Executive summary of UGC MRP (F. 42-486/2013 (SR) dated 22.03.2013) sanctioned to Dr. Rajeev Kumar Kapoor

Anthropogenic EDCs are broadly dispersed and elimination of these compounds is considered as one of the main worries for the sustainable development of our planet in this 21st century. Endocrine disruptor chemicals, including phenols and aromatic amines, congregated major class of pollutants which found in the wastewaters of mostly in the industries including petroleum extraction and refining, coal conversion, plastic, resins and wood preservations, metal coating, textile and dye making, mining and dressing, and pulp and paper. All most all of the EDCs compounds are contaminated and must be eliminated from wastewaters before they are discharged and settled into the environment. Bioremediation with enzymatic treatment is a generally safe, cost-effective and least disruptive strategy as over traditional physicochemical methods.

The search for new technologies suitable for the treatment of wastewater containing EDCs is a challenge because; existing treatment methods are not able to eliminate them completely. Even at concentrations of ng/L, EDCs have an impact on the endocrine system of the fauna producing morphological deformities, reduced overall growth, reduced sperm quality and delayed ovulation, reduced numbers of mature spermatozoa, sex reversal male to female among others. The biological alternative or modification of conventional methods with use biological agents may be used to treat wastewater containing EDCs including BPA and TCA.

Enzymatic treatment of EDCs has a minimal environmental impact with low energy consumption, easy to control and can work over a wide range of conditions with a wide range of target substrate. Enzymatic treatments may offer many advantages: i) enzymes are highly selective and can effectively treat even dilute wastes ii) biotransformation does not generate toxic side products as it is often in the case of physicochemical methods iii) the requirement to enhance the bioavailability of organic pollutants by the introduction of organic co-solvents or surfactants is much more feasible in enzymatic reactions than using whole cells and iv) enzymes are less likely to be inhibited by substances that are toxic to living organisms.

Laccase enzyme with above-mentioned benefits has been widely examined for the decontamination of various pollutants because it oxidizes a broad range of toxic compounds.

However, there are several holdups including high cost, low activity and/or stability, low reaction yields in given conditions, which needs extensive efforts from the scientific community to be overcome.

In the present study, the laccase producing fungal strains were isolated from collected fungal samples from north Indian forest area (Uttarakhand). The strain that produced higher laccase activity was identified by the morphological and phylogenetic method. The extracellular laccase production by the isolated strain was optimized using the statistical design of experiments. Further, the enzyme was partially purified and characterized. The effects of physicochemical factors were optimized in SmF for higher laccase production. In SSF the influence of particle size and moisture content on selected *Parthenium* substrate was accounted to maximize the laccase production. In addition, immobilization of partial purified laccase was performed and Laccase was further tested for its ability to degrade BPA and TCA.

1. In this study, 48 samples were collected from the northern forest area of India (Uttarakhand) and among these 11 isolates were found positive for lignolytic activity. The most potential laccase producing isolate was identified as *T. versicolor*JSRK13 using morphological and molecular characteristics.
2. Enzymatic profiling was evaluated during the fermentation of the *T.versicolor*JSRK13, only cellulase and proteases were detected in the enzymatic crude along with laccase.
3. In this study, the production of laccase from *T. versicolor* JSRK13 was achieved through both SSF and SmF strategies. To achieve the maximum production in SmF, optimization was carried out using one factor at time (OFAT) approach followed by statistical experiments with Placket-Burman Design and Central Composite Design (CCD) of Response Surface Methodology (RSM) (Figure 5.1).
4. Using RSM approach, it was predicted that at the 4.8 pH, 28°C temp, 2% malt extract concentration and 410 μM $CuSO_4$, the laccase response should be 87.75 UmL^{-1}.
5. The validation experiment was performed in triplicate for further confirmation as suggested by the model. The laccase production from the fungal isolate at optimized process parameters was 85.91UmL^{-1} which shows the usefulness of the given Model.

