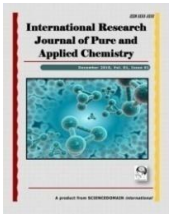

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Thermo-stable, Calcium Independent Alpha Amylase from Two *Bacillus* species in Afar, Ethiopia

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Authors' contributions

This work was carried out in collaboration between all authors. Author GDH designed the study. Author TWA managed the literature search, executed the experiments and wrote the protocol and the first draft of the manuscript with assistance from author GDH. Authors TBG and TWA preformed the statistical analysis. Author SA read and approved the final manuscript.

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ABSTRACT

Thermostable enzymes play a major role in the food industry. Two isolates (named as TGS1 and TGS2), which were later identified as *Bacillus* sp., were able to produce alpha amylase at a higher temperature (45-60ºC). The enzymes from the microorganisms were characterized at different temperatures (45-95ºC). Enzyme activity at different concentrations of metal ions (Ca^{2+}, Mg^{2+} and Zn^{2+}) and different times (10-40 minutes) of incubation was also tested. The optimum temperature for the enzyme isolated from TGS1was between 55 and 65ºC (2.722 and 3.134 U/mg,

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respectively), while the enzyme from TGS5 was active at temperatures of between 75 and 85ºC (2.786 and 2.904 U/mg, respectively). Both enzymes were found to be calcium independent.

Keywords: Thermostable amylase; ethiopian hot springs; alpha amylase.

1. INTRODUCTION

Thermophiles are microorganisms that have optimal growth temperature between 50-64ºC and a maximal temperature at which growth occurs below 70ºC. Extreme thermophiles are those microorganisms whose optimal growth temperature is between 65-85ºC and a maximal temperature above 70ºC and lastly, hyperthermophiles are microorganisms that have an optimal temperature above 85ºC and a maximal temperature above 90ºC. The highest temperature presently known to support biological life is 121ºC [1].

Industries get a variety of biotechnology tools like outstanding stable enzymes which remain active in a wide range of pH, temperature and extremes saline concentrations from Extremophilic microorganisms. Extreme environments include conditions such as high salinity, acidity, alkalinity and extreme temperature which are predominant conditions in a variety of industrial processes. Microbial population that can live and reproduced in this kind of environment are called extremophiles and its microbiology is being widely studied around the world, since they produce enzymes able to work under such conditions and can be used in biotechnological and industrial potential applications.

Amylases exhibit biotechnological applications as they can be used in various food, fermentation, textile and paper industries [2,3] and can be extracted from animals, plants and microorganisms [4]. The α-amylases isolate from bacteria differ in their physico-chemical properties (optimum temperature and pH, substrate specificity and end product of hydrolysis) [5,6].

Current starch industries use two ways for its hydrolysis. These are the acidic and enzymatic methods. Acidic hydrolysis requires mineral acids (pH, 1-2), enhanced temperature (150-230ºC) pressure. Acidic hydrolysis results unnecessary by products and thereby contaminate the hydrolysate. On the other hand enzymatic hydrolysis of starch requires milder conditions i.e., temperature (up to 100ºC), mild pressure and pH (6-8). In addition, hydrolysis using ensymes is characterized highly specific, high reaction rate and high stability to denaturation towards temperature and reaction solvents [7].

Afar region (located in Ethiopia) is one of the driest and hottest areas in the world. Continental breakup is caused by some combination of heating and stretching. The Afar Rift system in Africa is an example of active continental rifting, where a mantle plume probably weakened the lithosphere through thermal erosion and magma infiltration. In Afar the potential of geothermal sites as source of thermostable alpha amylase are not investigated. Therefore, this research is mainly to assess the potential of the geothermal site as source of enzyme and produce active, heat resistant, reusable alpha amylase for the hydrolysis of starch in starch industries.

