INT. J. BIOL. BIOTECH., 7 (4): 403-408, 2010.

BIODEGRADATION OF PHENOL USING CELLS AND CELL -FREE EXTRA CELLULAR CRUDE ENZYME PROTEIN PRODUCES BY NOVEL STRAIN OF *PSEUDOMONAS AERUGINOSA*

Sadia Sirajuddin, Omm-e-Hany, Moazzam Ali Khan and Suriya Jabeen

Institute of Environmental Studies, University of Karachi -75270, Pakistan.

ABSTRACT

Aromatic contaminants of the environment, to which belongs phenol and its derivatives, are toxic and in the most of the cases hard to degrade. A strain belonging to the genus *Pseudomonas* was characterized by high effectiveness of growth in different concentrations of phenol, which was used as carbon and energy source. The isolate of the novel strain of *P.aeruginosa* was used to minimize time for degrading environmental samples containing high content of phenol. Phenol utilization rates were estimated from the amount of phenol degraded. Experimental results offer the opportunity to compare the results of novel strain of *P.aeruginosa* cells, and cell-free extra cellular crude enzyme protein. The use of extra cellular and/or cell-free crude enzymes has been also proposed as an innovative remediation technique. They can offer some advantages over the use of microbial cells. The highest removal efficiencies 99% within 6h in terms of quantity of phenol removed were achieved when treating with cell-free crude enzymes, biodegraded phenol more rapidly than cells. The use of enzymes was desirable because they can perform the same function as many harsher chemicals. Protein concentration in cell-free crude enzymes was determined and was about 55µgm/ml. The Molecular weight of novel strain of *P.aeruginosa* cell-free crude enzymes protein separated by SDS-PAGE was approximately 51 kDa. Detoxification by enzymes rather than by the whole microbial cells is practically beneficial because enzymes sometime can tolerate environmental extremes better than the whole microbial cells.

Key words: Phenol, Cell Free Extra cellular crude enzyme protein, Microbial cells, Biodegradation, SDS-PAGE

INTRODUCTION

Aromatic compounds used as herbicides, pesticides, preservatives, solvents, and lubricants constitute a major class of environmental contaminants that are acted upon by bacteria. Phenol is one of the most common representatives of toxic organic compounds even at low levels. Most of the industrial effluents with high phenol levels (up to several grams per liter) come from petroleum refineries, phenolic resin production, plastic and coke oven industries *(*Gonzalez *et al.*, 2001*).* These effluents can be treated by physicochemical methods, such as activated carbon adsorption (Nakhla *et al*., 1990), solvent extraction (Patterson, 1975), chemical oxidization (Wang, 1992), and biological degradation. Among them, biodegradation is often the least expensive. Simple phenol could be removed aerobically (Sack and Bokey, 1979; Hughes and Cooper, 1996). Biodegradation is environmental friendly and cost effective, and is an increasingly important process in pollution control (Autenrieth *et al*., 1991). In the last two decades, several researchers have studied the use of enzymes in wastewater treatment Enzymes have many potential advantages over conventional biological treatment and/or chemical oxidation. Several extracellular enzymes are able to remediate phenol contamination. The use of extracellular enzymes has been standard in many industries for enhancing bioremediation. (Timothy *et al*., 2006) Enzymes can be used to ameliorate harsh conditions and harsh chemicals, thus saving energy and preventing pollution. Extracellular enzymes are able to increase the degradation rate of already biodegradable substances, allowing for more efficient treatment processes (Whitely *et al*., 2002). The use of enzymes is desirable because they can perform the same function as many harsher chemicals, such as solvents, but at a neutral pH, a moderate temperature, and without production of hazardous waste. Although enzymes tend to be expensive due to the extraction and purification costs, they can be very cost effective because they minimize waste disposal and heating needs (Gianfeda and Rao, 2004; Godfrey and Reichelt, 1996). The present study was conducted to explore the potential of bacterial strain for the biodegradation and biotransformation studies of phenol.

