

Biodegradation of halogenated phenol by immobilized phototrophic bacterium *Rhodopseudomonas palustris*.

Kushalatha M^{1*} and C S Karigar²

¹Department of Biochemistry, Maharani Lakshmi Ammanni College for Women, Malleshwaram, Bangalore 560012 INDIA

²Department of Biochemistry, Bangalore University, Bangalore - 560001 INDIA

Abstract

A phototrophic bacterial culture isolated from the contaminated soil around wood preserving industries was found to assimilate 2-chlorophenol (2-CP). The isolated bacterium was identified as *Rhodopseudomonas palustris* based on its morphological, biochemical and FAME-GC analysis. The cells were harvested in their log phase, immobilized in alginate and agar matrices. The immobilized cells were supplied with 0.6% (v/v) 2-CP to study the biodegradation potential. The cells were able to degrade 2-CP of about 95%. The degradation rate of 2-CP was monitored at 275nm using UV-Visible spectrometer at regular time intervals. The decrease in concentration of 2-CP and accumulation of metabolite was observed in the spectrum. The metabolites produced during degradation were characterized by TLC and GC-MS analysis. The immobilized cells were subjected to repeated batch culture technique. Both agar and alginate matrices were stable with degradation ability for 7 repeated cycles without decreasing its efficiency.

Keywords: *Rhodopseudomonas palustris*; 2-chlorophenol; UV-Visible spectra; immobilized cells

Introduction

Commercially produced 2-Chlorophenol (2-CP) is a chemical used entirely as an intermediate in the production of other chemicals. It is a basic chemical feedstock in the manufacture of higher chlorophenols for such uses as fungicides, slimicides, bactericides, antiseptics, disinfectants, and wood and glue preservatives [7]. 2-CP is also used to form intermediates in the production of phenolic resins, and has been utilized in a process for extracting sulfur and nitrogen compounds from coal. There are believed to be no significant natural sources of 2-CP. Volatility and ability to bioaccumulate gives rise to concern that 2-CP may be a global pollutant

2-CP has been shown to impair the flavor of the edible portions of fish at very low concentrations in aquatic life. 2-CP is a suspected carcinogen that may also have reproductive effects. Excessive exposure to 2-CP may affect the eye, skin and the unborn child. [12]

Immobilization of microbial cells has received increasing interest in the field of wastewater treatment [11]. Immobilized cells systems have the potential to degrade toxic chemicals faster than conventional wastewater treatment systems since high densities of specialized microorganisms are used in immobilized cell systems. Among the various cell immobilization methods that are available, entrapment in sodium alginate, and agar beads had been chosen for its ease of use, low cost, low toxicity and high operational stability.

The objectives of this study were to characterize the biodegradation of 2-CP using immobilized microbial cells of *Rhodopseudomonas palustris*.

Materials and Methods

Bacterial isolation and characterization

The bacterium degrading 2-CP was isolated from industrial effluent by enrichment culture technique. The bacteria was purified by adopting standard microbiological procedures and characterized according to the Bergey's Manual of Determinative Bacteriology [4] and fatty acid methyl ester analysis (FAME-GC) analysis.

Media and growth conditions

The culture was grown in mineral salt medium (MSM) in screw cap bottles. The MSM was supplemented with 2-CP (0.2% v/v) as an electron donor after autoclaving the medium. The bacterial culture medium (MSM) comprised (g/L) of NaHCO₃ (2.0), NH₄Cl (2.0), NaCl (2.0), MgSO₄ (0.4), K₂HPO₄ (0.4), Yeast extract (0.02%) and peptone (0.04%). NaNO₂ (2.0) served as an electron acceptor under microaerobic conditions. Bacterium was maintained as liquid cultures by transfer to fresh medium weekly [5], [6].

Immobilization of microorganisms

The 2-CP degrading bacteria were harvested in their log phase of growth from 1 liter of culture medium (MSM). The cell pellet obtained by centrifugation of the broth at 5000 rpm for 10min at 4°C, was used in immobilization experiments. Various methods and matrices were utilized for immobilization. Each one of these is described below.

