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Isolation, Screening and Characterization of Extracellular Enzyme Producing Thermophilic Bacteria from Suryakund Hot Spring

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Abstract

The present study was conducted to identify and characterize the extracellular enzyme producing thermophilic bacteria isolated from Suryakund hot spring of Yamunotri temple, in Uttarakhand by using phenotypic methods. Total 22 isolates were tested for enzymatic activity (amylase, Lipase, protease and L-asparaginase). Only four isolates (YII-1, YII-3, YII-10 & YII-14) were found capable to produce extracellular enzymes. The isolate YII-3 and YII-10 were positive for amylase and lipase. Isolate YII-1 was only showing amylase activity whereas YII-10 was capable to hydrolyze casein and forms a clear zone. Morphologically, YII-1 was filamentous Gram-negative, YII-3 and YII-14 were filamentous Gram variable and YII-10 was Gram-positive. The isolates were related to the genus *Bacillus.*

Keywords: Extracellular enzyme, Thermophilic bacteria, Amylase, Lipase, Protease.

Introduction

Geothermal reservoirs are naturally occurring areas of hydrothermal resources like volcanoes and fumaroles (holes in the earth where volcanic gases are released), geysers and hot springs. Hot springs and geothermal vents are found in several parts of the world *e.g.,* Iceland (Kristjansson & Alfredsson, 1983; Marteinsson et. al., 2001), Kamchatka Peninsula, Russia (Belkova et. al., 2007), Tunisia (Sayeh et. al, 2010), New Zealand (Niederberger et. al., 2008), India (Ghosh et. al., 2003), Jordan (Mohammad et. al., 2017), China (Xue et. al., 2001), Japan (Takai & Horikoshi 2000).

In India, there are many hydrothermal sites are well-known, found in different areas, Tattapani in Chhattisgarh, Chhumathang and Puga in Ladakh, Cambay Graben in Gujarat, Manikaran in Himachal Pradesh, Surajkund in Jharkhand, Bakreshwar in West Bengal, Jakrem in Meghalaya, Reshi Hot Water Spring in Sikkim, Aravali hot water springs in Maharastra, Dhuni Pani in Madhya Pradesh, Gaurikund and Tapovan in Uttarakhand. Along with the tourism and aesthetic values, geothermal sites are untamed energy resource that can be used as produce electricity, to heat buildings and to provide hot water for various purposes (Bhardwaj & Tiwari, 2008).

During the last two decades, scientists of different fields have fascinated to explore the microbial communities of habitats with extreme condition *e.g.* temperature, pressure, salinity, pH, radiation, pollution or toxin, lack of water or oxygen. These microorganisms, called extremophiles, produce biocatalysts that are functional under extreme conditions (Van Den Burg, 2003), such as thermophiles and hyperthermophiles (organisms growing at high or very high temperatures, Kristjansson, 1991), psychrophiles (organisms that grow best at low temperatures, D'Amico et al., 2006), acidophiles and alkaliphiles (organisms optimally adapted to acidic or basic pH values, respectively, Pikuta et al., 2007), barophiles (organisms that grow best under pressure, Horikoshi, 1998), and halophiles (organisms that require NaCl for growth, Oren, 2002). Besides, these organisms are normally polyextremophiles, being adapted to live in habitats where various physicochemical parameters reach extreme values. The geothermal sites are ideal source of thermophiles.

Thermophilic prokaryotes occupied the place as the deepest root in the "The Universal Tree of Life" (Bock & Goode, 2008). Thermophiles utilize a variety of adaptations to grow and thrive in their intense environments such as changes in the membrane lipid composition and use of sodium-ions rather than protons as coupling ion in energy transduction (Tolner et al., 1997), thermal-stability of proteins through amino acid changes in their primary structure and heat shock proteins (HSPs) including the chaperones DnaK, GroEL, and GroES to assist protein folding (Orellana et al., 2018). enough experimental pieces of evidences (e.g., sequence, mutagenesis, structure, and thermodynamics) have been accumulated on hyperthermophilic proteins in recent years to conclude that no single mechanism is responsible for the remarkable stability of hyperthermophilic proteins. Amino acid composition and intrinsic propensity, disulfide bridges, aromatic interactions, hydrophobic interactions, hydrogen bonds, ion pairs, intersubunit interactions and oligomerization, post-translational modifications, metal binding, docking of the N and C termini, and anchoring of loose ends and decreased the entropy of unfolding are well-known factors that play a very important role in the thermal stability of proteins (Razvi & Scholtz 2006). Intrinsically stable and active at high temperatures, thermophilic and hyperthermophilic enzymes offer major biotechnological advantages over mesophilic enzymes.

