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SELECTIVE AMPLIFICATION OF CATECHOL 2, 3 DIOXYGENASE GENE FROM PHENOL DEGRADING *ALCALIGENES SP* D_2 ISOLATED FROM SOIL

ASHIQ.M[1], INDU C NAIR [2], SAJUDEEN.P.A AND JAYACHANDRAN.K.*[3]

[1,3]School of Biosciences, Mahatma Gandhi University, Kottayam, Kerala, India 686560.
[2]SASSNDP Yogam College Konni, Pathanamthitta, Kerala, India- 689691

ABSTRACT

Catechol 2, 3-Dioxygenase transforms catechol by meta cleavage to 2-hydroxymuconate semialdehyde. The meta pathway is functional in the microbial degradation of phenol and many such organic compounds. In the present study an attempt was made to isolate and purify catechol 2, 3- dioxygenase from a phenol degrading *Alcaligenes* sp d_2. The enzyme in the culture extract was precipitated at 80% ammonium sulphate concentration and was dialysed overnight. The dialysed fraction was subjected to gel filtration using Sephadex G-100 using phosphate buffer of 50mM concentration and pH 7.5. The three fractions with an elution volume of 7.5ml carrying the enzyme were pooled, concentrated and the purity of the enzyme fraction was confirmed by PAGE. An attempt was also made to selectively amplify the gene encoding catechol 2,3 dioxygenase. The amplified product appeared in the Agarose gel was corresponding to a molecular weight of 200bp.

KEY WORDS: **Biodegradation, Catechol 2,3, dioxygenase, meta pathway,ortho cleavage**

JAYACHANDRAN.K.
School of Biosciences, Mahatma Gandhi University,
Kottayam, Kerala, India 686560.

Corresponding author

INTRODUCTION

Phenol is one of the important harmful aromatic compounds and is present in various industrial wastewaters. The main sources of phenol include the effluents of coal gasification, polymeric resin production, oil refining and paper pulp production.[5] Even at low concentration, phenol is toxic to aquatic flora and fauna. Phenol is toxic upon injection, contact or inhalation and is lethal to fish even at concentrations as low as 5 mg/ L [12]. Phenol imparts objectionable taste and odour to drinking water. Phenol pollution is a serious environmental problem. Many technologies have been evaluated for removing and for degrading the phenolic compound in wastewater. They include adsorption, biodegradation, oxidation using UV radiation etc. Among these biodegradation has received much attention. A wide variety of microorganisms are known to be capable of metabolising many of the organic pollutants or chemicals [4]. They include bacteria, fungi and actinomycetes. Bacteria like *Bacillus, Pseudomonas, Acinetobacter ,Achromobacter* and fungi like *Fusarium* sp.,*Phancrocheate chrysosporium, Coriolus versicolor, Streptomyces sp*. etc are proved to be efficient groups in phenol biodegradation. These microorganisms suffer from substrate inhibition at higher concentrations of phenol. [11]

Pathways for the aerobic biodegradation of aromatic compounds are diverse, but they usually involve the formation of Catechols or substituted catechols as common intermediates [2]. Catechol and its substituted derivatives are degraded through one of the two aromatic ring cleavage pathways, Ortho or Meta cleavage. Catechol 1, 2- dioxygenase (C12DO: EC 1.13.11.1) catalyses the ortho (intradiol) cleavage, while catechol 2,3-dioxygenase (C23-DO: EC 1.13.11.2) catalyses the meta (extradiol) cleavage. Functional genes encoding C12-DO and C23-O are suitable markers to detect a broad range of bacteria which degrade aromatic compounds as they are essential for breaking down the common intermediates. C2,3-DO genes also have a well characterized phylogeny that allows the systematic design of dioxygenase specific primer.

