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Bacterial degradation and decolorization of textile dyes by newly isolated *Lysobacter* sp.

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A bacterial strain, P28, with significant capability to decolorize the textile dye, Congo Red and Yellow HEGR was isolated from the effluent collected from a textile industry. Phenotypic characterization and phylogenetic analysis of the 16S rRNA sequence indicated that the bacterial strain belonged to the genus Lysobacter. Bacterial isolate P28 showed a remarkable ability to decolorize azo dyes, including Congo Red and Yellow HEGR. *Lysobacter* sp. P28 grew well in a concentration of dye 50 mg L^{-1} (Congo Red and Yellow HEGR) resulting in approximately 80.4 and 80.1% decolorization extent in 72 h, and could tolerate up to 200 mg L^{-1} of dye with 60.4 and 50.8% decolorization extent in 84 h. High decolorization extent and facile conditions show the potential for this bacterial strain to be exploited in the biological treatment of dyeing mill effluents.

Key words: Azo dyes, bacterial degradation, *Lysobacter*, decolorization.

INTRODUCTION

Dyes and dyestuffs are extensively used within the food, pharmaceutical, cosmetic, textile and leather processing industries. From the existing literature, it can be estimated that approximately 75% of the dyes discharged by textile processing industries belong to the classes of reactive (~36%), acid (~25%) and direct (~15%) dyes. Throughout the dyeing progression, about 10-15% of the dyes used are released into the wastewater. Textile dyes in the aqueous ecosystem are the cause of serious environmental and health concerns (Chen et al., 2015; Khan and Malik, 2014; Saratale et al., 2013; Sarayu and Sandhya, 2012; Fang et al., 2004; Asad et al., 2007; Clarke and Anliker, 1980; Pinheiro et al., 2004). Among these dyes, azo dyes are the most widely used; these account for over 60% of the total number of dye structures known to be produced (Zollinger, 1991). Azo dyes can be distributed in monoazo, diazo and triazo classes, and are available in six application categories: acid, basic, direct, disperse, azoic, and pigments. Pollution problems due to textile industry effluents have increased in recent years. Because color in wastewater is highly visible and affects esthetics, water transparency and gas solubility in water bodies, and particularly because many dyes are made from identified carcinogens, such as benzidine and other aromatic compounds, dye wastewaters have to be treated (Banat et al., 1996). Moreover, it is very difficult to treat textile industry effluents because of their high biological oxygen demand (BOD), chemical oxygen demand (COD), heat, color, pH and the presence of metal ions. Numerous methods

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are used to treat textile effluents to attain decolorization. A number of physical and chemical methods have been recommended for the treatment of dye-contaminated wastewater, but such methods are not widely used due to the high cost of input and secondary pollution that can be generated by the excessive use of chemicals (Jadhav et al., 2007). Unconventionally, biodegradation systems of color removal through the use of bacteria have been shown to be highly effective (Pearce et al., 2003). Micro-organisms are nature's original recyclers, converting toxic organic compounds to harmless products, often carbon dioxide and water. Ever since it was discovered that microbes have the ability to transform and/or degrade xenobiotics, scientists have been exploring the microbial diversity, particularly of contaminated areas in search for organisms that can degrade a wide range of pollutants (Jain et al., 2005). Although numerous microorganisms can decolorize such dyes, only a few are able to mineralize these compounds into CO_2 and H_2O (Junghanns et al., 2008). These include bacteria, fungi and algae, capable of decolorizing a wide range of dyes with high efficiency (Chen et al., 2015; Khan and Malik, 2014; Saratale et al., 2013; Sarayu and Sandhya, 2012; Fu and Viraraghavan, 2001).

In particular, systems based on biological processes using a large variety of bacterial strains, allow for degradation and mineralization with a low environmental impact and without the use of potentially toxic chemical substances, under mild pH and temperature conditions (Pandey et al., 2007). Microbial decolorization and degradation has appeared as an environmentally pleasant and cost-competitive alternative to chemical decomposition processes (Ozdemir et al., 2008). Bacterial strains that are able to decolorize azo dyes under aerobic (*Xenphylusazovorans* KF46F, *Bacillus* strain, *Kerstersia* sp. strain VKY1 and *Staphylococcus* sp.) and anaerobic conditions (*Sphingomonasxenophaga* BN6, *Eubacterium* sp., *Clostridium* sp., *Butyrvibrio* sp. or *Bacteroides* sp.) have been extensively reported (Olukanni et al., 2006; Dos Santos et al., 2007; Lin and Leu, 2008). Thus, the main objective of this study was to observe the degradation of azo dyes in a successive process using exclusively a novel bacterium isolated from textile dye effluents, process optimization and molecular characterization of the isolate.

