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Research Article

Study of Actinomycetes Isolated From the Thar Desert, India for Their Potential to Produce Extracellular Enzymes

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ABSTRACT
Actinomycetes are known to produce a wide array of extracellular enzymes of industrial importance. They are extensively studied in various mesophilic habitats to obtain verity of enzymes used nowadays, whereas thermophilic species of actinomycetes are comparatively less studied. However, thermophilic actinomycetes are anticipated to produce many novel thermotolerant or thermostable enzymes that could withstand elevated temperatures of various industrial processing techniques. Extensive screening of forty four soil and mud samples across the Great Thar Desert of Rajasthan was done for the isolation of thermophilic actinomycetes. Twenty different isolates of thermophilic actinomycetes were obtained from these samples and further analysed for their ability to produce various extracellular enzymes such as, proteases, amylases, lipases, and catalases. These isolates were found to produce various enzymes at elevated temperature of 65 ^{0}C. Enzymatic screening of isolates revealed that the protease and lipase enzymes were produced by majority of the isolated species in abundant quantities and these enzymes were found stable and functioning at temperatures 55-65^{0}C. These isolates were further characterized and studied for their enzymatic potential. This study concludes that the Thar Desert soils and Thermal plant discards are a potential source of novel and thermostable extracellular enzymes useful in many industrial applications.

KEYWORDS: Thermophiles; thermophilic actinomycetes; thermophilic enzymes; thermostable proteases; thermostable lipases

INTRODUCTION

Actinomycetes are a group of Gram positive bacteria; also known as pseudo-fungi due to their fragmented mycelia like appearance. This specific group of bacteria is commonly found in every corner of the world and is known to be most diversely distributed organism. The Actinomycetes have been extensively explored for their bioactive potential in past century. They are a potential source of naturally occurring antibiotics, enzymes of industrial importance, pigments and carotenoids amongst others [1]. Majority of naturally occurring commercial antibiotics are derived from species of Actinomycetes. Although these bacteria are majorly studied and utilized on large scale for their metabolic potential since last many decades in moderate environmental conditions, they have been lesser tapped in extremophilic environments. Thus, it makes the extremophilic environment a potential source of novel extremophilic Actinomycetes and their useful

metabolic compounds functioning at extreme conditions.

Extremophilic actinomycetes thrive in extreme environments and in order to adapt to these environments they evolve and produce compounds which remain active in these harsh conditions. Besides growth under the extreme conditions, production of industrially valuable compounds, such as enzymes, antibiotics, hormones etc. have fascinated and attracted attention of entire scientific community. The extremophiles majorly include; Halophiles, Thermophiles, Barophiles, Psycrophiles and Acidophiles [2]. Amongst these, thermophilic bacteria and actinomycetes are the organisms which can grow and produce valuable compounds at high temperatures. Thermophiles have evolved and are adapted to grow and function in high temperature conditions and thus make them an anticipated source of metabolic compounds with high temperature tolerance [3].

Enzymes obtained from thermophiles have been proved to be more useful for biotechnological applications than mesophilic enzymes due to their stability at high temperature. Thermostable enzymes allow a higher operation temperature, which is an advantage over normal mesophilic enzymes because of a higher reactivity due to higher reaction rate and lower diffusional restrictions. Thermostable enzymes have higher stability and higher process yield due to increased solubility of substrates and products at high temperature and favourable equilibrium displacement in endothermic reactions. Operating at high temperature lowers the viscosity of the medium and highly discourages the contamination problems also. Therefore, isolation of thermostable enzymes has become highly attractive in recent [1].

Thermophiles are categorized on the basis of their temperature tolerance: for instance, facultative thermophiles, can grow at temperatures between 50˚C-65˚C, but can also survive at 37˚C; obligative thermophiles have maximum growth temperatures of 65˚C-70˚C, and will not grow below 40˚C; extremely thermophiles can grow between 40˚C-70˚C with an optimal growth temperature of about 65˚C and hyperthermophiles, mainly comprising of archae, can grow over 90˚C with a range of optimal temperatures between 80˚C-115˚C.

Thermophilic actinomycetes (actinobacteria) have been less explored due to difficulties in isolation and maintenance in pure culture. It largely remains to explore their diversity and production of biotechnologically useful enzymes and other compounds [1].

This study focuses on habitats and methods for isolation and recovery of thermophilic actinomycetes and their enzymatic potential at high temperature conditions.