Due to their increasing importance and roles in different fields, potential applications in biotechnological processes, scientists have concentrated their studies to discover new genus and species thermophilic organisms across the world. In the state of Uttarakhand, India, many hot springs are available in different regions with temperatures ranging between 30^0C-90^0C (Joshi et al., 2011). Although interest in studying thermophiles from hot springs in Uttarakhand has been demonstrated by a few previous microbial studies, still no sustained research had focused on further utilization of these thermophiles. The present study aims to to establish a continuous research line for screening, isolation, and characterization of new extremophilic microorganisms that can possess high biotechnological and environmental potential.

Methods and Methodology

Sample Collection, Isolation and Culture Condition

The water samples were taken from the hot spring, Suryakund located at Yamunotri Temple, District Uttarkashi, Uttarakhand, India. In the western region of Garhwal Himalayas at an altitude of 3,291 meters and geographic coordinates $31^0$1'0.12"N $78^0$27'0"E, this temple is dedicated to Goddess Yamuna. The water samples were collected just before the starting of the pilgrimage to keep disturbance minimum at the sampling site. The temperature and pH of hot spring water were noted on the spot. Water samples were collected in sterile plastic vials and kept in the thermos flasks (Arya et al., 2015). The temperature of samples was maintained during transportation. The bacterial cultures were obtained by the pour plate methods. Samples were serially diluted than 1 ml of sample was poured on the plates containing trypton soy agar medium with the help of a glass spreader. The inoculated plates were kept in the incubator for 24-36hr at 70^0C. The temperature was set according the temperature of site. These distinctive colonies were subsequently streaked on agar plates to obtain pure culture. The culture colonies were differentiated based on of their morphological feature e.g. texture, color, margin, shape and forms (Cappuccino & Sherman 1996). The morphological characteristics are presented in the Table1. The 20% glycerol stock with overnight bacterial culture were prepared and preserved at -20^0C in deep freezer. The agar slants were prepared as working stock culture.

Assessment of Enzyme Production

Amylase Activity

For the detection of starch hydrolysis activity of bacterial isolates the trypton soya agar medium was supplemented with 10g/l soluble starch and mix well by heating and autoclave it at 15 lbs pressure (121°C) for 15 minutes. This media was poured into sterile petri plates. After the media became solidified the fresh bacterial culture was streaked on the plates and kept in an incubator at 70^0C. Amylase activity was detected by flooding the surface of 48 hr old culture on starch agar with Gram's iodine (Cappuccino & Sherman 1996). Amylase positive organisms show clearing around the colony while the development of blue to purple zone indicates starch is not hydrolyzed. Size of the clear zone is directly proportional to the starch hydrolyzing activity of the strain under study.

Protease Activity

A solution of 10% w/v skimmed milk powder was sterilized by autoclaving and after the cooling it was mixed in the sterilized trypton soy agar medium and poured in petri dishes (Cappuccino & Sherman 1996). The white opaque milk powder containing agar plates were streaked with the bacterial isolates and incubated at 70°C for 24 hr. Clear zones around the colonies indicated casein hydrolysis.

Lipase Activity

Tributyrin agar medium was prepared by adding 10ml/l tributyrin to the media for the detection of bacterial lipolytic activity (Carrazco-Palafox et al., 2018). Medium was mixed and heated to boiling to dissolve completely. Medium was sterilized by autoclaving at 15 lbs pressure (121°C) for 20 minutes. Mix well and pour into sterile petri plates and poured onto plates. Plates were streaked with fresh culture and kept in incubator at 70^0C for 24-36 hr. Clear zones around the colonies indicated tributyrin hydrolysis by extracellular lipase enzymes.

