J Ind Microbiol Biotechnol (2009) 36:955–960
DOI 10.1007/s10295-009-0574-3

ORIGINAL PAPER

Decolorization of Direct Red 28 by mixed bacterial culture in an up-flow immobilized bioreactor

Bella Devassy Tony · Dinesh Goyal · Sunil Khanna

Received: 21 October 2008 / Accepted: 5 April 2009 / Published online: 24 April 2009

Abstract Aerobic mixed bacterial culture comprised of five isolates (*Bacillus vallismortis, B. pumilus, B. cereus, B. subtilis* and *B. megaterium)* identified by 16srDNA analysis was developed from wastewater samples from the aeration tank of an effluent treatment plant of a textile and dyeing industry and evaluated for its ability to decolorize azo dye Direct Red 28 in an up-flow immobilized packed bed bioreactor using marble chips as support matrix. The bioreactor was operated under two parameters: an aeration rate of 0.4 and 0.6 mmol/min at a flow rate of 60, 90 and 120 ml/h, respectively. At a constant aeration rate of 0.4 mmol/min and with flow rates of 60, 90 and 120 ml/h, optimum decolorization of 91, 75 and 72% was observed, while at an aeration rate of 0.6 mmol/min and flow rates of 60, 90 and 120 ml/h, optimum decolorization of 93, 78 and 72% was observed over 10 days. The study concluded that across the two aeration rates and the respective flow rates, the higher aeration rate of 0.6 mmol/min along with a flow rate of 60 ml/h was best suited to decolorize Direct Red 28 in the packed bed bioreactor. Spectral changes of the input and output of the bioreactor by UV–visible spectroscopy indicated decolorization of the dye solution by degradation in addition to the visual observation of the biosorption process.

S. Khanna (✉)
Department of Biotechnology and Bioinformatics,
NIIT Institute of Information Technology,
Balaji Estate, Kalkaji, New Delhi 110019, India
e-mail: sunil.khanna@tni.edu.in; sunil.khanna@niit.com

B. D. Tony · D. Goyal
Department of Biotechnology and Environmental Sciences,
Thapar University, Patiala, Punjab 147004, India

Keywords Direct Red 28 · Biofilm · Mixed bacterial culture · Up-flow immobilized bioreactor · *Bacillus* sp.

Introduction

The huge growth in the textile dyeing and dyestuff manufacturing industries has resulted in an increase in the volume and complexity of the wastewater discharged into the environment. During textile processing, inefficiency in dyeing results in large amounts of dyestuff being directly lost in the wastewater, which ultimately finds its way into the environment. Wastewater characteristics from a dye house are highly variable from day to day, depending on the type of dye, type of fabric and concentration of the agents added. The presence of very small amounts of dyes in water is highly visible and affects the aesthetic merit, water transparency and gas solubility in lakes, rivers and other water bodies. Thus, loss of dyes to the environment has become an environmental hazard because of the low biodegradability and toxic nature of the dyes. Some dyes, dye precursors and their biotransformation products, such as aromatic amines, have been found to be toxic, mutagenic and carcinogenic in nature, apart from having the potential for bioaccumulation in the food chain [11]. Treatment of such wastewaters is therefore essential, but difficult.

Physico-chemical methods of decolorization have significant differences in color removal, excess amount of chemical usage and sludge generation with obvious disposal problems: costly plant requirements or operating expenses and sensitivity to a variable wastewater input and formation of hazardous by-products. Therefore, focus is now shifting to the development of environment-friendly biological treatment systems based on using microorganisms to decolorize/degrade recalcitrant compounds and

lead to mineralization of the target compounds. Aerobic decolorization of certain azo dyes through cell immobilization has been shown [2, 3, 7, 9, 13, 16, 18, 20, 24], but no study exits for azo dye Direct Red 28.

This work was undertaken to optimize the conditions for the decolorization of the azo dye Direct Red 28 by a packed bed bioreactor consisting of cell immobilization of the aerobic mixed bacterial culture which comprised of five bacterial isolates: *Bacillus vallismortis, B. pumilus, B. cereus, B. subtilis* and *B. megaterium.*

Materials and methods

Dyes and chemicals

The chosen azo dye Direct Red 28 with three naphthalene rings and a sulphonate functional group joined by an azo bond, commonly used in the dyeing and textile industries of the Punjab region, was generously provided by the Nahar Group of the Textile Dyeing Industry, Derabassi, India. Aqueous stock solution was filter-sterilized using a Millipore filter of 0.22 μm [7]. The wavelength maximum of the dye was spectrophotometrically measured by a wavelength scan against water as blank.

