Chapter VI

Isolation and characterization of catechol 1, 2 dioxygenase from alkaliphilic bacterial strain *Bacillus badius D1*

6.1 Introduction:

Majority of the enzymes used to date have been obtained from mesophilic organisms although the applications of these enzymes are restricted due to their limited stability to extreme temperature, pH, ionic strength, etc. On the other hand, extremophiles are microorganisms that are found in environments of extreme temperature (−2 to 15°C, 60–110°C), ionic strength (2–5 M NaCl) or pH less than 4.0 and greater than 9.0. The extremozymes possess extreme stability and activity to harsh condition. Therefore the applications of these enzymes as biocatalysts are attractive. These enzymes are stable and active under conditions that were previously regarded as incompatible with biological materials (443).

Studies of alkaliphiles lead to the discovery of many types of enzymes that exhibit interesting properties. The first report concerning an alkaline protease published in 1971 produced by *Bacillus* sp. 221. The alkaline protease had an optimal activity between pH 11.5–12 and retaining substantial activity at pH 13. Later on more than 35 other enzymes were isolated and purified by Horikoshi and proved its importance in industries (444). The unsurpassed chemical selectivity makes easy reactions. Their biggest advantage is differentiation between enantiomeric substrates and the enantio selectivities, that could be achieved > 99% routinely (445). Thus the alkaliphiles are unique microorganisms, having great potential for biotechnological and environmental applications with respect to extracellular enzymes and their genetic analysis. Alkaline enzymes should find additional uses in various fields of industry, such as chiral-molecule synthesis, biological wood pulping, and more production of sophisticated enzyme detergents. These can be good genetic resources for production of signal peptides for secretion and promoters for hyper production of enzymes (446). The oxidative cleavage is a remarkable in biological
chemistry which has little precedent in organic chemistry as well as it has a major role in maintenance of the global carbon cycle (447). Dioxygenases play an important role in the aerobic catabolism of a number of natural and manmade xenobiotic aromatic compounds in the environment. In these degradative pathways, a large array of aromatic compounds is converted into relatively few intermediates that contain two adjacent hydroxyl groups. These intermediates then undergo the critical step of ring cleavage, in which both atoms of molecular oxygen are incorporated concomitantly by dioxygenases (448). Pyrocatechase or catechol dioxygenases are the enzymes generally involved in it. Extremophiles, produce biocatalysts that are functional under extreme conditions and having applications in industries. Proteases, amylases, lipases and other enzymes which are resistant to harsh condition and active at high pH and high temperature or salinity. This has prompted the screening of alkaliphilic bacteria and archaea for their ability to produce such enzymes (449). Some enzymes can act on specific recalcitrant pollutants to remove them by precipitation or transformation to other products. They can also change the characteristics of a given waste to render it more amenable to treat or aid in converting waste material to value-added products. Cis-cis muconic acid is a value added product obtained from catechol by pyrocatechase or catechol 1, 2 dioxygenase (450). Cis, cis- muconic acid is precursor of adipic acid. Adipic acid is synthesized by hydrogenation of muconic acid and used as basic feedstock in the production of engineering plastics, polyurethane coatings, elastomers, biodegradable polymers in pharmaceuticals (451), tissue engineering (452). Adepic acid also has an importance in nylon synthesis (453). Currently adipic acid is prepared from a mixture of cyclohexanol and cyclohexanone. This process requires nitric acid. The nitrous oxide emission from this process measurably contributes to global warming and ozone depletion. Therefore, the development of an adipic acid production process leading to less damage or no damage to the environment is an important subject in chemical research. Cyclohexene can now be oxidized directly to colorless crystalline adipic acid with aqueous 30% hydrogen peroxide under organic solvent and halide-free conditions, which could provide an ideal solution to this serious problem (454).
6.1.1 Catechol dioxygenases:

It had been considered that the oxygen molecule is a terminal electron acceptor and is not incorporated into substrates. But by the use of $^{18}\text{O}_2$ and $\text{H}_2^{18}\text{O}$ in the reaction catalysed by mushroom phenolase, Howard S. Mason and his coworkers demonstrated the incorporation of an oxygen atom from molecular oxygen into 3,4-dimethylphenol, producing 4,5-dimethylcatechol (455). Concurrently, Osamu Hayaishi and his collaborators found that *pseudomonads* pyrocatechase incorporated two atoms of oxygen from $^{18}\text{O}_2$ but not from $\text{H}_2^{18}\text{O}$ into catechol and produced cis, cis-muconic acid (456). It was reported that cis–cis muconate is inducer of catechol 1, 2 dioxygenase (457). Mason in 1956 said the enzymes as oxygen transferase and mixed fuction oxidase respectively for one oxygen transferring and two oxygen transferring enzymes to the substrates (458).