MATERIALS AND METHOD

Sample collection

Total forty four different samples were collected from two major thermophilic environments. Firstly, from sand and rhizosphere soils from ten different areas of The Thar Desert Rajasthan and secondly from discards of Thermal plant situated at Kota, Rajasthan. These samples were collected and stored in sterile petri dishes and stored in iceboxes for transportation to laboratory. The samples were preserved at 4^0C in refrigerator for further experiments.

Sampling sites

Samples for the isolation of thermophilic actinomycetes are collected from different regions:

- Thermal Plant, Kota (effluent discharge and soil sample)
- Jodhpur sand
- Jodhpur soil (rhizosphere)
- Jaisalmer sand
- Jaisalmer soil (rhizosphere)
- Alwar sand
- Churu sand
- Pilani sand
- Bikaner sand
- Brick factory, near Alwar
- Jaipur soil

Isolation of Thermophilic Actinomycetes

The samples were treated at 100^0C for ten minutes in hot air oven before use. Actinomycetes Isolation Agar medium (Sodium caseinate 2.0 g/l, L-Asparagine 0.1 g/l, Sodium propionate 4.0 g/l, Dipotassium phosphate 0.5 g/l, Magnesium sulphate 0.1 g/l, Ferrous sulphate 0.001 g/l, Agar 15 g/l supplemented with 5ml/l Glycerol) and Nutrient Agar medium (Peptone 5 g/l, Sodium chloride 5 g/l, Beef Extract 1.5 g/l, Yeast extract 1.5 g/l and Agar 15 g/l) were used for isolation of actinomycetes.

Isolation of actinomycetes colonies from collected samples was done by serial dilution method on solidified agar plates of AIA medium and Nutrient Agar medium. One gram of dried soil was taken in 9 ml of distilled water and agitated vigorously using vortex mixer. Different aqueous dilutions: 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} of the suspension were spread onto plates containing AIA medium and Nutrient Agar medium. The plates were then incubated at different temperatures including 50^0C, 55^0C, 65^0C, 70^0C, 75^0C and 80^0C for selective isolation of thermophilic colonies. Actinomycetes colonies were differentiated by their unique colony structures and pure colonies were obtained by continuous sub culturing on isolation media [4].

Morphological Characterization of isolates

Sub cultured isolates of actinomycetes were morphologically characterized by the presence or absence of aerial mycelium, color of aerial/substrate mycelium, spore chain morphology [5], Gram's Staining and acid fast staining. These observations were analyzed using Bergey's Manual of Determinative Bacteriology, ninth edition [6].

Screening of isolates for production of caseinase enzyme

The isolates were screened for their potential to produce extracellular enzyme caseinase. Caseinases are protease enzymes known for breakdown of milk protein casein. The isolates were streaked on plates containing Skim Milk Agar medium and incubated at temperature 65^0C for 48 hours under sterile conditions. The isolates showing a distinct zone of clearance in this medium were reported positive for caseinase production [7].

Screening of isolates for production of gelatinase enzyme

Gelatinase production was screened using Gelatin medium containing 3% gelatin in sterile test tubes. The test tubes containing liquid medium were inoculated and incubated at high temperatures of 65^0C for 48 hours. The gelatin medium solidifies at 4^0C in 20 minutes. Isolates producing gelatinase enzymes degraded gelatin and thus these tubes did not solidify at 4^0C [7].

Screening of isolates for production of amylase enzyme

Production of amylase enzymes was screened using Starch Agar medium. After 48 hours of incubation at high temperature of 65^0C, the plates were flooded with iodine solution. The clear zone of hydrolysis around the colonies indicated the presence of amylase enzyme in the medium [8].

Screening of isolates for production of catalase enzyme

The medium containing freshly grown isolates (24 hrs cultures) were exposed with drops of pure hydrogen peroxide (H_2O_2). The formation of bubbles in the medium indicated the presence of enzyme catalase in the medium [9].

Biochemical characterization of potential isolates

Isolated colonies having enzymatic potential were selected for biochemical characterization. Selected isolates were tested and characterized using major biochemical tests that includes Carbohydrate fermentation test, MR-VP test, Nitrate reduction test, Indole utilization test, Phenylalanine utilization test, Urease test, Melanin production test, etc. [9].

RESULTS AND DISCUSSION

Screening of soil samples for isolation

A total of twenty isolated colonies of actinomycetes were obtained by screening of forty four different samples. The colonies were showing distinct morphological features, typical of actinobacteria and thus were isolated and sub-cultured to obtain pure colonies of bacteria. Continuous subculture of colonies ensured the purity of strains. These strains were then examined under microscope for confirmation.

