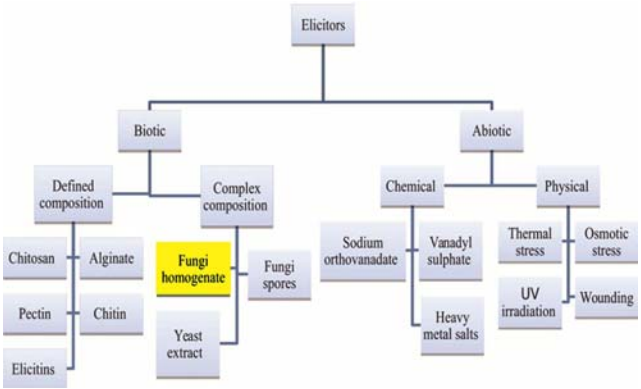

Fig. 1 — Classification of elicitors for production of secondary metabolites

Materials and Methods

Plant material

Indigofera tinctoria seeds were collected from local markets and plants were cultivated in 15"×15" size field plots in the CSIR-NEERI campus, Nagpur, India. Farm yard manure was added to the plots @ 1000 kg hectare^{-1} and ploughed. Seeds were shown in June, 2016 and watered as and when required. Leaf biomass from three months old plants were used in the experiments.

Fungal cultures

Three different cultures *Aspergillus niger*, *Trichoderma reesei* and *Sclerotium rolfsii* were procured from National Collection for Industrial Microbiology (NCIM-NCL), Pune. Each of these cultures were grown separately in 200 mL potato dextrose broth in 500 mL Erlenmeyer flask under aseptic condition and incubated for 15 days at 27^{o}C under dark. After 15 days, the fungal mycelia formed on the surface of the broth, were collected on filter papers through filtration.

Elicitor preparation

Known weight of each fungal biomass was subjected to acid wash neutralization and homogenized in mortar and pestle according to the reported method[22,23]. The homogenized mass was centrifuged at 10000 rpm for 5 min and the supernatant was used as fungal elicitors. Extract from each culture was added separately to the water used for steeping biomass in the concentrations of 1,5, and 10% (v/v).

Lab scale experimental setup

All experiments were carried out in a fabricated polypropylene reactor of 500 mL capacity. About 15 g leaves were placed inside the reactor and immersed in 150 mL tap water in the ratio of 1:10 (leaf biomass: water). The biomass was kept submerged under the water by putting weight over them so as to provide anaerobic environment for a steady duration. Different parameters *viz*. pH, redox potential (mV) and temperature (°C) of water were measured before adding to the fabricated reactor. The three different fungal extracts were added in separate experiments at doses of 1, 5 and 10% (v/v) as elicitors. In a typical fermentation setup for 1% elicitor dose, 1.5 mL of the fungal extract was added to 150 mL of fermenting water (v/v) with 15 g of plant biomass. The control experiments were devoid of fungal elicitors. Fermentation and subsequent steps for dye production were carried

out under fixed condition keeping all other parameters same. All experiments were carried out in triplicate and numerical data presented as mean ± SD.

Analytical method

The oxidation-reduction potential (ORP), pH and dissolved oxygen (DO) of the broth before and after fermentation was measured by an ORP meter (model: Hanna Instruments, HI98120 ORP/Temperature Tester), pH meter (model: EUTECH Instruments, Part of Thermo Fisher Scientific, Cyberscan DO 110, Serial No- 2061918) and DO meter (Model: EUTECH Instruments, Part of Thermo Fisher Scientific, Cyberscan PC 300, Serial No- 986792,). Indigo yield was quantified through spectrophotometry using dimethyl sulphoxide (DMSO) as the solvent at a wavelength of 620 nm[24] by a spectrophotometer (Shimadzu, Model no. UV 1800). Pure indigo (Sigma-Aldrich, R304949) was used to prepare the standard curve in the range of 0.01 to 0.2 ppm at the increment of 0.01 ppm.

