Estrone degrading enzymes of *Spirulina* CPCC-695 and synthesis of bioplastic precursor as a by-product

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A B S T R A C T

Estrone, a steroidal estrogen that is persistently contaminating the surface water has been classified as an endocrine disruptor and as Group-1 carcinogen by the World Health Organization. Long-term exposure to estrone-contaminated water disrupt physiology, behaviour and sexual development of living organisms that lead to many disorders. So, it has to be eliminated from our surrounding. Its biological degradation is a cost effective and eco-friendly approach. The present study targets to predict the degradation pathway and understand the role of cyanobacterial enzymes: oxidoreductases (laccase, peroxidase) and esterase in estrone degradation. Poly-β-hydroxy butyrate (PHB) was also quantified as a by-product of estrone biodegradation. The estrone degradation pathway was predicted using EAWAG-BBD/PPS database. *Spirulina* CPCC-695 was grown in different concentration of estrone (20 mg/l, 50 mg/l, 100 mg/l and 200 mg/l). The culture without estrone was considered as control. The culture supernatant was used for testing laccase and esterase activity whereas the biomass was used to test peroxidase activity and quantify by-product (PHB). The enzymes showed concentration-dependent activities. Maximum enzyme activities were seen at 20 mg/l estrone. *Spirulina* CPCC-695 utilizes estrone as a carbon source and degrades it to produce pyruvate which forms acetyl CoA that undergo condensation, reduction and polymerization to form PHB. Maximum PHB (169 μg) was also produced at 20 mg/l as a by-product during degradation.

1. Introduction

The development of new technologies, expansion of industries, extensive production and use of harmful chemicals can be attributed for the increased environmental pollution. Among these chemicals/xenobiotics, endocrine disrupting compounds are the most risky that effect health of the environment, wildlife and humans. These interfere with natural hormone systems and effect the endocrine system. Exposure to endocrine disruptors can have life-long effects and can even have consequences for the next generation [1]. Nowadays, the occurrence and persistence of estrogens in aquatic ecosystems has become a great concern to public health because its long-term exposure even at sub-nanogram-per-litre concentrations adversely affect animal behaviour and physiology [2–4]. One of the female estrogens, estrone (estra-1, 3, 5(10)-triene), is a 17-oxo steroid containing 18 carbons ($C_{18}H_{24}O_2$) and is known as C18 steroid. Its core structure consists of 17 carbon-carbon bonds arranged in the form of four fused rings. Out of the four, there are three cyclohexane

rings (Ring A, Ring B, Ring C) and one cyclopentane ring (Ring D). It is substituted by a hydroxyl group at the third position and an oxo-group (ketone group) at the seventeenth position. Estrone enters the environment through humans and animals excretion and shows persistence [5,6]. Further, the spreading of poultry and cattle waste on agricultural land increases the risk of groundwater contamination [7]. It remain stable in the environment and is a ubiquitous pollutant present in aquatic bodies [8,9]. It has been found that estrone exist up to 0.038 parts per billion (ppb) and 0.010 ppb in treated wastewater and in waters downstream of wastewater treatment plants respectively [10–12].

Bioremediation has been accepted as an effective tool for degradation of pollutants to non-toxic compounds [13,14]. Thus, due to environmental concern and effect of estrone on organisms, intensive research efforts have been focused on identification and isolation of estrogen-degrading microorganisms [15]. Various bacteria, mainly actinobacteria (e.g. *Nocardia* sp., Rhodococcus sp.) and proteobacteria (e.g. *Novosphingobium* sp.) have been isolated from different natural and engineered ecosystems like soils, seawater, sandy aquifers, compost and activated sludge that are capable of estrogen degradation [16–18]. It has been reported that *Scenedesmus quadricauda*, *Chlorella vulgaris*, *Ankistrodesmus*

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acicularis and *Chroococcus minutus* are capable of degrading nonylphenol [19]. *Scenedesmus obliquus* has been found to degrade climbazole-an antifungal drug [20]. Liu et al., (2018) observed that *Raphidocelis subcapitata* is capable of degrading 17β-estradiol and diethylstilbestrol [21]. Fu et al. (2019) have recently found that *Chlorella vulgaris* eliminates testosterone [22]. Though role of algae and cyanobacteria is well established for bioremediation of various organic pollutants but their role in estrone degradation has not been attempted thoroughly. Wang et al. (2019) reported that *Chlorella vulgaris*, *Haematococcus pluvialis*, *Scenedesmus quadricauda* and *Selenastrum capricornutum* effectively remove and transform 17α-ethynylestradiol, 17β-estradiol and estrone present in synthetic wastewater effluent [23]. Recently, we have screened cyanobacteria for estrone biodegradation potential. *Spirulina* CPCC 695 showed maximum growth and highest degradation potential at 20 mg/l estrone [24].

