Nature Environment and Pollution Technology
An International Quarterly Scientific Journal

ISSN: 0972-6268	Vol. 14	No. 2	pp. 319-324	2015

Original Research Paper

A Comparative Decolourisation of Rbbr Dye and Guaiacol Degradation by Free and Immobilized Laccase Producing *Bacillus Spp.*

Viraj Krishna Mishra†, Himani Sharma and J. Dubey
Department of Biotechnology Engineering, Ambala College of Engineering and Applied Research, Devsthali (Near Mithapur), Ambala-133101, Haryana, India
†Corresponding Author: Viraj Krishna Mishra

Nat. Env. & Poll. Tech.
Website: www.neptjournal.com
Received: 23-8-2014
Accepted: 13-10-2014

Key Words:
Anthraquinone dye
Immobilized cells
Laccase
Bacillus sp.

ABSTRACT

The aim of the research work was decolourisation of Remazol Brilliant Blue R (an anthraquinone dye) and guaiacol by free cells and immobilized strains of *Bacillus* sp. D1023, D1032 and D1033 which were isolated from field soil of Ambala College of Engineering and Applied Research. Comparison between free cells and immobilized cells was observed using 100 mg/L of Remazol Brilliant Blue R and efficiency of beads was observed by reutilization of beads using same concentration of dye. The selected strains D1023, D1032 and D1033 were able to decolourise RBBR dye up to 81.23%, 73.14% and 75.44% by free cells and 94.54%, 84.45% and 89.50% by immobilized cells respectively. These strains are also utilized to treat Guaiacol and corresponding colour change has been observed for free and immobilized strains. To increase the decolourisation process 2×SG medium was used which increases the spore production in culture.

INTRODUCTION

Among industrial wastewaters, the treatment of dye wastewater from textile and dyestuff industries is one of the most challenging tasks (Fu & Viraraghavan 2001). Besides the physicochemical methods such as adsorption, ion exchange, coagulation-flocculation and TiO_2 oxidation, microbial decolourisation process is more ecofriendly as it reduces the colour components to carbon dioxide, ammonia and water by initiating cleavage of bonds in dyes rather than creating possible toxic fragments (Mahvi et al. 2009, Bourbonnais et al.1997). The copper containing oxidase, laccase (EC 1.10.3.2) is a polyphenol oxidase that requires molecular oxygen (air) as a co-substrate and catalyses the oxidation of a great variety of phenolic and non-phenolic compounds (Ivana et al. 2006). Laccases have been reported for the treatment of wide spectrum of synthetic dyes having diverse chemical reactions that increases its applications in treatment of textile effluents as a low cost environmental technology (Macro et al. 2006, Ó'Fágáin 2003). Nevertheless, difficulties have been observed in treatment of wastewater as variation in treating conditions cause problems that lead to degradation of laccase. Use of enzyme and cell immobilization technology is a method to make the microbial system reusable, stable and more efficient (Zhang et al. 2007). Immobilized cell systems have the potential to degrade toxic chemicals faster than conventional wastewater treatment systems, because of high densities of specialized microorganisms (Wang & Liu 1996). Many natural and synthetic polymers are used for immobilization of cells and enzymes. In comparison to natural polymers (agar, agarose, alginate, kappa-carragenan), synthetic polymers have strong mechanical strength and durability but are often toxic to microorganisms (Wang & Liu 1996, Wang & Shi 1998). One of the most suitable methods for cell immobilization is entrapment in calcium alginate, because this technique is simple and cheap. Sodium alginate is a readily available non-toxic biological for bio-molecules and microorganisms (Wang et al. 2000).

Remazol Brilliant Blue R (RBBR) belongs to group of the second most important class of textile dyes i.e., anthraquinonic dyes. RBBR is an anthracene derivative and represents an important class of toxic and recalcitrant organopollutants (Milovanovic et al. 2007). There are several reports on decolourisation of RBBR by laccases (Kunamneni et al. 2008, Mechichi et al. 2006, Mohorcic et al. 2006, Palmieri et al. 2005, Peralta-Zamora et al. 2003, Susla et al. 2007 and Wang et al. 2010).