Results and Discussion

Dye yield from *Indigofera tinctoria* plant biomass through fermentation in addition with the fungal extracts have been investigated in this study. The main limitation in natural indigo production method is its extreme low productivity. Hence, it cannot be an alternative to chemical processes, to meet the commercial requirement. Elicitation of plant cells in culture has proved to be one of the most useful techniques to improve the production of valuable metabolites[25]. There are several reports of fungal elicitors successfully used to increase the yield of secondary metabolites from plant cell cultures enhancing the production of commercially important compounds[26].

Effect of elicitors on indigo yield

The three fungal extracts were prepared from *Aspergillus niger*, *Trichoderma reesei* and *Sclerotium rolfsii*, added separately to the fermentation water in 1%, 5% & 10% (v/v) of the broth and dye yield was estimated as described in materials and methods. Results indicate *A. niger* and *S. rolfsii* extracts enhanced indigo yield, whereas *T. reesei* had slight inhibitory effect. Elicitation with *A. niger* extract elicited maximum indigo (4.88 mg g^{-1}) at 10% v/v dose, which was 3.8 - 4 times higher than control (Fig. 2). There was also increase in indigo yield with *S. rolfsii* extract, but elicitation effect was

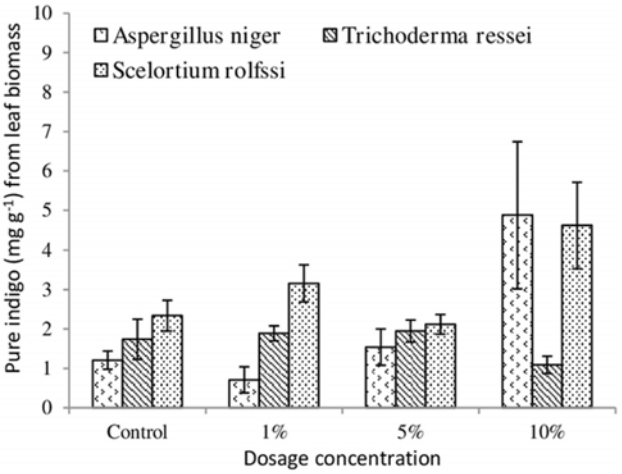

Fig. 2 — Effect of elicitor dosage on pure indigo yield (results are of three independent set of experiments)

comparatively lower in comparison to *A. niger* but twice in comparison with the control. These results indicate that addition of *A. niger* and *S. rolfsii* extracts during biomass fermentation elicits indigo productivity from the plant biomass, whereas that was not evident with the extracts of *T. reesei*. Kim *et al*.[22] have also reported application of chitosan as elicitor enhanced 2-3 folds indirubin yield in *Polygonum tinctorium* cell culture. Many techniques have been attempted for natural indigo production; out of which elicitation is one of the simple approaches[27,28]. It is reported that elicitors beyond a certain dosage could negatively affect a plant cell system and induce hypersensitive response leading to cell death[29]. Addition of *A. niger* elicitor to *Catharanthus roseus* cells has been reported to enhance the metabolite content with an increase in the dose up to 1.5% (v/v), above which plumbagin accumulation was decreased[30]. It is presumed that this could be the case with extracts of *T. reesei* which did not elicit indigo yield in our experiments. Specific nature and composition of active ingredients in the eliciting agent is the key component to elicit product formation in a bioprocess, and the optimum dosage for elicitation is worked out through dosage dependent response experimentation.

Effect of elicitors on redox potential

Oxidation reduction potential (ORP) of the fermentation broth was monitored after addition of different doses of the three types of elicitors at 1, 5 and 10% (v/v) during fermentation and oxidation process presented in Fig. 3 A and B, respectively. Redox is an indicator of the oxidation and reduction biochemical reactions and also governs the

