

## Exploration of Unkeshwar hot springs in Maharashtra for thermostable amylase producer

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### ABSTRACT

Five efficient amylase producing thermophiles were isolated and characterized from hot spring of Unkeshwar, District Nanded, Maharashtra. Of these, a gram positive rod shaped organism APP-1 was selected for amylase production. It was identified as *Paenibacillus alvei* using cultural, microscopic and biochemical characterization. Isolate APP-1 showed luxuriant growth and amylase production at temperature 55°C and pH 7.0. SDS-PAGE analysis revealed molecular weight of amylase was 65 kDa. Amylase was found to be stable in presence of commercially available detergents. Catalytic activity of amylase was enhanced in presence of 1 mM  $\text{CaCl}_2$  and 2-Mercaptoethanol. © 2014 Trade Science Inc. - INDIA

### KEYWORDS

Amylase;  
*Paenibacillus alvei*;  
Unkeshwar hot spring;  
Detergents;  
SDS-PAGE.

### INTRODUCTION

Unkeshwar thermal springs are located in Nanded district of Maharashtra state in India. The temperature of two springs ranges in between 48-62°C. The springs are frequently visited by people of Maharashtra, Karnataka and Andhra Pradesh state. A small shrine of Lord Shiva is associated with the spring. The spring water is believed to have curative properties especially against skin diseases.

In present investigation biotechnologically important thermophilic bacteria are isolated and screened for production of thermostable amylase producer. Identification and characterization of efficient amylase producer and determination of catalytic efficiency of partially purified enzyme is also attained.

### MATERIAL AND METHODS

#### Study of physicochemical properties

Composite water samples were collected in pre-sterilized bottles in August 2011. Temperature and pH of samples were measured during collection using digital thermometer and pH meter respectively. In order to study abiotic characters of water following physico-chemical parameters were determined viz colour, odour, Methyl Orange Acidity, Free  $\text{CO}_2$ , Dissolved Oxygen, BOD, COD, Total Hardness, Total Alkalinity, Phenolphthalein Alkalinity as  $\text{CaCO}_3$ , TDS, TSS, TS, Calcium, Magnesium, Residual Chlorine, Chloride, Phenolphthalein Acidity as  $\text{CaCO}_3$ , Salinity, Total acidity to pH 8.3 as  $\text{CaCO}_3$ , Calcium Hardness, Magnesium Hardness, OH Alkalinity as  $\text{CaCO}_3$ ,  $\text{CO}_3$  Alkalinity as  $\text{CaCO}_3$ ,  $\text{HCO}_3$  Alkalinity as  $\text{CaCO}_3$ ,  $\text{SO}_4$  [2,6,10].

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### Isolation of thermophilic bacteria

Water samples were spread on nutrient agar and incubated at 50°C for 24 h. Incubated plates were observed and isolated colonies were selected for further experiments. Morphological characterization of selected colonies was performed and five distinct isolates were inoculated on starch agar plates to confirm amylase production. All five isolates were subjected for biochemical characterization<sup>[8]</sup>.

### Identification of isolates

The selected isolates were subjected for Gram staining. Sugar utilization pattern of isolates was determined by inoculating pure culture in peptone broth containing dextrose, fructose, lactose, sucrose, mannitol, maltose, xylose, arabinose, galactose, glycerol, cellobiose, sorbitol, mellibiose, mannose, trehalose, ribose, salicin, rhamnose, inulin, adonitol, and raffinose individually. Various enzyme production was tested by inoculating isolate in medium containing substrate like casein, starch, gelatin, cellulose, urea, tween-80 and pectin individually. Antibiotic susceptibility of all isolates was determined using Vancomycin (30µg), Ofloxacin (5µg), Teicoplanin (30 µg), Gentamicin (10µg), Cefoxitin (30µg), Amikacin (30µg), Netillin (30µg), Piperacillin (100µg), Ceftazidime (30µg), Cefoperazone (75µg), Ampicillin (10µg), Penicillin (10 U/disc), Ceftriaxon (30µg), Chloramphenicol (25µg), Ciprofloxacin (5µg), Cotrimoxazole (25µg) and Tetracycline (30µg) antibiotic discs<sup>[8]</sup>.