2. MATERIALS AND METHODS

2.1 Sample Collection

Samples (soil and water) were taken from hot springs and soils of Afar namely; from Afdera, and Awash national park, after measuring temperature of water. Sample collection procedure was as indicated in our earlier report [3]. A sterilized steel pipe (50 mm diameter) was pressed 50-100 mm down into the soil. Sample was then transferred to a new plastic container and closed. Each sampling is preceded by washing of the pipe in 70% alcohol. From each location five soil samples were withdrawn randomly over an area of $1m^2$. Samples were then well mixed by shaking the container. Water samples were taken using a 2 meter long plastic pipe. The pipe was pressed 10-20 cm into the bottom. Similar to the soil samples five water samples (500 ml) were taken from each hot springs.

2.2 Total Microbial Count

A well mixed 1 gm portion of sample was aseptically transferred into test tube containing sterile 9 ml water and was mixed by magnetic stirrer for 10 min until a homogenized suspension was obtained. The suspension was allowed to

settle down large particles before sub samples were taken. Samples of the solution were diluted in sterile water up to 10^{-6} and appropriate dilution (0. 1 ml) was spread plated onto pre dried surfaces of standard plate count (PC) agar plates to investigate the total microbial load. The sample was incubated at 37ºC for 48 hrs and the total microbial colony was counted [8].

2.3 Isolation of Thermophilic Amylase Producing Bacteria

The stages in the isolation of thermostable bacteria are shown in Fig. 1. Serial dilution method was employed to isolate bacteria from the samples [9]. The nutrient broth-starch-agar medium was used for the isolation of bacteria. one g of the soil and 1ml of water samples were dissolved in 100 ml of sterilized saline water in different containers. Suitable dilution (10^{-1} - 10^{-6}) was prepared, given heat shock at 90ºC for 15 min followed by cooling to room temperature [3]. A 0.1 ml of each diluted suspension was transferred to the Petri plates containing nutrient broth-starch-agar medium. The Petri dishes were gently rotated clockwise and anticlockwise, spread by glass road to facilitate a uniform spreading of diluted suspension on nutrient broth starch agar medium and was incubated at 40°C in the incubator for 48 hrs. The colonies showing clear difference in cultural characteristics were further purified by sub culturing on nutrient agar, coded and preserved in a refrigerator using slants of nutrient agar [10].

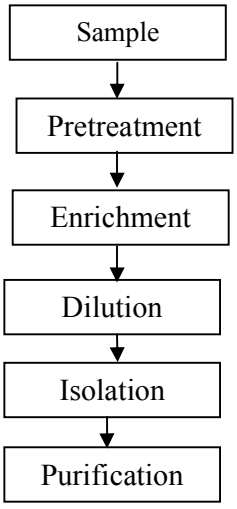

Fig. 1. Isolation of thermostable Bacteria

2.3.1 Screening of thermophilic amylase producing bacteria

The isolated bacteria were primarily screened for α – amylase synthesis after incubation at 60ºC for 48 hr. The petridishes were washed using I_2 solution (0.5% (w/v)) and KI (5.0% (w/v)) [3,11]. Bacterial colonies with a clear were then picked as they indicate starch hydrolysis. Measurement (diameter) of the clear zone was also recorded in millimeters.

2.4 Taxonomic Classification of Bacteria

Identification of the selected bacteria was done at the Ethiopian health and nutrition research institute on the basis of standard morphological tests according to the method described in Bergey's Manual of Determinative Bacteriology [12].

2.5 Screening for Amylase Producers

Screening of thermostable microorganisms that can produce thermostable α-amylase was done by enriching the sample in sterile saline water, isolating in nutrient broth starch agar (Fig. 2). The selected bacteria which were stored in nutrient broth-starch- agar slants were transferred to test tubes that contain nutrient broth and incubated at 65ºC for 48 hrs. The incubated culture were streak plated into nutrient broth –starch – agar medium and incubated at 60ºC for 48 hrs. After incubation the plates were flooded with iodine- iodine solution and colonies showing clear halos on the agar plates were selected as promising amylase producing strains. Primary screening of bacterial isolates for production of alpha amylase was done by the starch agar plate method [13]. The starch hydrolysis test was performed by adding a few drops of freshly prepared iodine on the petridishes at the end of the incubation time. Where starch was not hydrolyzed a blue-black color was observed due to the formation of starch-iodine complex. A zone of clearance was observed around the cultures that were producing amylase.

