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ORIGINAL PAPER

Optimized culture conditions facilitate the estrone biodegradation ability and laccase activity of *Spirulina* CPCC-695

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Abstract Endocrine disrupting compounds (EDCs) are emerging contaminants that persist and contaminate the environment. They mimic hormones, block hormones, or modulate their synthesis, metabolism, transport, and action, affecting living organisms and their progeny. Steroid hormones from exogenous sources like water bodies are important EDCs. Their biodegradation is an urgent global need. The present study is a preliminary work to maximize the estrone degradation potential of *Spirulina* CPCC-695 and study the effect of optimized conditions on its laccase activity. It was observed that the exponential phase culture at pH 10.0, 30 ℃, and 200 rpm of agitation speed resulted in the maximum growth, estrone degradation efficiency (93.12%), and highest laccase activity (74%) of *Spirulina* CPCC-695.

Keywords Estrone · *Spirulina* CPCC-695 · Temperature · pH · Agitation · Laccase activity

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Introduction

Steroid hormones, including both natural (endogenous hormone) and anthropogenic hormones (synthetic drugs), are known as the major endocrine disruptors (Wise et al. 2011). They get excreted through human urine and livestock manure (exogenous hormone), which enters into the environment through various routes like wastewater treatment plants, agricultural runoff and are recurrently detected in the environment (Shore and Shemesh 2003; Lorenzen et al. 2004; Fan et al. 2011). Johnson et al. recorded that expecting mothers excrete estrogen as high as 7 mg/day (Johnson et al. 2000). The occurrence and persistence of steroid sex hormones in surface water bodies have become a key concern in environmental research and policy. These hormones are persistent in the environment and their long-term exposure, even at extremely low concentrations, results in undesirable effects on animal behavior and physiology (Teles et al. 2004; Ghayee and Auchus 2007). Exogenous estrogen pollution harms the normal endocrine system function of aquatic wildlife by upsetting and mimicking the endogenous estrogen hormones as well as disturbing their metabolism. It exhibits competition with the intracellular estrogen receptors (ERs) that control the transcription of responsive genes and binds to it which initiates fast and non-genomic reactions (Liu et al. 2016). The erroneous ERs interferes with the normal functioning of the endocrine system and disrupt homeostasis in fish (Pinto et al. 2014).

Further, estrone accumulates through the aquatic food chain and reaches animals at higher trophic levels like humans where it affects the neurological, immune, and cardiovascular, systems (Wocławek-Potocka et al. 2013). This also predisposes humans to various cancers such as prostate cancer in men and breast cancer in women (Adeel et al. 2017). The other significant effects include lower sperm count, a decline in male reproductive health, and feminization (Sumpter and Jobling 2013). Among all the steroid hormones, estrone (E1), 17β-estradiol (E2), estriol (E3), and 17α-ethinylestradiol have been often detected in manure composts, soil, and water; and estrone has been regarded as the most estrogenic (Kim et al. 2015; Zhang et al. 2014). Thus, its safe degradation is a foremost important task.

Bioremediation is known as one of the useful and inexpensive methods to achieve the degradation of xenobiotics. Microorganisms play a substantial role in bioremediation due to the presence of degradative enzymes that helps in utilizing these pollutants as carbon and energy sources. Recently, we have reported the estrone degradation ability of cyanobacteria and found that oxidoreductases like laccases play an important role in its degradation (Sami and Fatma 2019; Sami et al. 2020). In the present study, we extended our work to enhance the estrone degradation ability of *Spirulina* CPCC-695 by optimizing the culture conditions (time, pH, temperature, and agitation).

Materials and methods

Chemicals

Estrone (97% purity) was procured from Sigma-Aldrich (USA). All other chemicals and reagents used in the present study were of pure analytical grade and purchased from HiMedia (India). The stock solution of estrone (1000 ppm) was prepared in acetone, sterilized by cellulose acetate filter (0.22 µm), and stored at 4 °C in dark bottles (Walker and Watson 2010). It was diluted to 20 ppm using culture media before use.