L-Asparaginase Activity

Modified Czapek Dox's medium (Pradhan et al., 2013) was supplemented with different concentrations of the phenol red dye. A 2.5% stock of the dye was prepared in ethanol and the pH was adjusted to 7.0 using 1 mol/L NaOH. The stock solution of the dye, ranging from 0.04 ml to 0.3 ml, was added to 100 ml of modified M9 medium, giving final dye concentrations of 0.001–0.009%

respectively. After the streaking, plates were then incubated at 70°C for 24 hr, to observe colonies with pink zones around them.

Biochemical Characterization of the Enzyme Producing Isolates

The biochemical characteristics for preliminary identification of the isolated bacterial strains were carried out according to the Bergey's manual. The results of the biochemical characteristics of the selected bacterial isolates were presented in the Table 2.

Results and Discussion

Isolation and Assessment of Enzyme Production

The hot spring, located in Yamunotri temple represent a thermophilic (80^0C) and neutrophilic (pH 6.8-6.9) environment. Total 22 bacterial isolates were selected on the basis of their distinctive morphological features. All the isolates were screened for amylase, protease, lipase and L-asparaginase enzyme production. Only four isolates were capable of producing extracellular enzyme. The isolate YII-3 and YII-14 were showing the extracellular enzymatic activity for amylase and lipase. Isolate YII-1 was showing the amylase activity while YII-10 was able to produce extracellular protease. None of the isolates showed L-asparaginase activity.

S/N	Isolate	Size	Colony form	Elevation	Margin	pigmentation
1.	Y9I	Small	Irregular	Flat	Serrate	Cream
2.	Y9II	Small	Irregular	Umbonate	Serrate	White
3.	Y7	Moderate	Irregular	Umbonate	Loabte	Yellow
4.	Y14I	Small	Irregular	Flat	Undulate	Yellow
5.	Y12II	Moderate	Irregular	Umbonate	Lobate	Cream
6.	Y10	Large	Circular	Umbonate	Entire	Yellow
7.	Y13	Large	Circular	Umbonate	Serrate	White
8.	Y8I	Large	Circular	Umbonate	Entire	Cream
9.	Y12I	Small	Circular	Umbonate	Undulate	White
10.	Y2	Small	Irregular	Umbonate	Lobate	Yellow
11.	Y16	Small	Irregular	Umbonate	Undulate	White
12.	Y1	Moderate	Irregular	Umbonate	Undulate	Cream
13.	YII-3	Large	Irregular	Umbonate	Serrate	Yellow
14.	YII-19	Small	Irregular	Flat	Undulate	Cream
15.	YII-2	Large	Circular	Umbonate	Serrate	White
16.	YII-15	Large	Irregular	Umbonate	Entire	Yellow
17.	YII-10	Large	Irregular	Convex	Entire	Yellow
18.	YII-20	Moderate	Circular	Convex	Entire	White
19.	YII-14	Small	Irregular	Convex	Undulate	Cream
20.	YII-1	Moderate	Irregular	Convex	Undulate	Yellow
21.	YII-16	Small	Circular	Flat	Entire	White
22.	YII-4	Moderate	Circular	Flat	Entire	yellow

Table1. The Microscopic and Colonial Morphology of Bacterial Isolates from The Suryakund Hot Spring

