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Research Article

Isolation, Identification and Oil Resistance of Protease Producing *Bacillus Subtilis* from Automobile Repair Centre Soil, Nanded (India)

Bhagwan N Rekadwad*, Laxmikant V Gumte and Chandrahasya N Khobragade

School of Life Sciences, Swami Ramanand Teerth Marathwada University, Nanded, India

***Corresponding Author:** Bhagwan N Rekadawd, School of Life Sciences, Swami Ramanand Teerth Marathwada University, Maharashtra, India.

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Abstract

The natural phenomenon of microbial bioremediation is an eco-friendly measure by which one can clean up petroleum hydrocarbon pollutants from the environments. We have isolated a total 12 isolates capable to degrade variety of hydrocarbons from automobile repair centres, Nanded (India). Of these 12 species, the protease producing bacterium Bacillus subtilis (HD1) was capable to grow and degrade kerosene, diesel, tar alone and in combinations. It is a potent degrader of hydrocarbon such as Diesel and Kerosene + Diesel + Tar hydrocarbon. The ability of the isolated Bacillus subtilis HD1 to grow in the presence of different hydrocarbons is clear evidence that its genome harbour the relevant resistant gene. The isolated strain HD1 may be useful in remediating oil polluted sites. Further research in this area is needed and will make contribution for marked improvement.

Keywords: *Oil pollution; Hydrocarbons; Bioremediation; Oxygenases; Bacillus subtilis*

Abbreviations: MSRTC: Maharashtra State Road Transport Corporation; CFU: Colony forming unit; MSB: Mineral salt broth; °C: Degree Celsius

Introduction

Petroleum products are one of the major sources of energy for industries and in daily life. Various petroleum products are directly released into environment. This incidence occurred while exploration, production, refining, transport handling and storage of petroleum products. These components also released into environment weather accidently or due to manmade activities take place at filling station repairing of motor vehicles, servicing etc. These activities are the main cause of soil and water pollution. The hydrocarbon containing soil and water causes extensive damage to the entire ecosystem and damaging human health leading to mutation in microorganisms or may cause death of higher organisms. To avoid such consequence, various technologies have been adapted to minimize its harm to the entire ecosystem such as mechanical burning, exploration, dispersion and washing. These useful methods for soil remediation are expensive and are characterized by incompletely removal of hydrocarbon contaminants, additionally, can also cause air pollution [1].

The process of bioremediation is one of the solutions to detoxify or remove pollutants using microorganisms. It is defined as "the use of microorganism to remove pollutants owing to their diverse metabolic capabilities" it is an evolving method for the removal and degradation of many environmental pollutants including the petroleum products. The success of bioremediation depends on environmental/abiotic factors such as temperature, pH, acidity, alkalinity, salinity etc. and biotic factor such as one's ability to establish and maintain stable condition (because of the presence of stable biomolecule) [2].

The complex process microbial degradation of petroleum compounds depends on the form (nature) and quantity of the hydrocarbon present in environment. Petroleum hydrocarbon is divided into four types such as saturated, aromatics, asphaltenes (phenols, fatty acids, ketones, esters and porphyrins) and resins (pyridines, quinolones, carbazoles, sulfoxides and amides). Degradation of hydrocarbon depends on their size and susceptibility to microbial attack can be generally ranked as follows: linear alkanes > branched alkanes > small aromatics > cyclic alkanes. Microbial bioremediation can be achieved by the alkanes degrading profile such as bacteria *(Arthrobacter, Burkholderia, Mycobacterium, Pseudomonas, Sphingomonas, Rhodococcus, Alcaligenes, Acinetobacter lwoffi, Flavobacterium sp., Micrococcus roseus, Corynebacterium ,Gordonia, Brevibacterium, Aeromicrobium, Dietzia)*; Fungi *(Amorphoteca, Neosartorya, Talaromyces, and Graphium)and yeasts (Candida, Yarrowia, and Pichia)*. These microorganisms were isolated from petroleum contaminated soil and proved to be the potential organisms for hydrocarbon degradation. Similarly, microalgae also take part in hydrocarbon degradation e.g. *Protheca20pfi*, Green algae, Red algae, Brown algae and Diatoms. These also have the ability to degrade hydrocarbons [3-7].

Microbial biotransformation has been performed by these microorganisms with the help of various enzymes produced during metabolism. Various enzyme produced by microorganisms such as dioxygenase, monooxygenase, hydroxylase, bacterial P450 oxygenase system, eukaryotic P450. In addition to these enzymes, amylase, protease, gelatinase, catalase, oxidase etc., are also produced by these microorganisms (Table 1) [8-11].