2.6 Production of Alpha Amylase

Bacterial culture isolated and stored were streak plated onto agar plates containing 0.5% meat extract, 1% poly peptone and 2% wheat raw starch. These plates were incubated at 60ºC for 24 hrs and exposed the petridishes to vapors of

iodine or washing colonies with a solution of iodine [3,11]. These colonies showing clear halos on the agar plates were selected as they are amylase producing strains and inoculated in to test tubes that contain nutrient broth and incubated at 65ºC for 48 hrs. A liquid culture medium containing 0.5% meat extract, 1% poly peptone and 1% soluble starch was prepared. One hundred milliliters of medium in 100 ml baffled flasks were inoculated with 4 ml of an overnight culture and incubated at four different temperatures (45, 50, 55 and 60ºC) with rotary shaking. The culture was harvested after 6 hr in the first run and every 6hr for the first temperature in order to select two high amylase producer bacteria. For the other temperatures samples were taken every 3 hrs up to 36th hr and cells were separated by centrifugation at 4000 rpm for half an hour. The culture supernatant which is cell free was used as the crude enzyme source. Optimum production time and temperature of the enzyme was analyzed based on the mount of product of hydrolysis. The mount of hydrolysis product was calculated in reference to its absorbance at 540 nm from standard maltose absorbance.

2.7 Characterization of Alpha Amylase

2.7.1 Effect of temperature

Partially purified enzyme was incubated at temperatures of between 35-95ºC.

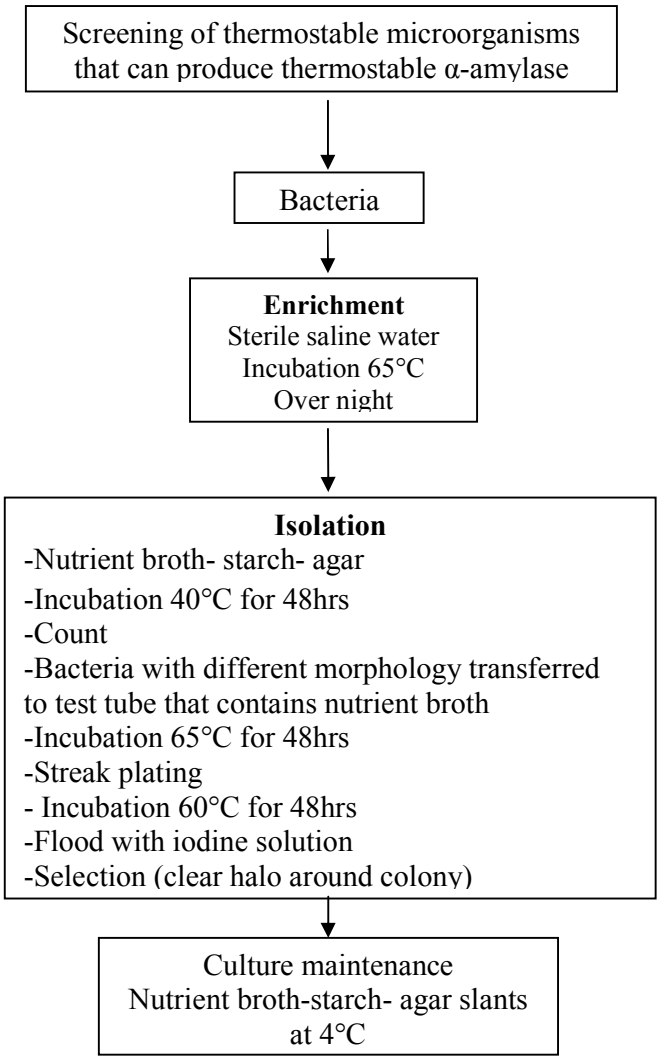

Fig. 2. Screening of amylase positive organisms [23]

This is followed by the measurement of the enzyme activity using a starch substrate which is prepared in 20 mM of sodium phosphate buffer at pH 6.0 [3]. High temperature stability of enzyme solutions was determined at 45, 55, 65, 75, 85 and 95ºC for 10 min [14].