Spirulina strain

The freshwater cyanobacterium *Spirulina* CPCC-695 obtained from the University of Madras was used as the test organism and grown in BG 11 growth media (pH 7.3) under the control conditions i.e. at a temperature of 27 ± 2 ℃ under a 12:12 light: dark photoperiod provided by cool white fluorescent tubes at 25 µmol photonsmin^{-1} light intensity and were stirred daily manually (Sami and Fatma 2019; Sami et al. 2020; https:, , www.fao.org).

Determination of optimal growth and estrone degradation conditions

The effect of different parameters like time (0 to 20 days), pH (2.0 to 11.0), temperature (30 °C to 60 °C), and agitation (100 rpm to 250 rpm) was observed on the growth, estrone degradation, and laccase activity of *Spirulina* CPCC-695. The present study was planned in a way that one factor was optimized at one time and the best condition so obtained was used while optimizing other individual factors (At first, time was optimized, and then the culture of the best day was taken for optimizing pH, then the temperature was optimized at best pH, and finally, agitation was optimized at the best of all these obtained conditions). Eventually, the effect of optimized conditions on the laccase activity was checked.

Experimental set-up

Spirulina CPCC-695 culture was regularly revived and maintained in the logarithmic phase before starting the experiment. Experiments were conducted in triplicates in 250-ml flasks. On the day of setting up the experiment, the initial absorbance of the cell culture was set to 0.1 at 750 nm. Two different sets of control were prepared for assessing growth (Control 1) and estrone degradation (Control 2):

Control-1 Spirulina CPCC-695 cells in the BG-11 culture medium.

Control-2 20 ppm estrone added to BG 11 culture medium.

However, the test samples was same in both the cases that consisted of *Spirulina* CPCC-695 culture exposed to 20 ppm estrone. All the flasks were incubated under control conditions as mentioned above in 2.2. After every 24 h, the samples were collected and the growth was monitored by recording the optical density (OD_{750}) of the culture (Fatma et al. 1994). For estrone degradation, absorbance of spent medium was taken at 296 nm.

Quantification of estrone

Estrone stock solution (1000 ppm) was diluted to concentrations of 20, 40, 60, 80, and 100 ppm using BG 11 medium. Absorbance was taken at 296 nm against acetone as blank, using the MS UV Plus, UV–Vis Spectrophotometer (Motras Scientific, India). Using these absorbance values, the standard curve of estrone was prepared. The regression equation so obtained was used to calculate the amount of estrone. The calibration curve was described by the equation given below with the regression coefficient (r^2) being 0.9894.

$$Y = 0.02791 \times X + 0.1238$$

where Y is the absorbance at 296 nm and X is the amount of estrone in ppm.

Degradation efficiency was calculated using the given formula:

$$Degradation\ efficiency(\%) = \left[\left(A_c - A_t\right)/A_C\right] \times 100$$

where A_c is the initial amount and A_t is the amount after time t.

Laccase activity

Laccase (EC 1.10.3.2) activity was assessed under the control condition (mentioned in 2.2) and the obtained optimized conditions using the modified protocol of Bourbonnais et al. (1998). The cell cultures were centrifuged at 8000 rpm for 20 min at 4 ℃ after every 24 h. The supernatant was collected and laccase activity was determined by performing the enzyme assay. The reaction mixture (in a total volume of 1 ml) contained 100 mM citrate buffer (pH 4.0), 2 mM ABTS and culture supernatant which was incubated for 10 min. The development of green colour due to oxidation of ABTS confirmed the presence of laccase.The reaction was monitored by measuring the absorbance at 420 nm using the MS UV Plus, UV–Vis Spectrophotometer (Motras Scientific, India).