Enzyme	Substrate	Microorganisms	References
Soluble methane monooxygenases	C_1-C_8 alkanes alkenes and cycloalkanes	*Methylococcus,Methylosinus, Methylocysts Methylomonas, Methylocella*	McDonald., *et al*. 2006
Particulate methane monooxygenases	C_1-C_5 (halogenated) alkanes and cycloalkanes	*Methylobacter, Methylococcus, Methylocyctis*	
AlkB related alkane hydroxylases	C_5-C_{16} Alkanes, fatty acids, alkyl benzenes, cycloalkanes and so forth	*Pseudomonas , Burkholderia, Rhodococuus, Mycobacterium*	van Beilen., *et al*. 2002
Eukaryotic P450	C_{10}-C_{16} alkanes, fatty acid	*Candida maltose, Candida tropicalis, Yarrowialipolytica*	Iida., *et al*. 2000
Bacterial P450 oxygenase system	C_5-C_{16} alkanes, cycloalkanes	*Acinetobacter, Caulobacter, Mycobacterium*	van Beilen., *et al*. 2006
Dioxygenases	C_{10}-C_{30} alkanes	*Acinetobacter sp.*	Maeng., *et al*.1996

Table 1: *Enzyme involved in biodegradation of petroleum hydrocarbon.*

The complex structure and high molecular weight hydrocarbon, degradation of petroleum occurred very slowly and hence creates serious problem to the living things. Some living organisms are resistant to hydrocarbon pollutant metabolize these compounds by producing hydrocarbon degrading enzyme. This process is called microbial transformations. The use of microbial catalyst (enzymes) in biodegradation of organic compounds has advanced significantly during the past three decades [12].

In the present research, we have isolated and identified enzyme producing eubacteria and showed their biodegrading ability.

Materials and Methods

All chemicals used were purchased from Hi Media laboratories Ltd., Mumbai (India).

Collection of sample

The composite soil samples were collected in sterile polythene bags from long seasoned automobile repair centres in Nanded city (India) such as Bafna, MSRTC workshop.

Isolation of petroleum degrading bacteria

Step I: The under mentioned petroleum products (at 1% concentration) were combined with enrichment medium such as

a. Kerosene
b. Diesel
c. Kerosene + Diesel (1:1)
d. Kerosene +Diesel + Tar (1:1:1)

Step II: The collected soil sample (1g) was suspended in 100 mL sterile Zobell broth and Bushnell Haas medium containing 1% of the above mentioned petroleum hydrocarbons separately and incubated in a shaker (at 120 rpm) at 30°C.

Step III: 100 µL of enriched broth were spread onto the Zobell Marine agar plate and then incubated for 24h at 30°C. After incubation period, the colony forming units (CFU) was recorded. The organism showing diverse characteristics has been selected and obtained as pure culture for further study.

Morphological, biochemical characterization and identification of isolated species

All selected isolates were examined for their colony, cell morphology, motility, gram's staining and biochemical characteristics as per standard methods (Aneja, 2003). Isolated species were identified using Bergey's Manual of Systematic Bacteriology [13].

Production extraction and partial purification of protease enzyme

For the production of protease enzyme under submerged state fermentation, Mineral salt medium supplemented with casein (2%) inoculated with 5% 18 hr activated Bacillus subtilis culture and kept in a shaking incubator at 120 rpm for 5 days at 37°C. After completion of incubation period, produced enzyme was harvested by centrifugation of medium at 10,000 rpm for 10 min. at 4°C. The supernatant was collected and the enzyme was precipitated using ammonium sulphate (at 65% saturation and temperature of 4°C). The content was kept in refrigerator at temperature 4°C overnight to precipitate residual protease enzyme. On next day, the precipitated enzyme was separated by centrifugation at 10,000 rpm at 4°C for 20 min. Pellet was collected and kept at 4°C. The collected pellet was dissolved in 5 ml of phosphate buffer (0.1mM, pH 7), filled in dialysis bags (30 KDa) and dialysed against the same buffer (0.05 mM) for 12 hours [14].

Characterization of dialysed protease produced by HD1

Protease assay

Protease was assayed by using casein as a substrate. The protease enzyme activity was determined in sodium carbonate buffer (0.5 M, pH 10) using casein at 1% concentration. The 2 mL reaction mixture containing 0.5 mL casein and 1 mL of appropriately diluted enzyme solution were incubated at 30°C for 10 min, and the reaction was stopped with 1 ml of 10% tri-chloro-acetic acid (TCA). The tubes were centrifuged at 15, 000 rpm for 10 min and the degraded products were measured by modified Lowry method. One unit of alkaline protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine/min/ml under the standard assay conditions. The estimations were based on a tyrosine calibration curve [15].