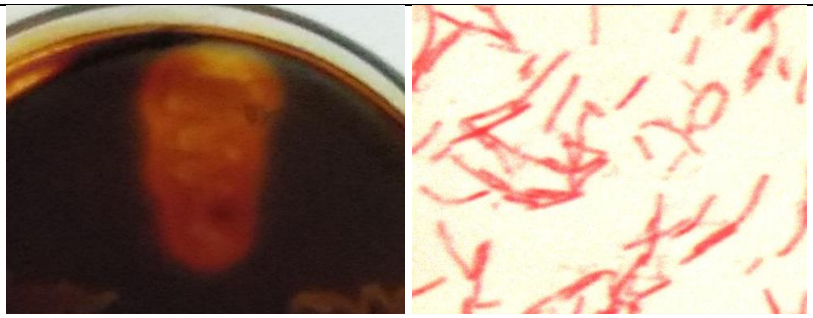
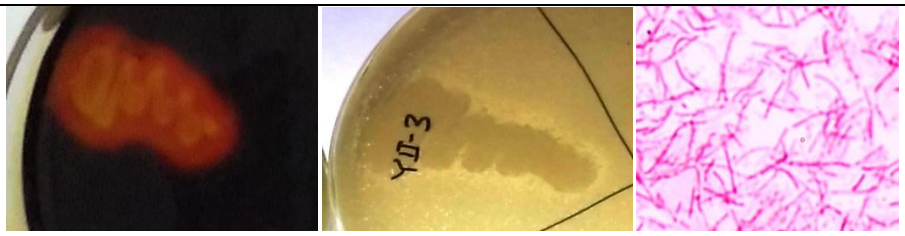
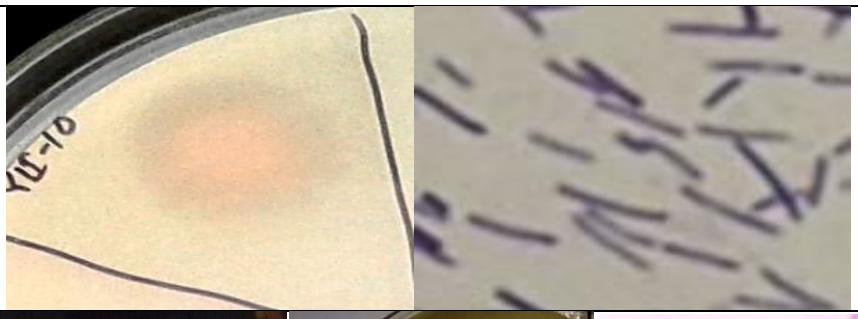
Isolates	Enzyme activity and Gram's reaction
	Figure showing the starch degradation by the isolate YII-1. Gram's reaction: negative
	Figure showing the starch and tributyrin degradation by the isolate YII-3. Gram's reaction: variable
	Figure showing the casein degradation by the isolate YII-10. Gram's reaction: positive
	Figure showing the starch and tributyrin degradation by the isolate YII-14. Gram's reaction: variable

Figure: Zone Formation and Gram's Reaction of Enzyme Producing Isolates

Biochemical Characterization

The investigation of biochemical and physiological properties of enzyme producing bacterial isolates showed that all the strains (YII-1, YII-3, YII-10 & YII-14) were negative for the IMViC and H_2S production test. These isolates were positive for catalase test. The isolate YII-10 was found non-fermentative for all tested carbohydrates (dextrose, sucrose and lactose) while YII-14 was capable to ferment all the tested carbohydrates. Isolate YII-1 was able to ferment dextrose and YII-3 was able to ferment dextrose and sucrose. The optimum pH and temperature for all strains were determined as 4-10 and 50-80°C, respectively. All strains were able to grow in the range of salt concentration of 2-4%. These fulfilled the criteria of thermophilic bacteria, which grew at temperatures above 50°C.

S/N	Isolate	Biochemical test								
		MR test	VP test	Indole production	Citrate Utilization	Catalase	H_2S production	Carbohydrate fermentation test		
								Dextrose	Sucrose	Lactose
1	YII-1	-ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve
2	YII-3	-ve	-ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve
3	YII-10	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve
4	YII-14	-ve	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve

Table2. Biochemical Characteristics of Enzyme Producing Isolates, -Ve Indicates The Absence of Reaction And +Ve Shows The Presence of Reaction

Conclusion

In this study total 22 bacteria were isolated and characterized phenotypically. The isolated bacterial strains showed significant enzyme producing ability. This ability may make them an important biotechnological tool for thermostable enzyme production. The investigations clearly indicate that the Suryakund hot spring is a rich source of many thermophilic bacteria and need to be explored for the industrially important enzymes by further studies on the microbiological aspects and genomics to explore the organisms. As the thermophiles grow at high temperature, so they must contain metabolites that can function at high temperature. The enzymes isolated from some thermophiles have proven to be of great use in the modern fields of biological sciences e.g. heat stable DNA polymerase for polymerase chain reaction, surfactants and in medicine as they can work under such conditions that would denature enzymes taken from most normal organisms. These isolates can be exploited in various useful processes after studying and cloning their gene.

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