In the current study, a bacterial strain, P28, with remarkable ability to decolorize the Congo Red and Yellow HEGR, was isolated from the textile dye contamination sites collected from a textile mill. Our results also showed that *Lysobacter* sp. P28 exhibited decolorizing activity through biodegradation.

MATERIALS AND METHODS

Dyestuff, chemicals and microbiological media

In order to obtain a high-performance bacterial decolorization, Congo red and yellow HEGR, commonly used azo dyes were chosen for the screening of dye degradative bacteria. All the chemicals were of analytical grade. Textile dyes and textile effluent were obtained from local textile industries (Shivalik Polymer Ltd.) Faridabad (Haryana), India. Reagents and other fine chemicals were obtained from Hi-media Laboratory, India.

Revival of reference culture

The reference culture (*Pseudomonas putida* MTCC 2445) was obtained from Institute of Microbial Technology (IMTECH), Chandigarh. The seal packaged ampoules containing reference culture was broken under aseptic conditions and the culture was revived in nutrient broth.

Isolation and screening of the microorganism

The soil and effluent samples were collected from various sites of textile industry. Samples were collected in pre-sterilized plastic and polyethylene bags and stored at 4°C. From serially diluted samples, 1 ml of diluted sample was spread on nutrient agar plates incorporated with azo dyes and then incubated at 37°C for 4-5 days. For primary screening of isolates, test tube containing 25 ml of mineral salt broth with azo dyes at 10 mgL^{-1} concentrations were prepared. Autoclaved broth was inoculated with 1% inoculum and then incubated at 37°C for five days. Reduction in color was observed after every 12 h.

During secondary screening, selected isolates from primary screening were further checked against various concentrations of textile dyes (10-200 mgL^{-1}). Decolorization of dye was measured as the decrease in optical density of the supernatant obtained upon centrifugation (10,000 rpm for 20 min) at their respective peak maxima (Y. HEGR-400 nm, C.R.-530 nm). Isolate with the maximum decolorizing extent, designated as P28, was preserved at -20°C in LB medium with 10% glycerol.

Measurement of decolorization extent

Samples (500 µL) were collected after every 12 h and centrifuged at 5000 g for 5 min. Decolorization extent was determined by measuring the absorbance of the culture supernatant at 560 nm using a double beam spectrophotometer (Systronics). Decolorization extent was calculated using the following equation:

$$\text{Decolorization extent (\%)} = (OD1 - Odt)/OD1 \times 100$$

Where, OD1 refers to the initial absorbance, ODt refers to the absorbance after incubation; and t refers to the incubation time.

Optimization of cultural conditions for textile dye decolorization

The bacterial isolate showing efficient decolorization activity in secondary screening was selected for the optimization of cultural conditions. Azo dye decolorization process was optimized by the conventional strategy; varying one factor at a time. Effect of temperature on decolorization of textile dyes was observed. The inoculated media containing flasks were incubated at different temperatures (25 to 45°C) for 4 days. The effect of pH on decolorization was observed by incubating flasks containing dye media and appropriate inoculums at different pH values at optimum temperature for 4 days. The effect of aeration on decolorization of textile dyes was observed by incubating media flasks containing dye in both static and shaking conditions at optimum temperature and optimum pH. The effect of non-living culture (Adsorption effect)

on decolorization of textile dyes was observed by inoculating the media flasks containing dye with both living and autoclaved cells (dead cells).

Effect of textile effluent concentrations on decolorization

Various concentrations of textile effluent (25, 50, 75 and 100%, v/v) were used. Each flask (250 ml) containing 100 ml textile effluent (pH-7.0) of respective concentration was autoclaved and inoculated with 1% inoculums and incubated at temperature 37°C for five days. Decolorization activity was observed.

Effect of inoculum size on decolorization activity and dry weight of cells

The effect of inoculum size on textile effluent (100%) decolorization was observed by inoculating the isolates at various concentrations in the textile effluent. Different concentrations (1.0, 2.0, 3.0, 4.0 5.0, 6.0, 7.0, 8.0, 9.0, 10.0%, v/v) of 24 h old cultures were inoculated in textile effluent and then incubated for 3 days. After interval of 12 h, 4 ml of sample was used for decolorization extent measurement. Simultaneously dry weight of each sample was also recorded. After centrifugation, pellets were suspended in saline and dried under 45°C temperature.

