Identification of catechol dioxygenases of acinetobacter species, with improved ability to degrade crude oil

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Abstract

Background: Various microbes involved in alkanes degradation or reducing organics oil products have been identified among. The goal of this work was to screen the soil bacteria with potential ability to degrade alkanes and characterized using a combination of genetic and physiological methods.

Methods: The microbial species of soil sample was isolated and the loss of hydrocarbon was calculated periodically by gravimetric analysis. Isolated bacterial strain with potential ability to degrade the engine oil in Baghdad city was further tested for hydrocarbon-degrading abilities using 2, 6-DCPIP. Colonies of bacteria were counted using serial dilution methods of the soil sample. One of the highly bioactive degrading strains ISN53 was selected and a different fraction of crude extract were analyzed for their hydrocarbon degrading activity. The purified active fraction characterised in LC/MS and SDS PAGE. Presence of chlorocatechol dioxygenases was checked by PCR and expression was analyzed in RT PCR. The envelope of ISn21 isolate was checked for any changes under different conditions using microbial adhesion to the hydrocarbon test (MATH).

Results: Serratia marcesens (Sm53) and Acinetobacter venetianus (ISn21) were isolated and ISn21 showed maximum growth on the third day and up to 72% degradation on the 7th day of incubation. LC-MS/MS spectrometry analysis identified the bioactive enzyme catechol dioxygenases in crude extract. The purified catechol dioxygenases enzyme had 35 kDa weight which showed the maximum activity between 48 to 72 hour of incubation. The expression of its gene was 2 fold more than control the maximum expression was observed at 60 to 72 hours of incubation. ISn21 was extremely hydrophobic both in MMV and in LB medium.

Conclusion: Using a naturally available resources would be better with efficiency at degrading diesel. Use of like microorganisms during bioremediation operation might supply worthy advances in this important biotechnological/ biological domain.

Introduction

Crude oil or petroleum products are complex hydrocarbons and various organic mixtures which are a serious threat to the environment if spills occur [(1).] The Incomplete ignition of petroleum produces toxic byproducts including carbon monoxide. The running vehicles with high temperatures and high pressures exhaust gases include nitrogen oxides main cause of photochemical smog [(2)]. Its naturally occurring products, therefore, its presence is not bothering but it affects the surroundings when released into the environment accidentally and inappropriately [(3, 4)]. Bioremediation uses microorganisms already present in environments to biodegrade was tot harmful contaminate materials.Bioremediation is always preferred way to treat the oil spillage and contamination and an efficient method could be worth saving millions of resources. There have been extensive researches focused on oil bioremediation, and degradation by use of mixed bacterial culture or isolates which can grow in oil-contaminated soils [(3)] [(4)]. In an attempt to find oil degradable bacteria, various bacterial inhabitance in oil-contaminated soil have been characterized

and their degradation competences were evaluated including the fraction of bacterial product which is individually degrading the oil [5].

The Mechanism of Petroleum alkane's degradation: Microbes decompose most organic compounds into carbon dioxide, water and mineral matter, such as nitrate, sulphate and different inorganic substance. The aerobic pathway proceeds most rapidly and most efficiently. Microbes break hydrocarbons by secreting enzyme or by producing biosurfactants [6]. Microbial biosurfactants are mainly produced by aerobic microbes, using as carbon sources carbohydrates, hydrocarbons, vegetal oils or animal or a mixture of those organic compounds. Biosurfactants can be intracellular meaning that it remains attached to the cell wall or can be excreted to the environment [1, 7].

Figure (1) show the degradation pathway for an alkane involves sequential formation of an alcohol, followed by an aldehyde and a fatty acid. The fatty acid is cleaved, releasing carbon dioxide and forms a new fatty acid. The new fatty acid is two carbon units shorter than the parent molecule in a process known as beta-oxidation. *Acinetobacter Spp.* Use an alkane monooxygenase (terminal oxidation) to convert the hydrocarbon to a primary alcohol to allow for the subsequent breakdown and employment of the hydrocarbon. The initial enzymatic attack involves a group of monooxygenases [1, 8].

In past decades, various microbes involved in alkanes degradation or reducing organics oil products have been identified among which degradation of n-alkanes has been massively investigated in *Pseudomonas putida* GPo1 [9–11], including *Acinetobacter spp.* bacteria [12, 13]. With regard to the first stage, various bacteria capable to grow on hydrocarbons as a unique source of carbon and energy have improved special strategies for cooperating with biofuel [14–16]. For example, various strains of *Acinetobacter venetianus* have adopted altering strategies to adhere to diesel fuel drops [17].