### Screening for efficient amylase producer

Selected isolates were spot inoculated on starch agar plate and amylase production efficiency was examined and isolate showing highest catalytic efficiency was selected for further production<sup>[7]</sup>.

### Effect of pH, temperature and incubation period on growth of selected isolate

Effect of pH on growth of selected isolate was determined using 0.2 M acetate buffer, 0.1 M Citrate buffer, 0.2 M Phosphate buffer, 0.2 M Tris buffer, 0.2 M Glycine-NaOH buffer of various pH values ranging from pH 4 to 10. Effect of temperature on growth of selected isolate was determined by incubating culture at various temperatures ranging from 35 to 75°C. Effect of incubation period on growth of selected isolate was determined by incubating inoculated samples for various incubation periods viz 24, 48, 72 and 96 h<sup>[9]</sup>.

### Enzyme production and optimization of parameters

Medium containing Starch 1.0%, Yeast extract 0.3%, peptone 0.5%,  $\text{KH}_2\text{PO}_4$  0.2%, NaCl 0.3%,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.005%<sup>[12]</sup> was inoculated with 5% inoculum containing  $3 \times 10^9$  cells/ml of selected strain. Medium was incubated in Orbital Shaking Incubator (CIS-BL 24, Remi Make, Mumbai) at 55°C and 120 rpm speed for 72 h for optimum enzyme production. Effect of pH on enzyme production was determined using appropriate buffers of various pH values ranging from pH 4 to 10. Effect of temperature on enzyme production was determined at various temperatures ranging from 35 to 75°C. Effect of incubation period on enzyme production was determined at various incubation periods viz 24, 48, 72 and 96 h.

### Extraction and purification of amylase

After 72 h incubation, the culture broth was cooled up to 4°C temperature and then filtered through Whatman No.1 filter paper and filtrate was centrifuged at 10,000 rpm for 10 min at 4°C in cooling centrifuge. Supernatant was used as crude enzyme extract. Solid ammonium sulphate was added slowly to the crude enzyme extract to attain 60% saturation. It was then kept for overnight at 4°C. The precipitate was collected by centrifuging at 10,000 rpm for 10 min and dissolved in 0.2 M phosphate buffer of pH 7.0 and dialyzed against the same buffer at 4°C for 24 h.

### Qualitative detection of amylase activity at 55°C

Partially purified amylase was further subjected to determine stability and efficiency at high temperature. 100 µl of partially purified enzyme was placed in the well in starch agar plate and incubated at 55°C temperature for 24 h.

### Enzyme assay

Crude enzyme extract (1 ml) was added to the test tube containing 1 ml of 1 % starch solution prepared in 0.2 M phosphate buffer pH 7.0. This mixture was incubated at 55°C for 10 min. The produced reducing sugar was measured using the dinitrosalicylic acid method<sup>[11]</sup>. One unit activity was defined as the amount of enzyme required to liberate  $1 \mu\text{mol min}^{-1} \text{ml}^{-1}$  of reducing sugar expressed as maltose equivalent, under the assay conditions.

### Molecular weight determination

Molecular weight of partially purified amylase was

determined by the sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE; 12%) as described by Laemmli<sup>[11]</sup>. Protein broad range molecular marker (Merck Biosciences) was used to determine molecular weight of amylase.

### **Effect of pH and temperature on catalytic efficiency of amylase**

Effect of pH on catalytic efficiency was determined using buffers of various pH values ranging from pH 4 to 10. Effect of temperature on catalytic efficiency was determined at various temperatures ranging from 35 to 85°C<sup>[7]</sup>.

### **Effect of temperature and pH on stability of amylase**

Thermal stability of amylase was examined by incubating the enzyme at 35 to 85°C for 3 to 9 hours in 0.1 M potassium phosphate buffer, pH 7.0 without substrate and then measuring the enzyme activity. The pH stability was determined by incubating the enzyme at pH of 4-10 in the 0.1 M acetate buffer (pH 4, 5), 0.1 M potassium phosphate buffer (pH 6, 7, 8) and 0.1 M Glycine-NaOH buffer (pH 9, 10) for 3 to 9 hours without substrate and then measuring residual (remaining) enzyme activity<sup>[11]</sup>.