Dry weight of cells = weight of dried pellets with china dish - weight of empty china dish

Effect of different concentration of textile effluent on seed germination and toxicity test

Toxicity of raw textile effluent and treated textile effluent was observed by seed germination experiments (Rehman et al., 2009). Mung bean (*Vigna radiata*) seeds were surface sterilized with 0.1% $HgCl_2$ for 2 min and then washed repeatedly with sterilized distilled water. Initially, the effect of different concentrations of untreated (25, 50, 75 and 100%, v/v) textile effluent was observed on seeds of mung beans. The surface sterilized seeds were spread on sterilized Petri dishes lined with sterilized filter paper. Then, the seeds were treated with equal volume (10 ml) of raw and treated textile effluent. The Petri dishes were kept at room temperature (25 ± 2°C) and the seed germination percentage was recorded after every 24 h. The emergence of radical of 2 mm length was taken as criterion for germination. After 7 days, 3 ml of textile effluent was added to the Petri plate and for the seedling growth, 7 days old seedlings were measured. After 15 days, seedlings were taken out from each Petri dish and their respective root and shoot lengths were measured. Seedling components were separated into root and shoot and measured separately for their fresh weight. These seedling parts were dried in an oven for 12 h at 45°C and their dry weight was taken. The plant growth measurements included shoot length, root length, wet shoot weight, wet root weight, dry shoot weight, dry root weight and vigor index as noted in 15^{th} day old seedlings with various seed germination parameters.

Comparison of live and autoclaved cell for decolorization

Two fresh culture broth of each strain was prepared, half of them were autoclaved. Both the autoclaved (inactive) and living cells were centrifuged at 5000 g for 4 min. To determine if extracellular byproducts or bacterial cells are involved in decolorization, the supernatant and pellets of the living and nonliving cells were incubated with the dye, absorption was used as a measure of their decolorization activity (Khehra et al., 2005).

Morphological and biochemical identification

The isolated organism was further identified on the basis of morphological and biochemical characteristics from MTCC, Institute of Microbial Technology (IMTECH), Chandigarh.

Molecular identification

Total genomic DNA of bacteria was isolated by using method of Charles and Nester (1993) with slight modifications. Pure cultures of bacteria were raised in 10 ml of nutrient broth medium for 18 - 24 h to obtain cell O.D of 0.6 at 600 nm and 12,000 rpm. The bacterial pellet was washed in 1.5 ml of 0.85% NaCl, centrifuged for 2 min at 12,000 rpm and was resuspended in 0.4 ml Tris-EDTA buffer (T_{10} E_{25}). Cell lysis was done by adding 20 µl of 25 % SDS, 50 µl of 1% lysozyme and 50 µl of 5 M NaCl followed by incubation at 68°C for 30 min in a circulatory water bath. For protein precipitation, 260 µl of 7.5 M ammonium acetate solution was added to the micro centrifuge tubes and tubes were kept in ice for 20 min followed by centrifugation at 13,000 rpm for 15 min at 20°C. Supernatant was carefully pipette out in another fresh sterile micro centrifuge tube in which 1 µl RNase (4 mg ml^{-1}) was added followed by incubation at 37°C for 20 min. Equal volume of chloroform was added in the tubes and proper mixing was done by inverting the tube up and down several times. RNA was precipitated by centrifuging for 1 min at 12,000 rpm. The top layer containing total cell DNA was pipette out in fresh microfuge tube and used for the next step. DNA was precipitated by adding 0.8 volume of iso-propanol followed by incubation on ice for 30 min and pelleted by centrifuging at 10,000 rpm for 15 min. DNA was further washed with 0.5 ml of 70% ethanol and spun down at 10,000 rpm for 1 min. Traces of ethanol were removed by air drying the tubes in inverted position. Pure DNA sample was then suspended in 20 µl Tris-EDTA buffer ($T_{10}E_1$) or deionized water and stored at 4°C for further use.

Quantification through agarose gel electrophoresis

The genomic DNA samples of bacteria were quantified through agarose gel electrophoresis by analyzing their migration on 0.8% agarose gels prepared in 0.5 M Tris-borate-EDTA (TBE) buffer and run in an electrophoresis tank filled with the same concentration of TBE buffer. The genomic DNA was diluted with Tris-EDTA buffer so as to achieve a concentration of 50 ng in 10 µl to be used as a template DNA in PCR amplification reaction.