White rot fungal strain has also been utilized to treat textile dyes. Strains of *Schizophyllum commune* and *Lenzites eximia* had been used to decolourise azo dyes of textile waste (Selvam et al. 2012). *Aspergillus niger* has also been used to decolourise reactive dye (Muthukumaran 2013). The purpose of this study was to isolate, screen and characterize laccase producing *Bacillus* sp. The selected strains D1023,

D1032 and D1033 were further used to observe RBBR dye decolourisation and guaiacol degradation by free and immobilized cells. Enhancement in decolourisation and degradation was observed when selected strains were cultured in 2×SG medium.

MATERIALS AND METHODS

Isolation, screening and characterization of isolates having dyes decolourisation activity: Strains D1023, D1032 and D1033 were isolated from soil of decomposing roots of a tree (Mishra & Sharma 2014). Soil samples were heat treated. Heat treated soil samples were serially diluted and spread on LB agar plates. 0.2 mmol $CuSO_4$ (Mongkolthanaruk 2012) and 0.1% guaiacol were used as primary indicator for screening of laccase producing bacteria. Morphological and biochemical characterization was performed. Gram staining and endospore staining were also performed according to standard protocol. Secondary screening of the strain was done on the basis of Remazol brilliant blue-250 and methylene blue dye decolourisation. To increase the spore quantity and to fasten the sporulation process along with to increase the decolourisation effect, selected isolates were transferred to the 2×SG medium in sterile conditions (Takamatsu et al. 2000).

Percentage decolourisation = {(Blank O.D – Sample O.D)/ Blank O.D} × 100

Preparation of spores: The isolates were cultured in 2×SG medium at 37°C and 120 rpm. Mature spores were harvested after 24 hrs and washed once with 10 mmol sodium phosphate buffer (pH 7.2). To remove the cell debris and vegetative cells, the pellets were suspended in 1-2 mL lysozyme buffer {10 mmol sodium phosphate (pH 7.2), 1% (w/v) lysozyme} and incubated at room temperature for 10 min. The suspension was centrifuged at 5000 rpm for 10 min, supernatant was decanted and resultant pallet was then washed repeatedly with buffer [10 mmol sodium phosphate (pH 7.2), 0.5 M NaCl] at room temperature (Gupta & Farinas 2010).

Immobilization of Cells in Calcium Alginate Beads

Preparation of inoculum: Content of flask was centrifuged at 5000 rpm for 20 minutes after 72 hours when spores were found in abundance. Cell biomass was washed thoroughly with 20 g/L sterile potassium chloride solution followed by 0.9% sterile NaCl solution and sterile distilled water. Finally cell mass was suspended in 0.9% sterile NaCl solution (Adinarayana et al. 2005).

Entrapment in calcium alginate beads: Sodium alginate (2%) solution was prepared by dissolving 2g sodium alginate in boiling distilled water and autoclaved at 121°C for 15 min. Both alginate and cell suspension (3mL) were mixed and stirred for 10 minutes to get uniform mixture. Slurry was taken into sterile syringe and added drop-wise into 0.2 M $CaCl_2$ solution from a height of 5 cm and kept for curing at 4°C for 1 hr. Cured beads were washed with sterile distilled water 3 to 4 times. When beads were not being used, they were preserved in 0.9% of NaCl solution at 4°C. All operations were carried out aseptically under LAF unit (Hullo et al. 2001)

Transfer of beads to media and decolourisation of synthetic dyes: 2×SG media was autoclaved with RBBR dye (100 mg/L) and beads were transferred into media. Difference between decolourisation efficiency of free cells and immobilized cells was studied at wavelength of 578 nm.