The colonies were isolated from variety of regions of Thar Desert and a thermal plant discharge, Thus they are designated accordingly. Four strains were isolated from Thermal plant, Kota and designated as "TP1", "TP2", "TP6" and "TP9". Similarly three isolates were obtained from Jaisalmer (Rajasthan) sand samples and designated as "JLS1", JLS2" and "JLS3" and "JPR1", "JPR2" and "JPR3" were obtained from Jaipur (Rajasthan) soil.

Five isolates namely "JDS1", "JDS3", "JDS5", "JDS8" and "JDS11" were isolated from sand samples collected from Jodhpur region, whereas

"JDR2", "JDR5" "JDR6" "JDR8" "JDR11" were isolated from samples of rhizosphere (soils around root areas of plants) soils of Jodhpur region.

Out of these twenty isolates five (25%) were moderately thermophilic as their growth temperature ranged between 45^{0}C to 65^{0}C with growth optima at 55^{0}C. Remaining fifteen (75%) (Fig 1) isolates were thermophiles as their growth temperature ranged between 50^{0}C to 75^{0}C with growth optima at 65^{0}C (Table 1).

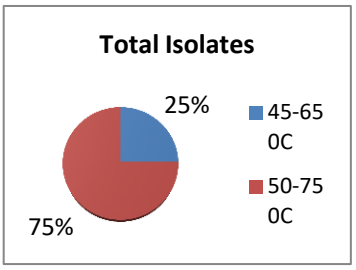


Fig 1: Showing diversity of thermophilic and moderately thermophilic isolates

Table 1: Isolates are categorized on the basis of their growth temperature

Incubation temperature	Total isolates	Category
45-65 ^{0}C	5	Moderately thermophilic
50-75 ^{0}C	15	Thermophilic
80 ^{0}C and above	None	Hyperthermophiles

Morphological characterization of isolates

The twenty isolated colonies were characterized morphologically using light microscope. All the isolates were able to produce distinct aerial mycelium and substrate mycelium. Aerial mycelium was powdery and chalky in nature and substrate mycelium was typically embedded on surface of agar medium (Fig 2). All the isolates were aerobic and Gram positive in nature; typical characteristics of actinomycetes bacteria. Isolates were reported acid fast negative (Table 2).

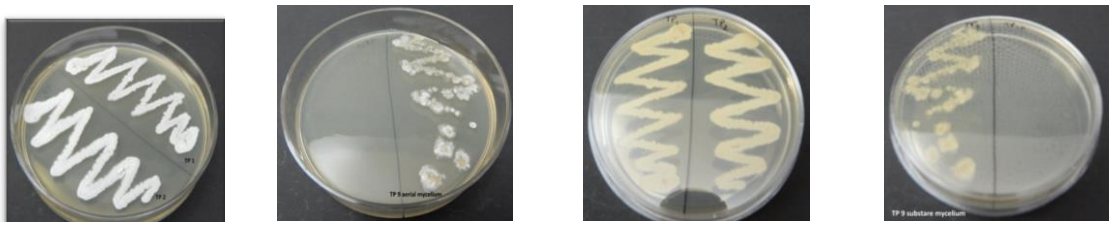


Fig 2: (a) and (b) are showing Aerial mycelium; (c) and (d) are showing Substrate mycelium

Table 2: Morphological characteristics of isolated colonies

Isolated Colonies	Characteristics					
	Colony Morphology		Microscopic Characterization	Optimum Growth Temperature	Gram Staining	Acid Fast Staining
	Aerial Mycelium	Substrate Mycelium				
TP 1	White & Chalky	Crème	**Rectus**: Straight filaments	65 ^{0}C	+	-
TP 2	White & Chalky	Crème	**Rectus**: Straight filaments	65 ^{0}C	+	-
TP 6	White & Chalky	Crème	**Spira**: Long spiral filaments (reproduce into chain of coccoids)	65 ^{0}C	+	-
TP 9	Powdery Light grey	Grey	**Spira**: Long spiral filaments (reproduce into chain of coccoids)	65 ^{0}C	+	-
JLS 1	Powdery creamy color	Crème	**Monoverticillus**: Straight branched filaments (coccoidal	65 ^{0}C	+	-