16S rRNA PCR-amplification of isolates

Primers

The forward and reverse primers were custom synthesized from "Ocimum link biotech" Hyderabad, India. The sequences of the oligonucleotide primers used for amplification of 16S rRNA genes are:
Forward primer 41f (5'-GCTCAAGATTGAACGCTGGCG-3'); Reverse primer 1488r (5'-GTTACCTTGTTACGACTTCACC-3') (Maatallah et al., 2002). The stock solution (100 ng ml^{-1}) of primers was prepared by reconstituting lyophilized primers in sterilized deionized (milliQ) water and stored at 20°C.

Amplification of 16S r-DNA was carried out by polymerase chain reaction using a thermal cycler (BioRed). The amplifications were carried out with 50-90 ng of pure genomic DNA. The amplification reactions were performed in a 25 µl mixture containing 0.6 U of Tag DNA Polymerase (Genei from 3U µl of 10X Tag Polymerase buffer, 0.4 µl of d NTP mix and 0.3 µl each of the two primers described above). For every PCR reaction, a negative control (no template

DNA) and a positive control (template DNA giving amplified product) were invariably maintained. The amplified product was run on a 1.2% agarose gel along with 1 kb MW marker (Genei), at a constant voltage, and visualize under uv light.

Bacterial identification by 16S rRNA sequences and phylogenetic relationship

Phylogenetic identity of bacteria was determined by BLAST result, sequences were aligned by using alignment software, that is, Clustal W. Phylogeny calculations and dendrogram was constructed by Mega 4.0 software package using neighbor joining (NJ) methods (Saitou and Nie, 1987). Bootstrap analysis (Felsenstein, 1985) was conducted using 1000 replicates samplings of data.

RESULTS AND DISCUSSION

Isolation of decolorizing bacteria

A strain of bacterium, P28, with strong decolorizing ability on Congo red and yellow HEGR was isolated. A significant decolorization extent (73.5%) was observed after 24 h incubation and a maximum value (80.4%) was achieved after incubation for 72 h at 50 mgL^{-1} w/v concentration. Further, a significant decolorization extent (60.4%) was achieved after incubation for 84 h at 200 mgL^{-1} w/v concentration. In case of Yellow HEGR, a significant decolorization extent (80.1%) was observed after 24 h incubation and a maximum value (80.6%) was achieved after incubation for 84 h at 50 mgL^{-1} w/v concentration. Further, a significant decolorization extent (50.8%) was achieved after incubation for 84 h at 200 mgL^{-1} w/v concentration. Whereas, *P. putida* MTCC 2445 (reference culture) showed decolorization extent (29.8%) after 24 h at 50 mgL^{-1} w/v and a maximum value (56.8%) was achieved after incubation for 84 h at 50 mgL^{-1} w/v concentration. Further, the extent of decolorization was decreased (30.8%) after incubation for 84 h at 200 mgL^{-1} w/v concentration (Figure 1 and Table 1).

The colony of bacterial isolate P28 showed swarming colony morphology on agar plates. The colony of bacterial isolate P28 was highly mucoid and cream colored. P28 was observed to be a non-spore forming Gram negative rod. Sequence analysis of 16S rRNA showed that strain P28 had highest similarity with the species *Lysobacter* sp. (99%) which has been proved to have decolorizing ability against azo dyes.

Decolorization of Cong Red and Yellow HEGR under shaking and non-shaking conditions

Decolorization of Congo red was more than 71% in static conditions and only 66.7% in shaking conditions (150 rpm). In the case of Yellow HEGR dye, decolorization extent was more than 62% in static conditions and only 55% in shaking condition. Whereas, reference culture also showed maximum decolorization extent (41.5, 45.6%) in static conditions and only (32.8%, 40.6%) in shaking conditions for Congo red and yellow HEGR, respectively (Figure 2). This result suggested that *Lysobacter* sp. P28 was shown to have slightly higher decolorization activity in conditions as compared to shaking conditions.