Media components were purchased from Himedia Laboratories, Mumbai, India, and other chemicals used were of analytical grade. Marble chips used for immobilization support were purchased from a hardware store (Patiala, Punjab, India) (Fig. 1).

Development of dye decolorizing microorganisms

Wastewater samples collected from the aeration tank of an effluent treatment plant of the Nahar Group of the Textile and Dyeing Industry, Derabassi, Punjab, India, was used for the development of the mixed bacterial culture SKB-II, capable of dye decolorization. After ten transfers on the basal medium containing the particular dye, the stabilized mixed bacterial culture SKB-II was used for further studies. The culture was routinely grown at 37°C in the basal culture medium, Bushnell and Hass Broth medium (BHB), containing the following in g/l: $MgSO_4$, 0.2; $CaCl_2$, 0.02; KH_2PO_4, 1.0; K_2HPO_4, 1.0; $(NH_4)_2NO_3$, 1.0; $FeCl_3$, 0.05

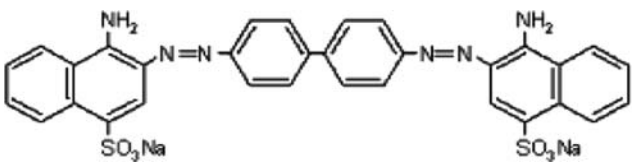

Fig. 1 Chemical structure of Direct Red 28, empirical formula ($C_{32}H_{22}N_6O_6S_2Na_2$)

supplemented with starch 1.3 g/l as carbon and energy source and 40 g/l NaCl. The final pH of the medium was adjusted to 7. The stock culture of the developed mixed bacterial culture was preserved in 50% glycerol stock and stored at −80°C and subsequently sub-cultured on basal medium.

Decolorization assay

At regular time intervals, aliquots (5 ml) from the output of the bioreactor were collected and centrifuged at 10,000×*g* for 15 min [6], and the decolorizing activity was expressed in terms of percentage decolorization determined by monitoring the decrease in absorbance of cell free supernatant at 499 nm (λ_{max} of the dye) using a UV–visible spectrophotometer (Hitachi U200, Tokyo, Japan) and monitoring the absorbance of aliquot from the outlet of the abiotic bioreactor as blank. Decolorization was calculated from the difference between the initial and the final absorption values of the supernatant at the λ_{max} for each dye.

$$\text{Decolorization } (\%) = I_i - I_f/I_i \times 100$$

where I_i and I_f are initial and final absorbance of the dye solution [15]. Each decolorization value is a mean of two parallel experiments. Another bioreactor was operated under the same conditions as an abiotic control to compare the total decolorization of the dye in the biotic reactor.

Molecular characterization of dye decolorizing bacterial isolates

Mixed bacterial culture SKB-II was serially diluted and then spread on Bushnell Haas agar plates and incubated at 37°C for 18 h. Fifty-three (53) distinct isolates were selected for decolorization studies of Direct Red 28 dye at batch scale. Five isolates that were morphologically distinct were then characterized by 16 s rDNA [14]. The 16S rDNA gene was amplified with primers 8-27f (5′-AGAG TTTGATCCTGGCTCAG-3′) and 1500r (AGAAAGGA GGTGATCCAGGC-3′). The amplified DNA fragment was separated on 1% agarose gel, eluted from the gel and purified using Qiaquick gel extraction kit (Qiagen, Germany). The purified PCR product was sequenced with four forward and three reverse primers, namely 8-27f (5′AGAGTTTGATCCTGGCTCAG-3′), 357f (5′-CTCCT ACGGGAGGCAGCAG-′), 704f (5′-TAGCGGTGAAATG CGTAGA-3′), 1114f (5′- GCAACGAGCGCAACC-3′), 685r (5′-TCTACGCATTTCACCGCTAC-3′), 1110r (5′-G GGTTGCGCTCGTTG-3′) and 1500r (5′-GAAAGGAGG TGATCCAGGC-3′), respectively (*Escherichia coli* numbering system). The rDNA sequence was determined by the dideoxy chain-termination method using the Big-Dye

