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UGC letter No. File No: 47-428/12 (WRO) (date: 16-03-2013)

SUMMARY OF THE FINAL REPORT OF THE WORK DONE ON THE PROJECT

1. Project report No. : Submission of project completion report
2. UGC Reference No.F. _ F. File No: 47- 508/12(WRO) dated 7 March, 2013
3. Period of report: from April 2013 to March-2015
4. Title of research project **" Biotechnological applications of ligninolytic fungi"**
5. (a) Name of the Principal Investigator: AJIT M. PATEL

(b) Department: Microbiology

(c) College where work has progressed: J. and J. College of Science, Nadiad-387001, Gujarat.

6. Effective date of starting of the project: April 2013
7. Grant approved and expenditure incurred during the period of the report:

   a. Total amount approved: Rs. 1,39,000 (Rupees one lakh and thirty nine thousands only)

   b. Total expenditure: Rs.1,39,000 (Rupees one lakh and thirty nine thousands only)

   c. Report of the work done: (Please attach a separate sheet) : **Separate sheet is attached**

   i. Brief objective of the project

   ii. Work done so far and results achieved and publications, if any, resulting from the work (Give details of the papers and names of the journals in which it has been published or accepted for publication

iii. Has the progress been according to original plan of work and towards achieving the objective? if not, state reasons

iv. please enclose a summary of the findings of the study. One bound copy of the final report of work done may also be sent to the concerned Regional Office of the UGC.

v. Any other information

SIGNATURE OF THE PRINCIPAL INVESTIGATOR

SIGNATURE OF THE PRINCIPAL

(Seal)

FINAL REPORT

UGC Minor Research Project: No. F. _ F. File No: 47- 508/12(WRO)

dated 7 March, 2013

Title of the project: " BIOTECHNOLOGICAL APPLICATIONS OF LIGNINOLYTIC FUNGI"

Submitted to

UNIVERSITY GRANTS COMMISSION

Western regional office

Ganeshkhind, Pune-411007

By

Dr. Ajit M. Patel

Department of microbiology,

J and J college of science,

Nadiad-387001

FINAL REPORT

NAME AND ADDREESS OF THE PRINCIPAL INVESTIGATOR:

Dr. Ajit M. Patel

Department of microbiology, J and J college of science, Nadiad-387001

Work done: April-2013 to April-2015

Title of the project: " BIOTECHNOLOGICAL APPLICATIONS OF LIGNINOLYTIC FUNGI"

Objectives of the project:

A. Screening of efficient ligninolytic fungi from various sources.

B. Identification of selected organisms and their enzymes.

C. Optimizations of the cultural condition for the growth and enzyme production.

D. Partial purification of Lignin modifying enzymes.

E. Evaluation of the selected organism and their enzymes for bioremediation.

C. Report of the work

Screening of ligninolytic fungi was carried out from various natural samples collected from diverse regions of Gujarat using different solid agar media containing guiacol as an indicator. Forty one fungal cultures were screened and evaluated for ligninolytic enzyme production. Remazol Brilliant Blue (RBBR), Poly B-411 and Azure B (for lignin peroxidases) were employed to study the dye decolonization whereas Asther medium was used for the production of ligninolytic enzymes under submerged condition. Fungal cultures tagged as DN and DW were selected for further study as they produced higher amount of laccase in Asther medium under static condition.

Identification of the isolated fungal cultures (DN and DW) was carried out by 18S rDNA sequence using a polymerase chain reaction (PCR) with universal primers of NS1 (F: 5' – CTG GTT GAT CCT GCC AGT AG -3', R: 5' – CCG CGG CTG CTGGCA CCA GA -3'). A 617 bp (DN) and a 641 bp (DW) sized 18S rDNA sequence was obtained. The PCR fragments which were sequenced by Bangalore Genei, India, were compared with the database retrieved from gene bank database of the National Center for Biotechnology Information (NCBI) by a multiple sequence alignment program (BLAST) of NCBI. DN sequence showed 99 % homology with *Coriolopsis caperata* while DW showed a homology of 99 % with *Coriolopsis trogii*. The sequences were deposited in the GenBank of the NCBI (Accession No: KF564288 (DN) and

KF910678 (DW)). The isolates DN and DW were thus named as *Coriolopsis caperata* DN and *Coriolopsis trogii* DW.