It has been reported that these xenobiotics get first adsorbed on the surface of the cell membrane, enters into the cell after establishing an equilibrium and gets degraded into non-toxic intermediates by extra-cellular and intra-cellular degradative enzymes [25]. Extra-cellular enzymes include a large range of oxidoreductases and hydrolases. Both these enzymes transform xenobiotics into partially degraded or oxidized products that can be easily taken up by the cells that can be further degraded by intra-cellular enzymes like peroxidases [26,27]. Enzymes like laccase and manganese peroxidase have been found to be active during estrone degradation in *Phanerochaete sordida* YK-624 [28]. They add hydroxyl group at the C-4 position in ring-A producing 4-hydroxy estrone [29,30]. Laccase-assisted (immobilised enzymes) removal of steroidal estrogens are also being explored nowadays [31,32]. The ester hydrolyzing enzymes i.e. esterase from *Nocardia erythraea*, cholesterol esterase from porcine and bovine pancreas, and esterase from *Candida cylindracea* and a cutinase from *Fusarium oxysporum* f. sp. pisi, 44 have been reported to degrade phthalates, an endocrine disruptor [33–35].

The role of these enzymes in estrone degradation has not been studied yet. Moreover, the pathway through which estrone degradation occurs in microalgae and cyanobacteria still needs to be explored. The present study targets to predict the degradation pathway using EAWAG-BBD/PPS database, examine the activity of catabolic enzymes (laccase, peroxidase and esterase) and to find out probability of PHB (bio-plastic precursor) synthesis.

2. Materials and methods

2.1. Chemicals

All the chemicals used in this study were of analytical grade and were purchased from Sigma (St. Louis, USA). All the buffers and reagents were prepared in Milli.Q.

2.2. Culture maintenance and experimental design

Spirulina CPCC-695 was procured from University of Madras, Chennai, India. It was grown in BG-11 medium supplemented with sodium nitrate as nitrogen source in 500 mL Erlenmeyer flasks. The culture condition was set as: 27 ± 2 °C under a 12:12 light:dark photoperiod supplied by cool white fluorescent tubes at 25 μmol photons min^{-1} light intensity [36]. The cells were maintained in the exponential phase by repeated inoculation into the fresh medium before being used in the experiments.

For experiments, the mid-exponential phase cyanobacterial cells were taken and grown in presence of different concentrations of estrone (20 mg/l, 50 mg/l, 100 mg/l and 200 mg/l). The estrone stock solution (1000 mg/l) was diluted with growth medium to attain these concentrations. The culture without estrone was considered as control. Cells were harvested by centrifugation at 10,000 x g for 10 min at 4 °C. The supernatant was filtered using 0.22 μm filters and then used for estimating the enzyme activity. The experiment was done in triplicates and activity was estimated after every 24 h for seven days (Day 0-Day 6).

2.3. Prediction of estrone degradation pathway

EAWAG Biocatalysis Biodegradation Database and pathway prediction system (EAWAG BBD/PPS) was used to predict the estrone degradation pathway by utilizing the substructure searching, a rule-base, and atom-to-atom mapping [37]. The system recognizes organic functional groups found in estrone and predicts its transformations based on the reactions found in the scientific literature or in the EAWAG-BBD database. The structure of estrone and its corresponding SMILES string was imported from PubChem Structure Database (SMILES is a typical specification for the structure of chemical molecules using short ASCII strings) to the EAWAG BBD/PPS that predicts the degradation pathway [38].

Canonical SMILES of Estrone: CC12CCC3C(C1CCC2=O) CCC4=C3C=CC(=C4)O

2.4. Identification of cyanobacterial enzymes responsible for estrone degradation

The role of cyanobacterial enzymes including oxido-reductase (laccase and peroxidases) and esterase was determined in estrone degradation as detailed below-

2.4.1. Estimation of laccase activity

Laccase (EC 1.10.3.2) activity was determined by modified protocol of Bourbonnais et al., (1998) that is based on the oxidation of ABTS [39]. The assay mixture (1 mL) contained 2 mM ABTS, 100 mM citrate buffer (pH 4.0), 500 μL of the culture supernatant and was incubated for 10 min. Oxidation of ABTS was monitored by determining the absorbance at 420 nm using Labomed UV–vis spectrophotometer (UVS-2700) and activity was calculated by taking ε420 as 3.6×10^4 M^{-1} cm^{-1} [40,41]. The citrate buffer was used as control. The laccase activity was expressed in international units per litre (U/L), defined as the amount of enzyme needed to produce 1 μmol product i.e. ABTS radical cation (ABTS$^{\bullet+}$) per minute at 30 °C.