terminator kit using ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

The 16S rDNA sequence of the strains generated in this work was aligned with the 16S rDNA sequence of other closely related *Bacillus* species retrieved from the GenBank data base. A sequence similarity search was done using GenBank BLASTN [1]. Sequences of closely related taxa were retrieved and aligned using the Clustal X program [21], and the alignment was manually corrected for the neighbor-joining analysis [17]; the distances between the sequences were calculated using Kimura's two-parameter model [8]. Bootstrap analysis was performed to assess the confidence limits of the branching [5].

Bioreactor

The bioreactor used for decolorization studies was built of borosilicate glass (44.5 × 6 cm, empty bed volume 1,260 ml) and packed with support material (marble chips of 2–5 mm size) for biofilm formation (Fig. 2). The support material (marble chips) was saturated with Direct Red 28 to avoid discrepancy in results due to adsorption of dye. The bioreactor was operated under parameter of aeration rates 0.4 and 0.6 mmol/min and flow rates 60, 90 and 120 ml/h, respectively. Washout from the outlet of the column was analyzed for stable biofilm formation by collecting samples at regular time intervals and by checking optical density spectrophotometrically at 550 λ max till a constant value was obtained, and the process continued until a chemostat arrangement was attained wherein the rate at which the cells were removed was the same as the rate at which new cells were formed.

Scanning electron microscopy

For scanning electron micrographs, the support material (marble chips) was collected from the bioreactor after stable biofilm formation and was fixed with 4% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 1 h and subsequently washed with 0.1 M phosphate buffer for 10 min. The fixed samples were then dehydrated through a graded series of ethanol solutions (30, 50, 70, 90 and 100% ethanol) for 30 min each. The samples were then dried in a critical point drier and coated with gold [7]. The uninoculated support material sample was also treated as per the described procedure. Scanning electron micrographs were taken on a JSM-840, JEOL (Japan) scanning electron microscope.

Results and discussion

Isolation and molecular identification of dye decolorizing microorganisms

Mixed bacterial culture SKB-II comprised of five distinct bacterial strains, which were identified by 16 srDNA analysis as *B. pumilus* (GenBank accession no. FJ386542), *B. megaterium* (GenBank accession no. FJ386540), *B. cereus* (GenBank accession no. FJ386543), *B. vallismortis* (GenBank accession no. FJ386541) and *B. subtilis* (GenBank accession no. FJ386544), was developed from the wastewater of a dyeing industrial unit as these microbes are naturally adapted to survive in the presence of toxic dyes (6, 15). The individual isolates showed only 75%

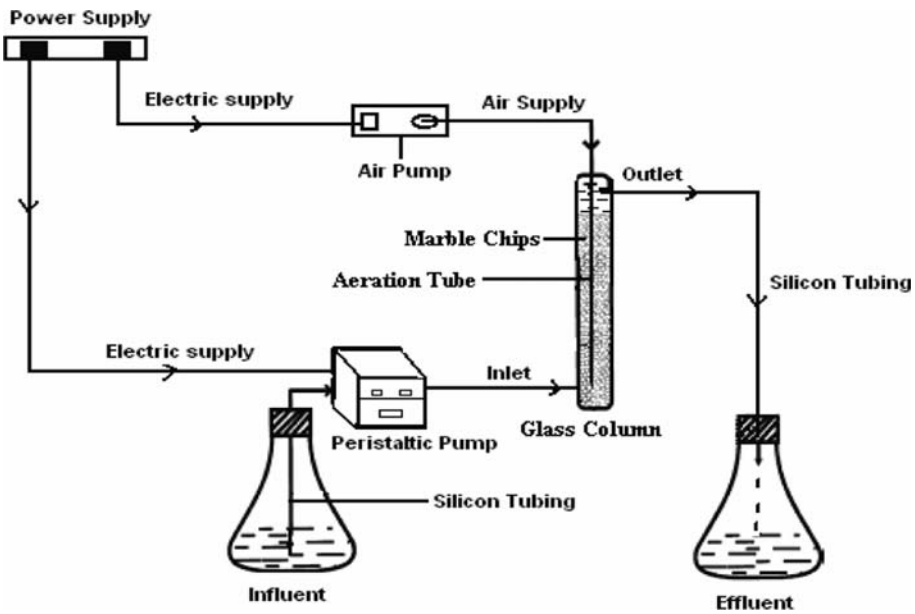

Fig. 2 Schematic representation of the up-flow immobilized cell bioreactor, with marble chips as support material