2.7.2 Effect of metal ions

Activities of α-amylase was done using 1.0 mM, 5 mM and 10 mM of salts of $CaCl_2$, $MgCl_2$ and $ZnCl_2$. Enzyme activity was also measured without any ions to be considered as reference.

2.7.3 Enzyme activity

To 2 ml of soluble starch 0.5 ml of amylase and 4.5 ml of 0.1 M phosphate buffer (pH 6.0) were added and incubated at 30ºC for 30 min in a shaking water bath. The reaction was interrupted by adding 2 ml of 3-5-Dinitrosalicylic acid. Then the test tubes were left in boiling water for 15 min to allow the development of color, followed by cooling to room temperature. Absorbance was then measured (540 nm) using a Spectrophotometer after adjusting the volume to 10 ml. For the preparation of the standard curve maltose was utilized as a standard. A unit of amylase activity the quantity of enzyme releasing 1 µmol maltose per min under the assay conditions Specific activity is expressed as amylase activity per mg of protein.

3. RESULTS AND DISCUSSIONS

3.1 Screening of Thermo-stable α-amylase Producing Microorganisms

The sampling areas are within the Ethiopian rift valley and involve the northern portion of the Great East African Rift system. The temperature of the waters varied from 44.36ºC to 48.33°C. The water temperature and pH of hot springs cover a wide range (30 - 100ºC and a pH of 1-15) and such a natural geothermal environment is usually diverse as a habitat [15,16]. The colonies grown in the media were counted and expressed as average colony number (Table 1).

3.2 Thermo-stable α-amylase Producing Bacteria

Five strains named as TGS14, TGS5, TGS1, TGS7 and TGS11were selected as the best promising amylase producers based on the halo diameter formed (Table 2) from which TGS1 and TGS5 were selected for enzyme production and characterization. In addition, to the halo diameter the time to discolor iodine by the microorganisms in nutrient broth was recorded as average. The average time recorded was 6, 7, 10, 15 and 18 for TGS1, TGS5, TGS14, TGS7 and TGS11, respectively.

Table 1. Average number of total colony from selected hot springs and soils of Afar

Hyper thermal springs and soils	Mean ± SD
AFW	169.625 ± 30.9005^{a}
AFS	89.875 ± 21.7678^{c}
ANW	111.375 ± 15.3803^{b}
ANS	121.125 ± 4.9117^{b}

Values are mean ±standard deviation. Values followed by different letters within a column indicate significant difference (P < 0.05). AFW: Afdera Water; Afdera Soil; ANW: Awash national park Water and ANS: Awash national park Soil

Table 2. Average halo diameter and time taken to discolor iodine solution of the isolated bacteria

Bacteria	Halo diameter(mm)	Time of discoloration of iodine (seconds)
TGS1	1.50±0.10	6.0±1
TGS5	1.23±0.11	7.0±1
TGS14	0.92±0.03	10±1
TGS7	0.84±0.03	15±1
TGS11	0.79±0.01	18±1

3.3 Taxonomic Classification of Bacteria

Both of the microorganisms namely, TGS1 and TGS5 were identified as gram positive rod shaped bacteria belonging to genus of *Bacillus*. Gram staining of the isolates showed that the selected isolates were gram positive rods.

3.4 Production of Alpha Amylase

Type of microorganism (mesophilic or thermophilic) determines the best temperature for enzyme extraction [17]. Relatively large quantity of enzyme was synthesized by TGS1 and TGS5 bacteria at the 30^{th} and 12^{th} hr respectively and were selected for further enzyme production test at higher temperatures (Table 3). The concentration of maltose by TGS1E and TGS5E at higher temperatures is plotted in Fig. 3.

The growth of TGS1and TGS5 for production of α-amylase was optimum at 60ºC and was preferred for amylase production. According to Asif et al. [18] 45ºC is optimum for *Bacillus* species. However, Vieille et al. [19] indicated that thermophiles formed active enzyme at temperatures near to the producing organism's optimal growth temperature.
One unit of enzyme activity is defined as the amount of enzyme releasing 1 µmol maltose per min under the assay conditions Specific activity is expressed as amylase activity per mg of protein [3].