Alginate entrapment of cells

Alginate entrapment of whole cells was carried out according to [1]. Alginate (4% w/v) was dissolved in boiling water and autoclaved at 121°C for 15 min. A 50ml bacterial cell suspension (1g wet weight cells in 2ml) was added to 10ml of sterilized alginate solution and mixed by stirring over a magnetic stirrer. This alginate-cell mixture was extruded drop by drop in to a cold, sterile 0.1M calcium chloride solution through a burette. The gel beads formed were left in the solution for one hour before being filtered off. The beads were washed with distilled water and used for further experiments.

Agar entrapment of cells

Agar entrapment of cells was carried out according to [11] using 4% (w/v) agar saline solution. 2.0ml of bacterial cell suspension (1g wet cell weight in saline) was added to 10ml agar 4% (w/v) solution, with continuous magnetic stirrer. The beads were prepared as described for alginate entrapment but with paraffin oil as immobilizing phase instead of CaCl₂. These agar-cell beads were washed successively with distilled water and saline finally for further applications.

Batch culture of immobilized cells

A 10g of the alginate or agar immobilized beads containing 1.6×10^8 cfu g^{-1} of beads were transferred to 250ml serum bottles containing 125ml of mineral salt medium to which 0.6% (v/v) 2-CP was supplemented. Bacterium was cultured microaerobically in serum bottles. Samples of the culture broths were taken at regular time intervals. The decrease in concentration of the 2-CP was determined as described in Standard Methods for the Examination of Water and Wastewater [9].

Repeated batch culture of immobilized cells

For establishing the long term stability of the catabolic process of 2-CP degradation in the matrices by immobilized cells, repeated batch fermentations were carried out. After every incubation period of 5 days, the spent medium was decanted and beads were washed with sterilized water and transferred into a fresh MSM supplemented with 2-CP. Immobilized beads were incubated further under the similar conditions as mentioned above.

Results

The microorganism degrading 2-CP was isolated from industrial effluent by enrichment culture technique. The bacteria were identified as *Rhodopseudomonas palustris*. (Data not shown)

Immobilization of *Rhodopseudomonas palustris*

The immobilized *R. palustris* cells (Fig.1) mediated the degradation of 2-CP in batch cultures (Fig.2 & 3). The alginate gel beads were found to be stable for more than 115 days as observed using repeated batch cultures of the immobilized cells. This also demonstrated that the 2-CP metabolism in these cells occurs even at concentrations between 0.4 to 0.8% (v/v) which is higher than toxic concentrations. 2-CP metabolism rates in both cases on continuous measurement for 115 days showed the accumulation of metabolite in the spent broth.

The agar immobilized *R. palustris* cells were however stable and retained the degradability for 105 days. Hence alginate is good matrix for the immobilization of 2-CP degrading bacterial cells in comparison to agar as immobilization matrix (Fig.4).



Fig.1. *Rhodospseudomonas palustris* degrading 2-CP immobilized in alginate matrix