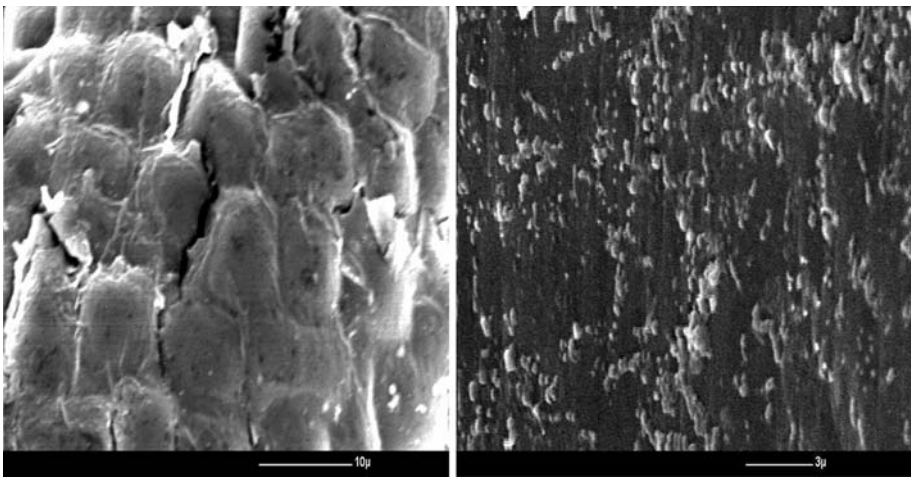

Fig. 3 Scanning electron micrographs. **a** Sterile uninoculated support particle showing different surface zones (×1,500). **b** Support particle completely covered with a layer of rod-shaped bacterial cells (×4,000) formed by mixed culture comprised of five bacterial isolates, namely *B. vallismortis, B. megaterium, B. cereus, B. subtilis* and *B. pumilus*

decolorization of Direct Red 28 dye (10 mg/l) in shake flask studies, whereas the mixed culture of these isolates under shake flask resulted in 96% decolorization (data not shown). Thus, the mixed culture was used as seed inoculum for the bioreactor studies as some strains can collectively carry out such biodegradation tasks that no individual pure strain can achieve [12] and the complexity of such a microbial consortium enables them to act on a variety of pollutants [23].

Development of immobilized cell bioreactor

The mixed bacterial culture comprised of *B. vallismortis, B. pumilus, B. cereus, B. subtilis* and *B. megaterium* (cell concentration 2.5×10^8 cfu m/l) was introduced into the bioreactor filled with marble chips (2–5 mm) for the formation of biofilm. Upon biofilm formation the mixed bacterial culture was fed with 10 mg/l Direct Red 28, and the experiments were conducted at three different flow rates of 60, 90 and 120 ml/h and at two aeration rates of 0.4 and 0.6 mmol/min. It was observed that with 0.4 mmol/min air and flow rates of 60, 90 and 120 ml/h, washout stabilization was achieved in 62, 94 and 105 h, respectively, while with 0.6 mmol/min of air and a flow rate of 60 ml/h, washout stabilization was achieved in 85 h; however, with flow rates of 90 and 120 ml/h, stable biofilm formation was achieved in longer time periods of 107 and 114 h, respectively (data not shown). During washout stabilization, at higher flow rates (90 and 120 ml/h), it was observed that the turbidity of the outflow increased due to the microbial cell washouts that came along in the sample as the biofilm mass was probably lost by the erosion of the biofilm surface due to shearing by the liquid passing by or by the detachment of the biofilm from the attachment surface due to sloughing. Therefore, washout stabilization at these two flow rates was achieved at longer time periods. Scanning electron micrograph (SEM) studies of marble chips of uninoculated bioreactor showed a large rough surface area for bacterial growth and attachment (Fig. 3a). SEM of marble chips of the inoculated bioreactor showed the surface area covered with bacterial cells (Fig. 3b).