Effects of initial dye concentration, pH and temperature on decolorization

With increase of the initial dye concentration, the decolorization extent over the same time interval decreased. When the effect of different initial dye concentrations of Congo Red and Yellow HEGR on decolorization was observed using 25, 50, 100 and 200 mgL^{-1}, the required times to reach a maximum decolorization extent were 12, 24, 36, 48, 60, 72, 84, 96, 108 and 120 h, respectively. It was reported that dye decolorization can be strongly inhibited when a high concentration dyestuff was used to examine the poisonous effect of the dye on the degrading microorganisms (Khalid et al., 2008). To accurately appraise the decolorizing ability of *Lysobacter* sp. P28, decolorization was investigated by assessing the amount of dyes decolorized. The decolorization extent was only 58.3% (Congo Red) at 200 mg l^{-1} dye and 49.5% (Yellow HEGR) after 120 h incubation. This result indicated that *Lysobacter* sp. P28 showed high decolorizing performance even in high initial dyestuff concentrations. The best decolorization was achieved at pH 7.0-8.0, with ~81% decolorization in 72 h and 84 h (Figure 3). This could be due to the optimum pH for the growth of *Lysobacter* sp. P28. pH has a major effect on the efficiency of dye decolorization, and the optimal pH for color removal is often between 7.0 and 9.0 (Guo et al., 2007). The pH tolerance of decolorizing bacteria is quite important because reactive azo dyes bind to cotton fibers by addition or substitution mechanisms under alkaline conditions and at high temperatures (Aksu, 2003). It should be mentioned that the pH of the wastewater samples used for isolation of *Lysobacter* sp. P28 was about 8-9.

The fact that *Lysobacter* sp. P28 could decolorize reactive dyes in a relatively different range of pH, make it suitable for practical bio-treatment of dyeing mill effluents. *Lysobacter* sp. P28 showed strong decolorizing activity from 37°C. Although a lag phase was observed and the decolorization rate was comparatively low at 25°C, the decolorization extent increased to a similar level from 25 to 37°C (Figure 4). Decolorizing activity was significantly suppressed at 45°C, which might be due to the loss of cell viability or deactivation of the enzymes responsible for decolorization at 45°C.

Effect of living and autoclaved cells and size of inoculum of isolates on decolorization of Congo Red and Yellow HEGR dye

It was observed that autoclaved cells exhibited no

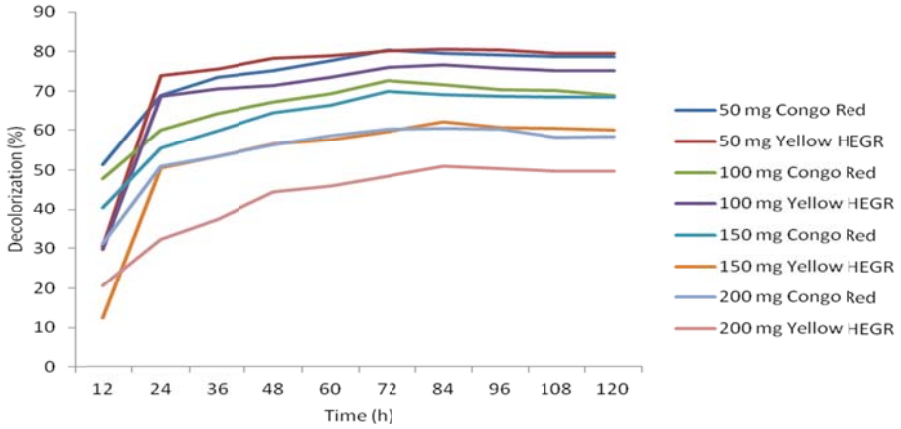

Figure 1. Growth of *Lysobacter* sp. P28 cultures and dye decolorization.

Table 1. Decolorization of textile dyes at different time intervals.

Time	Decolorization (%)			
	P28	P28	*P. putida* MTCC 2445	*P. putida* MTCC 2445
	Congo Red	Yellow HEGR	Congo Red	Yellow HEGR
12 h	47.6	29.7	10.6	22.5
24 h	60.1	68.7	19.8	45.6
36 h	64.2	70.5	28.5	56.4
48 h	67.1	71.4	32.4	62.1
60 h	69.3	73.4	38.2	64.8
72 h	72.6	76.0	44.3	65.3
84 h	71.5	76.6	50.5	65.7
96 h	70.2	75.7	50.6	64.8
108 h	70.1	75.2	50.9	64.5
120 h	68.9	75.2	50.3	64.5