The goal of this work was to screen the soil bacteria contaminated with crude oil to isolate bacteria with potential ability to degrade alkanes and characterized utilizing a collection between physiological and genetics methods. Characterization included biochemical identification, degrading capability, and detection of degrading enzymes.

Methodology

Isolation and identification of bacterial strains

The microbial species of different sources including from soil of nearby petrol pump, the vehicle engine service center where petroleum products leakage are constant in Baghdad city, were grown on culture media (Luria-Bertani (LB) including 0.4% diesel fuel and 0.4% succinate as a unique source of carbon and energy. The soil sample was inoculated in broth and was incubated at 37°C for 2 days. The organisms that established or formed clear or pure zones around the colonies were considered as crude oil degraders. Those isolates were identified and further characterized till species level, were also further confirmed through genetic detection using housekeeping gene *16S rRNA* [18].

Characterization of the degradation potential of bacterial strains

A single colony of each isolated bacterial strain was inoculated into a 10ml nutrient broth including 0.4% diesel fuel and 0.4% succinate at 35^{o}C overnight. The cell pellet was collected from overnight culture after centrifuged for 15 min at 5000 rpm and washed twice. The pellet of bacteria was resuspended with Bushnell Hass medium until OD(595) (1OD (600) equivalent) was moved into 10 ml of Bushnell Hass (BH) medium containing 1 ml of used engine oil. It was incubated for four weeks at 30^{o}C at 200 rpm. Then the loss of hydrocarbon was calculated periodically 0, 1st, 2nd, 3rd and 4th week by gravimetric analysis. The analysis of Gravimetric was performed according to Mandri & Lin [(19)]. The liquid phase of BH media was dispensed in a Schott bottle and the organic hydrocarbon was extracted with 1/10 dichloromethane, an organic dissolvent. The organic phase was work through sodium sulfate (Kept in pre—weighed flasks) and left in the fume cupboard to allow evaporation of the organic dissolvent. To ascertain the weight of the oil Combined of the pre-weighed flask and oil was listed and the pre-weight subtracted. This experiment was repeated four times and the average value was depended for interpretation. At the same time colonies of bacteria were counted using serial dilution methods of the soil sample.

Valuation of hydrocarbon-degrading efficiency using 2, 6-DCPIP (2, 6-Dichlorophenol indophenol) assay

Few of the selected isolated who was having the potential ability to degrade the engine oil were further tested for hydrocarbon-degrading efficiency using 2, 6-DCPIP (2, 6-Dichlorophenol indophenol) assay.

In brief, selected isolates were cultured in 10 ml of LB broth at 35°C and 200 rpm. Once the density of culture acquired an OD of 1.0 at 595 nm the bacterial cells were pelleted and washed thrice before testing. The cells were suspended into buffered saline to adjust optical density at 595 nm to become (1.0). In a microtube 1000 µl of W medium (Fe-free), 100 µl of FeCl3.6H2O solution (150 µg/ml) then 100 µl of 2,6-DCPIP solution (37.5 µg/ml) was procured and 100 µl of individual bacterial cell suspension together with 10 µl of sterilized hydrocarbons such as Benzene, Phenolphthalein as substrates were added, the color of the medium was noticed and estimated as positive or negative depending on the color change, positive (degraded) for microbial hydrocarbon degrading ability if colorless or negative (not degraded) for microbial hydrocarbon-degrading ability if blue [(20)].

Purification of the bioactive enzyme

One of the highly bioactive degrading strains ISN53 was selected and grown in Luria broth supplemented with 0.4% diesel fuel and 0.4% succinate. The culture was grown for different time periods, centrifuged, and run during a PD–10 gel filtration column (Pharmacia Inc., Uppsala, Sweden). The crude or raw enzyme was purified by adding $CaCl_2$ to a final concentration of 1 mM and the total enzyme was accumulated or precipitated by adding of 3 volumes of cold (220°C) acetone with fixed shaking overnight. The precipitate was dried on room temperature precipitated with ammonium sulfate at 85% saturation.