Upon stable biofilm formation, BHB medium containing 10 mg/l Direct Red 28 was introduced through the bottom of the reactor in an upward direction to avoid channeling effects and increase in retention time [2] at the respective flow rates. For decolorization studies, in continuous operation, the first initial hours of dye decolorization were not accounted because of the mixing of effluent with the contents of the reactor and also to avoid the accounting of the decolorization contributed by matrix adsorption of dye, which is negligible in a continuous flow fixed bed process [2, 10]. With constant aeration of 0.4 mmol/min, optimum growth of 0.290 (OD 550 nm) with 91% decolorization at 499 nm was observed at a flow rate of 60 ml/h. An increase in flow rate from 60 to 90 and 120 ml/h resulted in a decline in growth (0.250–0.220), while not much variation of 75 and 72% decolorization in the two flow rates (90 and 120 ml/h) was observed. Upon increasing the aeration rate from 0.4 to 0.6 mmol/min growth (0.260, 0.250 and 0.240) at OD 550 decreased at all the three flow rates of 60, 90 and 120 ml/h. Maximum decolorization of 93% was observed at flow rate 60 ml/h (Fig. 4) followed by a gradual decline to 78 and 72% decolorization at 90 and 120 ml/h, respectively (Table 1). In both cases of aeration (0.4 and 0.6 mmol/min) maximum decolorization was observed at flow rate of 60 ml/h, which declined at flow rates of 90 and 120 ml/h as higher flow rates limit the growth of the biofilm and also the time for contact between the dyes and the organism is less, thereby resulting in a comparatively lesser rate of decolorization. This could be due to the fact that at higher volumetric flow rate, the anchoring of the biofilms on the support particles is affected, thus, affecting the overall efficiency of the process. The decolorization when compared across the two aeration rates 0.4 and 0.6 mmol/

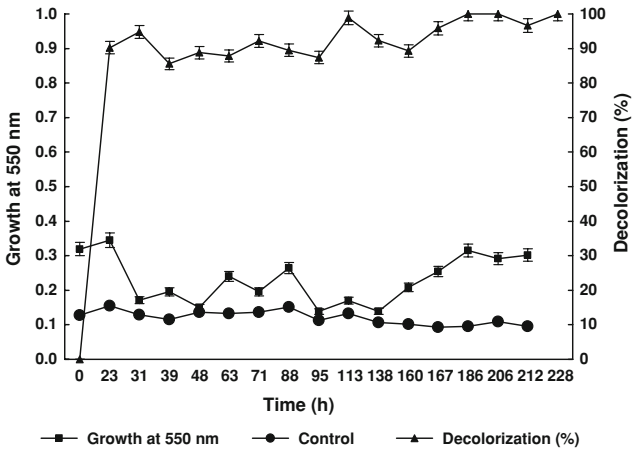

Fig. 4 Decolorization (%) of Direct Red 28 by mixed culture at an aeration rate 0.6 mmol/min and flow rate of 60 ml/h. The azo dye was treated in the bioreactor upon biofilm formation by mixed culture, comprised of five bacterial isolates, namely *B. vallismortis, B. megaterium, B. cereus, B. subtilis* and *B. pumilus* on support material (marble chips). From the outlet of the bioreactor samples were collected to estimate growth and decolorization as described in the text

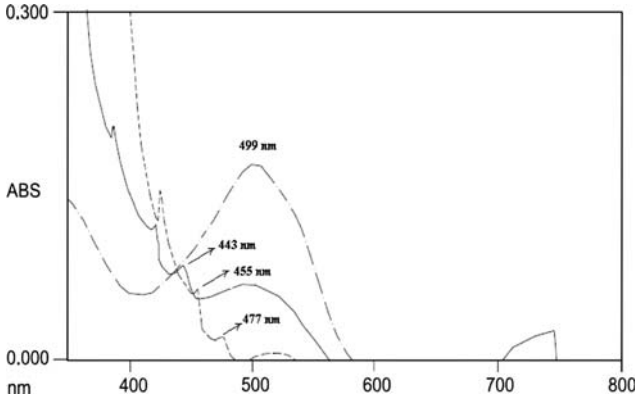

Fig. 5 Absorption spectra of Direct Red 28 dye treated in the bioreactor. *Broken line* Input feed of the bioreactor; 499 nm k_{max} of the dye; *continuous line* output feed of the bioreactor; *dotted line* output feed of the duplicate bioreactor