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

2.4.2. Estimation of peroxidase activity

Catalase peroxidase (CPX) (EC 1.11.1.21) was estimated according to the method proposed by Mutsuda et al. (1996) [42]. Algal biomass (20 mg) was homogenized with 1 mL extraction buffer (0.5 M Phosphate buffer, pH 7.5). Homogenate was centrifuged at 12,000 x g for 20 min. The supernatant (enzyme extract) was used for the assay. The reaction mixture contained 100 μL of enzyme extract, 50 mM of 1.6 mL phosphate buffer (pH 7.0), 3 mM EDTA and was incubated for 3 min.

$$Peroxidase\ activity\ (U/L)$$
$$= \frac{Absorbance\ \times\ Total\ volume\ \times\ Incubation\ time}{Sample\ volume\ \times\ 43.6}$$

1U of the enzyme is the amount necessary to decompose 1 μL of hydrogen peroxide per minute at 25 °C. The optical density was measured at 240 nm and catalase activity was calculated by taking

ε_{240} as 43.6 M^{-1}. cm^{-1} against a blank of same reaction mixture except hydrogen peroxide [43].

2.4.3. Estimation of esterase activity

Esterase activity (EC 3.1.1.1) was determined by the hydrolysis of 4-nitrophenyl acetate method by measuring the released para-nitrophenol [44]. The reaction mixture [50 mM of potassium phosphate buffer (pH 7.0), 250 μL of culture supernatant and 100 μL of 25 mM 4-nitrophenyl acetate] was diluted to a total volume of 1 mL and incubated at 37 °C for 30 min. The para-nitrophenyl acetate substrate was hydrolyzed to yield the acetic acid and para-nitrophenol. Absorbance was recorded at 400 nm against potassium phosphate buffer as blank.

$$Esterase\ activity\ (U/L)$$
$$= \frac{Absorbance \times Total\ volume \times Incubation\ time}{Sample\ volume \times 1.36 \times 10^{-2}}$$

The enzyme activity was presented in the unit of U/L. One unit of enzymatic activity is defined as the amount of the enzyme that releases 1mmole p-nitrophenol per minute at 37 °C [45].

2.5. Quantification of polyhydroxybutyrate (PHB)

While degradation of estrone, PHB may be produced as a by-product. To ascertain its production and accumulation in the cyanobacterial cells, the amount of PHB was quantified after every 24 h [46]. For estimating the production of PHB, the cyanobacterial biomass (10 mg) obtained after centrifugation was suspended in methanol (5 mL), kept overnight at 4 °C for removal of pigments and then centrifuged at 8000 x g. The pellet obtained was dried at 60 °C. The polymer was extracted in hot chloroform (2 mL) followed by precipitation with cold diethyl ether (4 mL) and centrifuged (10,000 g for 20 min). The pellet was washed with acetone (5 mL) to remove impurities, dissolved in hot chloroform (2 mL) and then transferred in a glass test tube. The chloroform was evaporated and conc. sulphuric acid (5 mL) was added. The solution obtained was heated in a boiling water bath. After cooling and thorough mixing, absorbance was measured at 235 nm against sulphuric acid as a blank.

In order to quantify the amount of PHB accumulated, the standard curve of PHB was prepared [46]. PHB (200 mg) was dissolved in concentrated sulphuric acid (10 mL). The mixture was boiled for 10 min and then cooled. The concentration of this stock solution was 20 mg/mL. It was diluted with distilled water to obtain different PHB concentrations (10−100 μg/mL). Absorbance was taken at 235 nm against distilled water as blank. The calibration curve was described by the equation given below with regression co-efficient (r^2) being 0.9904. This equation was used to calculate the amount of PHB.

$$Y = 0.0055 \times X + 0.0253$$

where, Y is absorbance at 235 nm and X is the amount of PHB in μg.

