academicJournals

Vol. 7(12), pp. 983-989, 19 March, 2013
DOI: 10.5897/AJMR2013.2537
ISSN 1996-0808 © 2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Decolorization of methyl red by an isolated *Pseudomonas putida* strain MR1

Neetika Mathur* and Ashwani Kumar

Department of Botany, University of Rajasthan, Jaipur, Rajasthan- 302 004, India.

Accepted 8 March, 2013

The dye methyl red was completely decolorized at 24 h of incubation by *Pseudomonas putida* MR1 isolated from the dyestuff contaminated soil, collected from the textile industrial area of Sanganer, Jaipur, Rajasthan. Maximum decolorization was achieved when the isolate was incubated at 34°C and pH 7. The decolorization was confirmed by studying the spectral analysis of the dye. 16S rRNA partial gene sequencing (772 bp) of isolate *P. putida* MR1 was also performed and a phylogenetic tree was prepared.

Key words: *Pseudomonas putida* MR1, methyl red, decolorization.

INTRODUCTION

With the increasing use of a wide variety of dyes, pollution by wastewater contaminated with dyestuff is becoming increasingly alarming (Moosvi et al., 2005). Synthetic dyes are extensively used in textile dyeing, paper printing, colour photography, pharmaceutical, food, cosmetic and other industries (Rafii et al., 1990; Singh et al., 2012; Sahasrabudhe and Pathade, 2012). Approximately 10,000 different dyes and pigments are used industrially, and over 0.7 million tonnes of synthetic dyes are produced annually worldwide. Major classes of synthetic dyes include azo, anthroquinone and triaryl methane dyes, and many of them are toxic or contain carcinogenic compounds with long turnover times (Hartman et al., 1978). It has been estimated that 10 to 15% of dyes are lost in the effluent during dying processes (Zollinger, 1987; Olligaard et al., 1999; Mathur, 2012).

Colour is the first contaminant recognized in textile wastewater which affects aesthetics, water transparency and gas solubilities in water bodies (Faraco et al., 2009; Satyawali et al., 2009) and has to be removed before discharging the wastewater into a receiving water body (Vijaya and Sandhya, 2003).

Effluent discharged from the textile industries has variable characteristics in terms of pH, dissolved oxygen, organic and inorganic chemical content, etc. Pollution caused by dye effluent is mainly due to durability of the dyes in wastewater (Jadhav et al., 2007). Existing effluent treatment procedures utilize pH neutralization, coagulation followed by biological treatment, but they are unable to remove recalcitrant dyes completely from effluents. This is because of the color fastness, stability and resistance of dyes to degradation (Anjaneyulu et al., 2005).

Bioremediation is the microbial clean up approach which can transform various toxic chemicals to less harmful forms. Several reports suggest the degradation of complex organic substances, which can be brought about by bacterial enzymes like oxygenase (Ren et al., 2006), laccase (Hatvani and Mecs, 2001), lignin peroxidase (Shanmugam et al., 1999), etc. Many microorganisms capable of decolorizing the dyes include

*Corresponding author. E-mail: neetikamathur@yahoo.co.in. Tel: 91-9983797849.

Gram- positive and negative bacteria **(**Sani and Banerjee, 1999) and fungi (Balan and Monteneiro, 2001; Verma and Madamwar, 2005). Gram-negative bacteria are *Pseudomonas*, *Acinetobacter*, *Alkaligenes*, *Moraxella*, *Achromabacter* and *Flavobacterium* spp. The Gram-positives include all in the actinomycete line and they are *Mycobacterium*, *Nocardia*, *Rhodococcus* and *Arthrobacter* spp. (Alexander, 1994).

Pseudomonas putida is a rod-shaped, flagellated, gram-negative bacterium that is found in most soil and water habitats where there is oxygen. It grows optimally at 25 to 30°C and can be easily isolated. This bacteria is unique because it has most genes involved in breaking down aromatic or aliphatic hydrocarbons. Thus, researchers are attracted to using *P. putida* as the "laboratory 'workhorse' for research on bacteria-remediated soil processes" (Kowalski, 2002). There is great interest in sequencing the genome of *P. putida* due to its strong effect in bioremediation (Marcus, 2003).