Reusability of immobilized beads: Reusability of beads was checked after 50 hrs of beads utilization for 100 mg/L dye concentration. The beads were washed with sterile distilled water for 3 to 4 times and transferred into fresh autoclaved media with 100 mg/L of RBBR dye and decolourisation was observed for 50 hrs.

Guaiacol utilization by free cells and immobilized cells: Inoculum was prepared and cells were immobilized in calcium alginate beads following same steps as described before. 2×SG media was autoclaved with 0.1% guaiacol. Immobilized cells and free cells were transferred to media separately. Changes in colour development were observed by increase in absorbance for both free cells and immobilized cells at wavelength of 450 nm.

RESULTS AND DISCUSSION

Isolation and screening of laccase producing bacterial strains: Twenty five colonies were screened out by 0.1% guaiacol utilization from cultures in LB medium supplemented with 0.2 mM/L Cu^{2+}. On basis of biochemical characterization, 6 colonies were selected.

Screening on the basis of dye decolourisation activity: Out of 6 isolates, 3 isolates D1023, D1032 and D1033 were screened out on the basis of higher percentage decolourisation activity of Remazol brilliant blue and methylene blue dyes. Selected strains D1023, D1032 and D1033 were able to decolourise 89.58%, 69.79% and 84.26% of RBBR and 44.96%, 62.59% and 85.25% of methylene blue respectively in duration of 8 days. The strains, D1023, D1032 and D1033 formed white colonies on agar plate, were gram-positive, spore forming, rod shaped, motile bacteria, and turned brown in colour in presence of guaiacol using LB and 2×SG media supplemented 0.2 mmol/L Cu^{2+}.

Observation for inhibitory concentration for selected isolates: Three indicators guaiacol, catechol and tannic acid

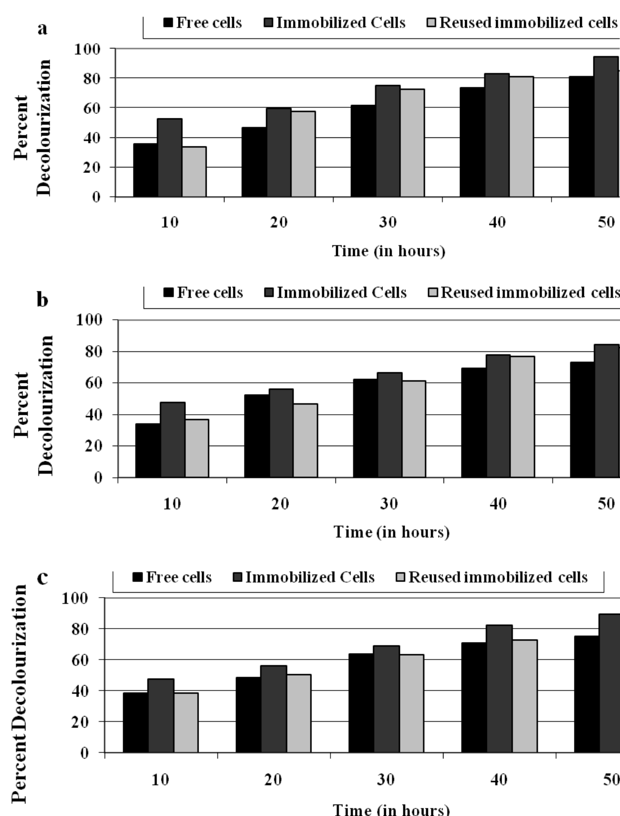

Fig. 1: Comparison of percentage decolourization of RBBR by free cells and immobilized cells and decolourization potential of immobilized cells when reused: (a) Isolate D1023; (b) Isolate D1032 and (c) Isolate D1033.

were used to observe inhibitory concentration. Changes in growth were observed at different concentrations of guaiacol, catechol and tannic acid. Different concentrations of guaiacol, catechol and tannic acid were 0.1%, 0.15%, 0.2%, 0.25%, 0.3% and 0.4%. Growth was observed in 18-24 hrs at 0.1% and 0.15% concentration of guaiacol. Late growth was observed at 0.2% and 0.25% of guaiacol. No growth was observed at 0.3% and 0.4% of guaiacol. Late growth was observed at 0.1% concentration of catechol. Except 0.1% no growth was observed at any of the concentrations of catechol. No growth was observed at any of concentrations of tannic acid.

