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# PRODUCTION AND OPTIMIZATION OF CATECHOL 1, 2-DIOXYGENASE FROM OIL CONTAMINATED SOIL ISOLATES OF *BACILLUS SUBTILIS*

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## ABSTRACT

This study aimed to evaluate the media conditions for enzyme activity of catechol 1, 2-dioxygenase (C1, 2O) produced by *Bacillus subtilis* in cell-free extracts. The optimum conditions of pH, temperature, Nitrogen and Carbon sources were determined. Peak activity of C1, 2O occurred at pH 7.0 and 37°C. Among the five nitrogen and carbon sources, highest activity was observed from yeast extract and glucose respectively. After optimization process, enzyme was purified with different method. The enzyme value was improved by gel filtration chromatography than other methods. Furthermore, the enzyme was subjected to molecular characterization with SDS PAGE analysis. The SDS-PAGE analysis reveals that the enzyme has a molecular weight of 30 k Dalton.

**KEYWORDS:** Biodegradation, Catechol 1, 2-dioxygenase, Catechol, *Bacillus subtilis*, SDS-PAGE.

## INTRODUCTION

Enzymes are the bio-catalysts playing an important role in all stages of metabolism and biochemical reactions. Certain enzymes are of special interest and are utilized as organic catalysts in numerous processes on an industrial scale. Many enzymes from microbial sources are already being used in various commercial processes. The use of microbial enzymes for biodegradation of toxic organic compounds such as PAH is promoting excellent results in the clean-up of different environments including water, sludge and soil.

Among the microbial enzymes, Catechol 1,2- dioxygenases play important roles in the degradation pathways of various aromatic compounds and are ubiquitous in microorganisms.[1,2,3]

Catechol 1, 2-dioxygenase (EC 1.13.11.1) has $Fe^{3+}$ as a prosthetic group and belongs to the enzymes that perform intradiol cleavage. Many bacterial species do not have identical α and β subunits $(\alpha\beta\text{-}Fe^{3+})_n$; while others have simple polypeptides (α α-$Fe^{3+}$) and present little or no activity to chloro-catechols. Catechol 2,3-dioxygenase (EC 1.13.11.2) however, belongs to the extradiol cleaving enzyme class and has four identical subunits of 32kda and contains a catalytic iron ion ($Fe^{2+}$) per subunit.[4]

Many bacterial strains such as *Pseudomonas* sp. BP10, *Stenotrophomonas nitritireducens*,[5] *P. aeruginosa* PSA5, *Rhodococcus* sp. NJ2 and *Ochrobactrum intermedium*[6] were reported to produce degradative enzymes during the biodegradation of hydrocarbons. According to previous studies, few authors only optimize the catechol 1, 2-dioxygenase production. Therefore, present study was to optimize the production of enzyme with different carbon and nitrogen source, T° and ph.

## MATERIALS AND METHODS

### Collection of soil samples

For the current study, 20 samples from petroleum contaminated soil were collected from 20 different locations of petroleum filling stations (Petrol Bunk) and workshops around the Namakkal area. The samples were collected in sterilized plastic bags and each bag was labeled to show the date and site of sample collection. The samples were then brought to the university laboratory for further processing.

### Screening of bacterial isolates from oil contaminated soil

The serially diluted soil samples were spread on Bushnell Haas agar plates overlaid with 100 µl of Hydrocarbon (Crude oil) and were incubated at 25°C for 14 days, any isolate which grow on Bushnell Haas agar plate were confirmed as degraders. Same colonies were inoculated into nutrient agar plates and incubate at 24hrs to 48hrs for further analysis.

### Screening of isolates for enzyme production

The potential isolates (6 isolates) were selected based on the highest millimeter of the zone of clearance, which were carryout for the production and optimization of Catechol 1, 2–

Dioxygenase. Bacterial inoculums (1ml) was transferred into 5ml minimal salt medium and was supplemented with Catechol as the carbon source, incubated at 30°C at 170 rpm in shaker incubator for a period of 7days.