Catechol 2,3 dioxygenase (C23-DO) is a member of the superfamily of extradiol dioxygenases and catalyses the ring cleavage of catechol and substituted catechols. Therefore, C2, 3-DO is the key enzyme of many bacterial pathways for the degradation of aromatic compounds. This ring cleavage reaction has been studied intensively and a general mechanism for oxidative cleavage of catechol by extradiol dioxygenases has been proposed. The two atoms of the oxygen molecule are incorporated into the catechol substrate on two adjacent carbon atoms of the aromatic ring, one of which already carries a hydroxyl substituent of the diol and the other of which is unsubstituted. As with other extradiol dioxygenases, C2, 3-DO requires ferrous ions for its activity. At the active site, a single ferrous ion binds with the substrate and the oxygen to participate in the catalytic cycle. In the present study the C23- DO isolated from a phenol degrading *Alcaligenes* sp d_2 was partially characterized [9]. The selective amplification of C23-DO gene was also attempted using designed primers.

MATERIALS AND METHODS

Microorganism used in the study

Microorganism used in the study was phenol degrading *Alcaligenes* sp d_2 [8].The organism was available in the culture collection of School of Biosciences, Mahatma Gandhi University. The culture was maintained in both nutrient agar and phenol contained media. Sub culturing was done once in two weeks.

Medium

A synthetic medium MSPM(Mineral Salt Phenol Medium) which utilizes phenol as the sole carbon source is used here with the following composition: KH_2PO_4-0.1g, $(NH)_2SO_4$-0.1g, $MgSO_4.7H_2O$-0.05g,$CaCl_2$-0.0001g, phenol-100ppm, Distilled Water-100 ml [8].

Inoculum preparation.

One loopful of the culture was inoculated to 50ml nutrient broth containing 40mM phenol.

The flask was incubated at room temperature on an orbital shaker set at 150rpm. After overnight incubation the cells were harvested by centrifugation for 10 minutes at 10000 rpm. The pellets were collected and diluted using physiological saline (0.86% NaCl) till the OD becomes 1.This was used as the inoculum at 3 % concentration for biodegradation studies and enzyme assay.

Extraction of Catechol 2,3 dioxygenase

100 ml of the MSPM was inoculated with the above inoculum and incubated for 32 hours. The cell free supernatant was collected after 32 h of incubation by centrifuging at 10000 rpm for 10 minutes at 4°C. This was taken as crude enzyme extract.

Estimation of Catechol 2, 3 dioxygenase (C 23-DO)

For estimation of enzyme 300µl of extract was mixed with 700µl of 10 mM l^{-1} catechol solution in 50 mM l^{-1} phosphate buffer (pH 7.5) at 25°C and the increase in the absorbance at 375 nm wavelength was monitored. Enzyme activity was expressed as specific activity (units per mg protein): 1 unit of activity was defined as the amount of enzyme required for the formation of 1µg of respective product from catechol (375 nm, ε = 33000 mol^{-1}) [10] .Protein concentration was determined by Lowry's Method [6].

Purification of catechol 2, 3 dioxygenase.

Purification process involved ammonium sulphate precipitation, dialysis and gel filtration.80% ammonium sulphate was used for precipitation and the precipitant was dialysed at 4^0 C in an ice bath. The dialysed sample was subjected to gel filtration with 50mM phosphate buffer (pH 7.5) using G 100 sephadex(height of the column-10cm,diameter- 3cm)

Isolation of Bacterial Genomic DNA.

Bacteria from a saturated liquid culture are lysed and the proteins were removed by digestion with Proteinase K. Cell wall debris, polysaccharides, and remaining proteins were removed by selective precipitation with CTAB.High-molecular weight DNA was recovered from the resulting supernatant by isopropanol precipitation [3]

Detection of specific gene using the primer corresponding to Catechol 2, 3 Dioxygenase.

PCR detection of C 23 -DO genes was carried out using the following Primer.

Table 1
Primer for the amplification of C 2, 3-DO genes.