min was observed to be higher at an aeration rate of 0.6 mmol/min due to the aerobic nature of the mixed bacterial culture. These results are in agreement with those of Yang et al. [24], who showed that in a rotating biological contactor (RBC), a higher rotational speed of 40 rpm provided better aeration resulting in increased decolorization efficiency of phthalocyanine dye Basic Blue 22 (C·I. 61512) by the white-rot fungus *Phanerochaete sordida* ATCC90872, and also of Faison and Kirk [4], who reported that the increase in rotational speed from 10 to 40 rpm increased the decolorization from 35 to 77%.

The comparison of the UV–visible spectrum of the input feed and the output showed a decrease in absorption at 499 nm and a gradual shift towards shorter wavelength after the treatment with mixed bacterial culture SKB-II (Fig. 5), which indicates that decolorization of this dye solution occurred by degradation in addition to the visual observation of the biosorption process [22]. No color loss was observed in the abiotic control bioreactor, suggesting that the observed dye decolorization in the biotic reactor is due to the biological activity of the microbial mixed bacterial culture immobilized on the marble chips in the bioreactor. Similar to our study, Senan et al. [19] in their study concluded that continuous use of an immobilized aerobic cell bioreactor by passive immobilization of the bacterial consortium is a feasible approach for the textile azo dye degradation. Thus, results of this study show that a developed immobilized cell bioreactor can enhance the efficiency of the mixed bacterial culture comprised of bacterial isolates *B. vallismortis, B. pumilus, B. cereus, B. subtilis* and *B. megaterium* to decolorize azo dyes used in the textile industries.

Table 1 Growth of mixed bacterial culture and decolorization (%) of Direct Red 28 at aeration rates of 0.4 and 0.6 mmol/min and flow rates of 60, 90 and 120 ml/h

Aeration (mmol/min) and flow rate (ml/h)	Growth (OD 550)	Decolorization (%)
0.4 and 60	0.29 ± 0.02	91.46 ± 1.57
0.4 and 90	0.25 ± 0.01	75.59 ± 1.46
0.4 and 120	0.22 ± 0.01	72.66 ± 1.75
0.6 and 60	0.26 ± 0.03	93.08 ± 1.24
0.6 and 90	0.25 ± 0.01	78.08 ± 1.09
0.6 and 120	0.24 ± 0.01	72.62 ± 4.74

Conclusion

In this study, high-efficiency decolorization of azo dye Direct Red 28 was achieved by immobilization of azo dye-degrading mixed bacterial culture SKB-II comprised of five bacterial isolates, *B. pumillus*, *B. cereus*, *B. megaterium*, *B. vallismortis* and *B. subtilis*, in a packed bed bioreactor. The obtained results show that the best working parameter for decolorization was 0.6 mmol/min aeration along with a flow rate of 60 ml/h using marble chips as immobilization support material. The high specific decolorization rate obtained and the simplicity of the immobilization method suggest that this technique would be suitable for using the developed aerobic mixed bacterial culture to remove azo dyes from wastewater on an industrial scale.

Acknowledgment We express our gratitude to the Department of Biotechnology, Ministry of Science and Technology, Government of India, New Delhi, for financial support for this research. The authors are also thankful for the necessary equipment and facilities provided

by the Department of Biotechnology and Environmental Sciences and TIFAC-CORE, Thapar University, Punjab, where this work was carried out.