Optimized growth conditions: Selected strains were transferred to 2×SG medium supplemented with $CuSO_4$ to increase and fasten the spore production. Same medium was used to optimize pH and temperature conditions. Optimum pH observed was 7.0 and the optimum temperature observed was 37°C, though they were able at temperatures ranging from 27- 47°C and pH 5.0-8.0. Optimized sugar source was fructose and optimized nutrient broth concentration was 2.6%.

Dye decolourisation using sporulation media: Increase in sporulation rate and in number of spores was observed by using 2×SG media. To observe changes in decolourisation efficiency RBBR dye was used. Selected strains D1023, D1032 and D1033 were able to decolourise 93.53%, 70.64% and 85.44% respectively in duration of 4 days. Percentage decolourisation was calculated.

Entrapment of cells in calcium alginate beads: Number of beads calculated was 197 and size of bead was 5 mm. RBBR decolourisation by free cells was observed 81.23%, 73.14% and 75.44% by isolates D1023, D1032 and D1033 respectively. In case of immobilized cells 94.54%, 84.45% and 89.50% by isolates D1023, D1032 and D1033 respectively. When immobilized beads were reused after 50 hours in same conditions, decolourisation was observed up to 85.31%, 82.44% and 81.33% (Fig. 1a,b,c).

Percentage colour change of guaiacol by isolate D1023: Changes in optical density and percent colour change are shown in Figs. 2 and 3. Percent colour change in case of isolate D1023 has been observed from 66.94% by free cells and 69.92% by immobilized cells in first 10 hrs and recorded up to 97% for both free cells and immobilized cells after 150 hrs. From 10 to 20 hrs both free cells and immobilized cells were performing in a similar way. After 20 hrs free cells were performing better than immobilized cells up to 120 hrs, and 120 hrs ahead immobilized were performing better than free cells.

Percentage colour change of guaiacol by isolate D1032: Changes in optical density and percent colour change are shown in Figs. 4 and 5. Percent colour change in case of isolate D1032 was observed from 79.69% by free cells and 69.92% by immobilized cells in first 10 hrs and recorded up to 98.10% for both free cells and 97.53% immobilized cells after 150 hrs. From the first 10 hrs free cells were performing better than immobilized cells up to 140 hrs and 140 hrs ahead free cells and immobilized were performing in a similar way. After 150, there was no change in absorbance.

Percentage colour change of guaiacol by isolate D1033: Changes in optical density and percent colour change are shown in Figs. 6 and 7. Percent colour change in case of isolate D1033 was observed from 70.14% by free cells and 67.21% by immobilized cells in first 10 hrs and recorded up to 97.99% by free cells and 98.11% by immobilized cells after 150 hrs. From 10 to 20 hrs both free cells and immobilized cells were performing in a similar way. After 20 hrs free cells were performing better than immobilized cells up to 130 hrs, and 130 hrs ahead immobilized cells were performing better than free cells.

Laccase from different fungal, plant and bacterial sources has been an enzyme of interest for its remarkable property of oxidation of phenolic and non-phenolic com-

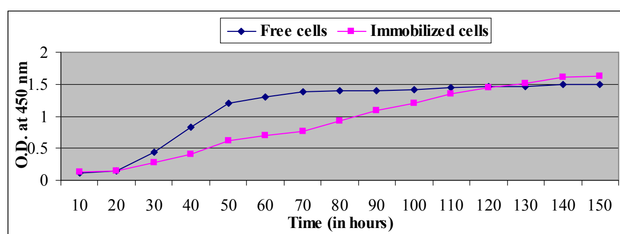

Fig. 2: Change in absorbance at 450 nm during colour change of guaiacol by isolate D1023.