The present study focuses on the use of *P. putida* strain MR1 isolated from the natural habitat of textile effluent environment, to degrade one of the frequently used textile dye, that is, methyl red. The effect of different physicochemical parameters on the growth and color removal were also studied.

MATERIALS AND METHODS

Isolation and identification

The textile effluent contaminated soil and sludge samples collected from textile industrial region of Sanganer, Jaipur, Rajasthan, were used for isolation of dye decolorizing culture in mineral base medium with dye methyl red. One gram different samples were used to inoculate the 100 mL of medium containing 10 mg of methyl red and the flasks were incubated in the orbital shaker at 37°C for 24 h. After 24 h of incubation in the liquid broth, the grown cells were streaked on the MBM agar plates with dye (100 mg L^{-1}) in order to obtain the isolated colonies. The plates were incubated for 24 h at 37°C. The dye decolorizing isolates were identified on the basis of the appearance of a clear zone around the colonies and purified by several streaking. The most promising bacterial isolate was used for further dye decolorizing studies. The isolate *P. putida* strain MR1 used in the present study is a Gram positive rod. Identification of isolate as *P. putida* strain MR1 was done by 16S r-RNA sequencing at Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh and deposited in Genbank with accession number MTCC 10,014. Basic Local Alignment Search Tool (BLAST) was also performed. The phylogenetic tree was constructed by neighbour-joining method.

Growth of pure culture of *Pseudomonas* was maintained on the mineral base medium with composition of: mineral base 200 ml/L; yeast extract 2.0 g/L; 100 mg/L of methyl red dye; trace element solution and thiamine HCl solution (300 µg/100 ml) and pH 7.0. Optimum temperature for growth was 37°C. The composition of final medium include: mineral base 200 ml/L; yeast extract 2.0 g/L and 100 mg/L of methyl red dye. The final volume was adjusted to 1000 ml with distilled water. The pH of the medium was kept at 7.0. The medium was autoclaved at 121°C for 20 min and then supplemented with 100 ml/L of previously autoclaved trace element solution having the composition (g/L): $CoCl_2$ 0.2; H_3BO_3 0.3; $ZnSO_4.7H_2O$ 0.1; $MnCl_2.4H_2O$ 0.03; $Na_2MoO_4.H_2O$ 0.03; $NiCl_2.6H_2O$ 0.02; $CuCl_2.2H_2O$ 0.01 and 100 ml/L of autoclaved and filtered thiamine HCl solution (300 µg/100 ml).

Chemicals

All chemicals used were of analytical grade. The methyl red was obtained from Hi Media, India.

Decolorization procedure

The flasks containing mineral base medium (100 mL each) and methyl red (10 mg) were inoculated using loopful of isolate *P. putida* strain MR1. The uninoculated flask containing mineral base medium (100 mL) and methyl red (10mg) was taken as control. These flasks were incubated at 37°C for 24 h. The decolorization was determined by measuring the difference between optical density of control and inoculated flasks at 473 nm. The decolorization (%) was calculated as:

Decolorization (%) = (Absorbance of control – observed absorbance) / absorbance of control × 100.

The dye decolorization efficiency of the isolate *P. putida* strain MR1 was also tested against other various dyes which included malachite green and some of the textile dyes like Red RH, Brown GR, Yellow FG, etc.

Study of physicochemical parameters

The isolated microbial strain *P. putida* MR1 was cultivated in liquid media under different physiological conditions to study the growth kinetics and also the decolorization process. These parameters included temperature, pH, dye concentration, addition of various carbon sources (sucrose, glucose, lactose, sodium acetate and starch) in the media, etc. The spectrophotometric analysis was carried out to study the effect of various parameters on the growth and the decolorizing ability of the isolate. The mineral base medium without the dye was used as a blank.