MATERIALS AND METHODS

ISOLATION AND MAINTENANCE OF PHENOL DEGRADING BACTERIAL CULTURE

The bacterial culture capable of degrading phenol was isolated from soil using enrichment technique, with varying concentration of phenol in the medium was inoculated into 250 ml of Nutrient containing 100-300 mg/l phenol. The flasks were incubated on a shaker operating at 120 rpm for 48 h at ambient temperature 25 °C. At daily interval one loopful of enrichment culture from the flasks was streaked on to N.A plates supplemented with phenol

and incubated at 35 °C for 24 h. Individual colonies were subculture into N.A plates containing phenol until pure cultures were isolated. Bacterial isolates that can handle relatively high concentration of phenol were subjected to morphological, cultural and biochemical tests. Colonies of interest were transferred from nutrient agar plate to nutrient broth medium containing different concentration of phenol and incubated at 35 °C for 24-48 h. Growth of the isolate was determined by viable cell enumeration immediately after inoculation. The isolated pure bacterial strain was also streaked on N.A slant, after incubation at 35°C for 24 h the culture was maintained at 4°C. The bacterial culture was sub cultured after every three months and used for growth and biodegradation studies.

ADAPTATION OF BACTERIAL ISOLATES FOR PHENOL DEGRADATION

A phenol degrading novel strain of *P. aeruginosa* developed at the Institute of Environmental Studies University of Karachi was inoculated into 250 ml N.B supplemented with different concentration of phenol and incubated for 48 h at 35°C. The resulting dense culture was streaked on nutrient agar plates supplemented with different concentration of phenol and incubated for 24-48 h. The potential isolate was stored at 4°C on slopes of nutrient agar containing 1 mg/l phenol.

ANALYTICAL METHODS

Phenol was determined spectrophotometrically by the modified 4-aminoantipyrin Standard Methods for the Examination of Water and Wastewater (APHA, 1985). 1ml of a sample from the test nutrient broth flask, 1 ml of 1 % (w/v) potassium ferricyanide and 1 ml of 1 % (w/v) 4-aminoantipyrine were added. The optical density of the resulting mixture was read at 500 nm with a Shimadzu UV-VIS 1201 spectrophotometer. Phenol concentration was determined with a calibration curve made from known phenol standard.

REAGENTS

The phenol used in the present study was reagent grade and purchased from Merck suppliers. Other reagents used were NH_4OH (Conc.), phosphate buffer solution (pH adjusted to 6-8), 4- aminoantipyrin solution and Potassium ferricyanide K_3 [Fe (CN) $_6$]

PREPARATION OF CELLS FREE EXTRACT (CFE)

For preparation of CFE novel strain of *P. aeruginosa* was cultivated in nutrient broth for 24 h and harvested by centrifugation at 6,000 rpm for 20 min .The pellets was suspended in 20 ml of distilled water and broken by sonication 10 times, with 30-s intervals Unbroken cells were removed by centrifugation at 6,000 rpm for 20 min and the supernatant was used as cell free extract crude enzyme protein.

PROTEIN DETERMINATION

Protein concentration was determined by the method of Lowry *et al*., (1951), BSA used as the standard, protein was detected with Shimadzu UV-VIS 1201 spectrophotometer at 750 nm.

BIODEGRADATION OF PHENOL

1% novel strain of *P. aeruginosa* cells, 1% CFE, inoculated into 250ml N.B flasks were exposed to different concentration of phenol (100, 200, 300 mg/l) one by one and analyzed the phenol degradation by measuring optical density at 500 nm. Flasks were incubated at shaking water bath 35°c 120 rpm for 48 h.

STATISTICAL ANALYSIS

For statistical analysis standard error of mean was calculated and Analysis of variance (ANOVA) was performed.

SDS- PAGE

Sodium dodecyle sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli, (1970) with 10% acrylamide gel. Stained were carried out with 0.2% coomassie blue, 7.5%acetic acid, 5% Methanol. The sample were dissolved in Sample diluting buffer (SDB) containing tracking dye, heated for 3 min and applied to the slab gel, using BSA protein as standard.