3. Results

3.1. Predicted estrone degradation pathway

The canonical SMILES of estrone was obtained from Pubchem Structure database and its biodegradation pathway was predicted using EAWAG-BBD/PPS. The predicted pathway was aerobic in nature and confirmed the breakdown of estrone into pyruvate (the major degradation product) with the release of acetate (Fig.1.). The obtained pathway elucidated the hydroxylation of A-ring at the C-4 position. The ring structure of estrone gets cleaved in the second step and formed an open ring structure which further undergoes
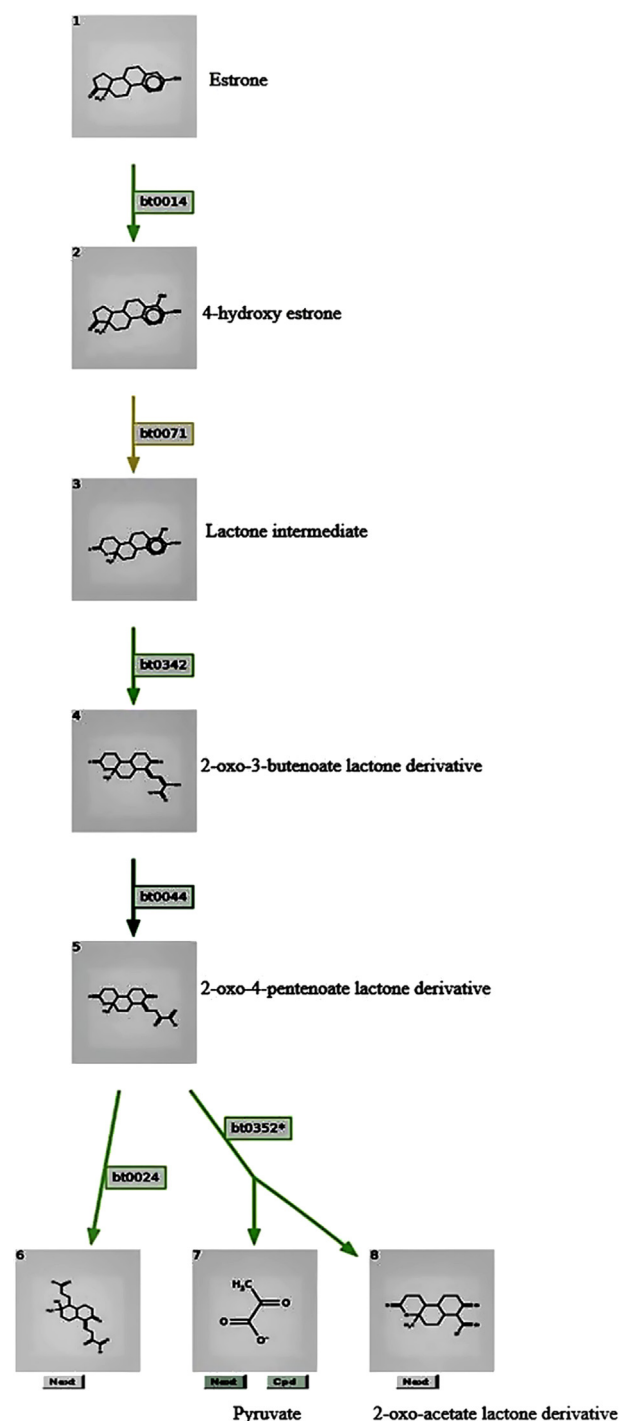

Fig. 1. Predicted estrone degradation pathway. (Green arrows show that the transformation is very likely aerobic and yellow arrow signify neutral). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