After few days Hayashi proposed the term oxygenase and then splited the term into monooxygenase and dioxygenase for them. Thus Oxygenases were first discovered in non-mammalian cells like the mushroom and a bacterium (459), but subsequent studies demonstrated their wide occurrences in mammalian tissues and their physiological functions in the metabolism of cholesterol, prostaglandin, tyrosine tryptophan and several others. Oxygenases also found playing important role in the metabolism of aromatic drugs and carcinogens. Catechol dioxygenases are metalloenzymes that carry out the oxidative cleavage of catechol (Hayassi 1955R). This class of enzymes incorporates dioxygen into the substrate. Catechol dioxygenases belong to the class of oxidoreductases and have several different substrate specificities, including catechol 1, 2-dioxygenase (EC 1.13.11.1), catechol 2, 3-dioxygenase (EC 1.13.11.2), and protocatechuate 3, 4-dioxygenase (EC 1.13.11.3). The active site of catechol dioxygenases most frequently contains iron, but manganese-containing forms are also known (461,462).
6.1.2 Structural features of catechol 1, 2 dioxygenase:

Extensive enzymological studies on various oxygenases after purification observed that their prosthetic groups and coenzymes were non-heme iron, copper, heme, flavin, pteridine, ascorbic acid etc. Muconic acid (hexa-2:4-diene-1:6-dioic acid) was first discovered to be a metabolite of benzene by Jaffe (1909), who isolated it from the urine of dogs and rabbits receiving benzene. It has three geometrical isomers namely, cis-cis, cis-trans and tran-trans (463).

This enzyme was purified from extracts of benzoate induced cells of *Pseudomonas arvilla*. The molecular weight was estimated to be approximately 90,000 D. Experiments with various chelating agents and the effects of oxidizing and reducing agents indicated that the iron in the native enzyme may be in the trivalent state. The trivalent iron bound to the enzyme found responsible for the visible red color, and absorption between 390 to 650 nm. It was also observed that by the addition of the substrate, catechol, under anaerobic conditions, the color of the enzyme solution changed to greyish blue with a concurrent increase of the absorbance at about 710 nm indicating the possible formation of enzyme-substrate complex. This change in absorption spectrum was restored to the original level after catechol was degraded to cis-cis muconic acid by the addition of oxygen (464).

Jamson and Raymond in 1969 reported that strain of *Nocardia corallina* accumulated α, α’-dimethyl cis-cis muconic acid under co-oxidation conditions employing n-hexadecane for growth and p-xylene as the co-oxidizable substrate (465). Enzymes in the soluble fraction of the *Arthrobacter* spc. rapidly metabolized several catechols like 3-methyl-, 4-methyl-, 4-chloro-, and 3,5-dichlorocatechols, with linear increase in UV-vis absorbance at respected acid as cis,cis-muconic acid, 2-methyl-, 3-methyl-, 3-chloro-, and 2,4-dichloromuconic acids etc. on incubation with it(466).

Alexander J. and Pandell in 1976 reported the chemical method for synthesis of muconic acid from phenol and per acetic acid by using Fe (III) and Cu (II) as catalyst. No
cis-cis muconate was formed in the absence of these metals or in the presence of other specified metals. Trace quantities of Fe (III) was effective in catalyzing the formation of cis-cis muconate (467).

The influence of halogen substituents on the 1, 2-dioxygenation of catechols was investigated in case of two isoenzymes of pyrocatechase I and II from the haloarene-utilizing Pseudomonas sp. B13. The pyrocatechase from benzoate-induced cells of Alcaligenes eutrophus B.9 were compared with it. Substituents on catechol were found interfering with O2 binding in the two isoenzymes of Pseudomonas sp. B 13. Electron-attracting substituent’s decreased the Km values for catechols. Studies with substituted catechols demonstrated narrow stereo specificities to pyrocatechase I of Pseudomonas sp. B 13 and Alcaligenes eutrophus B.9. Low steric hindrance by substituents in the binding of catechols with pyrocatechase II also observed. The low pK’ values of substituted catechols resulted in low Michaelis constants. Electron-attracting substituent’s like halogen decreased the reaction rates of pyrocatechase reaction (468).

Substituted muconic acids were prepared from the corresponding catechols by pyrocatechase II of Pseudomonas sp. B 13. The stabilities of substituted muconic acids were compared under different pH conditions. Three substituted cis-cis-muconic acids cycloisomerized readily in slightly acidic solutions, whereas 2-chloro and 2-fluoro-cis, cis-muconic acids were stable under these conditions. These could be isolated as crystalline compounds. They were isomerized to the cis-trans form in highly acidic solution particularly when heated to 80°C. Cycloisomerization of 2-chloro-cis-cis-muconic acid in 75% (v/v) H2SO4 yields 4-carboxymethyl-2-chloro-but-2-en-4-oxide (469).