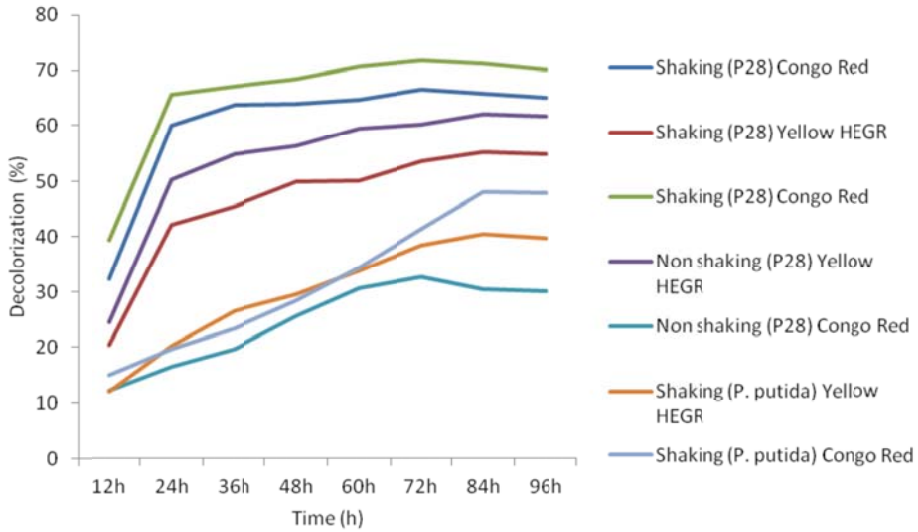

Figure 2. Growth of Lysobacter sp. P28 culture and dye decolorization in shaking and static conditions.

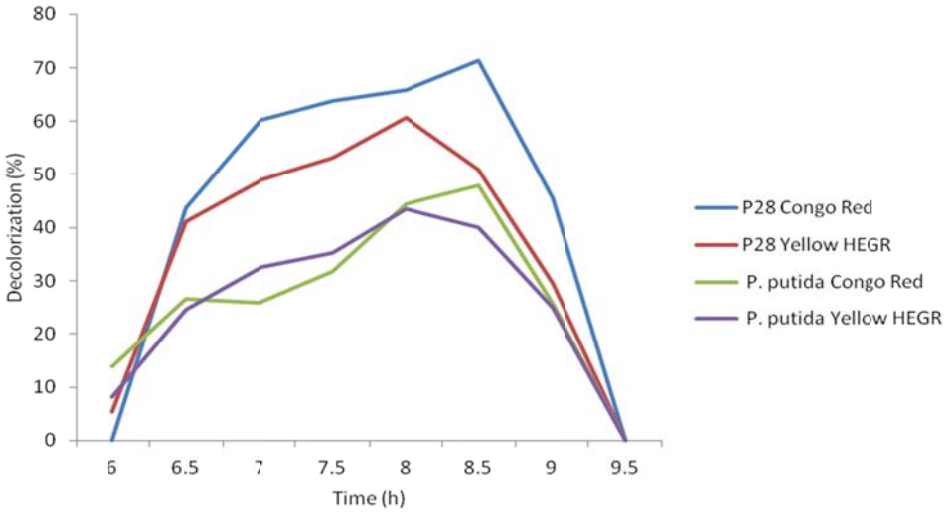

Figure 3. Effect of pH on decolorization of Congo red and yellow HEGR.

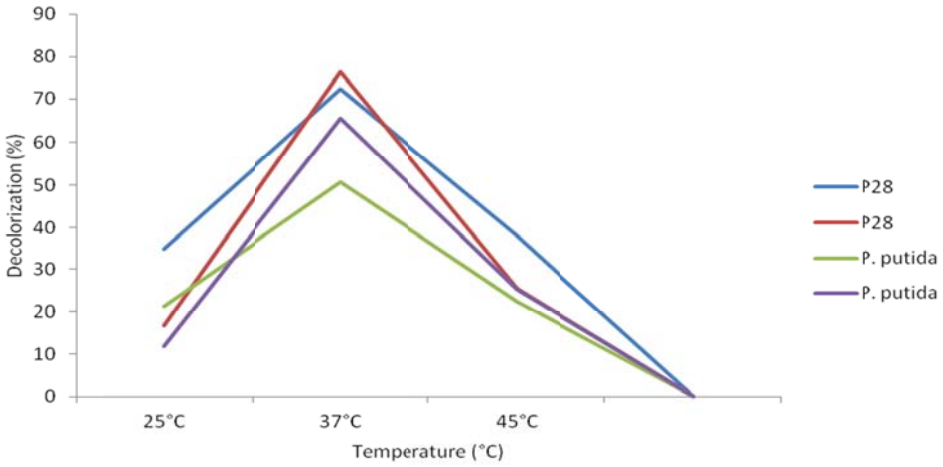

Figure 4. Effect of temperature on decolorization of Congo red and yellow HEGR.