### **Effect of substrate concentration on enzyme activity and determination of Km and Vmax values**

Amylase activity was determined at different starch concentration ranging from 5 to 30 mg/ml (pH 7.0) and after incubating at 65°C for 10 min enzyme activity per unit time was determined in each substrate concentration. Value of Km and Vmax were determined by plotting Lineweaver -Burk plot<sup>[3]</sup>. The starch concentration at which maximum enzyme activity obtained was used as 100% and relative enzyme activity was calculated.

### **Effect of commercially available detergents on enzyme activity**

Compatibility of amylase with commercially available laundry detergents was studied by using Ariel® (Procter and Gamble, Suisse), Tide® (Procter and Gamble, Suisse), Rin® (Hindustan Lever Ltd India), Wheel® (Hindustan Lever Ltd India), Surf excel® (Hindustan Unilever Ltd India), Nirma® (Nirma Lever Ltd India), Ghadi® (Kanpur trading Co. Pvt. Ltd. Kanpur, India), Sasa® (Sasa Detergent, Pune) and Vim® (Vim Co Ltd India) at final concentration 7 mg/

ml. Diluted detergents were boiled for 1h to denature the endogenous enzyme. Amylase preparation was preincubated with detergent solution and enzyme activity was determined in presence of detergents under standard assay condition<sup>[1]</sup>. The enzyme activity without detergent was taken as 100%.

### **Effect of metal ions on amylase activity**

The partially purified enzyme was incubated with the chloride salt of different metal ions at 30°C for 15 min and the residual enzyme activity was determined. The activity of enzyme in the absence of metal ion was taken as control (100%)<sup>[5,11]</sup>.

### **Effect of different solvents, surfactants, denaturing, chelating and oxidizing agents on amylase activity**

Catalytic activity of amylase was estimated in the presence of Ethanol, Methanol, Isopropanol, Acetone, Glycerol, Xylene, Hexane, Acetonitrile, Benzene at 1 and 10% concentrations, Tween-80, Tween-20 and Triton X-100, SDS, Hydrogen peroxide, DTT, 2-mercaptoethanol, EDTA at 1 and 5 mM concentration, Urea at 4 and 8 M. Amylolytic activity was studied by pre-incubating the enzyme in the presence of these additives for 30 min at 50°C, and then performing the assay in the presence of the same substances at the optimum temperature<sup>[1,4]</sup>. Residual activities were expressed as a percentage of the activity of the untreated control taken as 100%.

## **RESULTS AND DISCUSSION**

### **Study of physicochemical properties**

The temperature of water recorded was 48°C and pH was neutral. The water sample was colorless and odourless. TS, TSS and TDS recorded were 2, 1 and 1 mg/L respectively. Methyl orange acidity, phenolphthalein alkalinity, residual chlorine and salinity of Unkeshwar hot spring water was recorded as zero. Free CO<sub>2</sub> content, total hardness, chlorides, phenolphthalein acidity, Magnesium, calcium and sulphate were 8.8, 132, 42.6, 30, 7.7515, 40.08 and 32 mg/ml respectively. BOD, COD and DO values recorded were 2.04, 0.008 and 14.28 mg/L respectively.

### **Isolation of thermophilic bacteria**

Isolation of thermophiles was carried out using nu-

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trient agar and tryptone yeast glucose agar. Out of these nutrient agar has supported fast growth of diverse colonies. Total 160 colonies were appeared on nutrient agar plate after incubation of 24 h. Out of these 160 colonies, 20 distinct colonies were selected and further streaked on nutrient agar plates individually. From these five fast growing isolates were further selected and designated as APP-1 to APP-5. Colony

characteristics, sugar utilization pattern, enzyme profile and antibiotic sensitivity of selected five isolates are given in TABLE 1.