The single potential isolate was selected for the enzyme production. The bacteria inoculam (10%) from 7 days incubation of mineral salt medium culture were then transferred to 1000ml of freshly prepared same medium (2.75g/l of $K_2HPO_4$, 2.225g/l of $KH_2PO_4$, 1.0g/l of $(NH_4)_2SO_4$, 0.2g/l of $Mgcl_2.6H_20$, 0.1g/l of Nacl, 0.02g/l of $Fecl_3.6H_2O$ and 0.01g/l of $Cacl_2$) ph 7.0, supplemented with 1% of Catechol. The medium were incubated at 30° C on a rotary shaker at 200rpm.[7]

**Preparation of cell-free extract**

The cells were harvested by centrifugation at 10,000 × g for 10 min and washed twice with 2 volumes of 0.9% Nacl. Wet cells (10 g) were resuspended in 50 ml of 50 mm sodium phosphate buffer (pH 7.4), and cells were disrupted with a vortex for 2 min with ice-water cooling. The debris was removed by centrifugation at 12,000 × g for 20 min, and the supernatant solution was used as the cell-free extract.

**Catechol 1, 2 dioxygenase assay**

The activity of Catechol 1, 2 dioxygenase was prepared by mixing 2 ml of 50 mm Tris hcl buffer (ph 8.0), 0.7 ml distilled water, 0.1 ml of 100 mm 2-mercaptoethanol, 0.1 ml cell free extract and 0.1 ml catechol from 1 mm (Sigma) in sterile tube. The sample was read in a quartz cuvette. A control was prepared by replacing the catechol with distilled water. The formation of cis, cis muconic acid from ortho cleavage pathway was monitored using spectrophotometer at wavelength 260 nm for 10 minutes at 1 minute interval. The calculation was followed by according to Arezoo and Ainon's[8] procedure.

**Production and Optimization of antibacterial substance**

The single isolate was selected based on the highest enzyme production; this was utilized for the production and optimization process. The process parameters were optimized using mineral salt medium in a series of experiments to obtain higher productivity of the enzyme. The various carbon sources, nitrogen sources, pH and Temperature were utilized for optimization.

**Protein estimation**

Protein estimation was done by the Lowry method using Bovine Serum Albumin as a standard.[9]

## RESULTS AND DISCUSSION

Role of microbial activity in the biodegradation of hydrocarbons has been well recognized for more than a century. The native bacterial populations in the crude oil contaminated soil sites possess the capacity to mineralize hydrocarbons. In our study, 20 petrochemical contaminated soil samples were collected from Namakkal area. All samples were serially diluted with distilled water and spread on the oil contains media, among the 20 soil samples were collected; 9 (45%) of samples had the oil tolerating isolates. From the 9 samples, 6 isolates were selected based on the highest zone of clearance surrounding the colonies. The previous study of Marcelo[10] and Wang[11] were observed the hydrocarbon degrading bacteria with crude oil containing soil samples.

The previous study of Jayanthi and Hemashenpagam[12] were also observed the number of bacterial isolates from oil contaminated soil samples in two wheeler work shop area. Petroleum hydrocarbon is composed of carbon and hydrogen and many microorganisms have the ability to utilize the hydrocarbon as a sole source of carbon energy and these isolates are widely present in soil.

The use of microbial enzymes for biodegradation of toxic organic compounds such as PAH is promoting excellent results in the clean-up of different environments including water, sludge and soil. Among the different enzymes, catechol oxygenases have an important role in fundamental pathway in the carbon biochemical cycle and a high biotechnological potential in treatment of liquid wastes contaminated with aromatics compounds. Number of authors was observed enzyme producing isolates from oil containing soil samples (8, 13&14). In the present study, among the six isolates, single isolate was showed highest enzyme activity (0.288U/ml) (Table 1).

Several factors were involved in Catechol oxygenase activities. The product of enzyme can be changed based on used substrate, nutrient content, pH and T$^{o}$. Therefore, medium was improved with different pH, Temperature, carbon source and nitrogen source, which was considered to be optimized primarily as reported by many researchers.[15] Among the five types of nitrogen sources, Yeast extract was highly produces the enzyme (Fig.1). In 2015, Li

Han *et al*, produce the catechol 1-2 dioxygenases with yeast extract. In case of carbon source, glucose showed good enzyme production (Fig 2).