Effect of different temperature on protease activity and stability

Optimum temperature of protease was determined at pH 7 by incubating the enzyme for 10 min. at 10 to 60ºC temperatures. Thermal stability of protease was expressed as percentage of initial activity taken as 100%.

Effect of different pH on Protease activity and stability

Optimum temperature of protease was determined at pH values ranging from 5 to 10 (acetate, pH 5; citrate, pH 6; phosphate, pH 7; Tris-HCl, pH 8; carbonate, pH 9 and 10; sodium phosphate- NaOH). Stability of protease was examined by incubating the enzyme in different buffers at pH values ranging from 3 to 11 for 1 h. Residual activity was estimated as described earlier and expressed as percentage of the initial activity taken as 100% [16-17].

Biodegrading activities of bacteria on petroleum oil

The degrading activities of isolates were obtained by using Mineral salt broth (MSB) containing 1% of each hydrocarbon (petrol, kerosene and diesel) was added and incubated at room temperature for seven days. The optical density at optical density 600 nm was taken using spectrophotometer [18].

Biodegradation studies on minimal medium

94 mL of minimal medium was inoculated with 1 mL hydrocarbon (1%) and 5% inoculum in 250 mL Erlenmeyer flasks for seven days in a shaking incubator (set at 100 rpm) at temperature 30°C. After every 24h, 5 mL medium was taken out. The ability of the isolated strain to degrade hydrocarbon was studied by measuring pH and optical density of the medium [19].

Results and Discussion

Hydrocarbon degrading bacteria were isolated on Zobell Marine agar and Bushnell Haas Medium using petroleum hydrocarbon such as petrol, diesel, petrol and diesel along with tar included in the entire medium. Isolation of bacteria was carried out at 37°C. A total of 12 isolates capable of growth on petroleum products were selected and obtained in pure culture. Of these 12 isolates, nine isolate showing luxuriant growth and diverse characterization has been chosen for further study. These were named as HD1 to HD9. All nine isolate observed were rod shaped endospore forming bacteria. Of these, seven isolate were Gram positive and two isolates were Gram negative. All isolates were capable to grow at 20% NaCl concentration within their optimum temperature and pH. All isolates utilized a variety of sugars and showed diverse enzyme profile including amylase, protease, and urease (Table 2). All the isolate have been identified based on morphological and biochemical characteristics.HD1, HD6 and HD9 were identified as *B. subtilis* HD2 to HD4 were identified as *Bacillus larvae*. HD7 and HD8 were identified as *Enterobactoraerogenes*. Protease produced by *Bacillus subtilis* (HD1) has optimum temperature of 40oC and optimum pH 7 (Fig. 1-2). Similar type of results has been reported [20].

Parameter	Result								
Code	HD1	HD2	HD3	HD4	HD5	HD6	HD7	HD8	HD9
Cell shape	R	R	R	R	R	R	R	R	R
Gram staining	+	+	+	+	-	+	-	-	+
Endospore	P	P	P	P	P	P	P	P	P
Motility	M	M	M	M	NM	M	M	M	M
Colour of colony	PY	Re	W	W	Y	PY	Y	Y	W
Size of colony (mm)	1	1	1	2	2	1	1	1	1
Shape of colony	C	C	C	C	C	C	C	C	C
Margin of colony	E	E	Ir	Ir	E	E	E	E	E
Elevation of colony	El	F	F	F	Cn	El	El	El	El
Opacity	O	O	O	O	O	O	O	O	O
Tolerance of NaCl (%)	20	20	20	20	20	20	20	20	20
Optimum temperature (°C)	37	37	37	37	37	37	37	37	37
Optimum pH	7.6	7.6	7.6	7.6	7.6	7.6	7.0	7.0	7.0
Glucose	+	+	+	+	+	+	+	+	+
Lactose	+	-	-	+	-	-	+	+	+
Sucrose	+	+	+	+	-	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+
Rhamnose	+	+	+	+	+	+	+	+	+
Raffinose	+	+	+	+	-	+	+	+	+

Starch	+	+	+	+	-	+	+	+	+
Casein	+	+	-	+	-	+	-	+	+
Urease	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+
Indole production	-	-	-	-	-	-	-	-	-
Methyl red	-	+	+	+	-	+	+	+	+
Voges-Proskauer	+	-	-	-	+	-	-	-	-
Citrate utilization	+	-	-	-	+	+	+	+	+
H_2S Production	+	+	+	+	+	+	+	+	+

Note: + = Positive, - = Negative, R= rod, M=motile, NM= non-motile, P, Present, PY= Pale yellow, Re= Reddish, W= White, Y= Yellow, C= Circular, E= Entire, Ir= Irregular, El= Elevated, F= Flat, Cn= Convex, O= Opaque