Laccase activity was expressed in international units per litre (UL^{-1}), defined as the amount of enzyme needed to produce 1 µmol product min^{-1} at 30 °C. The extinction coefficient (ε) of ABTS was used as 36,000 M^{-1} cm^{-1}.

$$Laccase\ activity(U/L) = \frac{Absorbance \times Total\ volume \times Incubation\ time}{Sample\ volume \times \left(3.6 \times 10^4\right)}$$

Statistical analysis

The experiments were done in triplicate, and the mean and standard deviations (SD) were calculated (n = 3). Statistical analysis was performed using Graph Pad Software 8.1, San Diego, California, USA. To confirm the validity of the variability of results, data were subjected to paired t test analysis and one-sample t test (two-tailed).

Results and discussion

Association between *Spirulina* CPCC-695 growth and estrone degradation

Before optimizing pH, temperature, and agitation for accomplishing efficient estrone degradation, the association between the growth of *Spirulina* CPCC-695 and estrone degradation was assessed. Time course studies have shown that *Spirulina* enters into the stationary phase after 20 days (Shi et al. 2016). Thus, the experiment was planned for 20 days while optimizing time.

In control-1, it was observed that exponential phase was achieved on day-9. However, in the test samples, the growth was enhanced and the exponential phase was extended till day-10. The increase in growth was found to be statistically significant using a paired sample t test ($p < 0.05$) (Fig. 1). *Spirulina* CPCC-695 initially adapted to estrone and metabolized it as an additional source of carbon for its growth. The increase in growth was found to be statistically significant ($p < 0.05$).

The amount of estrone in the spent culture medium was determined with the help of a standard graph (Fig. S1). The spent medium of the test sample was spectrophotometrically monitored after every 24 h for estrone quantification. In the case of control-2, there was no significant decrease in the amount of estrone with time, suggesting that there were no abiotic factors involved in the degradation. However, in the case of test samples, it decreased significantly with time and the maximum amount

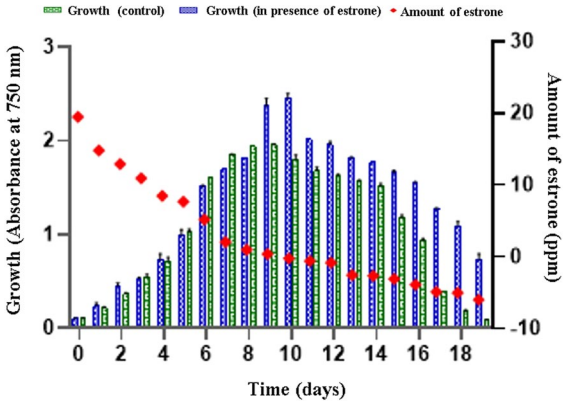

Fig. 1 Time course study of *Spirulina* CPCC-695 growth, estrone impact on its growth and estrone degradation

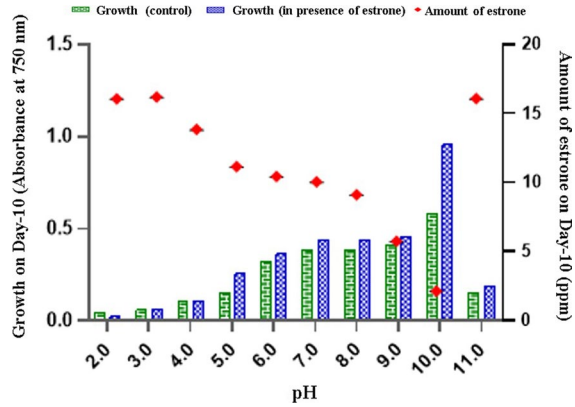

Fig. 2 Effects of pH (2.0–11.0) on growth of *Spirulina* CPCC-695 and estrone degradation

of degradation occurred on day-10, after which it moved towards saturation ($p < 0.05$).

Thus, maximum growth and degradation were observed on day-10 in estrone- exposed culture. This coincidence suggested that maximum estrone degradation was directly related with *Spirulina* CPCC-695 growth (Fig. 1).