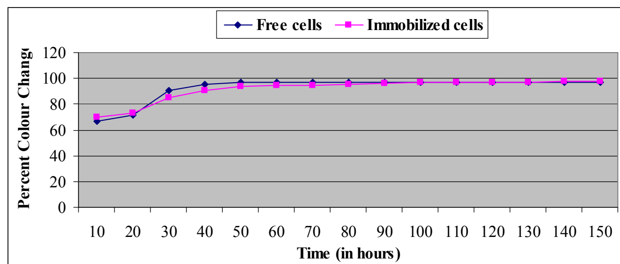

Fig. 3: Percent colour change during treatment of guaiacol by isolate D1023.

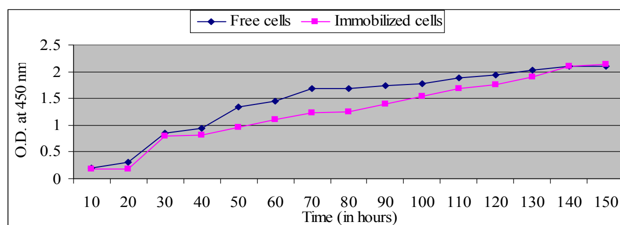

Fig. 4: Change in absorbance at 450 nm during colour change of guaiacol by isolate D1032.

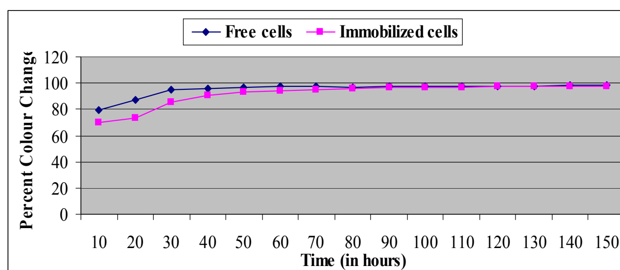

Fig. 5: Percentage colour change of guaiacol by isolate D1032.

pounds. Fungal laccase when compared to bacterial spore laccase is less active and stable at high temperature and pH conditions. A total of 25 isolates were screened using 0.1% guaiacol from heat treated soil samples at 80°C and unheated soil samples (Mishra & Sharma 2014). Soil samples were heat treated to screen out the spore forming bacilli.

Bacterial colonies utilizing guaiacol turns out of brown coloured so guaiacol acts as a indicator for laccase producing colonies; similar work has been described for screening of different bacteria producing laccase by Mongkolthanaruk et al. (2012). Identification was done on the basis of morphological characterization and biochemical characterization according to Bergey's manual. Isolates showing Gram +ve rods and endospores were selected for biochemical characterization. Results from biochemical characterization indicate that D1023 and D1033 relate to *B. subtilis* and D1032 relates to *B. polymyxa* possibly.

B. subtilis laccase protein existing as endospore CotA has been presented many workers (Won et al. 2005, Driks 2004, Claus & Filip 1997, Wesenberg et al. 2003 and Yu & Wen 2005). On the basis of biochemical characterization, 6 isolates exhibiting amylase activity were selected, as presence of amylase is the very first condition to be a *B. subtilis* according to Bergey's manual. Study was primarily aimed on bacteria showing characteristics same as *B. subtilis* but later on 3 isolates were selected on the basis of Remazol brilliant blue R and methylene blue decolourisation.

Strains D1023, D1032 and D1033 showed best decolourisation activity for Remazol brilliant blue and methylene blue. Same work for dyes decolourisation has been performed for screening and identification of yeast (Yu & Wen 2005) and decolourisation of spore laccase from *B. subtilis* SPR42 (Saharan & Ranga 2011). Isolates were transferred in 2×SG medium for increase and fasten up the process for spore production to have maximum laccase production. Media has been selected by the literature presented by Edgewood Chemical Biological Center (A U.S. Army RDECOM Laboratory) (Smith et al. 2011).