Effect of temperature

The effect of different incubation temperatures on growth and decolorization process was studied by keeping inoculated flasks, at different temperatures in the range of 30 to 40°C together with the control (0.1 g/L dye) for 24 h incubation.

Effect of pH

The effect of medium pH on the growth and decolorization efficiency of the isolate was investigated in the pH range of 6.5 to 7.5.

Effect of dye concentration

To find out the most appropriate concentration of dye that could be decolorized in a shorter duration. The concentration of the dyes used was 50, 100 and 200 mg/L, respectively. These flasks were

Table 1. Spectrophotometric analysis of different dyes used by the isolate.

Dye (0.1 g/L)	Wavelength (nm)	Absorbance		Decolorization (%)
		Control	isolate	
Methyl red	472	3.9133	No peak	100
Malachite green	619	2.3064	0.6252	72.89
Yellow FG	416	2.1210	2.0303	4.27
Red RH	314	1.7792	1.4821	16.69
Ponceau S	353	2.8929	2.8004	3.19
Brown GR	468	1.6187	1.5007	7.28

then incubated at 37°C.

Effect of various carbon sources

The decolorization efficiency of the isolate using methyl red (100 mg L^{-1}) was also evaluated in the presence of different carbon sources like glucose, lactose, starch, sodium acetate and sucrose in the mineral base medium at a concentration of 1g/L keeping all other parameters constant.

Growth kinetics

The isolate was also characterized in terms of their growth profile at optimal conditions in which the various parameters such as the increase in the cell biomass and the exact time of decolorization, etc, was monitored.

RESULTS

Isolation and identification

Soil and sludge samples collected from contaminated sites around various dye industries in Sanganer, Jaipur were used for isolation of dye decolorizing culture in mineral base medium at pH 7.0. The isolate was Gram negative rod shaped bacteria. The partial nucleotide base sequencing (1529 base pairs) of 16S rRNA of isolate was done at Institute of Microbial Technology (IMTECH), Chandigarh. Basic Local Alignment Search Tool (BLAST) search (Table 3) for sequence homology at GenBank (www.ncbi.nlm.nih.gov) was also performed which showed that the bacteria had 100% homology with *P. putida* strain BASUP87 16S ribosomal RNA gene partial sequence, *Pseudomonas monteilii* strain SB3091 16S ribosomal RNA sequence, *Pseudomonas* sp. J4(2008) 16S ribosomal RNA sequence and *Pseudomonas* sp. BJQ-D4 16S ribosomal RNA, *P. putida* strain LH-R1 16S ribosomal RNA gene and 99% identity with *Pseudomonas* sp. HB01 gene for 16S ribosomal gene partial sequence.

A phylogenetic tree (Figure 1) suggests that the isolate shows very near evolutionary relationship with *Pseudomonas oryzihabitans* IAM 1568T (D84004). Thus, the isolate was identified as *P. putida* strain MR1 and deposited at Microbial Type Culture Collection and Gene Bank (MTCC), IMTECH, Chandigarh, with accession number MTCC 10,014.

Decolorization performance

The isolate *P. putida* strain MR1 could decolorize the dye methyl red as well as some other dyes to an appreciable extent as shown in Table 1. The effect of various physicochemical parameters like pH, temperature, carbon source on decolorization of dye methyl red by the isolate was studied in mineral base medium with 100 mg/l methyl red.

Effect of temperature

The incubation temperature affected the growth and activity of the *P. putida* strain MR1. Based on the results of Figure 2, the maximum decolorization was obtained when the isolate was incubated at 34°C followed by 37°C. A very low efficiency of decolorization was obtained under lower and higher temperature of incubation.

Effect of pH

It was observed that maximum growth and maximum decolorization was achieved at pH 7.0. Even below and above the neutral pH, the isolate was able to grow and decolorize the methyl red (Figure 3).