Pseudomonas sp. strain P51 contains two gene clusters located on catabolic plasmid pP51 that encode the degradation of chlorinated benzenes. The nucleotide sequence of a 5,499-bp region containing the chlorocatechol oxidative gene cluster tcb CDEF was determined. The sequence contains five large open reading frames, which were all co-linear. The functionality of these open reading frames was studied with
various *Escherichia coli* expression systems and by analysis of enzyme activities. One of the genes, tcbC, encoded a 27.5-kD protein, chlorocatechol 1, 2 dioxygenase (470).

Joan Broderick and Thomas showed that purified chlorocatechol dioxygenase from *Pseudomonas putida* was able to oxygenate a wide range of substituted catechols with turnover numbers ranging from 2 to 29 s\(^{-1}\). This enzyme efficiently cleaved substituted catechols bearing electron-donating or multiple electron withdrawing groups in an intradiol manner with Kcat/KM values between 0.2 X 10\(^7\) and 1.4 X 10\(^7\) M\(^{-1}\) S\(^{-1}\). These unique catalytic properties prompted a comparison with related but highly specific enzymes catechol 1,2-dioxygenase and protocatechuate 3, 4-dioxygenase (471). *Psudomonas putida* PaW85 was able to use the hybrid plasmid chromosome encoded pathway for phenol degradation. The synthesis of the plasmid encoded phenol monooxygenase and catechol 1,2-dioxygenase was induced by cis-cis muconate. The transcription of the pheBA operon is positively controlled by a regulatory protein that is chromosomally encoded in *P. putida*. Cis-cis- Muconate in co-operation with transcription factor CatR activates the transcription of the chromosomal ortho-pathway by activating genes catA and catBC in *P. putida* (472). Nakai and others in 1990 determined *Pseudomonas arvilla* C-1 contains three isozymes αα, ββ, αβ, of catechol 1, 2 dioxygenase. Nakai and Yamazaki determined the amino acid sequence of the αα isozyme of catechol 1,2 dioxygenase by direct analysis. The sequence shared 77% homology with isozyme , and had conserved tyrosyl and histidyl residues which are thought to be involved in the binding of ferric ion (473). An oligonucleotide probe was used to clone the gene encoding catechol 1, 2-dioxygenase (catA) from *Rhodococcus rhodochrous* NCIMB 13259. It deduced 282 residual sequences corresponding to a protein of molecular mass 31539 D was sub cloned into the expression vector pTB361, which allowed the production of catechol 1, 2-dioxygenase making approx. 40% of the total cellular protein. The deduced amino acid sequence of the enzyme found 56% and 75% identical with the catechol 1, 2-dioxygenases of *Arthrobacter* mA3 and *Rhodococcus erythropolis* AN-13 respectively (474). Catechol 1, 2-dioxygenase enzymes can be divided into two types, I and II. According to Dorn and Knackmuss (475) both
type I and type II can occur in the same cell and even more isozymes of catechol 1, 2-dioxygenase can be found in one strain (476). Type I enzymes are relatively specific enzymes that use primarily catechol as a substrate while Chlorinated catechols are not used except 4-chlorocatechol. Type II enzymes are induced upon growth with a chlorinated carbon source, such as 3-chlorobenzoate, and are relatively nonspecific enzymes which have a wider substrate range. They converted chlorinated catechols more rapidly than catechol. The wider substrate range of the type II enzymes, however may have resulted in a substantially lower specific activity (477). Chlorocatechol 1, 2-dioxygenase from *Rhodococcus erythropolis* 1CP was purified to homogeneity. In contrast to chlorocatechol 1, 2-dioxygenase from Gram-negative strains having broad substrate tolerance, the *Rhodococcus* enzyme was relatively more specific and had a distinct preference for 4-substituted catechols (478 Olga Maltaseva 1994). Two catechol 1, 2-dioxygenase (C1, 2 O) isozymes (IsoA and IsoB) have been purified to homogeneity from a strain of *Acinetobacter radioresistens* grown on benzoate as the sole carbon and energy source. IsoA and IsoB were both homodimers composed of a single type of subunit with molecular mass of 38,600 D and 37,700, D respectively. In conditions of low ionic strength, Isozyme A can aggregate as a trimer, in contrast to IsoB, which maintains the dimeric structure, as also supported by the kinetic parameters (479). It was also found that glutamate used as a nitrogen source could optimize the production of cis-cis-muconic acid. In addition, an increment of 75–100% of cis-cis-muconic acid could be obtained by adding EDTA–FeCl₃ complex solution, since the enzyme of catA, a necessary oxygenase to convert catechol to cis-cis-muconic acid, consists of 2 Fe (III) ions as the cofactor (480). The intradiol cleavage active site involves an Fe III metal center co-ordinated by two histidine molecules, two tyrosine molecules and a hydroxide molecule in trigonal bipyramidal geometry (481,482).
6.1.3 Mechanistic approach of catechol dioxygenases:

In presence of the substrate catechol, one of the tyrosine molecule and hydroxide ligand get protonated and dissociated away from the iron center (483). The catechol substrate binds as bidentate dianion to the iron center. Once it bound the catechol, it react with molecular oxygen forming peroxide intermediate (484,485). The peroxide intermediate undergoes reaction with iron center forming cyclic peroxide which on further rearrangement turns into cis-cis muconate. One can easily come to know the difference in extradiol dioxygenase and intradiol dioxygenase by following reaction mechanism.