Extreme values of pH can inhibit microbial growth by interfere the microbial metabolism. Most natural environments have pH values between 5.0 and 9.0. In the present study, highest activity was observed when using pH 7. This result was contrary with previous study of Andrea;[8] they were observed better activity with pH 8 (Fig 3). But same time our finding was similar to the enzyme isolated by Kou and Li[16] who reported the C12D isolated from *Pseudomonas putida* 84103 to exhibit highest activity at the optimum pH 7.5-8.0. Most microbes prefer to grow at temperatures in a range of about 10 to 38°C. This activity slightly increased until 27°C, than strongly increased until the maximum activity at 27 °C. Above 37°C, it was drastically reduced the activity (Fig 4). Similar study was observed with Silva *et al* reports.[13]

After the optimization, the enzyme was produced with best parameters. Then enzyme was purified with ammonium sulphate precipitation, dialysis and coloumn chromatography. The enzyme value was improved compared than ammonium sulphate and dialysis (Table 5). In this study, SDS PAGE was carryout for the molecular characterization of enzyme. The SDS-PAGE analysis shows that the molecular weight of the Catechol 1, 2 dioxygenase was near about 30 KD.

Furthermore, enzyme producing isolate was identified with biochemical tests, according to that the isolate belongs to genera *Bacillus* and species as *subtilis.* In recently Jayanthi and Hemashnbagam (12) was isolated the Catechol 1, 2 dioxygenase producing soil isolate of *Bacillus cereus.* They were also observed the enzyme assay with same method.

**Table 1: Screening of catechol 1, 2-dioxygenase producing isolates.**

| S. No. | Isolates name | Results U/ml |
|---|---|---|
| 1. | C1 | - |
| 2. | C2 | 0.288 |
| 3. | C3 | - |
| 4. | C4 | 0.214 |
| 5. | C5 | 0.258 |
| 6. | C6 | - |