Increased growth of cyanobacteria on any organic pollutant signifies that the organism is degrading the target compounds/pollutants and using the same as an energy source. This also increases the algal biomass. To the best of our knowledge, there is no report on the optimization of *Spirulina* CPCC-695 growth in relation to estrone degradation. However, higher growth of *Spirulina platensis* has been reported on day-10 in Zarrouk's medium (Gabal et al. 2018). Recently, Wang et al. also reported higher growth in *Phaeocystis globosa*, *Nannochloropsis oculata*, *Dunaliella salina*, and *Platymonas subcordiformis* during the nonylphenol biodegradation or biotransformation (Wang et al. 2019).

Effect of culture condition on growth of *Spirulina* CPCC-695 and estrone degradation

After determining the peak growth day using growth curve, the effect of pH (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0), followed by temperature (20 °C, 30 °C, 40 °C, 50 °C, and 60 °C), and finally agitation (100 rpm, 150 rpm, 200 rpm, and 250 rpm) was determined successively for knowing the best condition at which maximum degradation can be observed. This was done by comparing growth and estrone degradation in control-1 and test samples under each of the above-mentioned conditions. It was observed that in the case of the test (estrone-exposed cultures), growth was more as compared to control.

Effect of pH

The extrinsic and intrinsic pH of microorganisms affects their metabolism (growth and degradation). The biodegradation ability of algae and cyanobacteria is affected by pH as it alters the enzymatic activities, nutrient transportation, and solubility (Subashchandrabose et al. 2013; Singh et al. 2016). The results obtained suggested that growth and estrone degradation were favored under alkaline conditions. Significantly less growth was observed at acidic pH (Table S1). Maximum growth and degradation of estrone (83.94%) occurred at pH 10.0 ($p < 0.05$) (Figs. 2, S2). Maximum growth was also exhibited by other algae and cyanobacteria under alkaline conditions like *Chlorella vulgaris* (Daliry et al. 2017) and *Spirulina maxima* (Richmond 2008) at pH 9.0–10.0, *Spirulina platensis* at pH 9.5 (Huang and Chen 1986; Soni et al. 2019; Joshi et al. 2014), and *Spirulina platensis* LN1 at pH 11.0 (Thirumala 2012). In all these studies, higher pH retarded the growth of the organism.

It has been reported that the increased cytosolic pH decreases the permeability of the bicarbonate pool to the cell membrane, reduces the amount of bicarbonate ion transport required per carbon fixation, and

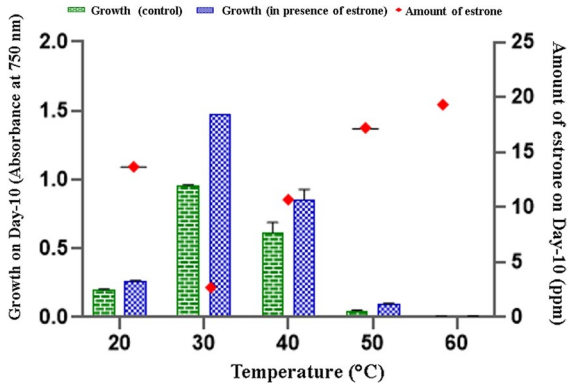

Fig. 3 Effects of temperature (20–60 ℃) on growth of *Spirulina* CPCC-695 and estrone degradation

decreases the total cost of carbon fixation through the Carbon Concentration Mechanism (CCM) in cyanobacteria (*Synechococcus elongates* 7942) (Mangan et al. 2016). Also, estrone having a pKa value of 10.34 exhibits higher aqueous solubility at alkaline pH which means there is more amount of inorganic carbon that facilitates carbon fixation (Calvin-Benson Cycle) through Rubisco and increases cyanobacterial metabolism that results in higher growth and biomass production at pH 10.0, which in turn would have facilitated estrone degradation (Shareef et al. 2006). However, at extremely high alkaline pH, the concentration of carbonate ion dominates and almost no carbon remains available for the cyanobacteria to combat oxidative stress, leading to cell death with a simultaneous gradual decrease in degradation efficiency (Choo et al. 2004). Additionally, under acidic conditions the enzymatic machinery becomes disturbed which result in the lowering of cell division, less growth, and degradation as observed in *Synechococcus* sp. strain Y-7c-s (Kallas and Castenholz 1982).