Various physiological and cultural parameters such as aeration, incubation period, medium composition, temperature, pH, carbon source, nitrogen source, addition of inducers, etc. were found to be influencing laccase production by these fungi. Maximum laccase production by DN (2.3 U ml^{-1}) and DW (2.1 U ml^{-1}) in Asther medium was attained after 16 days during the time course study of laccase production confirming that laccase is a secondary metabolite. Both the fungi produced maximum laccase at 30 ℃ temperature and 5.5 pH under static condition. Glucose and sucrose (carbon source); peptone and yeast extract (nitrogen source); $CuSO_4$, $MgSO_4$ and Tween 80 are essential for the production of laccase by DN and DW which was made clear by the results of one factor at a time (OFAT) experiments.

A statistical approach of Plackett-Burman design (PBD) was used to screen the most effective supplement and their concentrations to achieve highest possible laccase production by C. *caperata* DN and *C. trogii* DW under submerged condition. OFAT experimental results were fundamental in selection of the nine variables (glucose, sucrose, yeast extract, peptone, $CuSO_4.5H_2O$, $MgSO_4$, L-aspargine, Tween 80 and inoculums size) for statistical optimization of medium components. Glucose, peptone and $CuSO_45H_2O$ gave confidence level of 98.72 %, 95.15 % and 89.41 %, respectively for DN while for DW, glucose, yeast extract and Tween 80 gave confidence level 96.5 %, 95.1 % and 86.0 %, respectively. The application of statistical design (PBD) intended for screening of medium components was very beneficial in determining the relevant component for advanced optimization. These components were short-listed for further optimisation using response surface methodology (RSM). The results of Central Composite Design, aimed at studying the interactive effect and the optimum level of components screened by PBD for both *C. caperata* DN and *C. trogii* DW, was validated according to the conditions assumed by response surface model. The optimum concentrations estimated for each variable were 41.3 gm glucose, 12.7 gm peptone and 0.2 gm $CuSO_4.5H_2O$ L^{-1} which yielded the maximum (80.11 U ml^{-1}) laccase activity for *C. caperata* DN. In case of *C. trogii* DW, the optimum estimated concentration of each variable at which highest (49.01 U ml^{-1}) laccase activity was attained were 50 gm glucose, 15 gm yeast extract and 15.0 µl Tween 80 L^{-1}. These results were significant in formulation of statistically optimized medium (Confirmation medium) which was used for further experiments. After statistical optimization, *C. caperata* DN produced

80,000 U L^{-1} which was approximately 34.83 times higher than the initial and *Coriolopsis trogii* DW produced 49,000 U L^{-1} which was approximately 23.83 times higher than the initial. The present study is the first report of using statistical design (PBD and RSM) for optimization of medium for laccase production. *C. caperata* DN produced higher laccase activity which was comparatively better than most of the *Coriolopsis* strain reported by other authors. Thus *C. caperata* DN and *C. trogii* DW are potent cultures for large scale production of laccase.

Nine different inducers were analyzed for over production of laccase using optimised medium. The amount of *C. caperata* DN laccase production did not increase in presence of inducers while *C. trogii* DW produced higher amount of laccase in presence of 1.0 mM xylidine as compared to control. Additionally, in order to reduce the time period and cost for fungal growth, fresh medium was added to the biomass every time, for overproduction of laccase by *C. caperata* DN. Laccase production was also carried out under solid state fermentation process using seven agricultural wastes. Maximum laccase production by *C. caperata* DN (50 U gm^{-1}) and *C. trogii* DW (104.72 U gm^{-1}) was obtained using agro wastes like rice bran and rice straw, respectively. This process is utmost important, as the stubborn part, lignin was degraded whereas the cellulose and hemicellulosic part remained unaltered.