Primer	Sequence
2,3CAT- F	5' – CGA CCT GAT CTC CAT GAC CGA -3'
2,3CAT- R	5' – TCA GGT CAG CAC GGT CA -3'

PCR Condition for the Amplification of C 23- DO genes

Optimization of PCR conditions using primers 23CAT-F and 23CAT-R was attempted for *Alcaligenes* sp d_2. The PCR temperature program began with an initial 5 min denaturation step at 95°C; 30 cycles of 94°C for 1 min, 45- 52°C for 1 min, and 72°C for 2 min; and a final 10min extension step at 72°C [7]. All reaction mixtures were held at 4°C until analyzed. All experiments included controls without any added DNA 10µl of each PCR mixture was run on a 1.5% agarose gel in (TAE) buffer stained with ethidium bromide (0.5µg/ml) and visualized under UV light.

RESULTS AND DISCUSSION

There are reports on many microorganisms capable of degrading phenol through the action of variety of enzymes.These enzymes include hydroxylases, peroxidases, tyrosinases and phenol oxidases. Phenol degradation is mainly mediated through ortho and meta cleavage pathways. The important enzymes involved in these pathways are oxygenases which include monoxygenases

and dioxygenases. [9]. A common route for the degradation of catechol and its derivatives is the meta pathway. This pathway is mostly involved in the degradation of methylated aromatics. It involves an extradiol dioxygenase that cleaves the catechol ring derivatives at 2, 3 position, yielding a 2-hydroxymuconoic semialdehyde derivative. Most extradiol dioxygenases have two domain structures, in which C-terminal domain contains the active site. The enzyme usually require Fe^{2+} as cofactor [1].This organism predominantly follows meta pathway. As previously mentioned this organism carried high phenol degrading capacity than other phenol degrading organisms, it has to follow the meta pathway inorder to gain the high degrading capacity. Meta cleavage dioxygenase or catechol 2,3 dioxygenase are believed to be more capable than ortho cleavage dioxygenases [7]. The dioxygenase, catechol 2, 3 dioxygenase (C 23-DO) is an enzyme generally involved in the biodegradation of phenol and is considered to be highly conserved among different bacterial species capable of degrading phenol like compounds [13].Hence in the present study we have analysed the possibility of the presence of extracellular catechol 2, 3 dioxygenase in the phenol degrading *Alcaligenes* sp d_2. The supernatant after the removal of biomass showed catechol 2, 3 dioxygenase activity of 2.75U/ml (Table 2). The crude extract was subjected to ammonium sulphate precipitation at 80% fractionation followed by dialysis. The dialyzed fraction showed an enzyme activity of 7.04U/ml. The dialyzed fraction was purified by Gel filtration. The fractions collected were subjected to spectrophotometric estimation at 280 nm for the presence of protein. The samples with high OD value (Table 2) were pooled. This was concentrated in PEG 6000 and was subjected to native PAGE and the pooled samples gave an enzyme activity of 8.19 U/ml.

Table 2
Purification profile of Catechol 2, 3 dioxygenase isolated from phenol degrading Alcaligenes sp d2

Sample	Total volume (ml)	Enzyme U/ml	Protein mg/ml	Total Enzyme	Specific Activity	Fold	Yield (%)
Crude	100	2.75	100	275.2	0.0275	NA	100
Enzyme sample after Ammonium sulphate fraction and dialysis	5	7.04	15	35.24	0.0469	17.042	12.81
Enzyme sample after Gel filtration	1.5	8.19	1.5	12.28	5.46	116.14	13.4

The results of the present experiment indicated the presence of C23-DO in the culture supernatant. Ammonium sulphate precipitation followed by dialysis gave the partially purified enzyme fraction. The purified enzyme was tested for its activity and the purity was confirmed by native PAGE. In many of the earlier reports C23-DO was precipitated by ammonium sulphate saturation at 80% concentration and the sephadex gel matrix used was G100. The specific activity of enzyme in the crude extract was 0.0275 which got increased to 0.0469 on ammonium sulphate fractionation and dialysis. The dialysed fraction on gel filtration yielded a specific activity of 5.46 with a percentage of yield as 13.4. The overall fold of purification was 116.14. The fold of purification obtained in our strategy is extremely good and gives way for large scale purification of the enzyme.