6.1.3.1 Mechanism of catechol 1, 2 dioxygenase:

![Mechanism of catechol 1, 2 dioxygenase](image)

Fig-6.1.3.1. Mechanism of catechol 1, 2 dioxygenase

Dioxygen activation by metallozymes generally proceeds through one of two fundamentally different routes. Some enzymes, such as the ferric ion containing aromatic ring-cleaving dioxygenases like catechol 1, 2 dioxygenases activate oxygen by facilitating the localization of electron density on the substrate so that direct electrophilic
attack by $O_2$ can occur (Fig 6.4.1). Other metalloenzymes, such as catechol 2, 3 dioxygenase (extradiol) ring-cleaving dioxygenases, and cytochrome P450 monooxygenase (P450), activate $O_2$ by a reductive process in which the oxygen first binds to the ferrous form of the enzyme resulting in delocalization of electron density onto the bound oxygen (486) leading to form hydroxyl muconate semialdehyde (Fig 6.1.3.1).

6.1.3.2 Mechanism of catechol 2,3 dioxygenase:

![Mechanism of catechol 2,3 dioxygenase](image)

Fig 6.1.3.2 Mechanism of catechol 2,3 dioxygenase

6.1.4 Induction of Cat A gene: Biosynthetic regulation of catA, the gene encoding catechol 1, 2-dioxygenase (EC 1.13.1.1), was studied in an *Acinetobacter calcoaceticus* mutant strain unable to metabolize benzoate. Benzoate and muconate independently induced the enzyme. In glucose-grown cells, benzoate yielded higher enzyme levels than did muconate, whereas muconate was the more effective inducer in succinate-grown cells (487).
6.1.5 Pyrocatechase having extradiol cleavage activity:

It was reported that *Pseudomonas* pyrocatechase is a single enzyme, which catalyzes simultaneously both intradiol and extradiol cleavages of some substituted catechols (488). The isofunctional enzymes of catechol 1, 2-dioxygenase from species of *Acinetobacter, Pseudomonas, Nocardia, Alcaligenes*, and *Corynebacterium* oxidized 3-methylcatechol according to both the intradiol and extradiol cleavage patterns (489). However, comparison of substrate specificity among these isofunctional dioxygenases showed striking differences in the oxidation of 3-methylcatechol, 4-methylcatechol and pyrogallol. Two isomers of catechol 1, 2 dioxygenase were isolated by Dorn and Knkmuss as pyrocatachase I and II. Among these pyrocatechase I showed extradiol cleavage activity during kinetic study of 3-methyl catechol (490). In another study a series of mononuclear iron (III) complexes of the type [Fe(L)Cl3], where L is the linear N-alkyl substituted bis(pyrid-2-ylmethyl)amine, N-alkyl substituted N- (pyrid-2 ylmethyl) ethylenediamine, linear tridentate 3N ligands containing imidazolyl moieties and tripodal ligands containing pyrazolyl moieties had been synthesized and studied as models of catechol dioxygenase showed intradiol and extradiol property (491). The involvement of dioxygenases in ring fission of aromatic compound is well documented. Several of the investigators have isolated and characterized the dioxygenases from neutrophilic bacterial strains. No enough data is available on catechol dioxygenase from alkaliphiles.

Taking into account the importance of pyrocatechase, and the earlier enzyme reported from various strains of *Bacillus badius* such catalase from *Bacillus badius* -I73 by Kenji Sakai and others(492), Penicillin G acylase used for the synthesis of 6-aminopenicillanic acid(493), Phenyl alanine dehydrogenase from(494) *Bacillus badius* IAM 11059, restriction endonucleases from *Bacillus badius* and *Bacillus lentus* (495), azoreductase from alkaliphilic *Bacillus badius*(496), an attempt was made to isolate and purify catechol 1,2 dioxygenase from alkaliphilic *Bacillus badius* D1.
6.2 Materials and method:

6.2.1 Chemicals:

The chemicals such as DEAE–cellulose (DO909-100G), sephadex G-50, protease-free DNAse/RNAse mixture from sephadex G-100 (Sigma-Aldrich, U.K), lysozyme, glycerol, EDTA, DTT, MgSO4, MnSO4, FeCl3, CuSO4, H3BO3, HgCl, SDS, acrylamide, N-N-methylene bisacrylamide, TEMED, ammonium persulphate, coomassive brilliant blue, formaldehyde, silver nitrate, glacial acetic acid and glycerol, phenol from SRL (Mumbai, India), and triton X-100 (Merck, India), K2HPO4, K2HPO4, ZnSO4.7H2O, FeSO4.7H2O from Sd-Fine Chemicals (Mumbai), all other chemicals were of the highest grade of purity and commercially available. glacial acetic acid and glycerol EDTA, DTT, MgSO4, MnSO4, FeCl3, CuSO4, H3BO3, HgCl, from SRL (Mumbai, India), aniline, & triton X-100 (Merck, India), Catechol, Pyrogallol, L-DOPA, purchased from SRL, Mumbai

6.2.2 Cultivation and collection bacterial of biomass:

The nutrient media was prepared as explained earlier (chapter II). It was autoclaved at 121°C, 15 lbs pressure for 20 minutes. The 1% broth was inoculated with Bacillus badius D1 from a stock culture OD 1.6 at 600 nm and incubated at 37°C for 24 hours under shaking conditions at 110 rpm. The 24 hours grown culture was induced for catechol-1, 2-dioxygenase using 0.5 ml phenol to each flask having 250 ml nutrient broth. The flasks were again incubated at 37°C for 24 hours under shaking conditions. At the end of 24 hours, cell mass was harvested by centrifugation on DuPont Sorvall RC 5B refrigerated centrifuge at 10,000 x g for 20 minutes at 4°C. The cell mass was washed with physiological saline for 2-3 times.

6.2.3 Preparation of crude extract:

The cell mass(35g) was homogenized 1:4 w/v and dispersed in lysis buffer possessing potassium phosphate buffer: pH-7.4, 0.1 mM DTT, 0.1% Triton X-100, Glycerol 5% and (1%), 0.1M EDTA. The cells were then disrupted by Sonicator, Trans O Sonic using 5 strokes of 50 Hz for 20 sec with 30 second interval and centrifuged at 15,000 rpm.
for 20 minutes at 4°C to remove cell debris. Cytosolic fraction after discarding the cell debris was collected and used for enzyme purification. Protein content was determined by Biuret method (497,498). Catechol 1, 2 dioxygenase activity was checked by (Guzik Urszula2009), et al. The reaction mixture was prepared in 3 ml quartz cuvettes by mixing 2880 µl of phosphate buffer (0.05 M, pH8) and 60 µl of 10 mM catechol. Two such cuvettes were used to set the blank using double beam spectrophotometer JascoVarian. Then the increase in O.D. at 260 nm was monitored at time interval of 30 seconds. The activity of catechol 1, 2-dioxigenas was determined according to the formula given below by using 16.8 a molar extension coefficient of muconic acid.

\[
\text{Specific Enzyme activity (µmole/min/mg)} = \frac{(1/16.8) \times \text{average OD/min}}{\text{protein content (mg)}}
\]

6.2.4 Enzyme Purification

6.2.4.1 Ammonium sulfate precipitation:

The crude cell free extract was subjected to ammonium sulphate precipitation. Solid ammonium sulphate (NH₄)₂SO₄ was added to the cell free extract to obtain 30 % saturation. The precipitated protein was separated by centrifugation in cold condition at 15000 × g for 20 min and discarded as it did not show any activity. In the resulting supernatant, addition of ammonium sulphate was continued to get 70% saturation. The process was carried out in cold conditions. The precipitate was kept in cold condition and allowed to settle overnight. The precipitate was collected by centrifuging at 15,000 x g for 20 minutes at 4°C. The precipitate was dissolved in minimum amount of phosphate buffer (pH 8, 0.05 M).

6.2.4.2 Dialysis and Reverse dialysis:

The dissolved precipitate was extensively dialyzed against 0.05 M phosphate buffer of pH 8.0 with several changes of buffer. Dialyzed solution was then reverse dialyzed using solid sucrose, to reduce the volume of enzyme solution. The enzyme activity and protein content was determined.
6.2.4.3 Ion exchange chromatography:

After reverse dialysis the solution was loaded on pre-equilibrated DEAE cellulose (2X 25 cm). The column was thoroughly washed with 0.05M phosphate buffer pH 8.0 to remove the unbound protein. The enzyme was eluted with linear gradient of sodium chloride (0 to 500 mM) in phosphate buffer. Fractions of 4 ml were collected. The fractions which showed higher catechol 1, 2 dioxygenase activity were pooled and reverse dialyzed against solid sucrose to reduce the volume of preparation.