Both the enzymes were purified and further characterization studies were carried out. Ammonium sulphate precipitation and gel permeation chromatography using sephadex G-200 column was used to purify extracellular laccase enzyme. Laccase from both *C. caperata* DN and *C. trogii* DW were completely precipitated using ammonium sulphate in the range of 40.0 gm % to 60.0 gm % concentration at 4 ℃. The overall purification of laccase from DN, post gel permeation chromatography was 24.14-fold with 30.53 % recovery and it was 27.1-fold with 33.2 % recovery from DW. The specific activity of laccase was enhanced to 2902.52 U mg^{-1} protein for DN and 926.2 U mg^{-1} protein for DW. Laccase enzyme from both DN and DW were homogenous as determined by single-protein band on SDS-PAGE indicating that they are monomeric with the molecular mass of 61.0 kDa and 63 kDa, respectively and their zymogram by *o*-dianisidine showed a single band suggesting absence of isoenzymes.

The optimal pH of purified DN laccase (3.5) and DW laccase (3.5) were determined using ABTS as substrate. Activity of purified laccase from DN and DW spans over a wide temperature range from 20 ℃ to 80 ^{o}C with 70 ℃ and 40 ℃ optimum activity, respectively. The half-life of *C. caperata* DN laccase at 60 ℃ was 288 min suggesting thermostablility of enzyme. Both

enzymes were stable at neutral pH (phosphate buffer, pH 7.0) at 30 ℃ temperature for 48 h and at 4 ^{o}C for six months.

The purified laccase retained approximately 90.0 % of its initial activity in the presence of 10.0 % methanol, ethanol, acetone, butanol, DMSO and isoamyl alcohol after 1.0 h and retained more than 50 % of its activity after 24 h indicating that the enzyme might be suitable for use in reactions that require solvents.

Metal ions especially heavy metal ions are common environmental pollutants and can affect the production, stability and activity of the extracellular enzymes both negatively and positively. The laccase activity of DN and DW was reduced up to 90.0 % within 2.0 h in presence of 5.0 mM concentration of Fe^{+2}, Fe^{+3}, Ag^{+1} and Hg^{+2} while retained more than 90.0 % activity in presence of K^{+1}, Mg^{+2}, Ca^{+2}, Co^{+2}, Ba^{+2}, A^{+3}, Cu^{+2}, Mn^{+2}, Zn^{+2} and other metal ions.

The enzymatic activity of DN and DW laccase was completely inhibited by sodium dodecyl sulphate (1.0 gm %), DTT (5.0 mM) and sodiumazide (5.0 mM) (an inhibitor of oxidase), which suggested the function of laccase as an oxidase. DTT, which is a strong reducing agent on disulphide bonds, strongly inhibited the enzyme indicating the existence of a disulphide structure in the active domain. In addition, EDTA and ß mercaptoethanol partially inhibited the laccase, which suggested the existence of a metal-binding domain in the protein.

Kinetic analysis reveals the Michaelis-Menten constants for *C. caperata* DN and *C. trogii* DW laccase in the presence of different substrates. The *C. caperata* DN and *C. trogii* DW laccase exhibited the highest activity with *o*-dianisidine, the *K*m and V_{max} value were 0.02, 0.021 mM and 0.36, 0.66 mM, respectively. The apparent *K*m values that were determined for ABTS, DMP and guaiacol using *C. caperata* DN laccase were 0.04, 1.25 and 2.0 mM whereas V_{max} values were 1.42, 1.42 and 2.5 mM, respectively. For *C. trogii* DW laccase, the *K*m values with the same substrates were 0.03, 0.21 and 1.0 mM while the V_{max} values were 0.086, 0.76 and 1.81.

The ultraviolet-visible absorption and electron paramagnetic resonance spectra of the purified laccase indicated that both the laccase have copper molecules suggesting them as blue laccase.

Physiological optimisation of RBBR decolourization suggested that optimum pH and temperature and their range for laccase mediated process were 5.0 (3.0 to 7.0) and 40 ℃ (40 ℃ to 60 ℃).