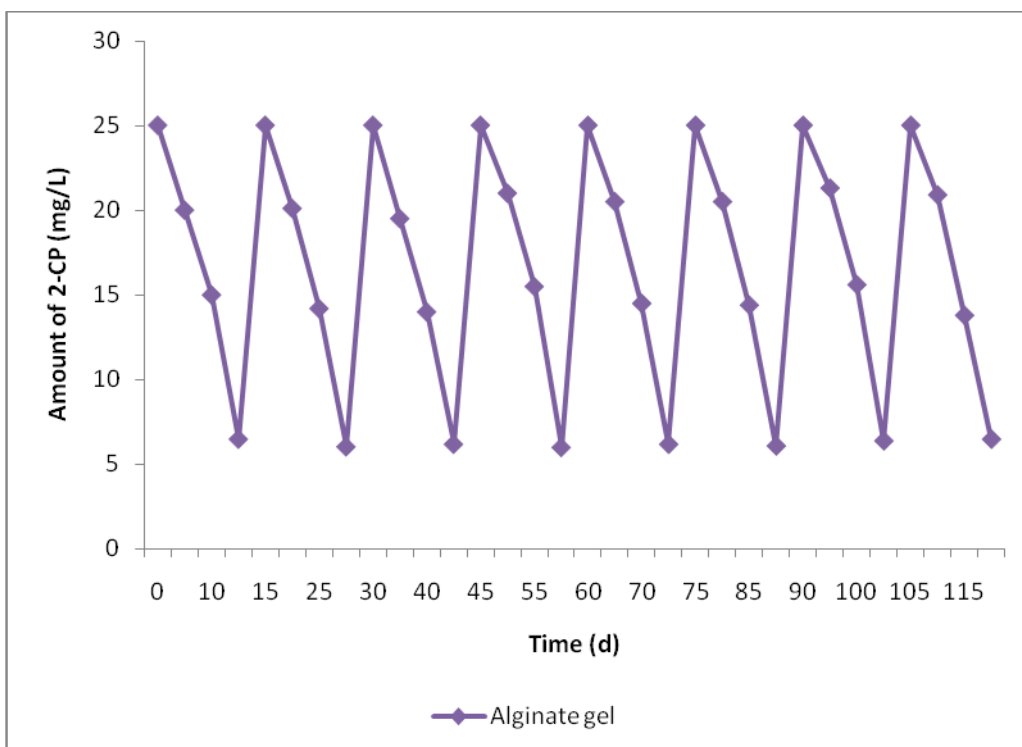


Fig.2. Degradation of 2-chlorophenol by *R. palustris* entrapped in alginate

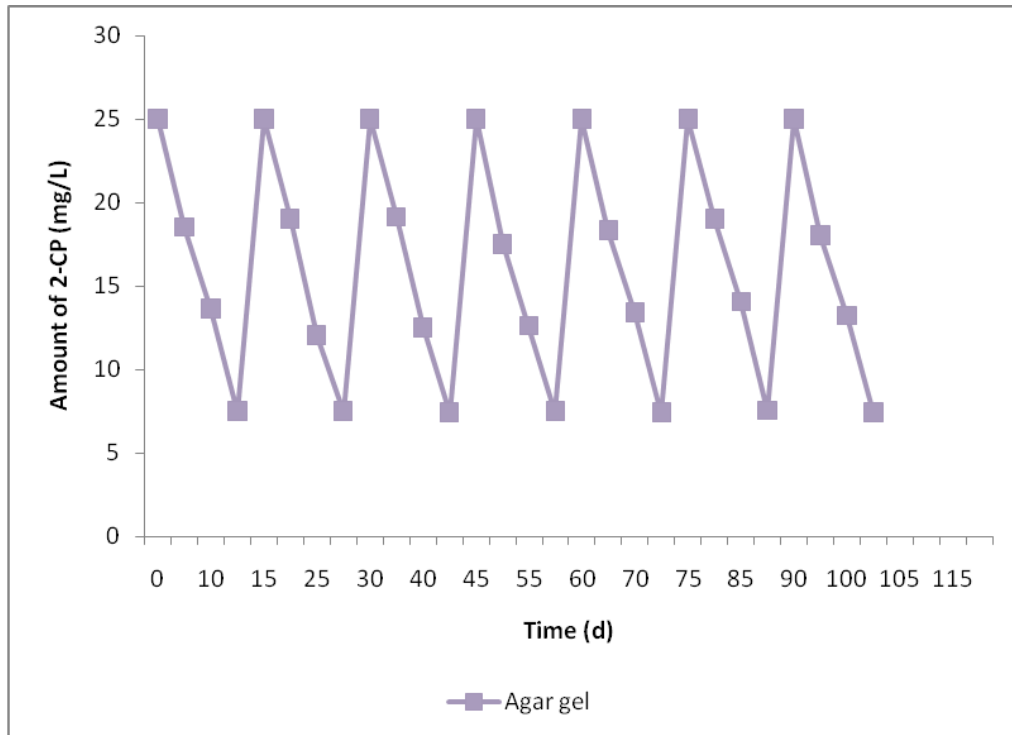


Fig.3. Degradation of 2-chlorophenol by *R. palustris* entrapped in agar

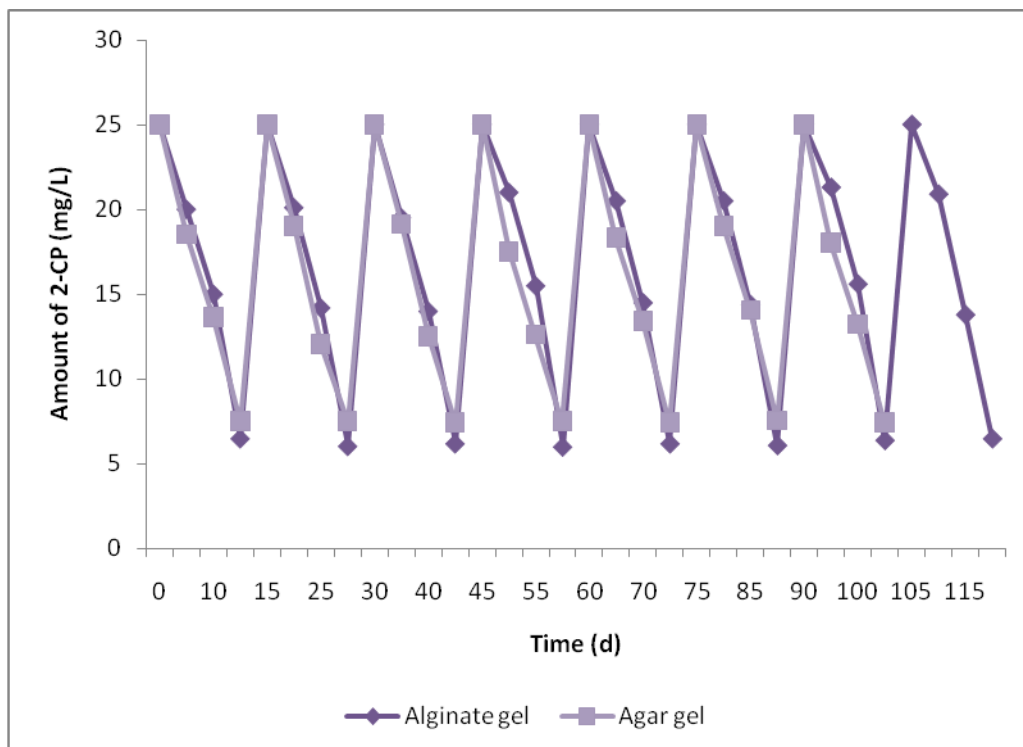


Fig.4. Comparison of the degradation of 2-chlorophenol by *R. palustris* entrapped in alginate and agar

Characterization of metabolites formed during the biodegradation of 2-chlorophenol by *Rhodopseudomonas palustris*.

Metabolites that accumulated in the spent medium during the growth of *R. palustris* on 2-CP were isolated and characterized. The thin layer chromatography (TLC) analysis demonstrated the accumulation of a metabolite with an R_f value of 0.62 that corresponded to phenol on co-chromatography (Data not shown).

This result established that phenol was one of the intermediary metabolite during the degradation of 2-CP by *R. palustris*. The identity of the metabolite was further confirmed by its GC-Mass spectrum (Data not shown). The involvement of this product in catabolism of 2-CP was further supported by its utilization studies in replacement studies.

The degradation of 2-CP by *R. palustris* was also monitored by following the change in its absorbance maxima (275 nm) consequent to microbial growth at different incubation times. The 2-CP concentration greatly decreased with increasing incubation periods and also showed accumulation of metabolites (Fig.5).