Effect of temperature

Temperature influences enzyme production and biochemical processes, including photosynthesis, growth, and degradation potential. Maximum growth and estrone degradation were observed at 30 °C in control as well as estrone (20 ppm) exposed cultures ($p < 0.05$) (Fig. 3; Table S2). The estrone degradation efficiency also increased to 86.04% at 30 °C which may be due to the increased activity of degradative enzymes (Fig. S3). At higher temperatures, the cyanobacteria tend to lyse, and thus reduced growth and degradation was observed as reported by Jetley et al. (2004).

Other microorganisms have also shown optimal growth at 30 °C like *Spirulina platensis* S-5 (Soni et al. 2019), *Spirulina platensis* (Huang and Chen 1986; Soni et al. 2019; Joshi et al. 2014; Chinnasamy et al. 2009), and *Chlorella vulgaris* (Eltoukhy et al. 2020). It has been found that biodegradation of other endocrine disrupting chemicals viz., Bisphenol A by *Pseudomonas putida* strain YC-AE1 (Kumar Rajendran et al. 2017) and 4-tert-octylphenol by *Candida rugopelliculosa* RRKY5 (Engqvist 2018) was also maximum at 30 °C.

Enzyme-controlled degradation activity prefers the temperature needed for optimal growth and physiological functions (Vinocur and Altman 2005). It is known that high temperatures cause shrinkage in cell size, adversely affect the metabolic activity of the microorganisms leading to the denaturation of the key enzymes associated with the carbon dioxide assimilation which inhibits growth and leads to algal death (Barati et al. 2019; Coleman and Colman 1980). Moreover, it may be possible that at higher temperatures the rate of photorespiration increases which reduces the affinity towards carbon dioxide that would have inhibited the CCM of *Spirulina* as observed in the case of *Chlaydomonas reinhardtii* (Colla et al. 2007) and *Spirulina platensis* (Boekel 2002). This would have resulted in slow growth and simultaneously reduces estrone degradation as well.

Similarly, at lower temperature, enzyme production reduces that affects photosynthesis by inactivating the photosynthetic proteins, reducing carbon assimilation and energy balance in the cell (Béchet et al. 2017; Sakamoto and Murata 2002). It also reduces membrane fluidity by enhancing membrane lipid desaturation through fatty acid desaturases which would have reduced degradation at lower temperatures (Lyon and Mock 2014; Sobczuk et al. 2006).

Effect of agitation

Up till now the cultures were manually shaken but to understand the effect of agitation on growth and estrone degradation, samples were incubated in the Refrigerated Shaker Laboratory Incubator (mrc, USA) at different agitation speeds. It is known that

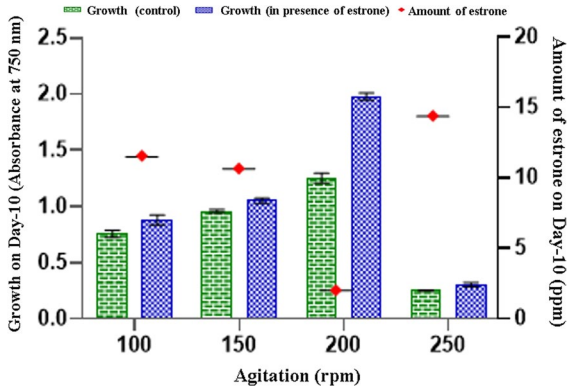

Fig. 4 Effects of agitation (100–250 rpm) on growth of *Spirulina* CPCC-695 and on estrone degradation