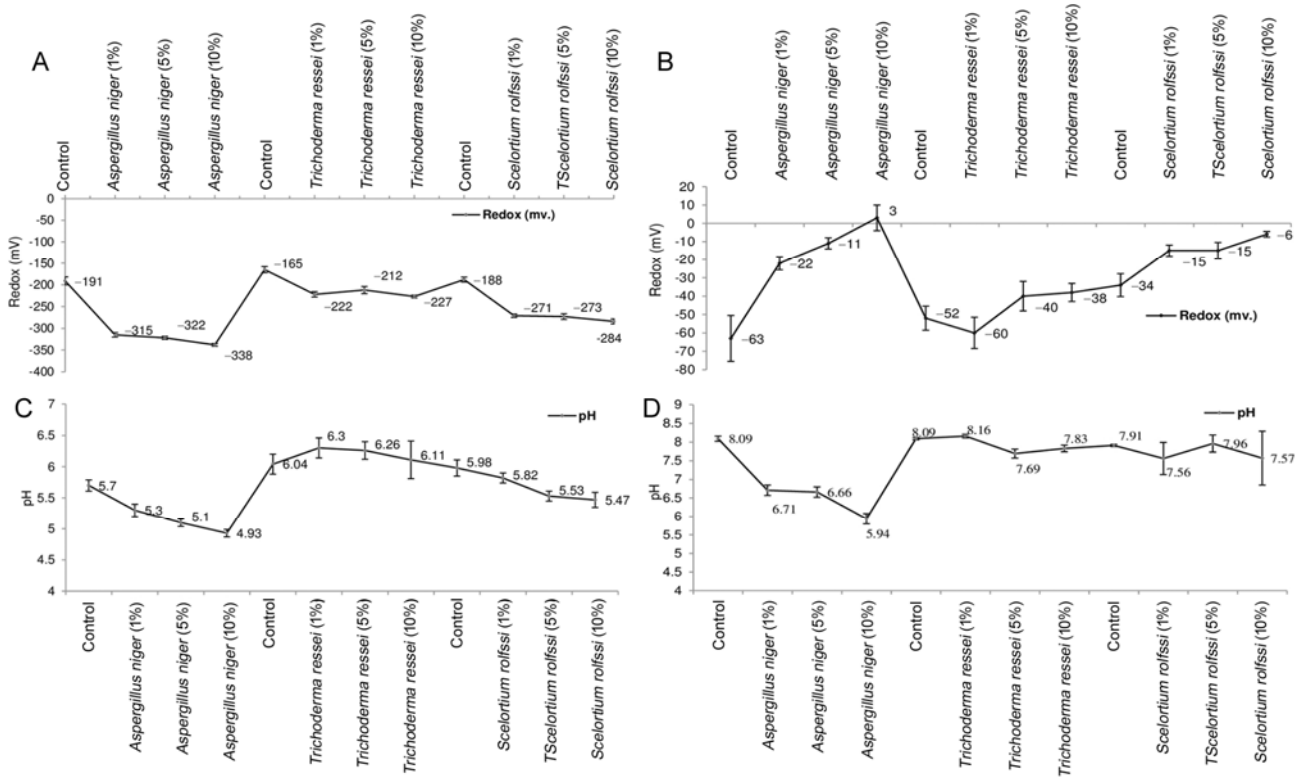

Fig. 3 — Effect of elicitor dosage on redox potential (A & B) during (A) fermentation process and (B) oxidation process; and pH (C & D) during (C) fermentation process and (D) oxidation process. [Results are of three independent set of experiments]

availability of metabolism of biologically important nutrients, such as carbon, nitrogen, oxygen, hydrogen and sulphur for microbial activity essential for optimum fermentation. Metabolic activity of microorganism depends on the redox potential of culture environment. An ORP profile, observed in fermented broth, indicates changes in metabolic flux due to microbial involvement in fermentation[31]. Measurement of ORP allows the fermenter operative to monitor the addition of reducing and oxidizing agents, while ensuring the redox potential to be in proper range for cell growth, especially when the dissolved oxygen (DO) level is very low[32]. In our experiments, ORP was high during the fermentation process for all the doses (1, 5 and 10%) of the three fungal extracts as compared to control, but during the oxidation stage of indigo production, redox potential decreased drastically for both, control and extracts treatment. Higher ORP values were obtained in case of *S. rolfsii* and *T. reesei* elicitation at all dosages as compared to their respective control experimental setup. But, the ORP was highest (−338 mV) with 10% extract of *A. niger* that also coincided with highest indigo yield, thus confirming the highest redox value with maximum yield. Ali *et al*.[33] suggested that elicitation with methyl jasmonate (MJ) and salicylic acid (SA) induced an oxidative stress in ginseng root (*Panax ginseng*) affecting the redox state of ascorbate. Investigation shows race specific elicitors brought changes in redox process with induced redox activity, stimulating defence mechanism in tomato against fungal pathogen[34,35].