MOLECULAR WEIGHT ESTIMATION

The Molecular weight of protein separated by SDS-PAGE was estimated by comparison with protein standard of known molecular weight. The protein standard used was BSA (66kDa).

RESULTS AND DISCUSSION

The work was conducted to produce reliable bioremediation data and to develop a better understanding of the microbial enzymes treatment process.

PHENOL DEGRADATION STUDIES

This study was aimed to determine the degradation of phenol using shaking water bath. Data taken during the study to assess the performance of novel strain of *P. aeruginosa* for phenol degradation are presented and discussed.

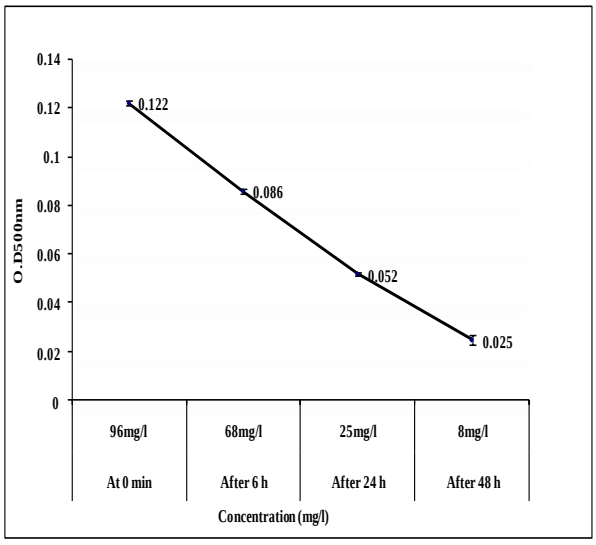

Fig.1. Degradation of phenol (100mg/l) using cells.

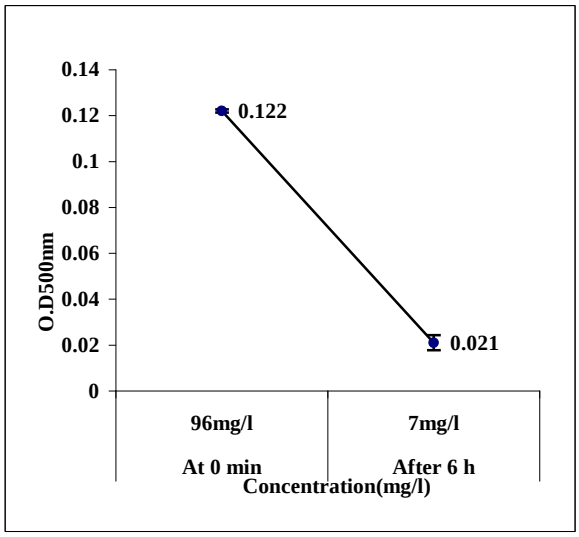

Fig.2. Degradation of phenol (100mg/l) using cell free extract crude enzyme.