keto-enol conversion to form 2-oxopent-4-enoate that finally forms pyruvate and acetate. The conversion rule (bt0352*) was a continuation of the extradiol (meta) ring cleavage pathway and handled all 2-oxopent-4-enoate derivatives produced by bt0351. Initially, the C4-C5 double bond (bt0021) gets hydrated to form 4-hydroxy-2-oxopentanoate derivative that subsequently leads to the aldolytic cleavage (bt0043) of the C3-C4 bond that yields pyruvate and acetate derivatives since C4 is unsubstituted.
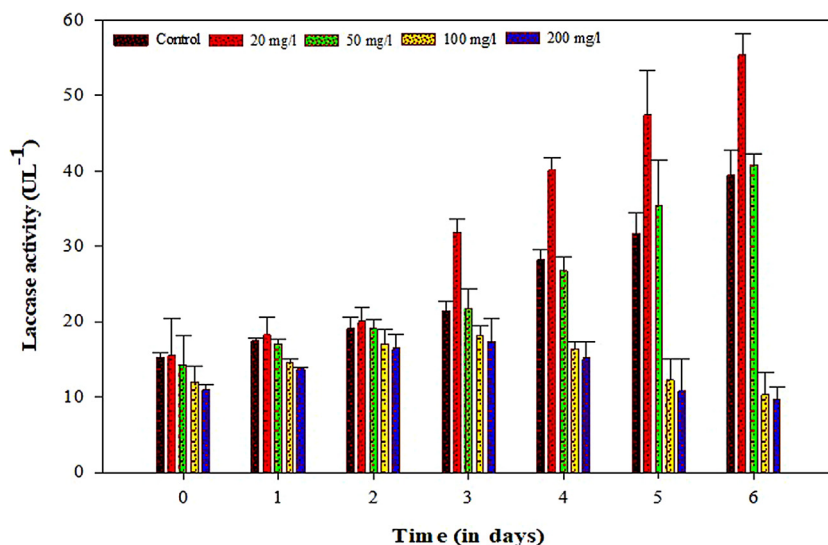
Fig. 2. Laccase activity in *Spirulina* CPCC 695 in presence of estrone.

3.2. Cyanobacterial enzymes involved in estrone degradation

To understand the role of cyanobacterial enzymes in degradation of estrone, *Spirulina* CPCC-695 was grown in presence of different concentrations of estrone and activities of laccase, peroxidase and esterase enzyme was checked.

3.2.1. Laccase activity

Laccase oxidises its substrate, the non-phenolic dye 2, 2′-azinobis (3-ethyl benzo thiazoline-6-sulphonic acid) (ABTS) to a more stable and preferred state of the cation radical (ABTS$^+$). The reaction sample developed a bluish green colour after 10 min incubation due to formation of ABTS$^+$ that directly correlated to enzyme activity. The activity increased in control as well as in estrone exposed samples (Fig. 2). The highest activity was recorded in cultures exposed to 20 mg/l estrone (55.4 U/L) which was even higher than the control (39.5 U/L) on day 6. However, culture exposed to higher estrone concentration ($\geq$ 50 ppm) showed much lower laccase activity.

3.2.2. Peroxidase activity

In order to evaluate the catalytic activity of peroxidase, the catalytic oxidation of phenolic compounds using H_2O_2 as the substrate was studied to prove the efficiency of the peroxidase [E.C. 1. 11.1.7]. The enzyme was active and showed concentration dependent activity. On day 6, the peroxidase activity was highest in the presence of 20 mg/l estrone (24.0 U/L) (Fig. 3). However, the activity was comparatively less in control (22.9 U/L). At higher concentrations of estrone, peroxidase activity reduced.

3.2.3. Esterase activity

The esterase activity was very less as compared to oxidoreductases. On day 6, the maximum activity was observed in case of culture exposed to 20 mg/l estrone (9.8 U/L)· The enzyme was comparatively less active in control (8.5 U/L) (Fig. 4). However, at higher estrone concentrations the enzyme activity reduced.

3.3. Accumulation of polyhydroxybutyrate

The degradation of estrone and simultaneous accumulation of PHB was also studied. The standard graph of PHB was plotted,
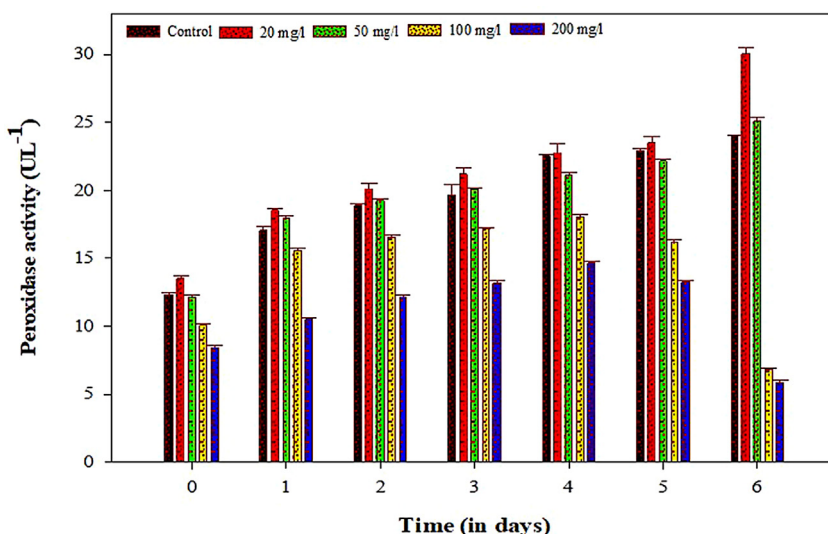