Response Surface Methodology has been executed to optimize decolorization and detoxification of a recalcitrant and toxic anthraquinone dye Remazol Brilliant Blue R (RBBR) using *C. caperata* DN laccase. Optimum concentration of laccase, pH and temperature for decolorization of RBBR dye (100 mg L^{-1}) were 0.5 U ml^{-1}, 3.5 and 40 ℃, respectively. Result of RSM showed that optimum value of tested variables for maximum dye decolorization was 1 U ml^{-1} enzyme, 1000 mg L^{-1} dye and 60 min and decolorization obtained was 542 mg L^{-1}. Result of kinetic study showed that the *K*m, V_{max}, *K*cat and *K*cat/*K*m values for RBBR decolorization were 1.06 mM, 0.226 mM U^{-1} min^{-1}, 135.0 S^{-1} and 1.27×10^5 S^{-1} M^{-1}, respectively. The thermodynamic studies (Ea, 10.87 kJ M^{-1}; ΔH, 7.408 kJ M^{-1} and ΔS, 80.06 J M^{-1} K) follow first order kinetics and the reaction is endothermic with negative value of ΔG suggesting spontaneous nature of reaction. Lower activation energy and Q_{10} value (1.49) are indicative of faster reaction rate and less sensitivity to change in temperature.

Present study evaluated the bioremediation capabilities of laccase enzyme from *C. caperata* DN for decolorization, degradation and detoxification of 21 synthetic textile dyes belonging to azo, anthraquinone, triphenyl methane dyes. Laccase enzyme as well as laccase mediator system (LMS) is regarded as a viable solution for bioremediation of synthetic textile dyes. Among the six laccase mediators, 1-Hydroxybenzotriazole (HBT) was most effective followed by ABTS and tempo as compared to other mediators which were assessed for improving the decolorization rate. The optimization of decolorization of azo dye Reactive Violet-5R was carried out by using Response Surface Methodology (RSM). 1.0 U ml^{-1} DN laccase decolorized 60.0 % reactive violet-5R (200 mg L^{-1}) in presence of 2.0 mM HBT concentration within 1.0 h. Application of HBT widens the specificity of laccase as well as increased the rate of dye decolorization. The optimized concentrations of dyes, laccase and HBT were useful to decolorize many different dyes by laccase.

Other dyes [azo {poly R-481(58.4 %), acid red (69.1 %), DR-81 (93.8 %), reactive red (78.3 %), reactive violet (96.3 %) and acid brown (75.8 %)}, triaryl methane {phenol red (83.0 %), malachite green (73.3 %) and crystal violet (59.8 %)} and triphenyl methane {aniline blue (84.0 %), CBB R-250 (86.7 %) and basic fuchsin (51.5 %)})were also decolorized using the above statistics. Poly B-411, congo red, reactive blue, bromophenol blue, bromothymol blue were decolorized equally in presence and absence of mediators, indicating insignificance of mediators in their decolorization.

Spectral scanning, HPTLC, FTIR and LC-MS analysis of laccase treated dyes mentioned above suggest their degradation after laccase treatment which confirmed oxidative cleavage of N-H bond of RBBR dye by laccase. Treatment of dyes with laccase or fungus *C. caperata* DN significantly reduced their toxicities which were confirmed using toxicity tests (microtoxicity and phytotoxicity). The toxicity (phyto, cyto and micro) of RBBR as well as other tested dyes was significantly reduced after laccase treatment.

Under submerged condition, *C. caperata* DN showed highest laccase production and decolorization (85.0 %) of RBBR dye in Coll medium after 192 h out of six different media studied. Mini Run of Design Expert software 11 (trial version) was used to optimize the RBBR dye degradation which has been used for the first time in the present study. It is single experimental design to analyze two different responses like dye decolorization and laccase production. The result of ANNOVA indicated that glucose, L-aspargine, yeast extract, $FeSO_4$ as well as glucose: yeast extract interaction were most significant for RBBR dye degradation. After statistical optimization, the optimum concentration of medium components (gm L^{-1}) for dye degradation were identified as glucose (11.0), L-aspargine (1.10), yeast extract, (1.10), $MgSO_4$ (1.10), $FeSO_4$ (0.0275) and $CuSO_4$ (0.275). Statistically optimized medium showed similar percentage of RBBR dye degradation after 96 h which is half the time taken using Coll medium. The optimized medium was successfully applied for bioremediation of 10 synthetic textile dyes belonging to azo, triphenyl methane and anthraquinone group and three poly aromatic hydrocarbons (phenanthrene, pyrene and naphthalene).