Effect of various methyl red concentrations

The rate and extent of decolorization were affected by increasing concentrations of dye ranging from 50 to 200 mg/L. The spectrophotometric analysis (scanned in a

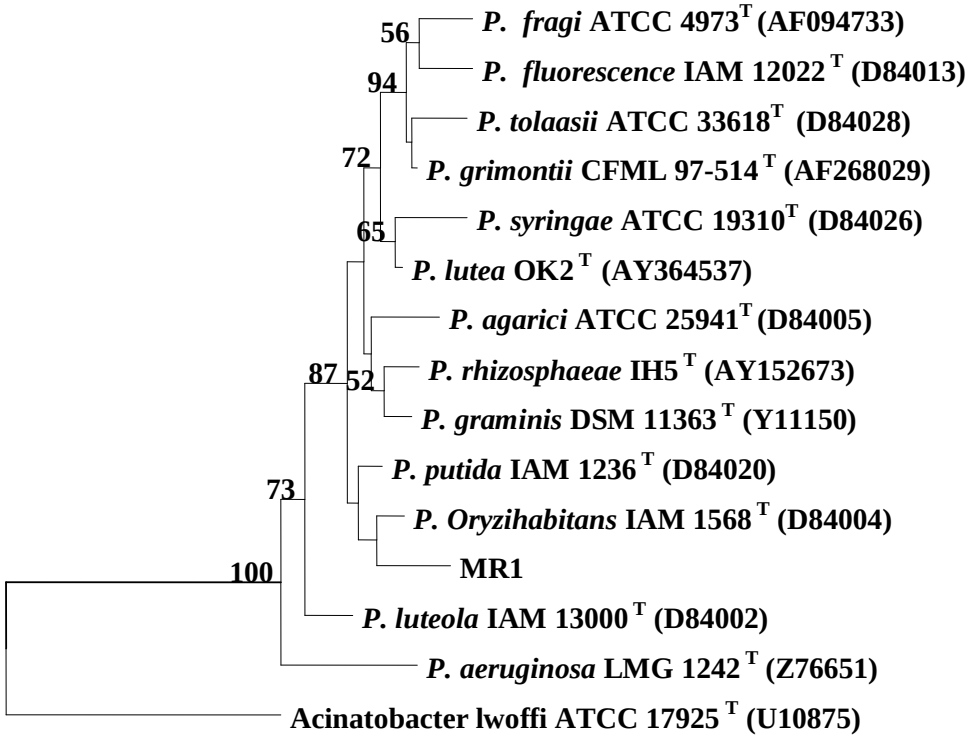

Figure 1. Phylogenetic tree of *P. putida* MR1.