### Identification of selected thermophiles

All isolates were Gram positive rods, spore former and catalase positive. All isolates have used dextrose as carbon source. All isolates showed amylase and

**TABLE 1 : Colony characteristics, sugar utilization pattern, enzyme profile and antibiotic sensitivity of selected five isolates**

Colony characteristics	APP-1	APP-2	APP-3	APP-4	APP-5
Shape	Irregular	Round	Irregular	Round	Round
Size (cm)	0.1-0.15	0.4	0.1	0.4	0.1
Margin	Cerate	Cerate	Cerate	Cerate	Entire
Colour	White	Dirty White	Dirty White	White	Off White
Elevation	Slightly Elevated	Elevated	Flat	Flat	Convex
Consistency	Dry	Dry	Dry	Non Sticky	Partial Dry
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
Microscopic features					
Gram Nature	Gram positive	Gram positive	Gram positive	Gram Positive	Gram positive
Cell Size (Micron)	L-2.0, b-0.5	L-2.0, b-0.5	L-1.0, b-0.5	L-0.9, b-0.5	L-1.0, b-0.5
Cell Shape	Thin rod	Rod	Rod	Short rod	Rod
Motility	Motile	Motile	Motile	Motile	Motile
Sporulation	+	+	+	+	+
IMViC Test					
Indole test	+	-	-	-	-
Methyl red test	-	-	-	-	-
VP test	+	-	-	+	-
Citrate utilization test	-	+	+	+	-
Utilization of sugars					
Dextrose	++	+	+	+	w+
Fructose	+	+	-	+	-
Lactose	-	-	-	-	-
Sucrose	-	+	-	+	-
Mannitol	-	+	+	+	-
Maltose	w+	+	-	+	-
Xylose	-	-	+	-	-
Arabinose	-	-	+	-	-
Galactose	-	+	+	-	-
Glycerol	-	+	-	+	-
Cellobiose	-	-	-	+	-
Sorbitol	-	-	-	-	-
Mellibiose	-	-	-	-	-
Mannose	-	-	+	+	-
Trehalose	-	+	-	-	-
Ribose	-	+	+	+	-
Salicin	-	+	-	+	-

Gram Nature	Gram positive	Gram positive	Gram positive	Gram Positive	Gram positive
Rhamnose	-	-	-	-	-
Inulin	-	-	-	-	-
Adonitol	-	-	-	-	-
Raffinose	-	-	-	+	-
Gas Production in all Sugar test	-	-	-	-	-
Enzyme profile					
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Caseinase	+	++	+	+	-
Amylase	++	+	+	+	+
Gelatinase	+	+	-	w+	-
Cellulase	-	-	-	-	-
Urease	-	-	-	-	-
Lipase	-	-	-	-	-
Pectinase	-	-	-	-	-
Antibiotic sensitivity (zone in cm)					
Vancomycin (30 µg)	2.1	2.8	2.6	2.3	2.5
Ofloxacin (5 µg )	3.2	4.2	3.5	3.4	4.2
Teicoplanin (30 µg)	2.2	2.2	2.5	2.4	2.9
Gentamicin (10 µg)	3.5	3.9	4.0	3.7	3.4
Cefoxitin ( 30 µg)	2.8	4.5	3.2	2.9	4.6
Amikacin (30 µg)	3.3	4.2	3.8	3.2	3.5
Netillin (30 µg)	3.0	3.3	3.5	3.5	3.4
Piperacillin (100 µg)	3.0	3.0	2.8	3.2	2.9
Ceftazidime (30 µg)	2.7	2.0	2.1	2.9	2.2
Cefoperazone (75µg)	3.7	3.0	2.7	2.8	2.9
Ampicillin (10 µg)	2.5	1.0	R	2.5	2.0
Penicillin 10 U/disc	2.9	1.8	2.5	1.5	1.9
Ceftriaxon (30 µg)	3.5	2.7	1.8	2.5	2.25
Chloramphenicol (30 µg)	3.5	2.0	1.5	2.6	2.40
Ciprofloxacin (5 µg)	4.2	3.5	3.5	2.4	3.6
Cotrimoxazole ( 25 µg )	3.7	3.2	3.5	R	4.0
Tetracycline (30 µg)	3.0	2.7	2.6	R	1.9
Identified as	<i>Paenibacillus alvei</i>	<i>Bacillus brevis</i>	<i>Bacillus sterothermophilus</i>	<i>Bacillus subtilis</i>	<i>Bacillus farraginis</i>

caseinase production. Sugar utilization patterns, enzyme profile of five isolates were compared with reference strain and isolates were identified as *Paenibacillus alvei* (APP-1), *Bacillus brevis* (APP-2), *Bacillus sterothermophilus* (APP-3), *Bacillus subtilis* (APP-4) and *Bacillus farraginis* (APP-5).