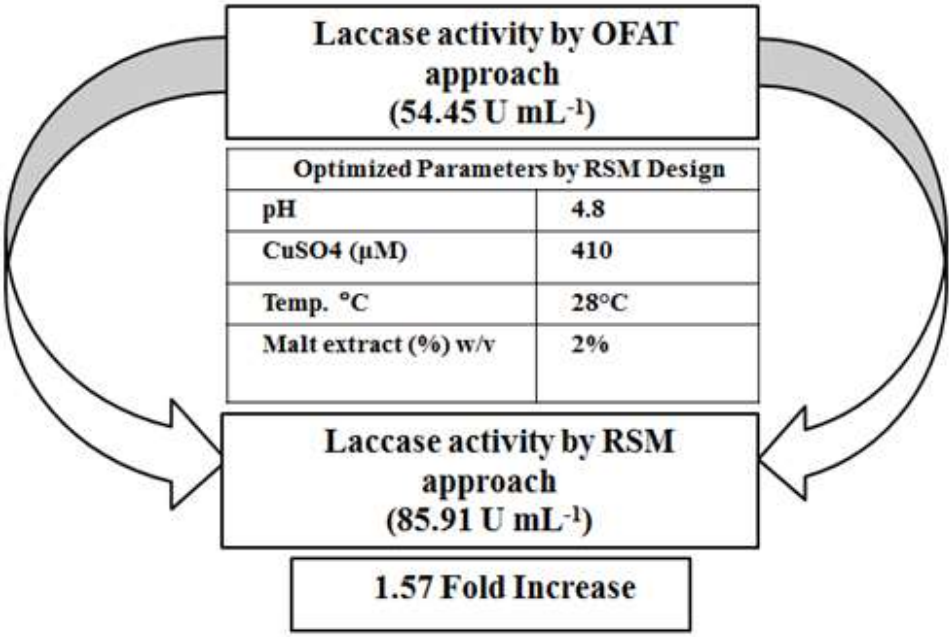

Figure 5.1 Analysis of Laccase activity by OFAT and RSM

6. *T. versicolor* JSRK13 was also produced in SSF using agro waste such as rice straw, wheat straw, sugarcane leaves, and parthenium (congress grass) as substrates. Laccase production was observed maximum on the 15th day of incubation in congress grass 185 (U/g) followed by sugarcane leaves with 165 (U/g). There was comparative low laccase production when wheat (150U/g) and rice straw (145U/g) were used as carbon substrate.
7. Five different moisture levels (40 %, 50 %, 60 %, 70% and 80 %) and differently sized, air-dried substrate having a size range of 0.5-1, 3-5 and 7-9 mM and powder form (below 0.5mm) were investigated with five grams of solid substrate. Particle size in the range of 3-5 mm was found to be optimum for laccase production with 189.40 U/g. while 60% initial moisture content was found optimum for highest laccase production up to 190.40 U/g.
8. Four purification steps, ammonium sulfate precipitation, dialysis, anion exchange, gel exclusion chromatography were necessary to purify the enzymatic crude. Approximately 55 fold purity with a yield of 2.3 % laccase was obtained after the final gel purification step which further weighted around 63KD. The optima and stability of partially purified laccase were evaluated against pH and temperature and observed the pH optima at 4.5 and highest stability was observed at pH 5 while 30˚C was reported for both optimal and

stability. Effects of various factors like metal ions, organic solvents and inhibitor compounds on the laccase activity were observed before going to the next step of immobilization of laccase.

9. Immobilization of laccase was carried out by adsorption, cross-inking, covalent and entrapment methods. These procedures were employed with the different polymers polypropylene, polystyrene, polyester, polyacrylamide, cotton and inorganic material glass beads.
10. The efficiency of reusability of immobilized laccase on these polymers was evaluated for up to 10 successive cycles by doing Guaiacol assay.
11. Glass beads were found most suitable with a 92% relative laccase activity followed by the polypropylene with an 86% activity as compared to the activity of the free enzyme.
12. The efficiency of immobilization on polypropylene was reduced from the first cycle (95%) to the 10^{th} cycle (15%). Similarly for glass beads, polyacrylamide gel, polyester and cotton threads the efficiency reduced from 85 to 5%, 75 to 12%, 92 to 55% and 80 to 30% but for polystyrene beads the efficiency was very low (45%) in first cycle and becomes zero in the 6^{th} cycle of use.
13. The potential of partially purified laccase in degrading two potentially harmful EDCs (Bisphenol and Triclosan) was used to develop a biological process to decontaminate our water bodies from EDCs. The significant rate of biotransformation of BPA & TCA after 24h of treatment with the free laccase was evident from the HPLC chromatograms which showed a decrease in the signal intensity. It was observed that around 24h of enzymatic oxidation is sufficient to achieve the transformation of about 70% for BPA and 60% for TCA.
14. The simultaneous transformation of BPA with laccase production in both submerged fermentation (SmF) and solid state fermentation (SSF) conditions was developed into a bioprocess which worked both on solid and liquid wastes containing BPA. Unfortunately, the said strategy did not work for the Triclosan as it was toxic for the growth of *T. versicolor* JSRK13. In SmF 10% of BPA removal was observed on 6^{th} days and 75 % after 10^{th} days of fermentation by *T. versicolor* JSRK13. Whereas, for SSF 12 % on the 7^{th} day, 50 % on 11^{th} and a maximum of 70 % on the 15^{th} day of fermentation were

observed. In both the fermentation strategies maximum laccase activity coincided with the highest BPA transformation.