PCR Detection of C 2, 3-O Gene for Specific Primer.

C23- D O genes are highly conserved among different bacterial species. C23-DO genes have well characterized phylogeny which allows for systematic design of specific primers and hence selected the given primer for the amplification of C23- DO in *Alcaligenes* sp d_2 (Table 1) The chromosomal DNA from the *Alcaligenes* sp d_2 was isolated by the previously mentioned protocol [3] and the purity was checked by taking OD at 260/ 280 nm. The isolated fraction was separated on (1%) agarose gel (Figure 1) To

detect the presence of catechol 2, 3-dioxygenase genes in these novel bacterial isolates, PCR amplification was performed using specific primers to identify the phenol degrading genes. Primers 23 CAT - F and 23 CAT-R were capable of amplifying the gene. Amplification was done using a gradient of annealing temperature 45^0C to 52^0C, with an optimal concentration of 1.5mM to 2.5mM $MgCl_2$. Based on the PCR product intensity, optimal amplification was achieved using DNA concentrations 20ng and 25ng, 2mM $MgCl_2$ and 20pm of each primer. The amplified product were analysed on 1.5% agarose gels stained with ethidium bromide and were visualised under UV light. The amplified band appeared in the range of 200bp molecular weight. (Figure 2)

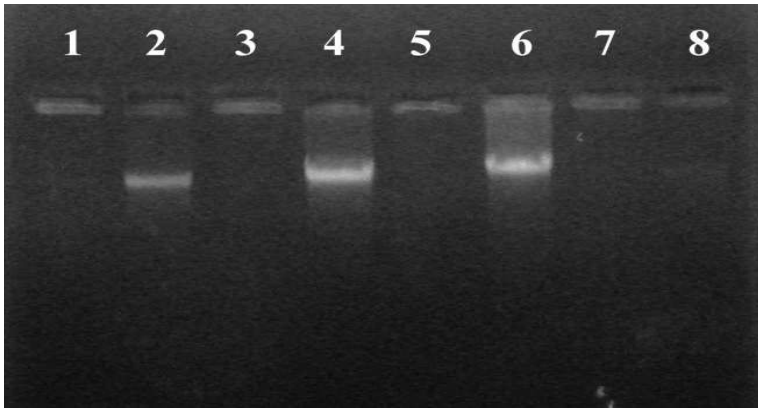

Figure 1
Chromosomal DNA on 1% Agarose gel, Lane 2, 4, and 6

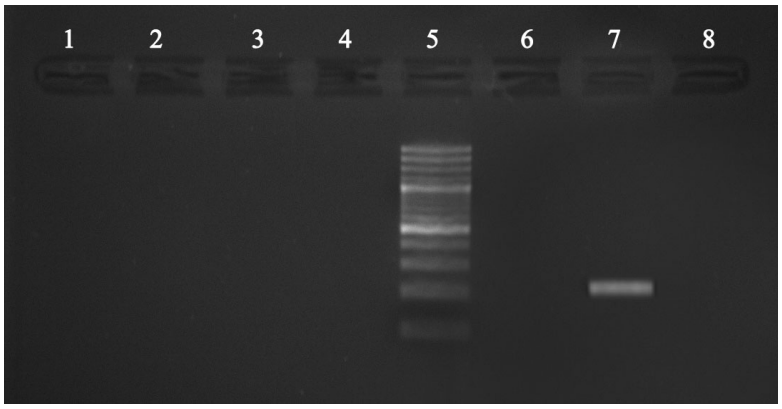

Figure 2
Agarose gel electrophoresis (1.5%) for PCR analysis of C 2, 3-DO gene using primer pairs 2,3 CAT Lane 7 - amplified DNA, Lane 5- Marker (100bp)