#### Screening for efficient amylase producer:

Remarkable amylase production was observed with APP-1, the Gram positive rod shaped bacterium. The APP-1 therefore used in further experiments.

#### Effect of pH, temperature and incubation period on growth

Optimum growth of APP-1 was recorded at 55°C temperature (Figure 1) and at neutral pH (Figure 2). APP-1 showed maximum growth after 72 h of incubation period (Figure 3).

#### Effect of pH, temperature and incubation period on amylase production

After inoculation of sufficiently grown inoculum,

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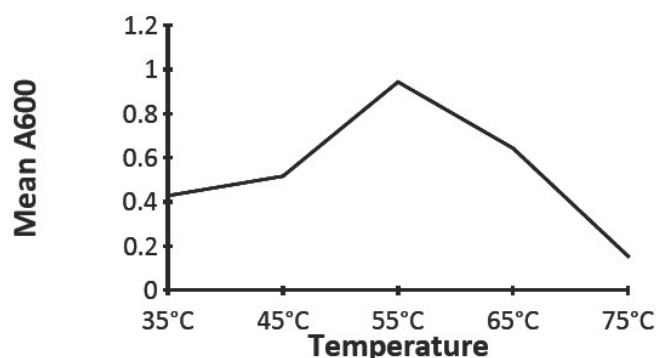


Figure 1 : Effect of temperature on growth of APP-1

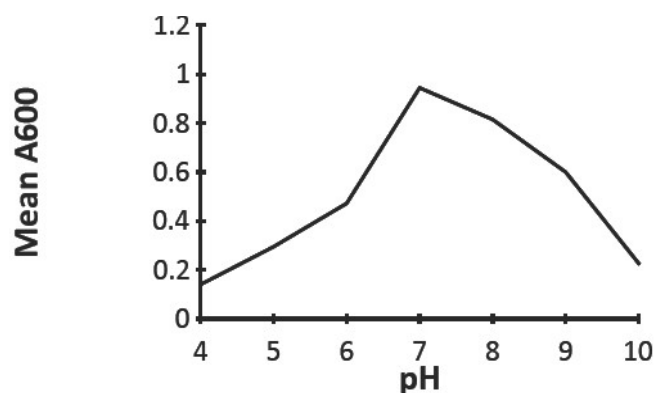


Figure 2 : Effect of pH on growth of APP-1

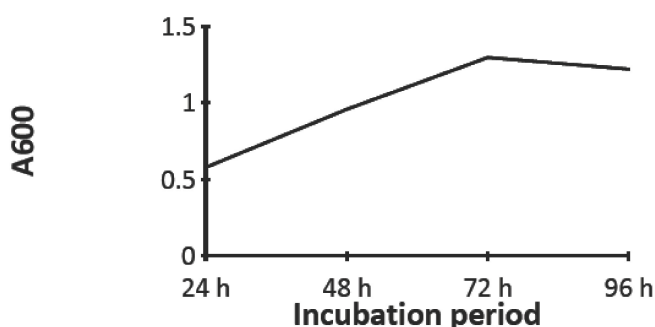


Figure 3 : Effect of incubation period on growth of APP-1

optimum amylase production was recorded at 55°C (Figure 4). Maximum enzyme production was obtained at pH 7.0 (Figure 5) and at 72 h of incubation period (Figure 6).

### Qualitative detection of amylase activity at 55°C

Zone of clearance of 20 mm diameter was formed around the well after flooding the plates with Grams iodine. In this way catalytic activity of partially purified APP-1 amylase was detected at 55°C.

### Molecular weight determination

SDS-PAGE analysis revealed that molecular weight of partially purified amylase was 65 kDa (Figure 7).