Separation and Identification of enzymes in the crude extract

To separate the different fraction of crude extract, microwave oven model Ethos combined with GC mass spectrometer chromatograph (Agilent Technologies) balanced with a split/splitless injector were utilized. The mass spectrometer was placed in the EI positive mode (70 eV), with (HP–5MS) fused silica capillary column (Agilent Technologies, Palo Alto, CA) with deactivated silica pre-column. The carrier gas (He, purity 99.999%) was used in the constant flow mode at (1.8 mL/min). For the enzyme amino acid sequence analysis, the injector was used in a splitless mode at 230°C. Each of distinct fractions was tested for their hydrocarbon-degrading abilities as described earlier. Identified the proteins by database search against the MASCOT database and the data of MS/MS raw were analyzed by using Agilent Ion Trap Analysis software version 5.2.

Molecular weight analysis in SDS Page

The purified active fraction was pass across a PD–10 gel filtration column (Pharmacia) into an adequate buffer and used. The molecular weight of the enzymes was determined by one dimensional SDS-PAGE.

Presence of chlorocatechol dioxygenases by PCR

As indicated by the results from an earlier experiment that chlorocatechol dioxygenases were present in the crude extract of ISN53, with degrading activity towards hydrocarbon. The expression of chlorocatechol dioxygenases were studied in real-time PCR syber green assay by depending the primer groups itemized in (Table 1), the primers depended were designed based on degenerate chlorocatechol dioxygenase [21] and amplified partial regions of each gene in Table 1. The endogenous *rpoB* gene was used to normalize the sample.

Investigation of adherence to hydrocarbon

The envelope of ISn21 isolate was checked for any changes under various conditions (i.e., in MMV or LB medium supplemented either with 0.4% diesel fuel or 0.4% succinate), using microbial sticking or adhesion to the hydrocarbon test (MATH) following the protocol as described by Hori [22].

Results

Isolation and identification of bacterial strains

From the taken soil sample, two different bacterial species were isolated and later identified as *Serratia marcesens (*Sm53)and *Acinetobacter venetianus* (ISn21). The maximum clearing zone in the plate was seen around the isolates Sm53 and ISn21 and selected for more screening of biodegradation rates (Figure 2). The petroleum hydrocarbon degradation efficiency was estimated by gravimetrical method. During the assay, after a week time of incubation, there were visual observations of biodegradation of used engine oil have been noticed. ISn21 showed maximum growth on the third day and up to 72 % degradation on the 7^{th} day of incubation (Figure 3 & 4). The degradation ability was evaluated using 2, a

6-DCPIP assay which showed that at a defined concentration of cells ISn21 was readily able to degrade the hydrocarbon. This effect was maximum between 7 to 9^{th} days (Figure 5).

Purification of the bioactive enzyme

CaCl2 was used in separation methods to reduce the activity of EDTA in buffers. The crude extract was purified from *ISn21*using PD–10 gel filtration column chromatography followed by saturation with ammonium sulfate. The degrading activity of the precipitate was confirmed before the further process. The fraction (n = 12) obtained through GC from the crude extract were tested for its degradation activity and two of this fraction found to have hydrocarbon degradation activity in gravimetrical method. LC-MS/MS spectrometry analysis identified this enzyme as phytase and catechol dioxygenases were the later exhibited high biodegradable activity (Figure 6 & 7). Phytase has been already reported in the literature for its biodegradable activity isolated from Acinetobacter species. The purified catechol dioxygenases enzyme was subjected to one dimensional SDS-PAGE and one large polypeptide band was detected by silver staining corresponding to 35 kDa and also the enzyme had an isoelectric point of 6.8 (figure 8). The ability of the catechol dioxygenases to degrade the petroleum product was tested (Figure 9). It was shown that the purified enzyme was showed the maximum activity between 48 to 72 hour of incubation. 0.5 µM concentration of the purified enzyme was found to show similar activity as the 10^7cfu/ml culture tested (figure 9). As expected the chlorocatechol dioxygenases gene was present in ISn21. The expression was checked and results showed that at any point of time it was expressed 2 fold more than control the maximum expression was observed at 60 to 72 hours of incubation (data not shown). Data obtained from the MATH test showed that ISn21 was extremely hydrophobic both in MMV and in LB medium.