References

1. Attschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402. doi:10.1093/nar/25.17.3389
2. Chen BY, Chen SY, Chang JS (2005) Immobilized cell fixed-bed bioreactor for wastewater decolorization. Process Biochem 40:3434–3440. doi:10.1016/j.procbio.2005.04.002
3. Coughlin MF, Kinkle BK, Bishop PL (2003) High performance degradation of azo dye Acid Orange 7 and sulfanilic acid in a laboratory scale reactor after seeding with cultured bacterial strains. Water Res 37:2757–2763. doi:10.1016/S0043-1354(03)00069-1
4. Faison BD, Kirk TK (1985) Factors involved in the regulation of a ligninase activity in *P. chrysosporium*. Appl Environ Microbiol 49:299–304
5. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evol Int J Org Evol 39:783–791. doi:10.2307/2408678
6. Khehra MS, Saini HS, Sharma DK, Chadha BS, Chimni SS (2005) Decolorization of various azo dyes by bacterial consortium. Dyes Pigments 67:55–61. doi:10.1016/j.dyepig.2004.10.008
7. Khehra MS, Saini HS, Sharma DK, Chadha BS, Chimni SS (2006) Biodegradation of azo dye C.I. Acid Red 88 by an anoxic–aerobic sequential bioreactor. Dyes Pigments 70:1–7. doi:10.1016/j.dyepig.2004.12.021
8. Kimura M (1980) A simple method for estimation of evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120. doi:10.1007/BF01731581
9. Kodam KM, Soojhawon I, Lokhande PD, Gawai KR (2005) Microbial decolorization of reactive azo dyes under aerobic conditions. World J Microbiol Biotechnol 21:367–370. doi:10.1007/s11274-004-5957-z
10. Lee LY, Ong SL, Ng WJ (2004) Denitrification of Nitrate wastewater using packed-bed columns. Water Environ Res 76(5):388–393. doi:10.2175/106143004X151653
11. Lourenco ND, Novais JM, Pinheiro HM (2000) Reactive textile dye color removal in a sequencing batch reactor. Water Sci Technol 42:321
12. Nigam P, Banat IM, Dalel S, Roger M (1996) Microbial process for the decolorization of textile effluent containing azo, diazo and reactive dyes. Process Biochem 31:435–442. doi:10.1016/0032-9592(95)00085-2
13. Park EH, Jang MS, Cha IH, Choi YL, Cho YS, Kim CH, Lee YC (2005) Decolorization of a sulphonated azo dye, Congo red, by *Staphylococcus* sp. EY-3. J Microbiol Biotechnol 15(1):221–225
14. Rainey FA, Ward-Rainey N, Kroppenstedt RM, Stackebrandt E (1996) The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of *Nocardiopsaceae* fam. nov. Int J Syst Bacteriol 46:1088–1092
15. Ramya M, Anusha B, Kalavathy S (2008) Decolorization and biodegradation of Indigo carmine by a textile soil isolate *Paenibacillus larvae*. Biodegradation 19:283–291. doi:10.1007/s10532-007-9134-6
16. Ren S, Guo J, Zeng G, Sun G (2006) Decolorization of triphenylmethane, azo, and anthraquinone dyes by a newly isolated *Aeromonas hydrophila* strain. Appl Microbiol Biotechnol 72:1316–1321. doi:10.1007/s00253-006-0418-2
17. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425
18. Senan RC, Abraham ET (2004) Bioremediation of textile azo dyes by aerobic bacterial consortium. Biodegradation 15:275–280. doi:10.1023/B:BIOD.0000043000.18427.0a
19. Senan RC, Shaffiqu TS, Jegan RJ, Abraham ET (2003) Aerobic degradation of a mixture of azo dyes in a packed bed reactor having bacteria coated laterite pebbles. Biotechnol Prog 19:647–651. doi:10.1021/bp020103g
20. Sharma DK, Saini HS, Singh M, Chimni SS, Chadha BS (2004) Biological treatment of textile dye Acid violet-17 by bacterial consortium in an up-flow immobilized cell bioreactor. Lett Appl Microbiol 38:345–350. doi:10.1111/j.1472-765X.2004.01500.x
21. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882. doi:10.1093/nar/25.24.4876
22. Wang Y, Yu J (1998) Adsorption and degradation of synthetic dyes on the mycelium of *Trametes versicolor*. Water Sci Technol 38(4–5):233–238. doi:10.1016/S0273-1223(98)00532-0
23. Watanabe K, Baker PW (2000) Environmentally relevant microorganisms. J Biosci Bioeng 89:1–11. doi:10.1016/S1389-1723(00)88043-3
24. Ge Y, Yan L, Qinge K (2004) Effect of environment factors on dye decolorization by *P. sordida ATCC90872* in an aerated reactor. Process Biochem 39:1401–1405. doi:10.1016/S0032-9592(03)00273-5