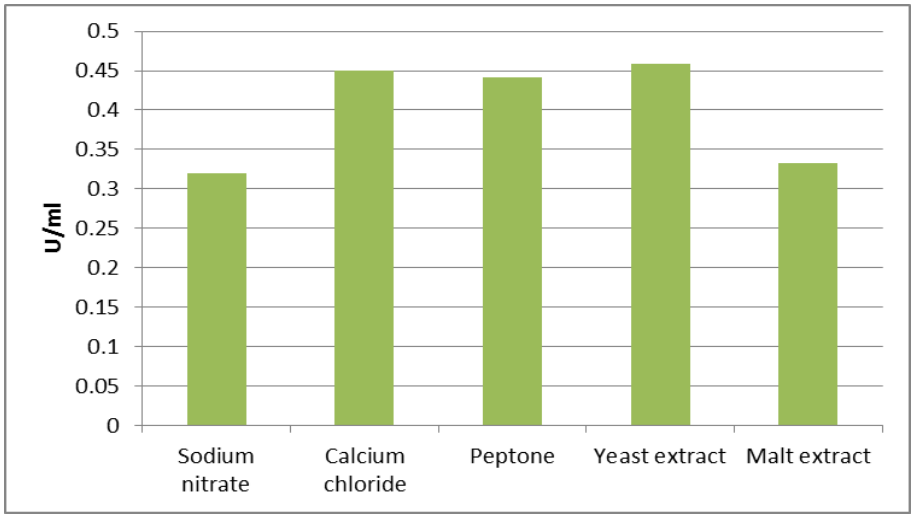


**Fig. 1: Effect of nitrogen source on production of Catechol 1,2-dioxygenase.**

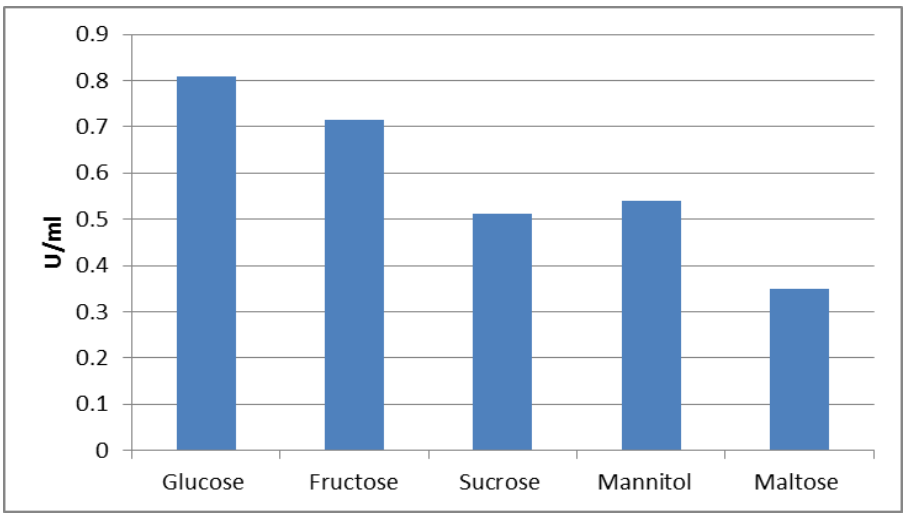


**Fig. 2: Effect of carbon source on production of Catechol 1,2-dioxygenase.**

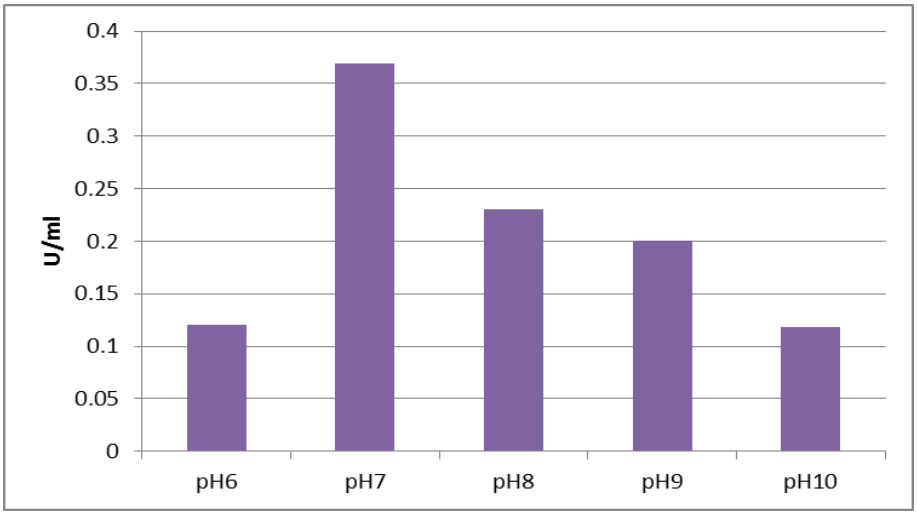


**Fig 3: Effect of pH on production of catechol 1,2-dioxygenase.**

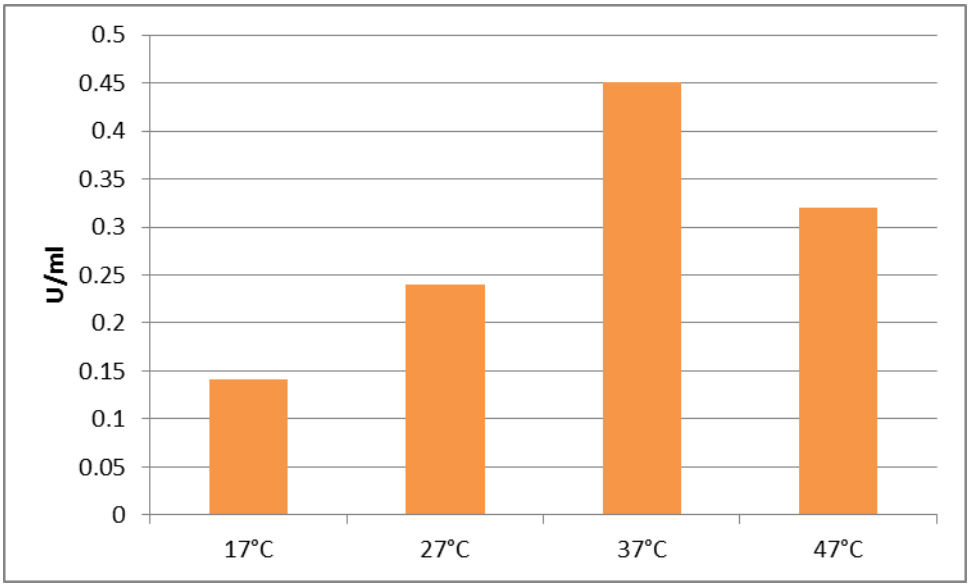


**Fig 4: Effect of temperature on production of Catechol 1,2-dioxygenase.**

**Table 2: Purification of Catechol 1, 2-dioxygenase.**

| Purification steps | Enzymes (U/ml) | Protein mg/ml | Specific Activity U/m | Purification Fold |
|---|---|---|---|---|
| Crude extract | 1.74 | 1.1 | 1.581 | - |
| Ammonium sulphate precipitation | 1.32 | 0.82 | 1.609 | 1.017 |
| Dialysis | 0.99 | 0.56 | 1.767 | 1.098 |
| Gel filtration | 0.61 | 0.30 | 2.033 | 1.150 |

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