Discussion

In the present era when the main resource for energy, the entire world is dependent on crude oil or petroleum product, the pollution due to its spillages becoming a major concern. Though there is no economical cheap technique to eradicate the accidental leakage of hydrocarbon products the research on using various microbial genera have been extensively being done to detect in petroleum contaminated soil or water, for its role in the transformation of hydrocarbons process [(23)]. Earlier it was found that microbial communities which are in constant exposure to hydrocarbon would have much more ability to degrade hydrocarbon contamination than communities with no history
of contamination with hydrocarbons [(24)]. The degradation quantity is dependent upon bio degradative activity and optimal environmental required for the conditions to stimulate the production of degrading enzymes of bacteria. Bioremediation is the most preferred option for degrading petroleum as the other industrial option may cause more pollution. The main parameter for typically measured to test efficacy include an enumeration of microbial populations [(25)] and rate by which hydrocarbon degrade [(26).] The most direct measure of the effectiveness of bioremediation
is the monitoring of disappearing hydrocarbons [(27)].

Conclusion

In this study, we propose to analyze the presence of soil bacteria contaminated with hydrocarbon, able to degrade long-chain n-alkanes. The soil sample was collected from an area near by petrol pump and the sample was cultured on LB media having hydrocarbon sources. Our study identified only two bacterial strain from soil sample having the degradation activity of hydrocarbon.It showed growth of only two distinct bacterial species which identified as *Serratia marcesens (*Sm53) and *Acinetobacter venetianus* (ISn21), and ISn21 being the potent degrader of hydrocarbon. The maximum degradation was seen for catechol dioxygenases compare to phytase and on day 7 or between 48–72 hours the degradation was on high.

This study shed light on the potential use of bacterial strain toward diesel fuel degradation. The commercially available technology for petroleum degradation is extensively costly and not environment-friendly. Therefore, using a biostrain already present in the environment would be better with efficiency at degrading diesel. Use of that microorganisms through bioremediation methods might supply oriental advances in this important biotechnological / biological field.

Declarations

Acknowledgement:

The authors would like to thank Mustansiriyah University (https://uomustansiriyah.edu.iq/) / Baghdad, Iraq for its support to complete this work.

Conflict of interest:

The authors declare no conflict of interest. The authors themselves are responsible for the content and writing of the paper.

Author contribution:

All authors contributed equally to the Literature review, Study plan, Lab work, fund generation, Manuscript writing, analyses data and proof.

Ethical statements for human/animal experiments:

The study was approved by institutional ethics committee “University of Mustansiriyah” and informed consent was obtained in written by each individual participants. Each participant was known about the study follow up before enrolling for the study. I confirm that all experiments were performed in accordance with relevant guidelines and regulations.

Declaration of interests:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Consent for publication: All authors Consent for publication in this journal.

Availability of data and material: All data available in manuscript.

Note: All pictures of this study taken by us only.

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Tables

Table 1. Two sets of primer for chlorocatechol dioxygenase genes

Gene	Sequence
Forward	5′GTITGGCA[CT]TCIACICCIGA[CT]GG3′
Reverse	5′CCICC[CT]TCGAAGTAGTA[CT]TGIGT3
Forward	5′AAGAGGCATGGGGGCGCACCGGTTCGATCA3′)
Reverse	5′CCAGCAAACACCTCGTTGCGGTTGCC3′