6.2.4.4 Gel filtration chromatography:

The reverse dialyzed enzyme preparation was applied to sephadex G-50 column (2 X 25 cm ) equilibrated with two bed volumes of equilibrating buffer. The enzyme was then eluted with equilibrating buffer with a flow rate of 20 ml per hour. Fractions of 3 ml were collected and those that showed higher catechol 1, 2 dioxygenase activity were pooled and reverse dialyzed against solid sucrose in cold condition. The purified enzyme was used for further study.

6.2.4.5 Electrophoresis:

The purity of isolated enzyme was judged by polyacrylamide gel electrophoresis according to Laemmli 1970 with slight modification (499). The molecular weight of the enzyme was determined by comparing the electrophoretic mobility of the enzyme with standard markers, ranging from 16-216 kDa. (Himedia). Gels containing 5% (stacking or spacer gel) and 10% (separating gel) acrylamide were prepared from stock solution of 24% by weight of acrylamide and 0.65% by weight of N,N\textquotesingle-bisacrylamide. The final concentrations in the separating gel were as follow: 0.375M Tris-HCl buffer pH. 8.8 and 0.1% sodium dodesyl sulfate (SDS). The gels were polymerized chemically by the addition of 0.025% by volume of tetramethylethylenediamine (TEMED) and ammonium persulfate.
Slab of gel was prepared in gel mould with a thickness of 1.5 mm. Before the gel hardens a few ml of water were layered on the top of the gel solution. After 10-20 minutes an interface the gel was solidified. Similarly the stacking gel (1cm) was prepared above the resolving gel. The stacking gel of 3% acrylamide containing 0.0125M Tris-HCL buffer pH6.8 and 0.1% SDS and were polymerized chemically in the same way as for the separating gel. Just before the use, water layer was sucked off and the slab with glass plates was placed in electrophoresis apparatus (Bangalore Genei Mini Vertical Unit). The electrode buffer 8.8 containing 0.025 M Tris, 0.192 M glycine and 0.1%SDS. The sample and marker proteins (20µl in 0.1 to 0.2 ml) containing the final concentrations (sample buffer) 0.0625 M Tris-HCL, pH 6.8, 2% SDS 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue as an indicator dye. The protein was completely dissociated (denature) by immersing the sample for 2 minutes in boiling water. Sample was loaded in gel wells (20 µl protein and 10 µl M.W marker). Gel was run on 100v for 2 hrs. Once gel dye reached 90% from anode to cathode Gel. Then protein was fixed in the gel with a mixture of 50% methanol and 10% acetic acid overnight.

6.2.4.6 Silver nitrate staining:

In silver staining, the gel was impregnated with soluble silver ions and developed by treatment with formaldehyde, which reduces the silver ion to form an insoluble precipitate of metallic silver promotes to from protein bands. The clear gel was transferred into a tray with cover containing fixative solution possessing 50% of ethanol and 100 µl formaldehyde for 2 hr in shaker. The gel was rinsed with distilled water once, and an aliquot of DTT at the final concentration of 10 µl /ml. The gel was incubated in shaker for about 30-45 min till it was swollen and got back to its original size. It was rinsed with distilled water once, then added 0.75 ml of 20% silver nitrate staining solution and incubated in shaker for 30 min. It was further washed thrice with 200 ml of water to completely remove the silver nitrate. Then it was rinsed with 4% sodium carbonate containing 0.076% formalin for 15-30 min in dark. The dark bands were
developed in 6% Na$_2$CO$_3$ and 0.05% formalin. The reaction was stopped by pouring a few drops of 52.52% citric acid solution on the gel and then it was washed with distilled water for 20 seconds. The gel was stored in 2% glacial acetic acid.

6.2.5 Determination of optimal pH and temperature of purified catechol 1,2 dioxygenase

6.2.5.1 Effect of pH and temperature temperature:

The influence of pH on catechol 1,2 dioxygenase was studied using potassium phosphate buffer and tris buffer in the range 6.5 to 9.5. The effect of temperature on enzyme activity was studied in the range of 20 to 40 °C using potassium phosphate buffer pH 8.0.

6.2.5.2 Effect of purified enzyme on spectral properties of various substrates:

The reaction mixture was prepared in 3 ml quartz cuvettes by mixing 2880 µl of phosphate buffer (0.05 M, pH8) and 60 µl of 10 mM catechol. Two such cuvettes were used to set the blank using double beam spectrophotometer JascoVarian. Then the change in spectra of parent molecule was recorded with specific time interval.