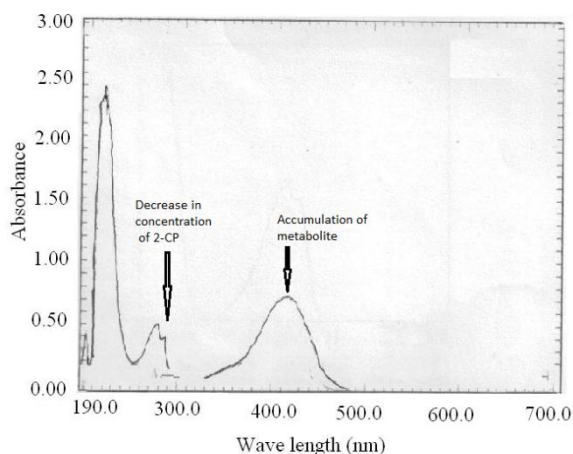


Fig.5. UV-Visible absorption spectra of organic extract of *R. palustris* grown spent medium

Discussion

Immobilization of microbial cells shows good prospects for improving the efficiency of certain bioprocesses. Compared with the use of suspended cells, immobilization has several potential advantages: (1)

simple and more reliable biomass retention, (2) increased volumetric biodegradation rate through the accumulation of high biomass, (3) high dilution rate without washout, and (4) enhanced retention and stability of slow-growing microorganisms, especially when growth rates are slowed down by inhibition. Biodegradation using immobilized cells has been widely investigated for several toxic compounds, including chlorophenol [10]. The immobilization of bacterial culture was more useful compared to free cells. However, the substrate toxicity is the major limiting factor in chlorophenol degradation by free cell culturing methods. This could be overcome by employing the immobilized cells. The immobilized cells have an important advantage of being employed as the components of bioreactors to treat the pollutants. In such instances the cells are not released to the environment and they also facilitate degradation of toxic pollutants at high concentrations, as may be observed in nature resulting due to the industrial effluent disposal practices. However, for immobilized cells the degradation rate was much higher than that of free cells. The immobilized cells could degrade a higher substrate concentration more efficiently and reached maximum degradation rates as compared to the free cells [2].

Our studies on the immobilization of 2-CP degrading *R. palustris* in alginate and agar as immobilizing matrices revealed that alginate matrix is more promising with respect to gel bead stability as well as the degrading ability of substrate in comparison to agar matrix. The experiments on 2-CP metabolism with the two gel bead types demonstrated the accumulation of phenol in the spent medium, thereby suggesting that rate of 2-CP metabolism in immobilized cells would drastically increase than those of the free cells. The higher 2-CP degradation rates in case of immobilized cells was probably due to the higher cell density as well as cells being exposed to the toxic higher substrate concentrations for long period and thus cells must have been adapted to degrade the high concentration of substrates in their environment. Entrapment of microbial cells in alginate has been well studied, since it is easy to perform, inexpensive and non toxic for organisms.

Several previous studies have shown the degradation of 2-CP by immobilized cells. Biodegradation of 2-CP was studied using immobilized activated sludge. Stoichiometric amounts of free chloride detected at the end of the experiments confirmed mineralization of 2-CP [8]. 2, 4-dichlorophenol was removed efficiently by immobilized pure culture and mixed culture of *Bacillus insolitus* [13]. A cell-free culture broth of *Phanerochaete chrysosporium* immobilized in polyurethane foam has been evaluated for the biodegradation of

chlorophenols [3]. Biodegradation of xenobiotics like 2-CP by alginate entrapped microbial cells have been reported [2].

The gel beads stability as well as 2-CP degradation capacity of alginate and agar immobilized *R. palustris* cells were studied. The immobilized cells mediated degradation of 2-CP in batch cultures. The alginate gel beads were found to be stable for more than 115 days as observed using repeated batch cultures of the immobilized cells. This also demonstrated that the 2-CP metabolism in these cells occurs even at concentrations between 0.4 to 0.8% (v/v) which is higher than toxic concentrations. The agar immobilized cells were however stable and retained the degradability only for 105 days. Hence alginate is good matrix for immobilization of bacterial cells in comparison to agar matrix. The reason for the low biodegradation activity of agar immobilized cells may be that an oily phase was used during the gel bead preparation by the interphase technique, and the oil adsorbed by gel beads may also result in impeding of the degradation rates for 2-CP. Moreover, the gel bead-forming procedure was complex and ineffective, which made them less suitable for industrial biodegradation processes [14]. 2-CP degradation rates of immobilized cells have been attributed due to the higher cell densities and cell adaptations. The immobilized *R. palustris* cells can be used for at least 7-8 times.