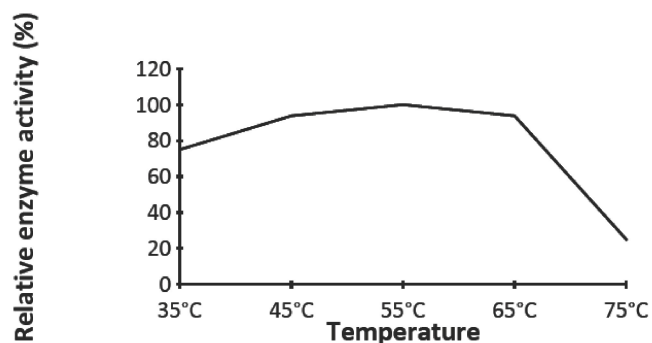


Figure 4 : Effect of temperature on production of APP-1 amylase

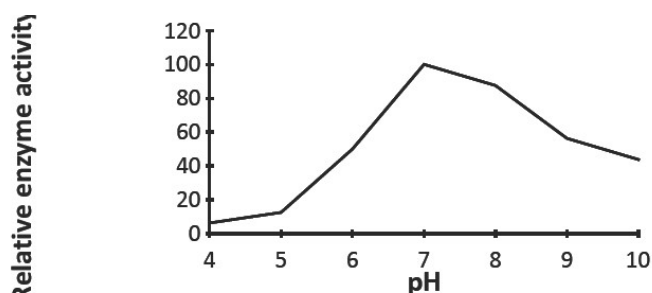


Figure 5 : Effect of pH on production of APP-1 amylase

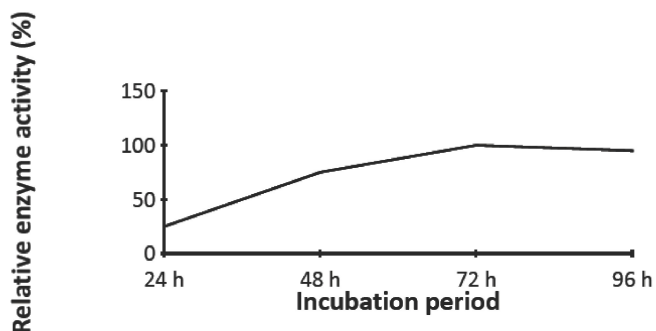
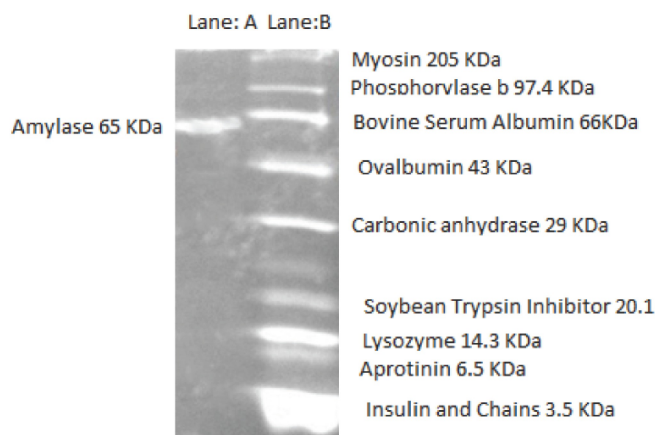


Figure 6 : Effect of incubation period on production of APP-1 amylase

### Effect of temperature and pH on activity and stability of amylase

In present study, an assay temperature of 65°C and pH of 7.0 were found to be optimal for catalytic activity of amylase (Figure 8 and Figure 9). The effect of temperature on activity and stability of partially purified amylase showed high stability for a wide range from 35 to 75°C up to 3 h of incubation period (Figure 10). The enzyme retained 42.65% of its original activity even after incubation period of 9 h at 75°C. The enzyme lost 60.01% and 84.61% of activity at 85°C after 3 and 9 h of incubation period respectively. The enzyme retained 91.63% and 62.48% of its original activity after incubation period of 3 h at pH 9 and 10 respectively (Figure 11).



Lane A: APP-1 amylase, Lane B: Molecular weight markers

Figure 7 : Molecular weight determination of APP 1 amylase

**Effect of substrate concentration on enzyme activity and determination of Km and Vmax values**

Vmax and Km calculated values were 1.6 μM/ml/min and 15.15 mg/ml respectively.