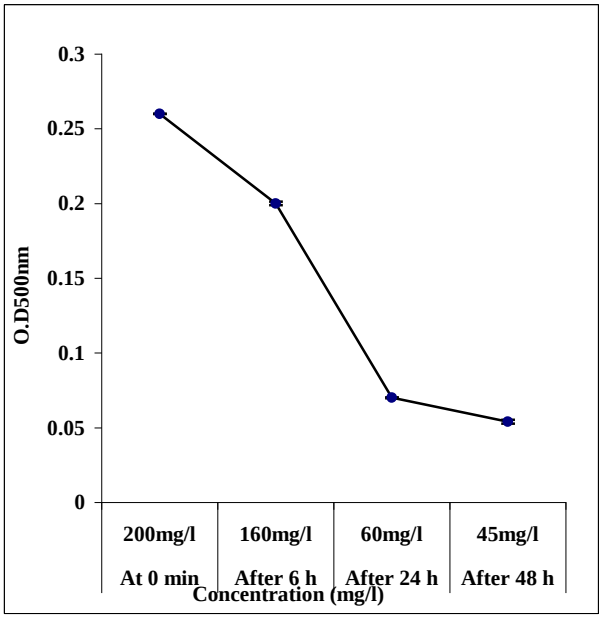

Fig.3. Degradation of phenol (200mg/l) using cell

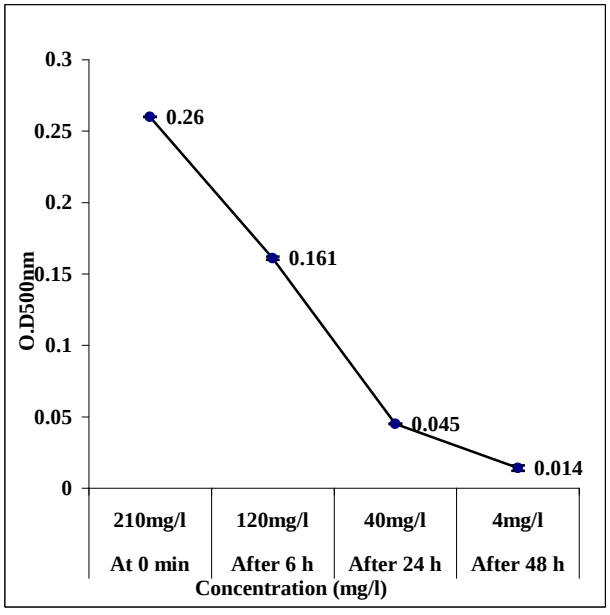

Fig.4. Degradation of phenol (200mg/l) using cell free extract crude enzyme

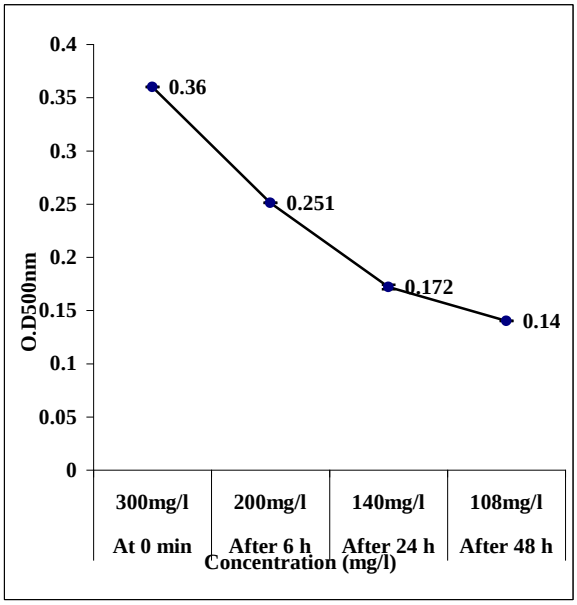

Fig.5 Degradation of phenol (300mg/l) using cells

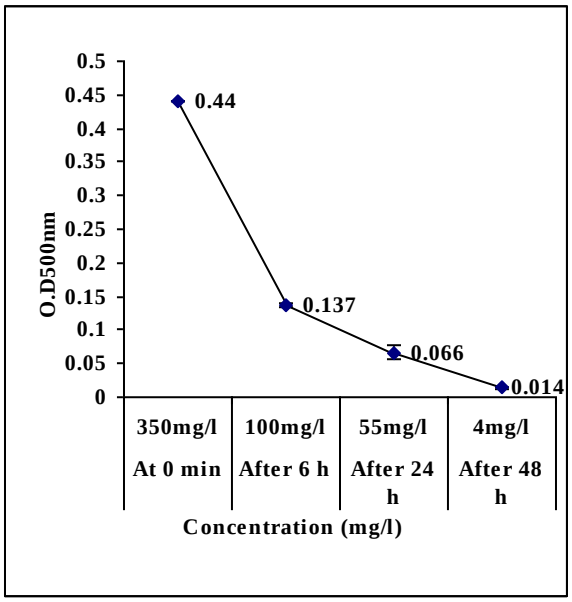

Fig.6 Degradation of phenol (300mg/l) using cells free extract

Table 1. Percentage Degradation of phenol.