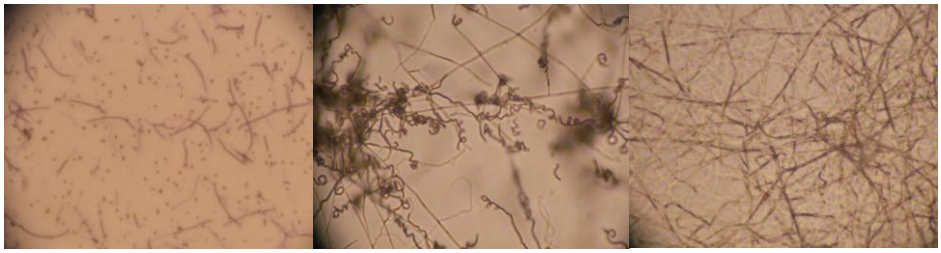
			spores)			
JLS 2	Dry White Powder	white	**Spira**: Short Spiral filaments breaks into spores	65 ^{0}C	+	-
JLS 3	Dry White Powder	white	**Monoverticillus**: Straight branched filaments (coccoidal spores)	65 ^{0}C	+	-
JPR 1	White & Chalky	Crème	**Flexibilis**: Network of flexible filaments (cocci shaped spores)	65 ^{0}C	+	-
JPR 2	White & Chalky	Crème	**Flexibilis**: Network of flexible filaments (cocci shaped spores)	65 ^{0}C	+	-
JPR 3	White & Chalky	Crème	**Flexibilis**: Network of flexible filaments (cocci shaped spores)	65 ^{0}C	+	-
JDS 1	Small flaky colonies	white	**Flexibilis**: Network of flexible filaments (cocci shaped spores)	65 ^{0}C	+	-
JDS 3	Light Grey Colonies	Light Grey	**Spira**: Long spiral filaments (reproduce into chain of coccoids)	55 ^{0}C	+	-
JDS 5	Dry White Powder	white	**Spira**: Long spiral filaments (reproduce into chain of coccoids)	65 ^{0}C	+	-
JDS 8	White powder	Crème	**Spira**: Long spiral filaments (reproduce into chain of coccoids)	55 ^{0}C	+	-
JDS 11	White & Chalky	Crème	**Flexibilis**: Network of flexible filaments (cocci shaped spores)	65 ^{0}C	+	-
JDR 2	Light cream powder	Crème	**Spira**: Short Spiral filaments breaks into spores	65 ^{0}C	+	-
JDR 5	White & Chalky	Crème	**Spira**: Short Spiral filaments breaks into spores	65 ^{0}C	+	-
JDR 6	White & Chalky	Crème	**Flexibilis**: Network of flexible filaments (cocci shaped spores)	65 ^{0}C	+	-
JDR 8	White & Chalky	Crème	**Spira**: Short Spiral filaments breaks into spores	55 ^{0}C	+	-
JDR 11	White & Chalky	Crème	**Spira**: Short Spiral filaments breaks into spores	65 ^{0}C	+	-

(+) = Positive result of test

(-) = Negative result of test

Colonies were grown in typical mesh like structure of fine filamentous mycelium. These filaments were either straight (Rectus) or spiral (spira) (Fig 3). Some isolates were showing branching of filaments (Monoverticillus) and some were unbranched flexible structures (Flexibilis) (Table 2).

All the isolates were showing sporulation, formation of coccoidal shaped spores at the ends of the filamentous growth.

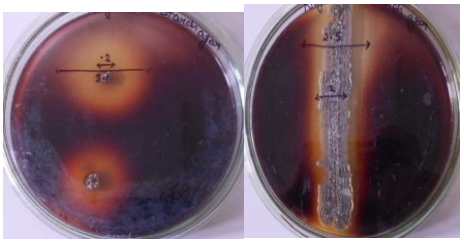


(a) (b) (c)

Fig 3: (a) Spiral filaments (Spira), (b) Straight filaments (Rectus) and (c) showing Branched filaments (Monoverticillus)

Enzymatic screening of isolates

Isolated colonies of actinomycetes were screened for the production of five extracellular enzymes *i.e*, Caseinase, Gelatinase, Amylase, Catalase and Lipase (Fig 4, 5 and 6). All the eighteen isolates were able to produce Proteases and catalase enzyme at high temperature conditions (65^0C) (Table 3). Amylase enzyme was produced by sixteen isolates (Table 3); similarly sixteen different isolates were able to produce lipases enzyme (Table 3) at elevated temperatures (65^0C). Amongst these eighteen tested isolates, six were producing all the five extracellular enzymes in abundant quantities. Those six isolates were "JPR2", "JLS2", "JDR2", "TP2", "JDS1" and "JDS11"; these isolates were then further characterized biochemically.