The present attempt steps into the course of biodegradation pathway of phenol by *Alcaligenes* sp d_2. Many environmental problems must be discussed within a conceptual framework which needs an extensive knowledge of microbial catabolism. Microorganisms are not only subjected to laws of chemistry but also to their evolutionary relations. The issue of biodegradability of a compound is best addressed by being able to deduce a pathway for catabolism and to find out its prevalence in evolutionarily related microbial communities. In the present study the previously reported phenol degrading *Alcaligenes* sp d_2 was further proved to produce C23- DO extracellularly. The enzyme was purified and the encoding gene was amplified with specific primers. This clearly established the presence of C23-DO in this isolate. The presence of C23-DO is always

considered as a requisite for all phenol degrading organisms and hence the sequence is also considered to be highly conserved. Further investigation into the sequence of this gene may throw light into the phylogenetic relationship of this isolate. Moreover, the comparison of the sequence of this gene with that of other phenol degrading organisms may also throw light on the percentage homology existing in the conserved sequence

REFERENCES

1. Loh KC & Tar PP., Effect of additional carbon sources on Biodegradation of phenol. *Bull. Environ. Contam. Toxicol*. 64, 756-763 (2000).
2. Saha, N C., Bhunia, F & Kaviraj., A Toxicity of phenol to fish and aquatic escosystems. *Bul of Envirol Contam Toxicol*; 63,195-202 (1999).
3. Koboyashi, H. & Rittman, B.E., 'Microbial removal of hazardous organic compounds', *Environ. Sci. Technol.,* 16, 170–183 (1982)
4. Prieto MB, Hidalgo A, Rodriguez FC, Serra JL & Llama.MJ, Biodegradation of phenol in synthetic and industrial wastewater by *Rhodococcus erythropitics*. UPV-1 immobilized in air-stirred reactor with clarifier. *Appl. Microbiol. Biotechnol* ; 58, 583-859 (2002)
5. Harayama S & Kok M, Neidle., ELFunctional and evolutionary relationships among diverse oxygenases, *Annu Rev Microbiol*;46,565–601 (1992).
6. Nair Indu.C, Jayachandran K & Shankar S Treatment of paper factory effluent using a phenol degrading *Alcaligenes* sp. under free and immobilized condition. *Bioresour. Technol;*98, 714-716. (2007).
7. Nair I C & Shashidhar S.,Microbial degradation of phenol by a species of *Alcaligenes* isolated from a tropical soil. *Soil sci*. T; (5, 3-(4): 47- 51 (2004).
8. Nishihara T., Yamada T,Takano K, Osada S,Nishikawa J & Imagawa M, Characterization of *phe B* gene encoding catechol 2,3-dioxygenase, *Lett in Appli Micro*;19 ,181-183 (1994)
9. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J., Protein measurement with the Folin phenol reagent. *J. Biol. Chem*;193, 265–275 (1951)
10. Kate,W, Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G. & Smith, J. A, Preparation of genomic DNA from bacteria, in *Current Protocols in Molecular Biology Wiley Publications,* New York, pp 2.4.1.-2.4.5. (1987)
11. Astrid E. Mars, Jaap Kingma, Stefan R. Kaschabek, Walter,Reineke & Dick B. Janssen., Dioxygenase Region of *Pseudomonas* Analysis of the Chlorocatechol Catechol 2,3 Dioxygenases and Sequence Conversion of 3-Chlorocatechol by Various *putida* GJ31, *J. Bacteriol;*181(4):1309 (1999)
12. Matthew B. Mesarch, Cindy H. Nakatsu, & Loring Nies., Development of Catechol 2,3-Dioxygenase-Specific Primers for Monitoring Bioremediation by Competitive Quantitative PCR *Appl and Environ Microbiol,* ,66, 678-683 No. 2 *Microbiol. Ecol*.;19 5–15 (2000).
13. Yung J.,Xianglong Y., Bin L., Huabing Z.,Qiuxiang C & Baolic C, Catechol 2,3-Dioxygenase from Pseudomonas sp. Strain ND6: Gene sequence and Enzyme Characterization,*Biosc.Biotechnol.Biochem*;68(8) 1798- 1800, (2004)