Phenol Conc. (mg/l)	Cells	Cell free extract crude enzyme
100	91	99
200	77	99
300	64	99

COMPARISON OF PHENOL DEGRADATION ABILITY

Bacteria belonging to the genus *Pseudomonas* spp. are highly oxidative, aerobic and metabolically versatile and have been reported to degrade aromatic hydrocarbons, oil, petroleum products, pesticides. (Choudhary *et al*., 1988; Kimbara *et al.*,1989).The phenol utilization rates were estimated from the amount of phenol degraded. The results of a phenol biodegradation experiment are shown in Fig 1 to 6. Experimental results offer the opportunity to compare the results of free cells and cells free extract of novel strain of *P. aeruginosa* for biodegradation kinetics of phenol. The highest removal efficiencies, in terms of quantity of phenol removed were achieved when treating with cells free extract biodegraded phenol more rapidly than cells. The more striking difference in biodegradation kinetics of phenol was by using cells free extract supplemented with different concentration (100, 200, 300 mg/l) of phenol. Rapid degradation was observed at 100mg/l in first 6hrs was about 99%. By using cells free extract shown in Fig. 2. Studies on phenol toxicity to bacteria have shown that bacteria can adapt to ambient phenol concentration but increasing phenol concentration appear to decrease the overall phenol biodegradation (Abd-el-Haleem *et al*., 2002). Extra cellular enzymes have been shown to be very effective in transforming and removing phenol. Cell free extract crude enzyme can offer several advantages over the use of microbial cells. The most significant features of cell-free crude enzymes are their unique substrate-specificity and catalytic power; their capability to act in the presence of many toxic, even recalcitrant, substances, and/or under a wide range of environmental conditions, often unfavorable to active microbial cells (i.e. relatively wide temperature, pH and salinity ranges, high and low concentrations of contaminants); and their low sensitivity or susceptibility to the presence of predators, inhibitors of microbial metabolism, and drastic changes in contaminant concentrations. Extra cellular enzymes enhance bioremediation are secreted from organisms during a metabolic activities and possess the ability to break down bonds within organic compounds and/or catalyze their transformation into less toxic and more biodegradable forms. Enzymes of novel strain of *P. aeruginosa* have been shown to be effective degrade different concentration of phenol. Enzymatic treatment to be feasible options for bioremediation the use of enzymes was effective because the transport of phenol

into whole microbial cells can be problematic, such membrane transport problem could be avoided when soluble enzymes are employed in disposal process. Detoxification by enzymes rather than by the whole microbial cells is practically beneficial because enzymes sometime can tolerate environmental extremes better than the whole microbial cells. Enzymatic technology is very promising, it has limitations. Microbes can reproduce and increase their population in order to consume a large amount of substrate, but extracellular enzymes cannot. Enzymes cannot reproduce themselves, meaning that any increase in enzyme population must come from outside of the system (i.e., humans adding more enzymes to the system). It has also been shown that enzymes may actually lose some reactivity after they interact with pollutants and could eventually become completely inactive (Gianfreda and Rao, 2004).

MOLECULAR WEIGHT ESTIMATION

This is done by SDS-PAGE of protein are shown in Table 2 and Fig.2. The *Rf* is calculated as the ratio of the distance traveled by the molecule to that traveled by a marker dye-front. The crude enzymes protein band of novel strain of *P. aeruginosa* visible in gel provides an effective tool for the identification of the proteins involved in the metabolic pathway. The molecular weight of the enzymes protein was approximately 51 kDa and the molecular weight of BSA was 66 kDa used as standard. On the basis of this research novel strain of *P. aeruginosa* extra cellular enzymes was considered to have a good potential for application in remediation of phenol contaminated environment and improvement of phenol removing treatment of industrial wastewater.

Table 2. Estimation of molecular weight.

	Protein	Molecular weight (kDa)	Distance travelled (cm)	*Rf*
Standard	BSA	66	3.7	0.61
Sample	Cell free extract crude enzyme protein	51	4.6	0.76

Fig.7. SDS –Page, Lane 1, 3, 5, 6 shows the bands of BSA protein used as standard, Lane 2, 4, shows the bands of cell free extract crude enzyme protein of novel strain of *P.aeruginosa*.