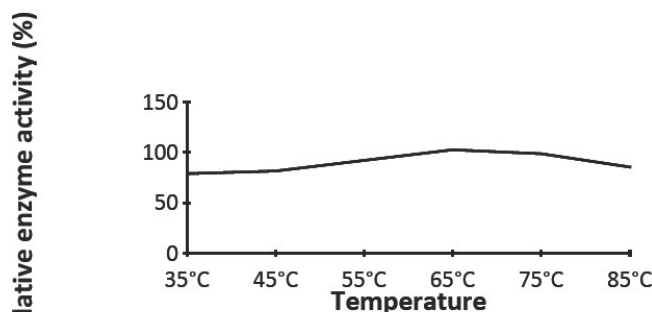


Figure 8 : Effect of temperature on catalytic activity of APP-1 amylase

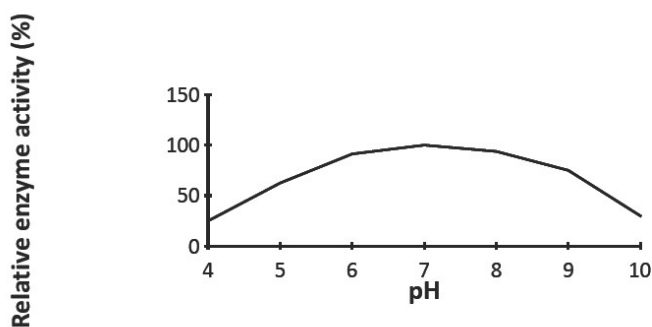


Figure 9 : Effect of pH on catalytic activity of APP-1 amylase

**Compatibility of amylase with commercially available detergents.**

APP-1 amylase was extremely stable in the presence of Sasa®, Vim® and Wheel®. Amylase retained about 99% its initial activity with Sasa® and 98% its initial activity with Vim® and Wheel®. Amylase was found to be least stable in the presence of Ariel®, re-

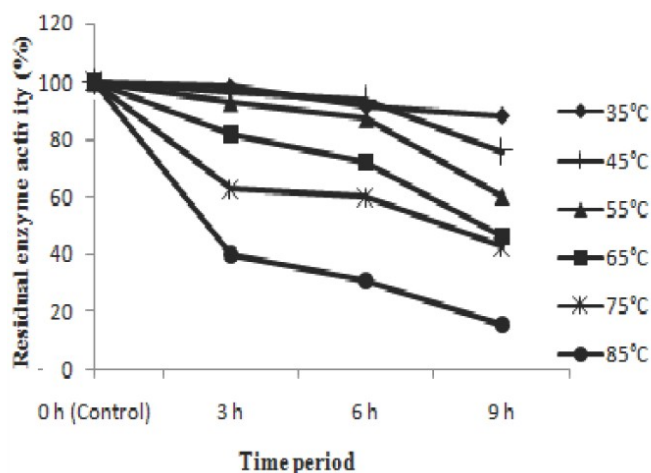


Figure 10 : Effect of temperature on stability of APP-1 amylase

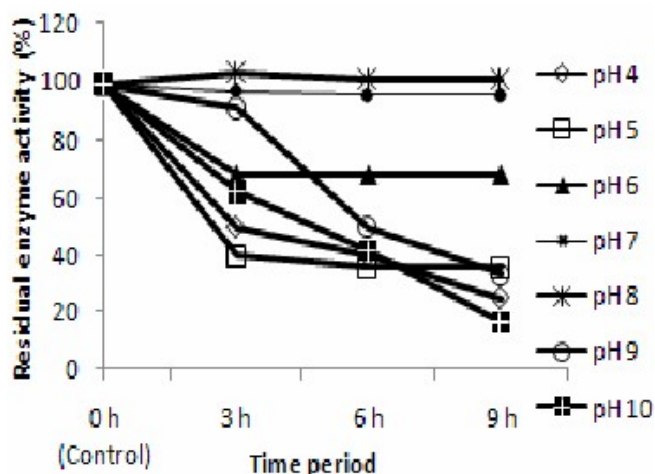


Figure 11 : Effect of pH on stability of APP-1 amylase taining 82.27% of its initial activity (Figure 12).