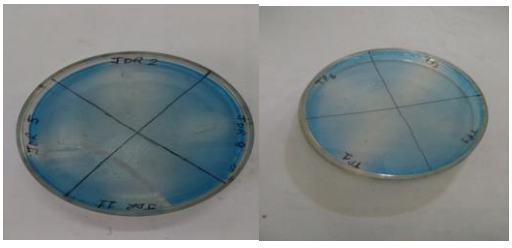


(a) (b)

Fig 4: (a) and (b) are showing positive result for amylase production

(a) (b)

Fig 5: (a) and (b) are showing positive result for protease production.



(a) (b)

Fig 6: (a) and (b) are showing positive result for lipase production

Table 3: Enzymatic screening of eighteen isolates

Sl. No.	Isolated Colonies	Production of Bioactive Compounds				
		Caseinase	Gelatinase	Amylase	Lipase	Catalase
1	**TP 1**	+++	+	-	++	+++
2	**TP 2**	+++	+	+	+++	+++
3	**TP 6**	+++	+	-	++	+++
4	**TP 9**	++	+	-	++	+++
5	**JLS 1**	+++	+	++	+++	+++
6	**JLS 2**	+++	+	++	+++	+++
7	**JLS 3**	+++	+	++	+++	+++
8	**JPR 2**	+++	+	+++	+++	++
9	**JDS 1**	+	+	++	+	+++
10	**JDS 3**	+	+	+	-	+++
11	**JDS 5**	++	+	+	+++	+++
12	**JDS 8**	++	+	+	+++	+++
13	**JDS 11**	+++	+	++	+++	+++
14	**JDR 2**	+++	+	+	+++	+++
15	**JDR 5**	+++	+	++	+++	++
16	**JDR 6**	++	+	++	-	++
17	**JDR 8**	++	+	+	++	+++
18	**JDR 11**	+++	+	+	+++	++

(+++) = very good quantity of enzymes
(++) = comparatively better quantity of enzymes
(+) = low quantity of enzymes
(-) = No results

Biochemical characterization of isolates

Six potential isolates were characterized for their biochemical properties and their results are summarized in a tabular format (Table 4).

Table 4: Biochemical characterization of potential isolates

Sl. No.	Biochemical test	JPR2	JLS2	JDR2	TP2	JDS1	JDS11
1.	Melanin production test	-	-	-	-	-	-
2.	Nitrate Utilization test	+	+	+	+	-	-
3.	Phenylalanine utilization test	-	-	+	-	+	+
4.	Oxidase Test	+	ND	+	+	-	-
5.	Motility Test	+	-	+	-	-	-
6.	Urease Test	+	-	+	-	-	+
8.	Gelatin liquification test	+	+	+	+	-	-
9.	H_2S production test	-	-	+	_	-	-
10.	Catalase test	+	-	+	+	-	-
	Carbohydrate fermentation						
	Sucrose	+	+	+	+	+	+
	Dextrose	+	+	+	+	+	+
	Fructose	+	-	-	-	-	+
	Galactose	-	-	-	-	-	-
12.	Indole Production test	-	-	-	-	-	+
13.	Methyl Red test	-	-	-	-	-	-

14.	Voges Proskauer test	-	+	-	-	-	-
15.	Citrate utilization test	-	-	+	-	-	-

(+) = positive result (-) = negative result

CONCLUSION

Different regions of The Thar Desert of Rajasthan and Thermal Plant, Kota were selected as sampling sites for the purpose of isolation of thermophilic actinomycetes with enzymatic potential at high temperature. Forty four different soil and sand samples were screened and twenty colonies of thermophilic actinomycetes were isolated from them. These colonies were found to be active elevated temperature conditions (65^0C). Out of these twenty isolates; eighteen isolates were found to be active producers of extracellular enzymes. Many isolates were showing high proteolytic, amylolytic and lipolytic activity. These enzymes were actively degrading their substrate at high temperature conditions. Thus, it is reasonable to conclude that these enzymes produced by thermophilic actinomycetes were thermostable enzymes and they show biocatalytic activity at high temperature. The present study successfully concludes that these thermostable enzymes can be potentially utilized in various industrial processes which require high temperatures conditions.

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CONFLICT OF INTEREST

The authors declare no conflict of interest in this research article.

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