According to literature, out of the four media used {Amended Nutrient Media, Synthetic Sporulation Media, 2×SG Media and Long Version Nutrient Media} for spore yield studies 2×SG media has shown >90% spore yield within 6 days and considered best media for sporulation yield as compared to others.

In the present study it was observed that in LB medium there was late sporulation as dye degradation began after 4th day of inoculation but in case 2×SG media dye decolourisation was started on 2nd day after inoculation when spore production was in abundance.

Immobilized cells were used to check increase in percentage utilization of guaiacol with increase in absorbance at 450 nm. Percent colour change observed for free cells and immobilized cells has remained same in case of both. Similar pattern has been observed by both free cells and immobilized cells from 10 hrs to 150 hrs. Percent colour change has been observed from 69-70% in first 10 hrs in case of all three isolates. In case of free and immobilized culture of all three isolates, after 70 hrs, 97% colour change has been observed and after 70 hrs O.D. has remained constant.

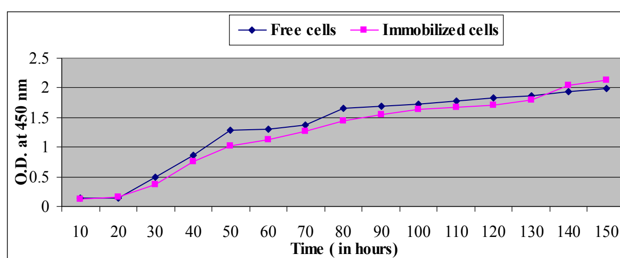

Fig. 6: Change in absorbance at 450 nm during colour change of guaiacol by isolate D1033.

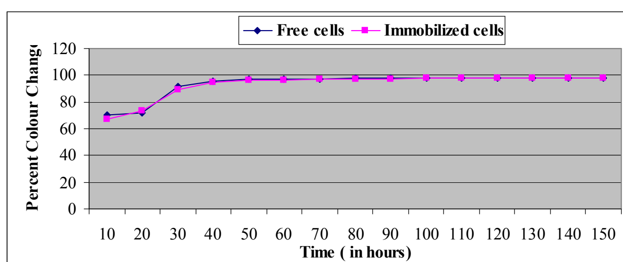

Fig. 7: Percent colour change during treatment of guaiacol by isolate D1033.

Effect of immobilization has been studied for decolourisation of different synthetic dyes. Isolates were immobilized using calcium alginate beads with the procedure described (El-Hadi & El-Minofi 2012). In present study, immobilization of cells has improved dyes decolourisation than free cells and reused beads have also shown decolourisation in similar manner when beads were used freshly.

Decolourisation of dyes by microorganisms can be due to adsorption of the dye to microbial cells or due to biodegradation. In adsorption, examination of the absorption spectrum will reveal that all peaks decrease approximately in proportion to each other. Dye adsorption can also be judged clearly by observing the colour of cell pallet. Cell pallets become deeply coloured because of adsorbing dyes, whereas those retaining their original colours do so as a result of biodegradation. Selected isolates have exhibited absorption for synthetic dyes. All the three isolates decolourised RBBR dye by adsorption as pallet attained blue colour of dye. Methylene blue was also adsorbed by cells of all three isolates as pallet was blue in colour. RBBR has been used at concentration of 100 mg/L to study decolourisation effects of immobilized cells. The maximum decolourisation has been observed in case of *Bacillus* strain D1023.

CONCLUSION

Spore laccase can be used for the treatment of dyes decolourisation. Immobilization has increased the decolourisation activity of *Bacillus* spore laccase. The main advantage of immobilization is that spore laccase is used directly so the time for enzyme production is neglected. Decolourisation rate gets faster when spore laccase is immobilized than in case of free laccase and whole organism.