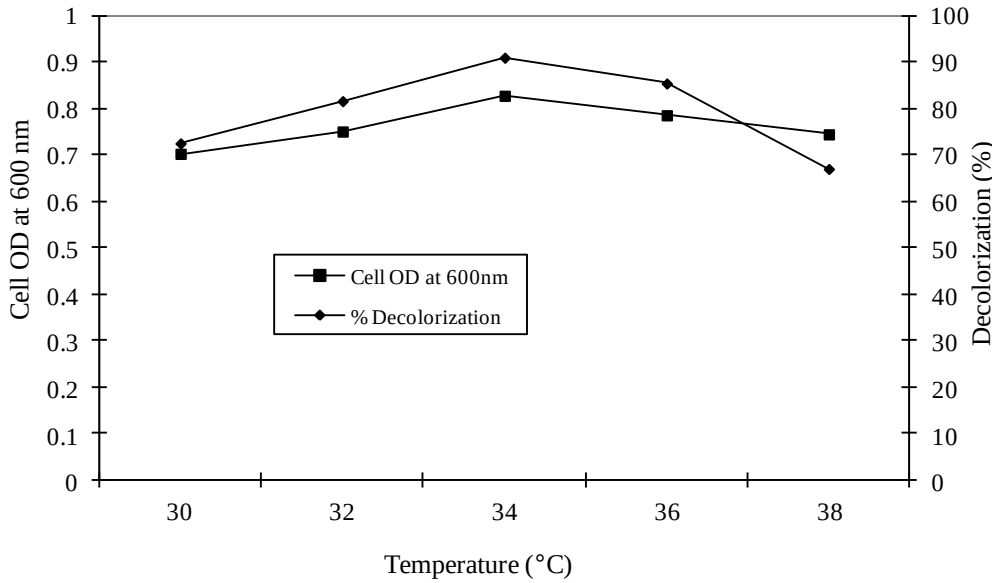

Figure 2. Effect of temperature on cell growth and decolorization of methyl red (100 mg/L).

spectrophotometer under the range of 200 to 600 nm) revealed that the maximum decolorization (99.65%) of the dye was seen at dye concentration of 100 mg/l within 24 h, whereas lesser decolorization of 92.72 and 63.67% was seen at dye concentration of 50 and 200 mg/l, respectively.

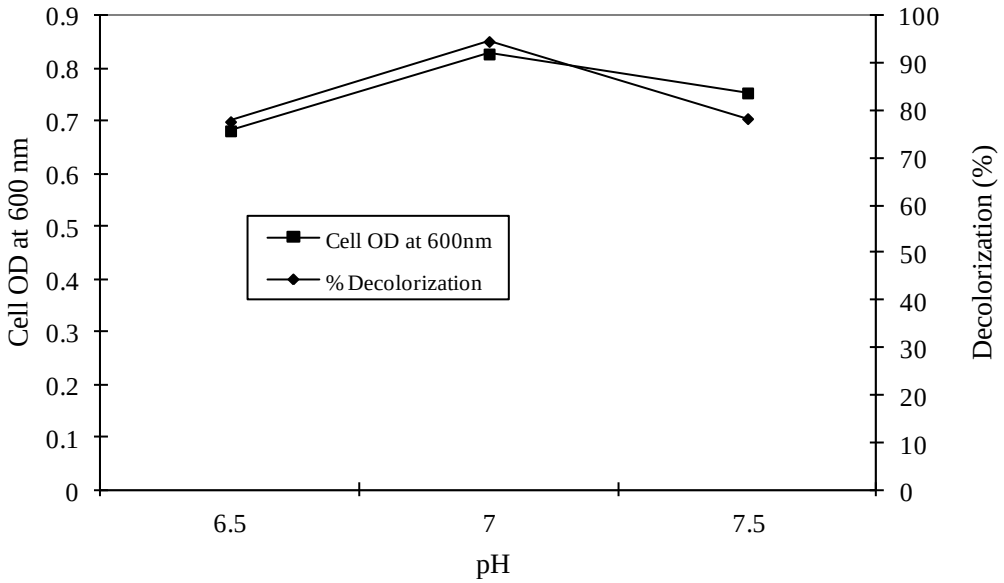

Figure 3. Effect of pH on cell growth and decolorization of methyl red (100 mg/L).

Table 2. Decolorization of methyl red by *P. putida* strain MR1.

Time (h)	Methyl red (control) 472 nm	Absorbance 472 nm	Decolorization (%)
6	3.2148	3.0679	4.57
9	3.2148	1.5686	51.21
12	3.2148	0.5461	83.01
18	3.2148	0.4224	86.86
24	3.2148	Not detected	100
36	3.2148	Not detected	100
48	3.2148	Not detected	100

Effect of various carbon sources

While trying to enhance decolorization performance of methyl red, extra carbon sources were added in the medium. The spectrophotometric analysis revealed that the percentage decolorization was maximum (95.39%) when no extra carbon source was added in the medium and 94.99 and 92% decolorization was seen with glucose and sucrose, respectively, while less decolorization was seen with starch (88%) and lactose (86%).

Growth profile of isolate

It was observed that with increase in incubation time, the decolorization efficiency of the isolate increased and the dye methyl red was completely decolorized at 24 h of incubation (Table 2) while the cell biomass also increased up to 12 h and then decreased (Figure 4).