15. In Laccase production and simultaneous transformation (LPST) strategy, the use of mediators like HBT and Vanillin is dispensable. Further, bio-monitoring studies for the determination of trace amount of most common EDCs like BPA and Triclosan in our water bodies and domestic supplies is the need of the hour, so that their influence on human health can be studied.
16. 70% BPA Transformation was observed without HBT, and 90% with HBT and TCA Transformation of ~60% was observed without HBT to ~75% with HBT.
17. Vanillin (VA) was not found to be much effective mediator for enhancing the transformation rate. Vanillin could enhance the transformation of BPA by 8% and 5% for TCA.
18. The effect of both the mediators on BPA transformation was also investigated in the SmF and SSF process with *T. versicolor* but the negligible increment in the transformation rate was compared to in vitro transformation with free laccase.

Scope for future study

The potentiality of Laccase enzyme discussed in this study and other studied enzymes are making the way to counter the emerging contaminants. Active membranes can be developed by grafting individual enzymes or consortium of biocatalysts. Though, there are various challenges in the grafting of the enzymes, stability of immobilized enzymes and process development for the water filtration. The process development of water treatment by grafted enzymes can lead through chiefly three levels which are described below: At small scale, the optimized process can be used to develop the enzymatic membrane used in the portable water purifier. Recently, some companies launched the portable water bottle with the filtration membranes used by the hikers, mountaineers, expeditioners, travelers and defense persons**.** Further, the process of water filtration based on the ultrafiltration technology can be developed for the household drinking water systems. The benefits of a Laccase immobilized ultrafiltration membrane (LIUF) system can also be added to a gravity-based water purifier (Figure 5.2). The adoption of hydrostatic pressure (gravity) to provide the driving force for permeation further reduces the system complexity and has resulted in widespread adoption of submerged hollow fiber ultra-filtration for drinking water treatment. The gravity-based water purifier is the cheapest water filtration unit

working without electricity. Such systems can be upgraded with the laccase immobilized membrane. Together with laccase immobilized membrane, gravity-based purification system may offer EDC free drinking water, chemical free purification, removal of cysts, protozoa, and bacteria and will have negligible running cost since it does not require electricity for the purification process.

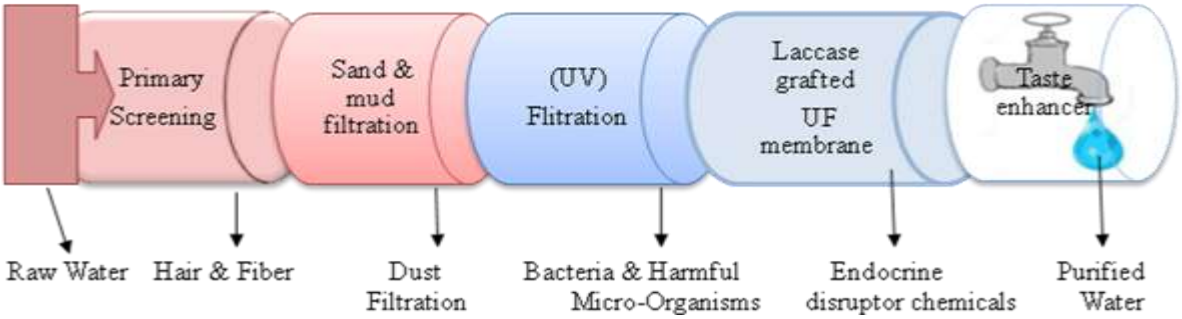

Figure 5.2: Laccase immobilized/grafted drinking water filtration scheme

In a membrane reactor, this will just be a replacement of the conventional membrane without disturbing the reactor format. Large-scale water treatment plants membrane bioreactors (MBR) use different arrangements of membrane bioreactors for biodegradation. Basically, MBR carries out the process using two separate units: a bioreactor tank and a joint membrane module/unit, but now, these two separate units are fixed in a single unit, where the membrane modules are submerged inside the bioreactor. Most of the MBR use hollow fiber ultra-filtration membrane for large-scale water treatment plants.