Fig. 3. Peroxidase activity in *Spirulina* CPCC 695 in presence of estrone.
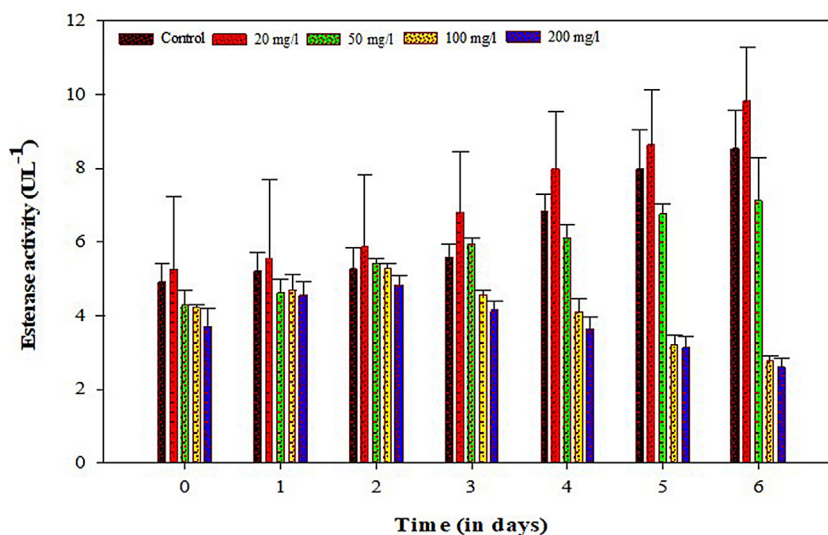
Fig. 4. Esterase activity in *Spirulina* CPCC 695 in presence of estrone.

using which amount of PHB accumulated on different days under different concentrations was calculated (Fig. 5a). The amount of PHB produced in control and in culture exposed to 20 mg/l estrone increased with time. But, at higher concentrations ($\geq$ 50 mg/l), the increase was not so substantial. The highest amount of PHB (169 μg) was recorded at 20 mg/l estrone on day 6 which was slightly higher than that observed in control (Fig. 5b).

4. Discussion

Estrone excreted by animals and humans enters into the environment through different modes including waste water treatment plants (WWTPs). It gets accumulated, bio-magnified during its passage through the food chain and ultimately disrupts the endocrine system of living organisms even at nanogram-per-litre levels by mimicking or antagonizing the estrogen signalling pathway. Bioremediation using micro-organisms offers a cheap and eco-friendly way to remove it from the environment. Our preliminary finding on cyanobacterial species (16 *Spirulina* species) showed the role of *Spirulina* CPCC-695 in estrone degradation which grew easily at 20 mg/l estrone [24]. The present study predicts the estrone degradation pathway, analyze the role of degradative enzymes (laccase, peroxidase and esterase) of *Spirulina* CPCC-695 in estrone degradation and simultaneously quantify PHB.

The first estrone degradation pathway was discovered in a soil bacterium, *Nocardia* sp. E110 by Coombe et al. (1966) [47]. Later on, degradation pathways have been further studied in bacterial species including *Comamonas testosteroni*, *Sphingomonas* sp. strain KC8, *Novosphingobium sp.* strain SLCC, *Novosphingobium tardaugens* strain ARI-1 [48–50]. Bacteria can degrade estrone via ortho- or meta- cleavage pathway. The ortho-pathway involves cleavage of ring D of estrone and converts C-17 to its keto-form which results in the formation of a hydroxyl acid. This acid loses water and converts the cyclic ketone to lactone that enters TCA cycle to produce carbon-dioxide and water [51]. The meta-pathway, instead involves the cleavage of benzene ring (Ring A) of estrone and oxidation of estrone by dioxygenase which adds hydroxyl group at the C-4 position in ring-A producing 4-hydroxy estrone [52]. Further, oxido-reductase decarboxylate this meta-cleavage product to form 3aα-H-4α (3′-propanoate)-7aβ-methylhexahydro-1,5-indanedione (HIP) that undergoes common HIP degradation to form succinyl CoA, three molecules of acetyl CoA and propionyl CoA. Propionyl CoA gets converted to succinyl CoA by propionyl

CoA carboxylase. Succinyl CoA further gets decarboxylated to form succinic semi-aldehyde which then gets oxidised to succinate that enters the TCA cycle [53].