ACKNOWLEDGEMENT

Authors are thankful to Ambala College of Engineering and Applied Research for providing their infrastructure and support to carry out their research work.

REFERENCES

Adinarayana, K., Jyothi, B. and Ellaiah, P. 2005. Production of alkaline protease with immobilized cells of *Bacillus subtilis* PE-11 in various matrices by entrapment technique. APPS Pharm. Sci.Tech., 6(3): 391-397.

Bourbonnais, R., Paice, M.G., Freiermuth, B., Bodie, E. and Borneman, S. 1997. Reactivities of various mediators and laccases with kraft pulp and lignin model compounds. Appl. Environ. Microbiol., 63: 4627-4632.

Claus, H. and Filip, Z. 1997. The evidence of a laccase-like activity in a *Bacillus sphaericus* strain. Microbiol. Res., 152: 209-215.

Driks, A. 2004. The *Bacillus subtilis* spore coat. Phytopathology, 94: 1249-1251.

El-Hadi, A. A. and El-Minofi, H. A. 2012. Enhanced bioconversion of lactose by immobilized cells of *Bacillus subtilis* using different matrices. Wudpecker Journal of Agricultural Research, 1(10): 415-423.

Ó'Fágáin, C. 2003. Enzyme stabilization recent experimental progress. Enzyme Microbiol. Technol., 33(2): 137-149.

Fu, Y. and Viraraghavan, T. 2001. Fungal decolourization of dye wastewaters: A review. Bioresour. Technol., 79: 251-262.

Gupta, N. and Farinas, E. T. 2010. Directed evolution of CotA laccase for increased substrate specificity using *Bacillus subtilis* spores. Protein Engineering, Design & Selection, 23(8): 679-682.

Hullo, M. F., Moszer, I., Danchin, A. and Martin-Verstraete, I. 2001. CotA of *Bacillus subtilis* is a copper dependent laccase. J. Bacteriol., 183: 5426-5430.

Ivana, E., Ladislav, H. and Frantisek, N. 2006. Synthetic decolorization capacity of white rot fungus *Dichomitus squalens*. Bioresour. Technol., 39: 51-55.

Kunamneni, A., Ghazi, I., Camarero, S., Ballesteros, A., Plou, F. J. and Alcalde, M. 2008. Decolorization of synthetic dyes by laccase immobilized on epoxy-activated carriers. Process Biochem., 43: 169-178.

Leighton, T. J. and Doi, R. H. 1971. The stability of messenger ribonucleic acid during sporulation in Bacillus subtilis. J. Biol. Chern., 246(10): 3189-3195.

Macro, S. L., Carla, A., Ana, S., Jose, A. P. and Albino, A. D. 2006. Biodegradation of the diazo dye Reactive Black 5 by a wild isolate of *Candida oleophila*. Enzyme Microb. Technol., 39: 52-55.

Mahvi, A.H., Ghanbarian, M., Nasseri, S. and Khairi, A. 2009. Mineralization and discoloration of textile wastewater by TiO_2 nanoparticles. Desalination, 239: 309-316.

Mechichi, T., Mhiri, N. and Sayadi, S. 2006. Remazol Brilliant Blue R decolourization by the laccase from *Trametes trogii*. Chemosphere, 64: 998-1005.

Milovanovic, A., Bozic, N. and Vujcic, Z. 2007. Cell wall invertase immobilization within calcium alginate beads. Food Chem. 104: 81-86.

Mishra, K. V. and Sharma, H. 2014. Decolourization of textile azo dyes by laccase producing *Bacillus* spp. isolated from soil. Asian Jr. of Microbiol. Biotech. Env. Sc., 16(2): 313-319.

Mohorcic, M., Teodorovic, S., Golob, V. and Friedrich, J. 2006. Fungal and enzymatic decolourization of artificial textile dye baths. Chemosphere, 63: 1709-1717.