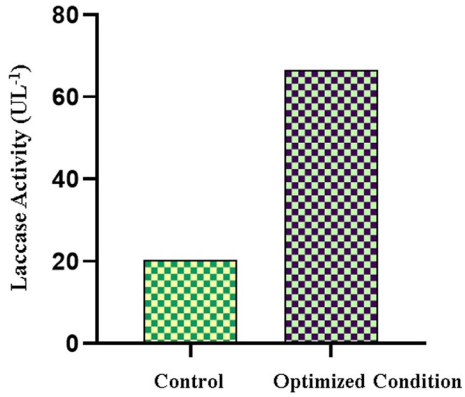

Fig. 5 Comparative laccase activity under control and optimized culture conditions (pH 10.0, 30 ℃ and 200 rpm)

agitation helps in nutrient mixing, proper aeration, and uniform accessibility to light that facilitates growth and prevents the biomass from settling and aggregating. Thus, growth and estrone degradation increased with increasing agitation speed until 250 rpm and was maximum at 200 rpm ($p < 0.05$) (Table S3). The estrone degradation efficiency also increased to 93.12% at 200 rpm (Figs. 4, S4). At 250 rpm (vigorous shaking) impaired cell growth is probably due to rupturing of cells that cause leakage of chemicals and cell death (Ohira et al. 2012; Mai et al. 2000). This would have reduced the degradation of estrone as well.

Laccase activity

Laccase has been known to play an important role in the degradation of many xenobiotics (Sei et al. 2007; Ting and Praveena 2017; Wang et al. 2018). Culture induced with guaiacol was grown under control (mentioned in 2.2) and optimized conditions (pH 10.0, 30 ℃, and 200 rpm) to find the relation between the optimized conditions and the laccase activity. Under optimized conditions, a significant increase in laccase activity (74%) was observed as compared to control ($p < 0.05$) (Fig. 5). This suggested that enhanced growth resulted in more enzyme production that eventually helped in degrading estrone efficiently.

Optimum laccase activity at 30 °C has been also reported in *Spirulina platensis* (Afreen et al. 2017), and in fungi including *Pycnoporus sanguineus* (Pointing et al. 2000), *Pleurotus*, *Dichomitus squalens*, and *Trametes modesta* (Zadrazil et al. 1999; Nyanhongo et al. 2002). Laccase having high activity in neutral or alkaline pH ranges is highly desirable for industrial applications. The highest extracellular laccase activity was found at pH 9.0 in the case of *Spirulina platensis* (Afreen et al. 2017). Laccase isolated from *Aspergillus flavus* showed the highest yield at neutral pH (7.0) (Kumar et al. 2016). To the best of our knowledge, the role of agitation on cyanobacterial laccase activity has not been studied to date.

Conclusion

Biological treatment is an eco-friendly and relatively inexpensive method for the remediation of many xenobiotics including endocrine disrupting compounds. Temperature, pH, and agitation are considered important factors that influence the degradation ability of microbes. In the present study, *Spirulina* CPCC-695 in exponential phase exhibited maximum growth and estrone degradation ability at pH 10.0, temperature-30 ℃, and agitation speed-200 rpm. At these conditions CCM was favoured that resulted in utilization of estrone as a carbon source and its metabolization through the Calvin-Benson Cycle. This supported the growth-linked degradation theory. Enhanced growth also increased laccase activity under optimized conditions that also helped in efficient estrone degradation.

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Author contributions NS and TF designed the experiment. NS carried out the lab work, data curation and wrote the initial manuscript that was corrected and finalised by TF.

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Data availability All data generated or analysed during this study are included in this published article.

Declarations

Competing interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval and consent to participate This study does not require approval from the local ethics committee. Studies involving plants must include a statement specifying the local, national or international guidelines and legislation and the required or appropriate permissions and/or licences for the study.

Consent for publication Not applicable.

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