decolorization activity, while decolorization of Congo Red dye by living cells appeared within 12 h of incubation. As seen in Figure 5, living cells of isolate *Lysobacter* sp. P28 showed 39.4 and 65.3% decolorization activity after 12 and 24 h of incubation (Figure 5). Maximum activity of 71.5% was observed in 72 h of incubation. Activity decreased to 70.6 and 70.2% in 84 and 96 h of incubation. In comparison with this, no activity was shown by autoclaved cells of *Lysobacter* sp. P28. Living cells of isolate *Lysobacter* sp. P28 showed 50.1% activity within 24 h of incubation and increased to 54.8, 56.3, 59.5 and 60.5% in 36, 48, 60 and 72 h, respectively. It showed maximum activity of 62.4% in 84 h of incubation. *P. putida* (MTCC 2445) showed maximum activity of 48.8% in 84 h of incubation with inoculums of living culture only. No activity was shown by autoclaved cells of *P. putida* (MTCC 2445). Maximal decolourisation was observed with the 9% inoculum size (Figure 7).

Effect of varying textile effluent concentrations

Effect of various concentrations of textile effluent (25, 50, 75 and 100%, v/v) on decolorization was studied. As shown in Figure 6, the decolorization percentage of selected isolates at 25% (v/v) concentration of textile effluent increased with increase in incubation period for each isolate. All the isolates showed decolorization and it increased variably till 96 h of incubation and after that slight decrease in decolorization was observed. It was found that in 24 h, isolate P28 showed 10.8% activity in 12 h, it increased to 28.2% in 48 h and maximum

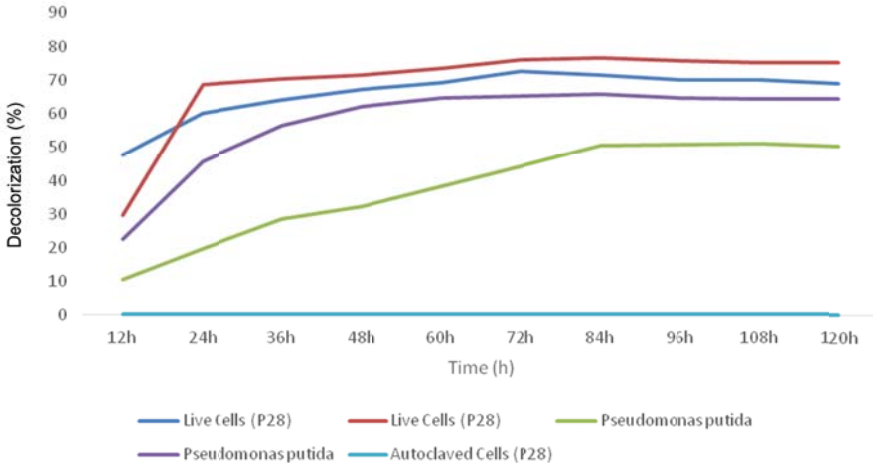

Figure 5. Decolorization of Congo Red and Yellow HEGR by live cells and dead cells (Autoclaved-killed) of Lysobacter sp. P28.

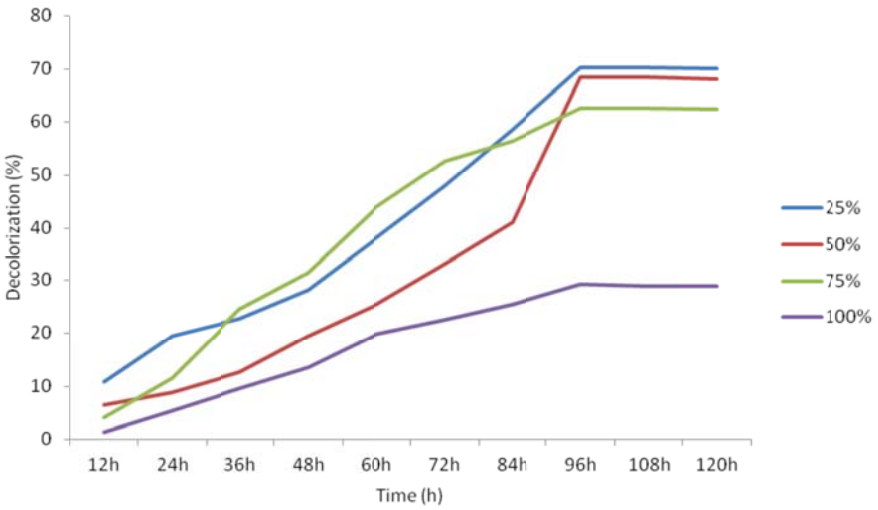

Figure 6. Effect of varying textile effluent concentrations on decolorization.