Figures

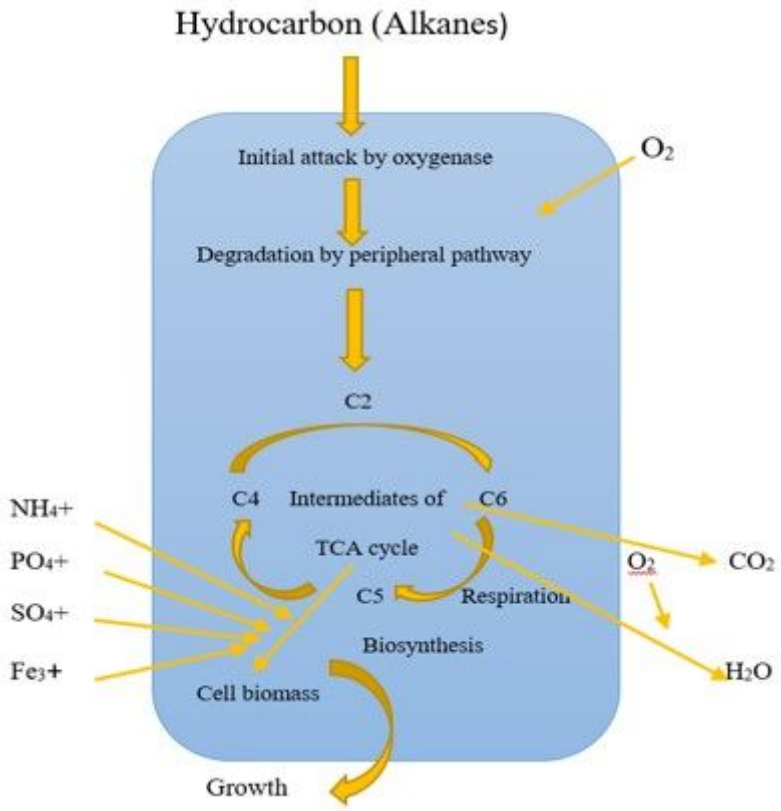

Figure 1

General pathway of oxygen degradation of petroleum alkanes by Microbes.

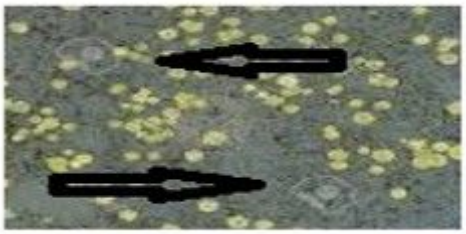

Figure 2

clear zone in the plate was seen around the isolates Sm53 and ISn21.

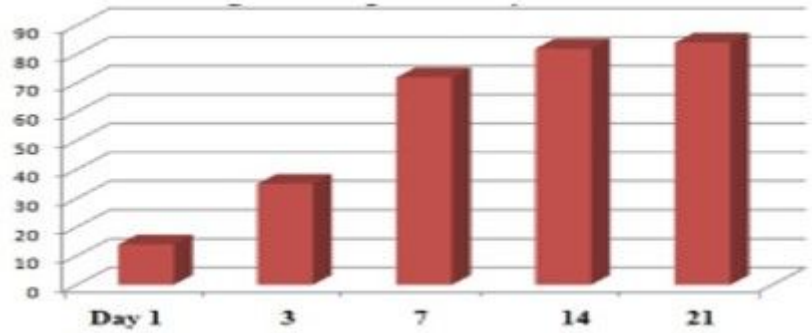

Figure 3

the percentage of degradation in gravimetrically method

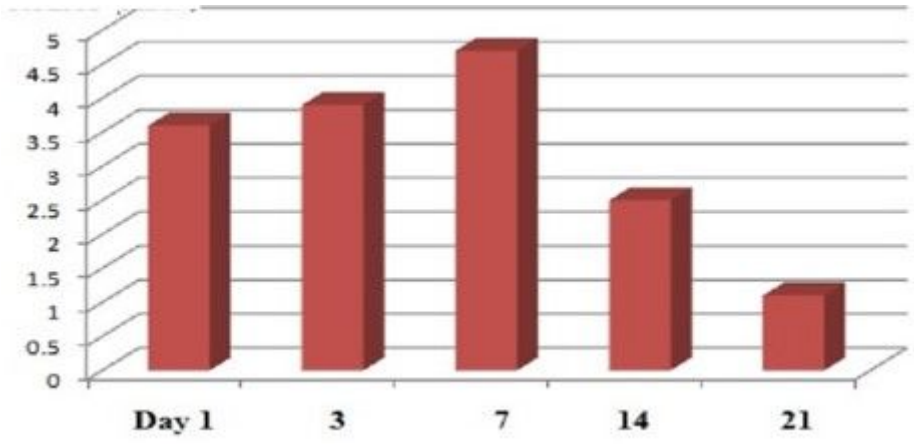

Figure 4

colonies growth (X106) on the third day and up to 72 % degradation on the 7th day of incubation of ISn2.