The estrone degradation pathway predicted by EAWAG-BBD/PPS in the present study was similar to the earlier reported pathways as discussed above. The obtained cascade of reactions explains the breakdown of estrone to 4-hydroxy estrone that finally forms pyruvate and gets further metabolized via TCA cycle to produce carbon-dioxide and water. The other product formed as predicted by the database is acetate which forms acetyl CoA that may undergo condensation to produce PHB [54]. The degradation pathway as predicted by the database is of meta-cleavage type. The common mechanism known to be involved in the degradation is side-chain breakdown, hydroxylation, dehydrogenation, oxygenation and hydrogenation [55–57]. These metabolic processes are governed by the action of important degradative enzymes like oxidoreductases and hydrolases [58]. Oxido-reductases like laccases (EC 1.10.3.2, benzenediol:oxygen oxido-reductases) and Peroxidases (EC 1.11.1.7) show great potential in bioremediation of organic pollutants [29]. Laccase is a metal-containing enzyme that catalyses oxidation of many electron-rich organic and inorganic compounds by degrading them using four-electron reduction of molecular oxygen to water [59–61]. It converts estrone into 4-hydroxyestrone and opens the ring structure of estrone resulting in the formation of 2-oxo-4-pentenoate lactone derivative. The heme-containing peroxidase enzyme oxidises estrone and produce insoluble oligo- or polymeric derivatives like lactone intermediate [62,63]. The ester hydrolyzing enzymes (esterases) (EC 3.1.1.2) acts on the lactone intermediate and induces de-esterification and hydrolyzes it to form alcohol and pyruvic acid [64].

Maximum activities of these degradative enzymes was found in the culture containing 20 mg/l estrone. This suggests that *Spirulina* CPCC 695 grows efficiently at this concentration and produces these enzymes (laccase, peroxidase and esterase) that help in estrone degradation. The order of enzyme activity at 20 mg/l estrone was found as: laccase (55.4 U/L) > peroxidase (24.0 U/L) >esterase (8.8 U/L). However, at higher estrone concentration (>50 mg/l), enzyme activities decreased in a concentration-dependent manner along with reduction in growth. These enzymes induce cleavage in the ring structure of estrone, opens it and result in its degradation. During the initial phase of the study (Day 0 to Day 2), enzyme activity was less which reached maximum on day 6. This may be due to the adaptation of enzyme machinery of *Spirulina* CPCC-695 on estrone exposure. Tamagawa, et al. (2006) also found
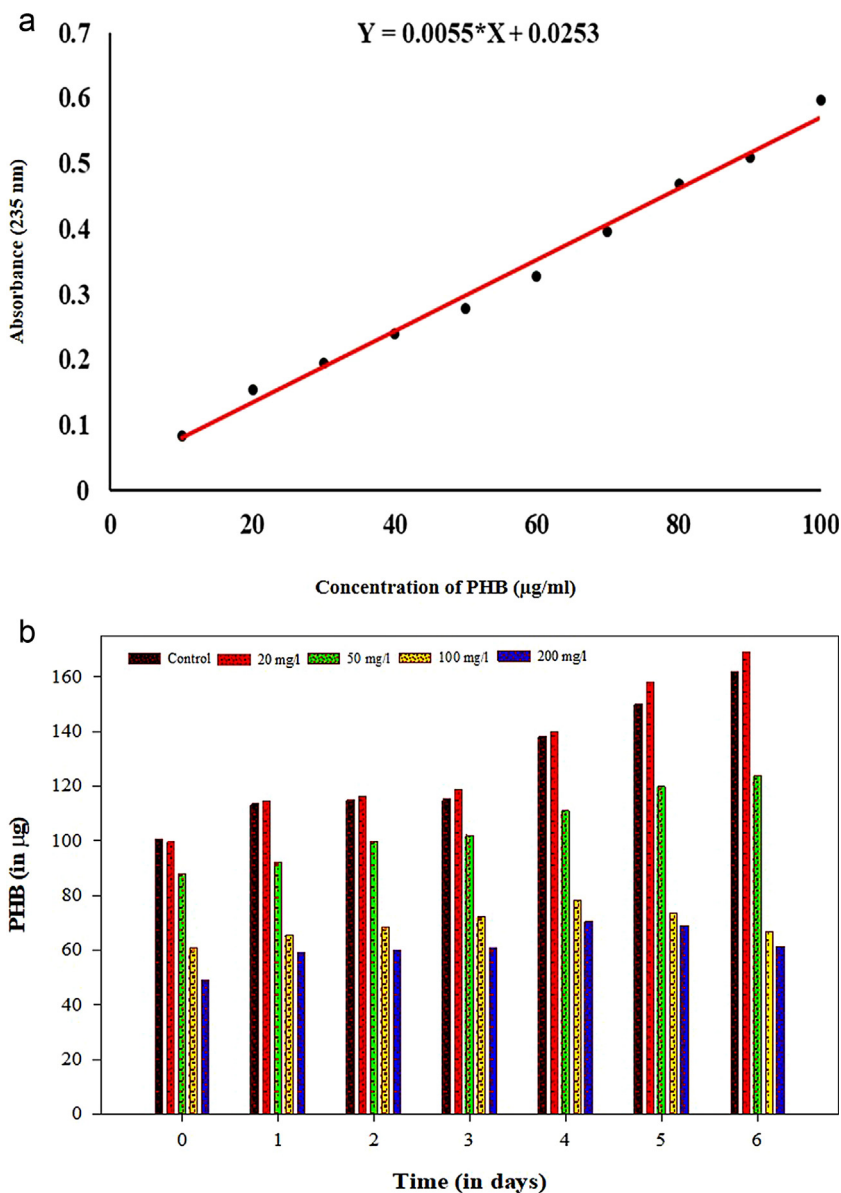
Fig. 5. (a) PHB standard curve. (b). *Spirulina* CPCC 695 potential to produce PHB in presence of estrone.