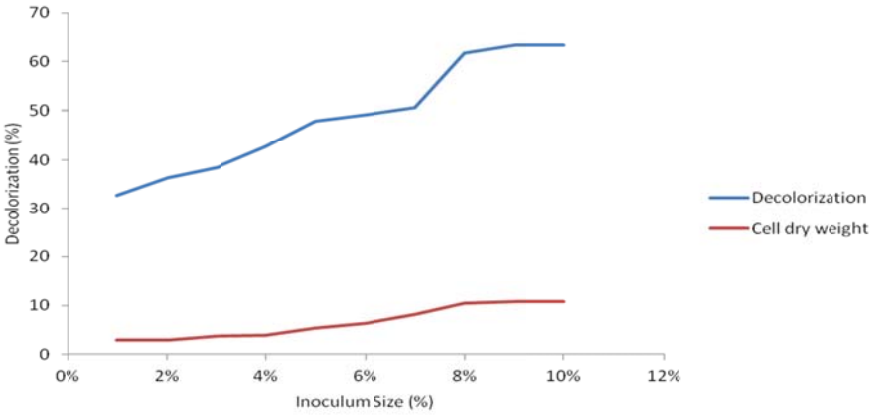

Figure 7. Effect of inoculum size on decolorization extent and dry weight of cells.

Table 2. Different parameters studied for germination treated with raw and bioremediated effluent.

Parameter	Raw effluent	Treated effluent	
		P28	*P. putida*
Germination (%)	30.3±0.67	83.1±0.77	70.9±0.61
Germination speed	3.03±0.51	7.30±0.48	5.16±0.44
Emergency index	2.0±0.40	8.3±0.32	7.10±0.51
Peak value	3.03±0.35	7.30±0.41	5.16±0.29
Vigor index	0.582±0.42	308.8±0.44	274.5±0.46
Shoot length (cm/seed)	0.067±0.32	3.710±0.55	3.110±0.44
Root length (cm/seed)	0.160±0.54	3.01±0.51	3.13±0.45
Wet root weight (mg/seed)	2.5±0.38	47.10±0.51	40.50±0.50
Wet shoot weight (mg/seed)	18.0±0.47	85.42±0.73	76.43±0.56
Dry root weight (mg/seed)	0.503±0.31	2.51±0.40	1.94±0.33
Dry shoot weight (mg/seed)	5.01±0.44	10.0±0.38	7.84±0.46

decolorization of 70.2% was observed in 96 h of incubation. Decolorization remained unchanged on 108 (70.2%) and 120 h (70.1%).

Effect of effluent/decolorized effluent on seed germination

Effect of raw effluent and treated effluent was studied and it was found that the seed germination rate was high (83.1 and 70.9) in the case of treated effluent as compared to the raw effluent. This is possibly due to the reduction in dyes concentration as degraded dye test organism (Table 2). Furthermore, vigor index, shoot length and root length were also observed better in the case of treated effluent.

Identification

Based on the phenotypic characteristics and phylogenetic analysis, strain P28 was identified as *Lysobacter*. Physiological characteristics were determined according to the procedures outlined in Bergey's Manual of Determinative Bacteriology.

Gene bank accession number

Phylogenetic position was carried out by searching the National Center for Biotechnology Information NCBI (BLAST). The 16S rRNA sequence of *Lysobacter* sp. P28 is availed under the GenBank accession number HQ316115.

Conclusions

In this study, a decolorizing bacterial strain, *Lysobacter* sp. P28, was isolated from textile industry effluent contaminated sites. *Lysobacter* sp. P28 showed decolorizing activity through a degradation mechanism rather than adsorption, and it could tolerate high concentrations (up to 200 mgL^{-1}) of Congo Red and Yellow HEGR. With high degradative and decolorizing activity against various azo dyes commonly used in the textile industries, it is proposed that *Lysobacter* sp. P28 has a practical application potential in the biotransformation of various dye effluents.

Conflict of interests

The authors did not declare any conflict of interest.

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