Effect of elicitors on pH

The pH values were monitored during at the end of fermentation and oxidation process, respectively after addition of different dosage of elicitors. *A. niger* extracts at different doses (1, 5 and 10%) showed gradual increase in indigo yield in the acidic range of pH. Indigo yield was maximum (4.88 mg g^{-1}) by elicitation with 10% *A. niger* extract at pH 4.93 and 5.94 in respective stages, represented in Fig. 3 C and D, respectively.

In case of *T. reesei* and *S. rolfsii* elicitation even with highest dosage, there was not much change in pH during both fermentation and oxidation of broth. This corresponds to meagre enhancement in indigo yield unlike to elicitation with extracts of *A. niger*. It is presumed that indoxyl liberated from indican, is oxidized spontaneously by air, thereby enhancing

indigo yield at pH >5[35]. It is known that pH of a reaction system is vital for much chemical and analytical scrutiny, hence often used as an indicative parameter of the process dynamics. The pH gradient present in vacuole, cytosol, and extracellular medium, plays an important role in both active and passive release of secondary metabolites in plant cells in vitro[36]. Marero *et al*.[37] reports the maximum yield of (4.99 mg g^{-1}) indirubin content at a specific pH of 5.7 achieved by treating *P. tinctorium* cell culture with cell wall released heat soluble elicitator from a fungal pathogen *Rhizoctonia- soloni* resulting in enhancement and accumulation of indirubin.

The indigo dye is derived from indican through several steps of extraction; beginning with fermentation to release the dye precursor from plant tissue to the fermentation broth, cleavage of the β-glucoside molecule from the precursor by the β-glucosidase enzymes to make free indoxyl molecules, dimerization of the free indoxyl with oxygen through a induced oxidation process by air sparging to form the dye molecule and separation of the dye molecule from the broth. The extraction process is a chemo-biochemical process, and biomass fermentation stage is the primary step to release indican from the biomass. High indigo yield depends on complete release of indican from the biomass for its subsequent processing. The biochemical reactions during fermentation are important to provide the optimum conditions for partial tissue digestion, and facilitate complete release of indican to the broth for further processing. The microbial enzymes work apposite biocatalysts to perform these intricate reactions. A broad multiplicity of chemical compounds comprising terpenoids, aromatics, coumarins, alkaloids and steroids can undergo biotransformation using plant cells; organ cultures. The biocatalyst mediated reactions are stereo specific reaction types include oxidations, reductions, hydroxylation, methylations, acetylation, isomerization, glycosylation and esterification. Use of fungal extracts as elicitors to facilitate indicant release offer great potential to enhance indigo dye yield from the plant biomass. Fungal elicitors have been widely and successfully utilized to facilitate or improve yield of the product from the biochemical reactions[38]. Our study has shown that *A. niger* extract is an effective elicitor to enhance indigo dye yield from the *Indigofera* plant biomass when added to the fermentation water.

Conclusion

Indigo dye production from *Indigofera tinctoria* plant biomass was elicited by addition of fungal extracts during biomass fermentation. Three fungal strains i.e. *A. niger*, *S. rolfsii and T. reesei* were used at doses of 1, 5 and 10% (v/v) of the fermentation water which enhanced dye yield after processing of the ferment through the extraction process. These strains were chosen due to their known ability in synthesizing *β*-glucosidase enzymes which act for hydrolyzing indican-glucose moiety releasing more indoxyl to the broth for further oxidation to form the dye. The results of pure indigo yield obtained in this study implied that among the three elicitors, *A. niger* was the best. pH and redox potential were monitored during the fermentation and oxidation stage to assess their role on dye yield in addition to the fungal extracts. At acidic pH, indigo yield is maximising. It is possible that, addition of fungal extracts created acidity of broth resulting in better plant biomass maceration followed by cell vacuole disruption leading to increased indican release to the broth. The negative value of redox potential suggests spontaneous metabolic flux indicating more the negativity of redox, higher is the indigo yield.

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Conflict of Interest

The authors declare no conflict of interest.

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