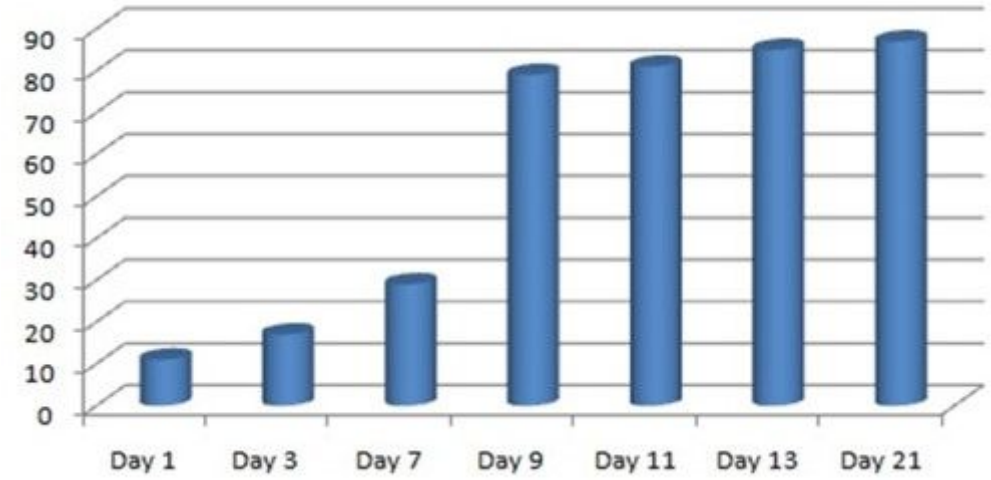

Figure 5

the percentage of degradation at cell density of cell sample (107cfu/ml)