REFERENCES

APHA (1985). Standard methods for the examination of water and Wastewater. XVIth ed. America. Health Association, Washingtomn, D.C., 00.556.

Choudhary G. R., A. N. Ali and W.B. Wheeler (1988). Isolation of methyl parathion degrading *Pseudomonas sp* that possess DNA homologous to the opd gene from a flavobacterium sp. *Appl.Environ.Microbial*., 54 (2): 288-293.

Abd-El-Haleem, D., H. Moawad, E.A. Zaki and S. Zaki (2002). Molecular characterization of phenol degrading bacterial isolated from different Egyptian ecosystem. *Microb Ecol*., 43:217-224.

Flock, C., A. Bassi and M. Gijzen (1999). Removal of aqueous phenol and 2-chlorophenol with purified soybean peroxidase and raw soybean hulls. *J. Chem. Technol. Biotechnol*., 74, 303–309.

Gianfreda, L. and M.A. Rao (2004). Potential of extra cellular enzymes in remediation: A review. *Enzyme Microb. Technol*., 35, 339–354.

Godfrey, T. and J. Reichelt (1996). *Introduction to industrial enzymology. Industrial enzymology:* The application of enzymes in industry.

González G., G. Herrera, M.T. García and M. Peña (2001). Biodegradation of phenolic industrial wastewater in a fluidized bed bioreactor with immobilized cells of Pseudomonas putida. *Bioresour Technol*., 80(2): 137-42.

Haribabu E., Y.D. Upadhya and S.N. Upadhyay (1993). Removal of phenols from effluent by flash ash. *Inst. J. Environ.Studies*., 43:169-179.

Hughes, S. and D. Cooper (1996). Biodegradation of phenol using the self-cycling fermentation process. *Biotechnology & Bioengineering*, 51 (1): 112-119.

Kimbara, K., T.Hashimoto, M. Fukuda, T. Koana, M. Takagi, M. Oishi and K. Yano (1989). Cloning and sequencing of 2 tendem gene involved in polychlorinated biphenyl degrading soil bacterium *Pseudomonas* sp. Strain KKS102. *Jour.Bacteriol*., 171 (5): 2740-2747.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227:680-685.

Lowry O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem*., 193, 265.

Nakhla G. F., M.T.Suidan and J.T. Pfeffer (1990). Control of anaerobic GAC reactors treating inhibitory wasteeaters. *J. Water Pollution Control*., 62(1): 65-72.

Patterson, J. W (1975) *Wastewater treatment technology*. Ann Arbor Science Publishers, Ann Arbor, Mich.

Autenrieth, R.L., J.S. Bonner, A. Akgerman and E.M. McCreary (1991). Biodegradation of phenolic wastes. *J. Hazardous Materials*., 28: 29–53.

Sack W. A. and W.R. Bokey (1979). Biological treatment of coal gasification wastewater. *Proc., 33rd Industrial Waste Conf. Purdue Standard Methods for the Examination of Water and Wastewater,* XVIth ed, American Public Health As-sociation,Washington., DC 1985, p. 556.

Timothy, P., Ruggaber, M. ASCE, W. Jeffrey, P.E. Talley and M. ASCE (2006). Enhancing Bioremediation with Enzymatic Processes. Practice Periodical of Hazardous. *Toxic, and Radioactive Waste Management*, 10 (2): 73-85.

Wang Y. T. (1992). Effect of chemical oxidation on anaerobic biodegradation of model phenolic compounds. *Water Environ. Res*., 64: 268-273.

Whiteley, C. G., P. Heron, B. Pletschke, P.D. Rose, S. Tshivhunge, F.P. Van Jaarsveld and K. Whittington-Jones (2002). The enzymology of sludge solubilisation utilising sulphate reducing systems: The properties of protease and phosphatases. *Enzyme Microb. Technol*., 31: 419–424.

(*Accepted for publication September 2010*)