Table 2: *Morphological and biochemical characteristics of isolates.*

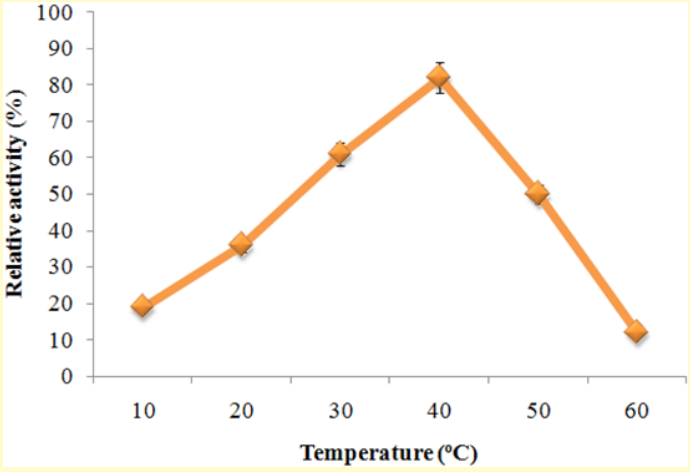

Figure 1: *Effect of different temperatures on stability and activity of HD1 protease.*

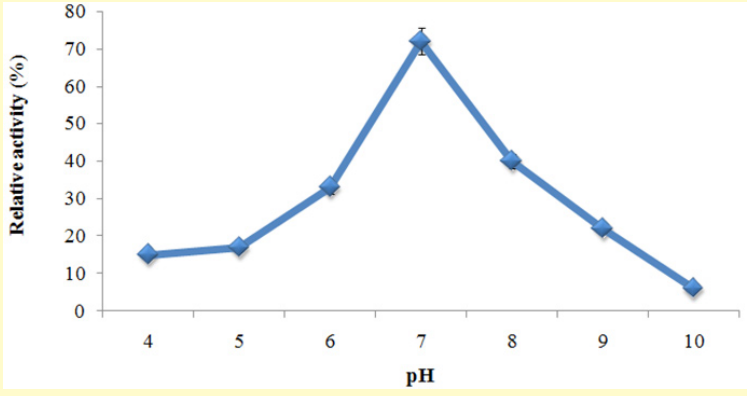

Figure 2: *Effect of different pH on stability and activity of HD1 protease.*

The biodegradation studies were performed using *B. subtilis* (HD1) using minimal medium and mineral salt broth containing 1% hydrocarbon with different combinations. The minimal medium supplemented by 1% kerosene + Diesel + Tar hydrocarbon, *Bacillus subtilis* showed maximum growth after 48 hours at its optimum temperature and optimum pH. Similarly, we have conducted studies using different combination at 1% concentration on Mineral salt broth (such as kerosene, Diesel, Tar hydrocarbon). HD1 showed noticeable resistant and showed growth in the presence of 1% diesel alone and 1% hydrocarbon mixture (kerosene + Diesel + Tar hydrocarbon) after 72 hrs. (Figure 3). Similar types of studies were performed by different research groups worldwide and reported that *Bacillus subtilis* species tolerate and grow on higher concentration of diesel, petrol and other hydrocarbon [21-23].

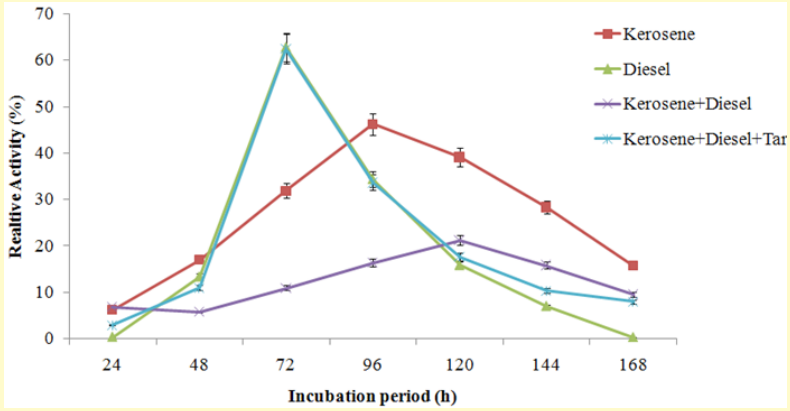

Figure 3: *Effect of incubation period on degrading ability of Bacillus subtilis on hydrocarbon.*

Conclusion

From the present research, it is clear that the protease producing *Bacillus subtilis* isolated by us have a high hydrocarbon degrading ability. *Bacillus subtilis* (HD1) showed as remarkable growth and resistance in the presence of diesel, petrol, and kerosene and tar. Hence, this strain may be used in cleaning oil polluted site.

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