that laccase and peroxidase of white rot fungi *Phanerochaete sordida* YK-624 were effective and efficient in degrading estrone [28]. Thus, both extracellular and intracellular enzymes of *Spirulina* CPCC-695 participate in estrone degradation.

Since the discovery of polyhydroxybutyrate (PHB) in the cyanobacterium, *Chloroglea fritschii*, the occurrence of PHB has been shown in many cyanobacterial species [65,66]. Moreover the accumulation of PHB and simultaneous degradation of phenolic contaminants has been observed in the bacteria *Cupriavidus taiwensis* 187 [54]. This incited us to check whether PHB (biopolymer) is formed as a by-product of estrone degradation or not i.e. if PHB accumulation is either facilitated or not in presence of estrone. Maximum PHB formation was observed in *Spirulina* CPCC-695 at 20 mg/l estrone exposure which was found to be slightly higher than control. Though the difference in the increase was marginal, however, it suggested that estrone breakdown by cyanobacteria increases the growth and simultaneous production of PHB which can be further increased by optimizing the degradation conditions. The cyanobacterial cells when exposed to higher concentration of estrone (200 mg/l) experiences stress

that inhibits the utilization of carbon source reducing growth due to which the amount of PHB produced decreases as compared to the control even at day 0. Cyanobacteria accumulate PHB as a storage compound in response to excess carbon source. They produce short chain length PHAs (3–5 carbon atoms) with the help of Class III PHA synthase encoded by *pha*C whose substrate is acetyl CoA [67]. Thus, it could be suggested that acetyl CoA produced as a degradation product of estrone undergoes condensation and reduction to form PHB via three enzymatic reactions. Firstly, 3-ketothiolase converts two acetyl-CoA molecules to one acetoacetyl-CoA molecule then NADPH-dependent acetoacetyl-CoA reductase converts acetoacetyl-CoA to D-3-hydroxybutyryl-CoA and finally PHB synthase catalyses polymeriztaion of D-3-hydroxybutyryl moiety via an ester bond to form PHB molecule [68].

5. Conclusions

Concurrent increase in growth, degradation and catabolic enzyme activities at 20 mg/l estrone exposed in *Spirulina* CPCC-695 suggests that laccase, peroxidase and esterase play crucial role

in degradation of estrone- an endocrine disrupting compound. It also produces PHB as a by-product. Therefore, *Spirulina* CPCC-695 can be used as a probable candidate to deal with biodegradation of toxic estrone present in aquatic bodies with simultaneous production of bio-plastic precursor (PHB). However, further research is required to identify the intermediate products formed during estrone degradation.

Author contributions

NS and TF designed the experiment, SA helped in the PHB estimation and quantification, DY helped in data curation. NS carried out the lab work and wrote the initial manuscript that was corrected and finalised by TF.

Declaration of Competing Interest

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.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2020.e00464.

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