DISCUSSION

The isolate *P. putida* strain MR1 could decolorize the dye methyl red (no peak) and some other dyes like Yellow FG (4.27%), Red RH (16.69%), Ponceau S (3.19%) and Brown GR (7.28%). The observed variation in percent decolorization of different dyes by the isolate was attributed to the difference in structure and complexity of each dye (Zimmermann et al., 1982; Sani and Banerjee, 1999; Khehra et al., 2004).

Different physicochemical parameters like temperature, pH, dye concentration and carbon source influence decolorization of textile dyes by the isolate. In our present investigation, the optimum pH and temperature required for the efficient decolorization of dye methyl red by the

Table 3. NCBI BLAST: Nucleotide Sequence 772 letters of isolate *P. putida* MR1.

GU396283.1	*Pseudomonas putida* strain BASUP87 16S ribosomal RNA gene, partial sequence	1426	1426	100%	0.0	100%
GU191925.1	*Pseudomonas monteilii* strain SB 3091 16S ribosomal RNA gene, partial sequence	1426	1426	100%	0.0	100%
EU372973.1	*Pseudomonas* sp. J4(2008) 16S ribosomal RNA gene, partial sequence	1426	1426	100%	0.0	100%
GQ284476.1	*Pseudomonas monteilii* strain PCWCW7 16S ribosomal RNA gene, partial sequence	1426	1426	100%	0.0	100%
GQ284471.1	*Pseudomonas* sp. PCWCW2 16S ribosomal RNA gene, partial sequence	1426	1426	100%	0.0	100%
GQ284465.1	*Pseudomonas* sp. TSWCW20 16S ribosomal RNA gene, partial sequence	1426	1426	100%	0.0	100%
AB456678.1	*Pseudomonas* sp. TIS1-127 gene for 16S ribosomal RNA, partial sequence	1426	1426	100%	0.0	100%
FJ032013.1	*Pseudomonas* sp. CTN-2 16S ribosomal RNA gene, partial sequence	1426	1426	100%	0.0	100%
FJ600361.1	*Pseudomonas* sp. BJQ-D4 16S ribosomal RNA gene, partial sequence	1426	1426	100%	0.0	100%
EU439420.1	*Pseudomonas putida* strain LH-R1 16S ribosomal RNA gene, partial sequence	1426	1426	100%	0.0	100%
EU103629.2	*Pseudomonas taiwanensis* strain BCRC 17751 16S ribosomal RNA gene, partial sequence	1426	1426	100%	0.0	100%
AM911664.1	*Pseudomonas* sp. RW2S1 partial 16S rRNA gene, strain RW2S1	1426	1426	100%	0.0	100%
AM911645.1	*Pseudomonas* sp. RD9PR3 partial 16S rRNA gene, strain RD9PR3	1426	1426	100%	0.0	100%
AM911633.1	*Pseudomonas* sp. RD5PR1 partial 16S rRNA gene, strain RD5PR1	1426	1426	100%	0.0	100%
AM911630.1	*Pseudomonas* sp. RD3SR3 partial 16S rRNA gene, strain RD3SR3	1426	1426	100%	0.0	100%
AM911629.1	*Pseudomonas* sp. RD3SR2 partial 16S rRNA gene, strain RD3SR2	1426	1426	100%	0.0	100%
AM911625.1	*Pseudomonas* sp. RD1PR2 partial 16S rRNA gene, strain RD1PR2	1426	1426	100%	0.0	100%
DQ060242.1	*Pseudomonas putida* 16S ribosomal RNA gene, partial sequence	1426	1426	100%	0.0	100%
AJ785569.1	*Pseudomonas* sp. Wt/3 partial 16S rRNA gene	1426	1426	100%	0.0	100%
DQ301785.1	*Pseudomonas* sp. PHD-8 16S ribosomal RNA gene, partial sequence	1426	1426	100%	0.0	100%
HQ270550.1	*Pseudomonas putida* strain GPSD-19 16S ribosomal RNA gene, partial sequence	1424	1424	99%	0.0	100%
AM911666.1	*Pseudomonas* sp. RW7P2 partial 16S rRNA gene, strain RW7P2	1424	1424	99%	0.0	100%
AM911639.1	*Pseudomonas* sp. RD7SR2 partial 16S rRNA gene, strain RD7SR2	1424	1424	99%	0.0	100%