On a comparative basis of the 2-CP degradation rates between free *R. palustris* cells and immobilized, the immobilized cells were approximately 10 fold higher. The immobilized cells also carried out maximum degradation of substrate at higher concentration than that of free cells, suggesting that the immobilized cells are resistant to higher toxic concentrations of 2-CP. Thus immobilized cells were better degraders of 2-CP than the free cells. Thus, the potential of the immobilized bacterial cells can be harnessed for developing biotechnologies for decontamination of 2-CP from the polluted sites as well as water bodies.

References

- [1]. Battmann H, Rehm H.J., (1984). The degradation of phenol by polymer entrapped microorganisms. *Appl. Microbiol. Biotechnol.* 20: 285-290.
- [2]. Chen F, Cui M, Fu J, Sheng G, Sun G, Xu M (2003) Biodegradation of quinoline by freely suspended and immobilized cells of *Comamonas* sp. strain Q10. *J. Gen. Appl. Microbiol.* 49:123-128.

- [3]. Choi SH, Seung-Hyeon Moon, Man Bock Gu (2002) Biodegradation of chlorophenols using the cell-free culture broth of *Phanerochaete chrysosporium* immobilized in polyurethane foam. *J. Chemical Tech. and Biotech.* 77:999-1004.
- [4]. Holt J. G., Kreg N. R., Sneath P. H. A., Staley J. T and Williams S. T., (1994) Berge's Manual of Determinative Bacteriology, Ninth edition. Williams and Wilkins, Baltimore. http://141.150.157.117:8080/prokPUB/chaphtm/239/06_02.htm
- [5]. Kushalatha M, Vidya G and Karigar C S, (2011) Biodegradation of 2-chlorophenol by *Bacillus subtilis* isolated from industrial sludge The Bioscan; *Inter-National J. Life Sciences* 6: 305-309 [ISSN 0973-7049].
- [6]. Kushalatha Mutharasaiah, Vidya Govindareddy, and Karigar Chandrakant (2012) Biodegradation of 2-chlorophenol by *Rhodopseudomonas palustris*. *Bioremediation Journal* 16:1- 8. [ISSN 1088-9868][IF-0.93]
- [7]. Muller F and Caillard L (1986) Chlorophenols. In Ullmann's Encyclopedia of Industrial Chemistry. 5th ed., Vol. A7. W.Gerhartz et al. (Ed.). VCH Publishers, New York, N.Y.
- [8]. Sofer Gordon SS, Lewandowski A, Lodaya MP, Lakhwala FS, Yang KC, Manjari singh (1990) Biodegradation of 2-chlorophenol using immobilized activated sludge *Research J. Water Pollu.* 62:73-80.
- [9]. Standard Methods for the Examination of Water and Wastewater 1998 eds. Greenberg, A.E., Clesceri, L.S. & Eaton, A.D., 20th edn. APHA, AWWA & WEF. ISBN 0875532357
- [10]. Sun Xiaoran and Shan Zhongjian (2007) Removal of Di-*n*-butyl Phthalate Using Immobilized Microbial Cells. *Chin. J. Chem. Eng.* 15:167-171.
- [11]. Suneetha P, Manjunath NH, Karigar CS (2008) Degradation of ethylbenzene by free and immobilized *Pseudomonas fluorescens*-CS2. *Biodegradation.* 19:137-144
- [12]. US-EPA (1980) Ambient water quality criteria for 2-chlorophenol. United States Environmental Protection Agency, Office of Water Regulations and Standards Criteria and Standards Division Washington D.C. 20460. EPA 440/5-80-034.

- [13]. Wang CC, Lee CM, Kuan CH (2000) Removal of 2, 4-dichlorophenol by suspended and immobilized *Bacillus insolitus*. *Chemosphere*. 41:447-452.
- [14]. Xia Wang, Zhonghui Gai, Bo Yu, Jinhui Feng, Changyong Xu, Yong Yuan, Zhixin Lin, Ping Xu (2007) Degradation of carbazole by microbial cells immobilized in magnetic gellan gum gel beads. *Appl. Environ. Microbiol.* 73: 6421-6428.