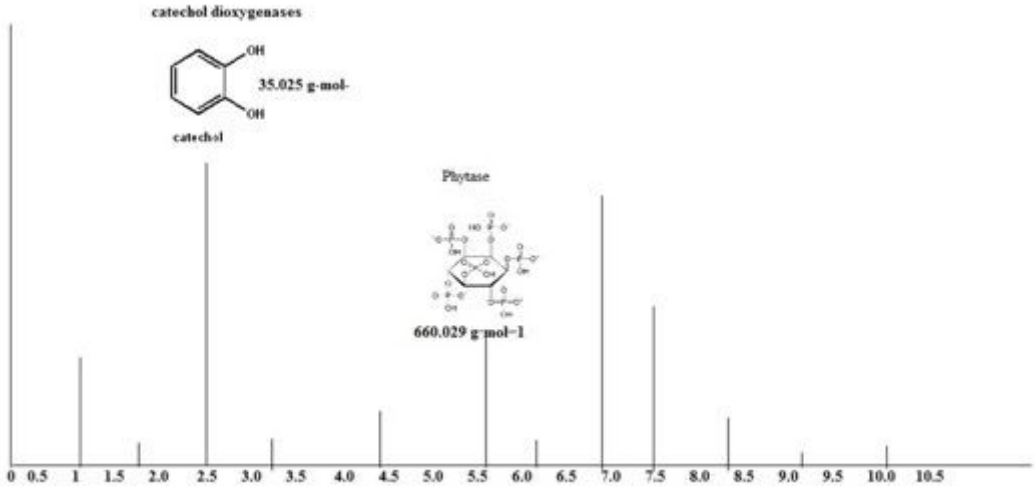

Figure 6

LC-MS/MS spectrometry analysis identified catechol dioxygenases

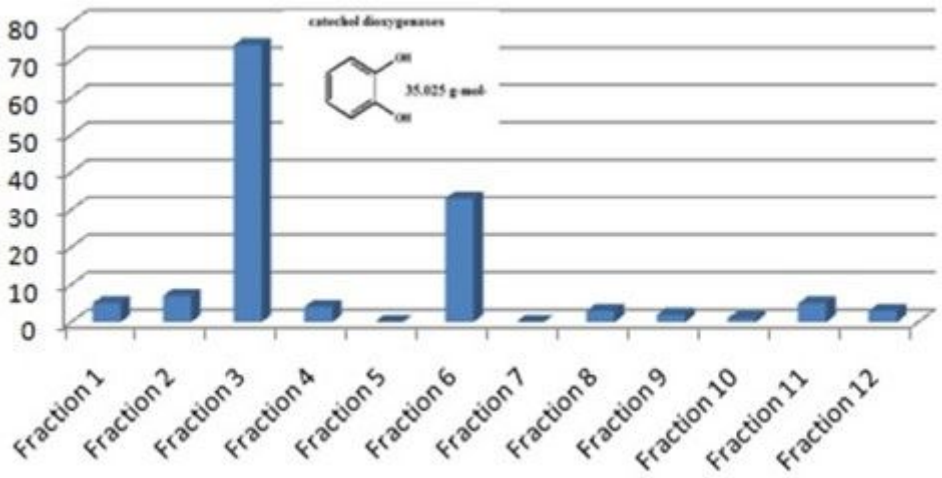

Figure 7

the percentage of degradation catechol dioxygenases by 2, 6-DCPIP assay

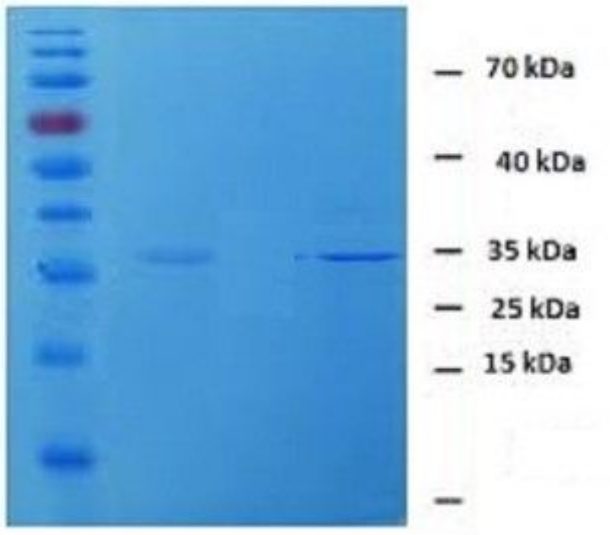

Figure 8

purified catechol dioxygenases subjected to one dimensional SDS-PAGE and one large polypeptide band was detected by silver staining corresponding to 35 kDa and also the enzyme had an isoelectric point of 6.8

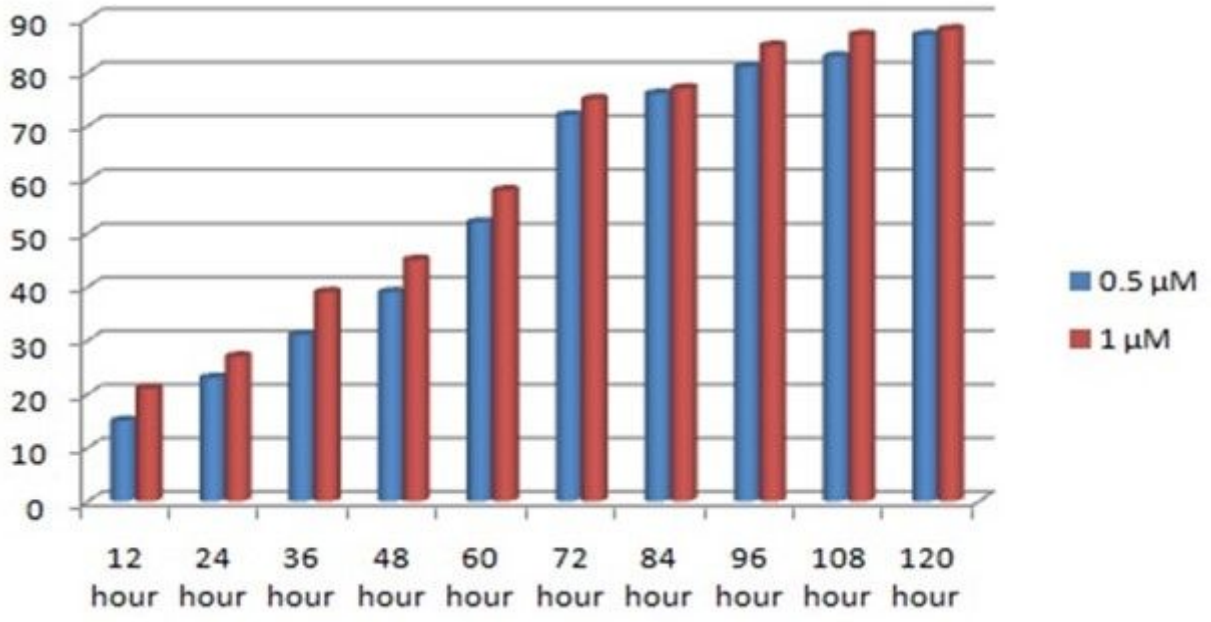

Figure 9

the percentage of degradation 0.5 µM concentration of catechol dioxygenases