6.2.5.3 Kinetic studies of purified catechol 1,2 dioxygenase:

Initial velocities of enzymatic reaction were performed by varying the substrate concentration from 10 mM to 70 mM. Michaels constant $K_m$ and maximal velocity $V_{max}$ was determined for the substrates catechol, pyrogallol and L-DOPA from Line Weaver-Burk plot.
6.3 Results:

6.3.1 Purification of catechol 1, 2 dioxygenase from alkaliphilic strain *Bacillus badius* D1:

At the initial stage of purification a total amount of 560 mg of protein was obtained from crude extract of disrupted cells of *Bacillus badius* D1 (36 g wet weight). In the purification steps of the enzyme, approximately 54% contaminating proteins were eliminated by ammonium sulfate precipitation. After DEAE cellulose column chromatography, the total amount of protein was found to be 13.2 mg and 79 fold increase in specific activity. The concentrated solution of enzyme loaded on Sephadex G-50 [Fig no.] yielding the purification fold of approximately 90. (Table No 6.12.2)

6.3.2 Summary of the purification of catechol 1,2 dioxygenase from *Bacillus badius* D1:

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Source</th>
<th>Vol ml</th>
<th>Total Protein Mg/V ml</th>
<th>Total Activity</th>
<th>Specific Activity µmole/mg/min</th>
<th>Purification Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>crude extract after sonication</td>
<td>140</td>
<td>560 mg</td>
<td>0.76</td>
<td>0.019063</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>(NH₄)₂SO₄ ppt after dialysis on DEAE</td>
<td>50</td>
<td>307 mg</td>
<td>0.497</td>
<td>0.081142</td>
<td>4.5</td>
</tr>
<tr>
<td>3</td>
<td>after DEAE separation</td>
<td>12</td>
<td>13.2 mg</td>
<td>0.466</td>
<td>1.507981</td>
<td>79</td>
</tr>
<tr>
<td>4</td>
<td>after Sephadex G-50</td>
<td>8</td>
<td>7.86 mg</td>
<td>0.46</td>
<td>1.7079</td>
<td>89.5</td>
</tr>
</tbody>
</table>
6.3.3 SDS-PAGE

Fig.6.3.3 SDS-PAGE pattern of purified catechol 1,2 dioxygenase (L1 - purified protein and L2 –marker proteins)

The SDS pattern purified of catechol 1,2 dioxygenase is demonstrated in Fig 16.3.3 with silver Staining. The single band in SDS PAGE suggest that a single polypeptide chain with molecular mass of approximately 50 Kd was present in the preparation.

6.3.4 Characterization of purified catechol 1,2 dioxygenase:

6.3.4.1 Effect of pH and temperature on catechol 1,2 dioxygenase activity:

The effect of pH on purified catechol 1,2 dioxygenase was determined at various pH from 7 - 9.5. The catechol 1,2 dioxygenase from Bacillus badius D1 has shown optimum pH at 8.5 (Fig 6.3.4.1) The effect of temperature on the activity of purified enzyme was studied with a wide range of temperature from 20-45°C. After incubating the reaction mixture at various temperature for 30 min in water bath, the residual activity of enzyme was measured. It was observed that the optimum activity was found at 30°C. (Fig 6.3.4.2)
6.3.4.1 Effect of pH on catechol 1,2 dioxygenase activity:

![Graph showing the effect of pH on catechol 1,2 dioxygenase activity](image)

Fig 6.3.4.1 Effect of pH on catechol 1,2 dioxygenase activity

The effect of pH on catechol 1,2 dioxygenase activity was determined by incubating the reaction mixture at pH values ranging from 6.5 to 9 using the standard buffer systems as potassium buffer. It showed maximum enzyme activity at pH 8.5.

6.3.1.2 Effect of temperature on catechol 1,2 dioxygenase activity:

![Graph showing the effect of temperature on catechol 1,2 dioxygenase activity](image)

Fig 6.3.1.2 Effect of temperature on catechol 1,2 dioxygenase activity
The optimum temperature for enzyme activity was determined by conducting the assay at various temperatures from 20, 25, 30, 32, 35, 37, and 40 °C in 50 mM phosphate buffer at pH 8.0. The residual activity was determined as per the standard assay procedure for the catechol dioxygenase by (214). Optimum temperature found 32 °C to *Bacillus badius* However Urszula Guzik, Izabela Gren recorded 35 °C to this enzyme isolated from *Pseudomonas putida* strain N6.

6.3.5 Kinetic study of purified catechol 1,2 dioxygenase:

To obtain Km and Vmax, the enzyme activity was measured by varying the concentrations of various substrates, catechol, pyrogallol and L-dihydroxy phenylalanine (L-DOPA). In case of catechol Fig 6.3.5.1 a and 6.3.5.1 b, the Km and Vmax values were Km 0.04, Vmax 0.3. While in case of pyrogallol (fig 6.3.5.2 a and 6.3.5.2 b), the Km and Vmax were found to be 0.09 and 0.32. In case of L-DOPA (fig 6.3.5.1 a and 6.3.5.1 b) the Km and Vmax were 0.078 and 0.8.