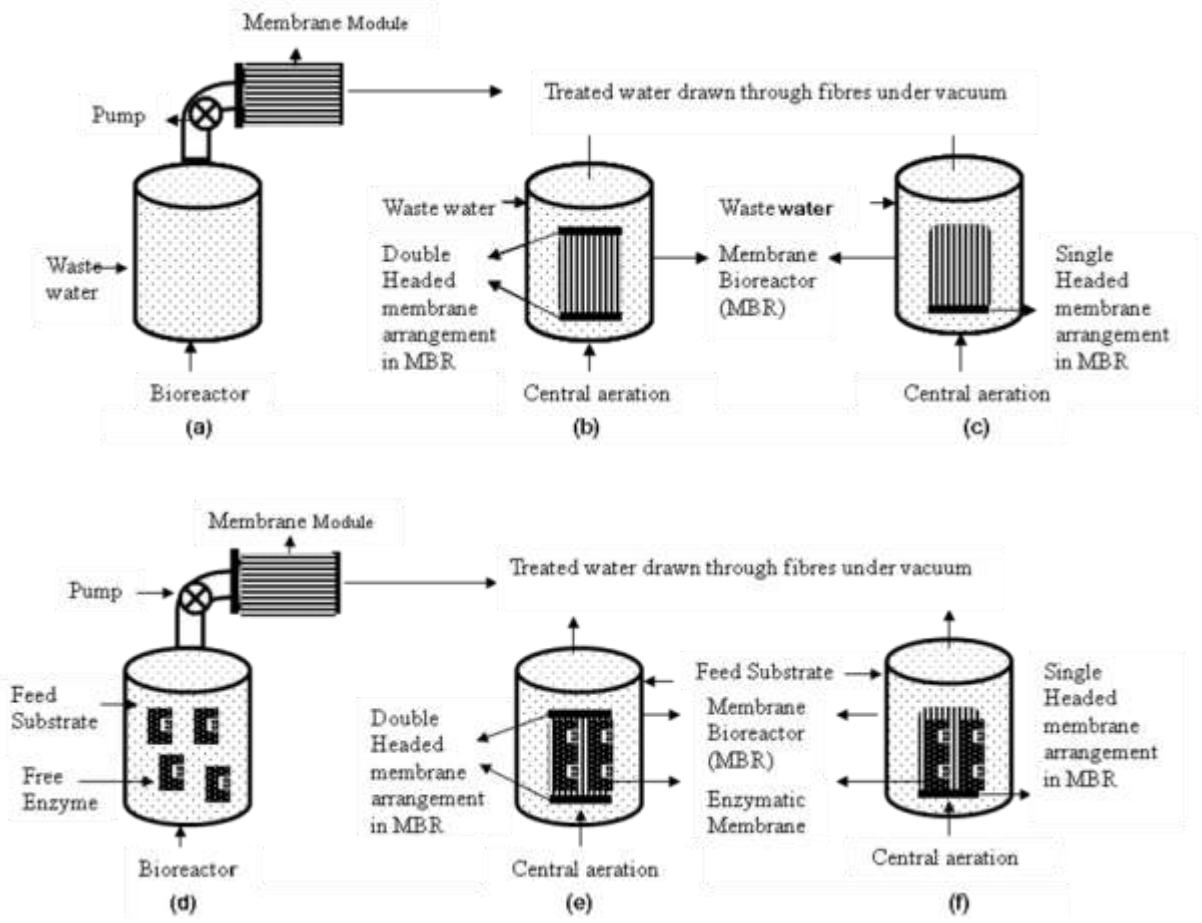

Figure 5.3: Different arrangements of membrane bioreactors (MBRs) and enzymatic membrane bioreactors (EMBRs)

Figure 5.3, describes the two formats in which the filtration process can be carried out using MBR, figure 5.3 (a, b and c) show the arrangement of membranes in the reactor whereas, figure 5.3 (d, e and f) show the arrangement of enzyme immobilized membrane in the same reactor. Figure 5.3 (a) shows a split bioreactor tank and membrane module; figure 5.3 (b) shows an arrangement where a double-headed hollow fiber membrane assembly is submerged into the bioreactor. Figure 5.3 (c) shows a single-headed membrane unit arranged in an MBR, to avoid the clogging problem. In Figure 5.3 (d) the enzymatic reactor with free enzyme and a separate membrane filtration unit are shown, here membrane allows the transfer of only reaction products but retains the enzyme inside the reactor throughout the process. Figure 5.3 (e) shows a double-headed enzymatic membrane reactor, the membrane acts as a selective barrier, and at the same time, it works as a support for the immobilization of single type of enzyme or group of associated enzymes. The module of enzyme grafted membranes is submerged inside the

bioreactor. When contaminated water will pass through the membrane pores, EDCs will come in contact with the grafted enzyme on and inside the pores leading to their degradation into the simpler form. To avoid the clogging of the MBR central aeration is applied which keeps moving the water. In the formats discussed above, single-headed enzymatic membrane reactors are preferred over double headed fiber arrangements (Figure 5.3(f)).

To conclude, the optimized processes for the production of enzymes at large scale and their immobilization in a manner which retains the activity and adds to the stability are required. There are only a few research studies on the enzymatic degradation of EDCs through membrane technology, more research studies or projects are required to be carried to develop greener and economical processes to complement the existing water treatment technologies.