Mongkolthanaruk, W., Tongbopit, S. and Bhoonobtong, A. 2012. Independent behavior of bacterial laccases to inducers and metal ions during production and activity. African Journal of Biotechnology, 11(39): 9391-9398.

Muthukumaran, P.M. and Mathumitha, M. 2013. Decolorization and degradation of reactive dyes by *Aspergillus niger*. Research Journal of Engineering and Technology, 4(4): 235-238.

Palmieri, G., Giardina, P. and Sannia, G. 2005. Laccase-mediated Remazol Brilliant Blue R decolorization in a fixed-bed bioreactor. Biotechnol. Prog., 21: 1436-1441.

Peralta-Zamora, P., Pereira, C.M., Tiburtius, E. R. L., Moraes, S.G., Rosa, M. A., Minussi, R. C. and Duran, N. 2003. Decolorization of reactive dyes by immobilized laccase. Appl. Catal. B: Environ., 42: 131-144.

Saharan, B. and Ranga, P. 2011. Optimization of culture conditions for decolourization of textile azo dyes by *Bacillus subtilis* SPR41 under submerged fermentation. International Journal of Advanced Biotechnology and Research, 2: 148-153.

Selvam, K. and Priya, M. S. 2012. Biological treatment of Azo dyes and textile industry effluent by newly isolated white rot fungi *Schizophyllum commune* and *Lenzites eximia*. International Journal of Environmental Sciences, 2(4): 1926-1935.

Smith, L. S., Wallace, L. and Rastogi, V. K. 2011. Studies of sporulation optimization and characterization of *Bacillus subtilis* spore quality. Edgewood Chemical Biological Center, U.S. Army Research.

Susla, M., Novotny, C. and Svobodova, K. 2007. The implication of *Dichomitus squalens* laccase isoenzymes in dye decolorization by immobilized fungal cultures. Bioresour. Technol., 98: 2109-2115.

Takamatsu, H., Kodama, T., Imamura, A., Asai, K., Kobayashi, K., Nakayama, T., Ogasawara, N. and Watabe, K. 2000. The *Bacillus subtilis* yabG gene is transcribed by SigK RNA polymerase during sporulation, and yabG mutant spores have altered coat protein composition. J. Bacteriol., 182: 1883-1888.

Wang, C. L., Zhao, M., Li, D.B., Cui, D.Z., Lu, L. and Wei, X.D. 2010. Isolation and characterization of a novel *Bacillus subtilis* WD23 exhibiting laccase activity from forest soil. African Journal of Biotechnology, 9(34): 5496-5502.

Wang, J. L. and Liu, P. 1996. Comparison of citric acid production by *Aspergillus niger* immobilized in gels and cryogels of polyacrylamide. Journal of Industrial Microbiology, 16: 351-353.

Wang, J.L. and Shi, H.C. 1998. The research and development of microbial immobilization using polyvinyl alcohol (PVA) gel. Journal of Industrial Microbiology, 28(2): 35-39.

Wang, J. L., Horan, N. and Qian, Y. 2000. The radial distribution and bioactivity of *Pseudomonos* sp. immobilized in calcium alginate beads. Process Biochemistry, 35: 465-469.

Wesenberg, D., Kyriakides, I. and Agathos, S.N. 2003. White-rot fungi and their enzymes for the treatment of industrial dye effluents. Biotechnol. Adv., 22: 161-187.

Won, K., Kim, S., Kim, K.J., Park, H.W. and Moon, S.J. 2005. Optimization of lipase entrapment in Ca-alginate gel beads. Process Biochem., 40: 2149-2154.

Yu, Z. and Wen, X. 2005. Screening and identification of yeasts for decolorizing synthetic dyes in industrial wastewater. International Biodeterioration & Biodegradation, 56(2): 109-114.

Zhang, L. S., Wu, W. Z. and Wang, J. L. 2007. Immobilization of activated sludge using improved polyvinyl alcohol (PVA) gel. Journal of Environmental Sciences, 19: 1293-1297.