6.3.5.1 a. Michaels Menton plot of catechol 1,2 dioxygenase for catechol as a substrate:

Fig.6.3.5.1 a) Michaels Menton plot of catechol 1,2 dioxygenase for catechol
6.3.5.1 b) Line Weaver Burk plot of catechol 1,2 dioxygenase for catechol as a substrate

![Line Weaver Burk plot of catechol 1,2 dioxygenase for catechol as a substrate](image)

Fig 6.3.5.1 b) Line Weaver Burk plot of catechol 1,2 dioxygenase for catechol

6.3.5.2 a) Michaels Menton plot of catechol 1,2 dioxygenase for pyrogallol as a substrate:

![Michaels Menton plot of catechol 1,2 dioxygenase for pyrogallol](image)

Fig 6.3.5.2 a) Michaels Menton plot of catechol 1,2 dioxygenase for pyrogallol
6.3.5.2.b) Line Weber Burk plot of catechol 1,2 dioxygenase for substrate Pyrogallol:

Fig 6.3.5.2.b) Line Weber Burk plot of catechol 1,2 dioxygenase for substrate Pyrogallol

6.3.5.3.a) Michaelis Menten Plot of catechol 1,2 dioxygenase for substrate L-DOPA

Fig 6.3.5.3.a) Michaelis Menten Plot of catechol 1,2 dioxygenase for substrate L-DOPA
Fig 6.3.5.3.b) Line Weaver Burk plot of catechol 1,2 dioxygenase for substrate L-DOPA

6.3.6 Invitro transformation of di and tri hydroxy compounds:
Various compounds like catechol, pyrogallol and L-DOPA were effectively transformed invitro by purified catechol 1,2 dioxygenase from Bacillus radius D1. Gradual decrease in absorption maxima at 217 nm was observed and at the end of 15 min incubation of reaction mixture complete, disappearance of this peak was observed in case of catechol. Fig.(6.3.6.1)
Fig.6.3.6.1 indicate invitro transformation of pyrogallol by purified enzyme catechol 1,2 dioxygenase. Incubatiion of 10 mM pyrogallol with purified enzyme in reactionmixture resulted in stepwise biotransformation within 6 min. The peak at 272 nm was markedly reduced within a min.and new peaks were appeared at 239 nm and 298 nm may be corelating with hydroy muconic acid and 2 pyrone 6 carboxylic acid.

![Fig.6.3.6.1](image1)

**Fig.6.3.6.2** Invitro transformation of pyrogallol

![Fig.6.3.6.2](image2)

**Fig.6.3.6.3** in vitro transformation of L-DOPA

![Fig.6.3.6.3](image3)
Invitro incubation of L-DOPA (10 mM) with purified enzyme has also resulted in stepwise transformation (Fig.6.3.6.3). The peak at 282 nm was drastically increased after five min. incubation and was completely disappeared after 10 min incubation period.

6.4 Discussion:

Microbial degradation of a wide variety of chemicals has been the topic of interest in the recent years as they are toxic in nature and their cleanup from the environment. Various reports on microbial degradation of these compounds is economically plausible and ecologically accepted cleanup method generally high resistant power of these compounds to the physicochemical degradation processes, microbial enzymes from various species have shown the simple biodegradation process which are responsible for complete remediation of these chemicals.

Therefore the purpose of this study was to provide insight into invitro transformation of aromatic hydroxyl compounds by purified catechol 1,2 dioxygenase. There are several reports on catechol 1,2 dioxygenase from nutrophilic bacteria isolated from different environment (501-508). But the extremophiles are mostly ignored. Therefore our efforts were directed towards the isolation and characterization of catechol 1,2 dioxygenase from alkaliphilic strain Bacillus badius D1. Initially the isolated alkaliphilic bacterial strain was aclamatized 0.5 g with phenol for 10 days. The purification of catechol 1,2 dioxygenase from this alkaliphilic bacterial strain was started with protein concentration 560 mg in crude extract and finally got 7.8 mg of purified protein. This protein has shown single band in SDS –PAGE, indicating that the active form of enzyme may consist only one polypeptide chain having molecular weight of 50 KD.

The catechol 1,2 dioxygenase from Bacillus badius D1 has a optimum pH 8.5. Although the pH of growth medium was above 9.0, this is due to the ability of...
alkaliphilic microorganism to survive in high pH condition by successfully lowering the pH across the cell wall and the membrane, so that the intracellular pH is kept neutral(509). The temperature optima for catechol 1,2 dioxygenase was 30 0C. This is in well accordance with the other reports. The affinity for the substrate like catechol, pyrogallol and L-DOPA were examined. On the basis of Km value it is observed that catechol 1,2 dioxygenase is having very high affinity for catechol as compared to other substrates.

The rate of transformation was vary for different dihydroxy and trihydroxy compounds. This could be due to variation in substituted groups or their numbers. The result of this study reveals that alkaliphilic microbial catechol 1,2 dioxygenase has a promising potential to degrade wide variety of aromatic compounds by ring fission. Similarly, this enzyme may be used in synthetic organic chemistry.