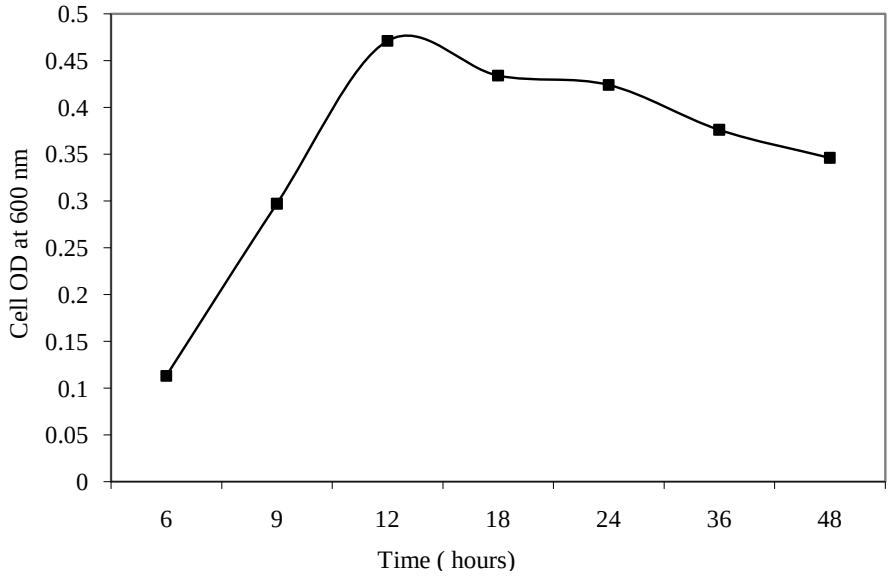

Figure 4. Growth profile of the isolate in methyl red.

isolate *P. putida* strain MR1 in the liquid culture was 7.0 and 34°C, respectively.

Normally, dye decolorizing bacteria have a narrow pH range (Chen et al., 2003; Moosvi et al., 2005). Presently, it was found that maximum growth of isolate and maximum decolorization (94.63%) was achieved at pH 7.0. This is in agreement with the studies previously conducted on degradation of methyl red by Adedayo et al. (2004) and Verma and Madamwar (2005).

Increase in temperature proved to have a positive effect on the growth of the isolate and methyl red decolorization, which was maximum (0.828 and 91%, respectively) at 34°C. These observations could be attributed to the increase in enzyme activity and growth increase with the temperature (Asad et al., 2006). However, further temperature increase proved to be quite limiting for the growth and related decolorization of methyl red by the isolate.

The isolate decolorized methyl red maximally (99.65%) at dye concentration of 100 mg/l. Further increase in dye concentration resulted in decrease in the percentage of decolorization and cell growth. This might be due to toxicity of dye through the inhibition of metabolic activities (Asad et al., 2006).

Conclusion

The dye methyl red was completely decolorized by a bacterium isolated from the soil samples collected from textile dyeing industrial region of Sanganer, Jaipur, Rajasthan. The isolate was identified as *P. putida* strain MR1 by MTCC, IMTECH, Chandigarh. The isolate decolorized dye methyl red within 24 h of incubation. Maximum decolorization was achieved at temperature 34°C and pH 7. The partial gene sequence of 16S rRNA, BLAST search and phylogenetic tree of the isolate were also made.

ACKNOWLEDGEMENT

This work was supported by the grant from Council of Scientific and Industrial Research (CSIR), New Delhi.

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