**Effect of metal ions on amylase activity**

Amylase was strongly inhibited by Al<sup>3+</sup> (41.24%) followed by Hg<sup>2+</sup> (37.49%), Ba<sup>2+</sup> (34.99%) and Cu<sup>2+</sup> (25%) at 1 mM concentration. Enhanced amylase ac-

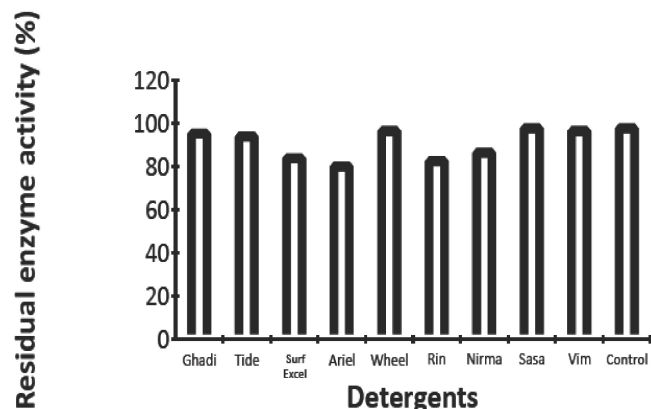


Figure 12 : Compatibility of commercial detergents with APP-1 amylase

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tivity was recorded by addition of  $\text{Ca}^{2+}$  followed by  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Ni}^{2+}$  and  $\text{Co}^{2+}$  at 1 mM concentration. Amylase activity in the presence of different metal ions at 1 and 5 mM concentration is showed in Figure 13.

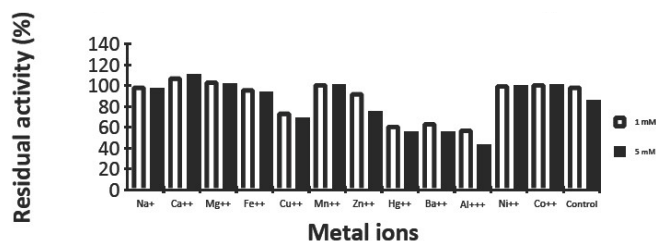


Figure 13 : Effect of metal ions on APP-1 amylase

### Effect of different solvents, surfactants, denaturing, chelating and oxidizing agents on amylase activity

Activity of amylase was increased 1.05, 1.04 and 1.025-times in presence of 1 mM of 2-Mercaptoethanol, Triton X-100 and SDS respectively. It was remarkably inhibited (93.74%) at 1 mM and completely inhibited at 5 mM concentration of DTT. 86.25 and 62.5% activity of amylase was recorded with 1 and 5 mM concentration of EDTA respectively. Amylase showed 93.75 and 80% activity in the presence of Tween-80 and Tween-20 at 1 mM concentration however the activity was decreased by 23 and 33% at 5 mM concentration respectively. Amylase showed maximum activity in the presence of Benzene (95%) followed by Isopropanol (93.5%), Hexane (87.5%), Ethanol (86.25%), Glycerol (85%), Acetone (81.25%), Methanol (75%), Xylene (62.5%) and Acetonitrile (50%) at 1% concentration. 87.5 and 62.5% of amylase activity was recorded in the presence of Urea at 4 and 8 M concentration respectively (Figure 14).

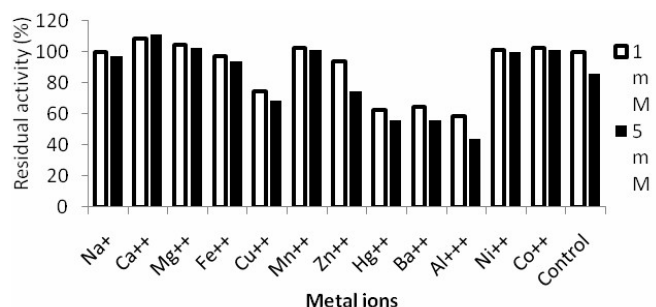


Figure 14 : Effect of different solvents, surfactants, denaturing, chelating and oxidizing agents on amylase activity

## CONCLUSION

In conclusion, five different amylase producing

bacteria were isolated and identified from the Unakeshwar hot spring. The efficient amylase producer was identified as *Paenibacillus alvei* APP-1. Amylase produced by APP-1 was highly stable and active at high temperature and showed optimum activity in the range 55-75°C temperature. The APP-1 originated amylase showed excellent stability and compatibility with commercial detergents. Thus APP-1 amylase may find the use in different industries, where starch degradation is carried out under high temperature.

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