The selected bacteria were capable to produce enzyme throughout the selected temperatures within the specified incubation time with the exception from 30-36 hr incubation time at 60ºC where no enzyme was produced. In this study, optimal enzyme production was carried out under optimized liquid culture medium (0.5% meat extract, 1% poly peptone and 1% soluble starch). As the time of incubation increases alpha amylase production decreases after the 6th hr. Long time incubation period caused in reduced synthesis of the amylase. A possible explanation for this is exhaustion of the nutrients, death phase of the organism or the manufacture of protease in the medium [20]. Bacterial amylases are produced at a much wider range of temperature. *Bacillus amyloliquefaciens*, *B. subtilis*, *B. licheniformis* and *B. stearothermophilus* are among the most commonly used *Bacillus* sp. reported to produce α-amylase at temperatures of 37–60ºC [21] which is similar to this study.

3.5 Characterization of Alpha Amylase

3.5.1 Effect of temperature on activity and stability of bacterial alpha amylase

Optimum temperature of the crude enzyme was 55 -65ºC for TGS1E and 75- 85ºC for TGS5E at 10 minutes incubation time, respectively.

Activities of the crude enzymes to hydrolyze starch (to produce maltose from the reaction mixture) were calculated and recorded (Table 4).

The enzymes were characterized at different temperatures (45-95ºC) for 10-40 minutes of incubation at each temperature. Even though the enzymes were active in all the temperatures TGS1E is more active at temperatures of between 55 and 65ºC (2.722 and 3.134, respectively) while that of TGS5E was active at temperatures of between 75 and 85ºC (2.786 and 2.904, respectively). Chakraborty et al. [22] found that maximum activity of α-amylase was can be observed at 55ºC. Yabuki et al. [23] have also reported similar results. Additional increment in the temperature of incubation ensued in a reduction in the activity of the enzyme. Fig. 4 shows the influence of temperature on the activity of crude amylase.

The optimum temperatures of the enzymes were 55-65ºC for TGS1E and 75- 85ºC for TGS5E at 10 minute incubation time (Fig. 5).

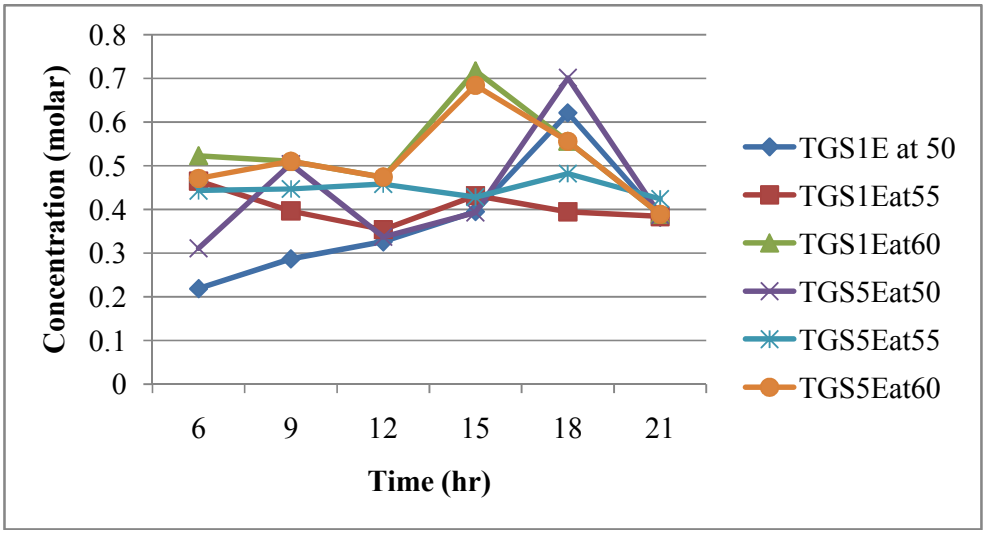

Fig. 3. Maltose produced by TGS1 and TGS5 crude enzymes at different temperatures

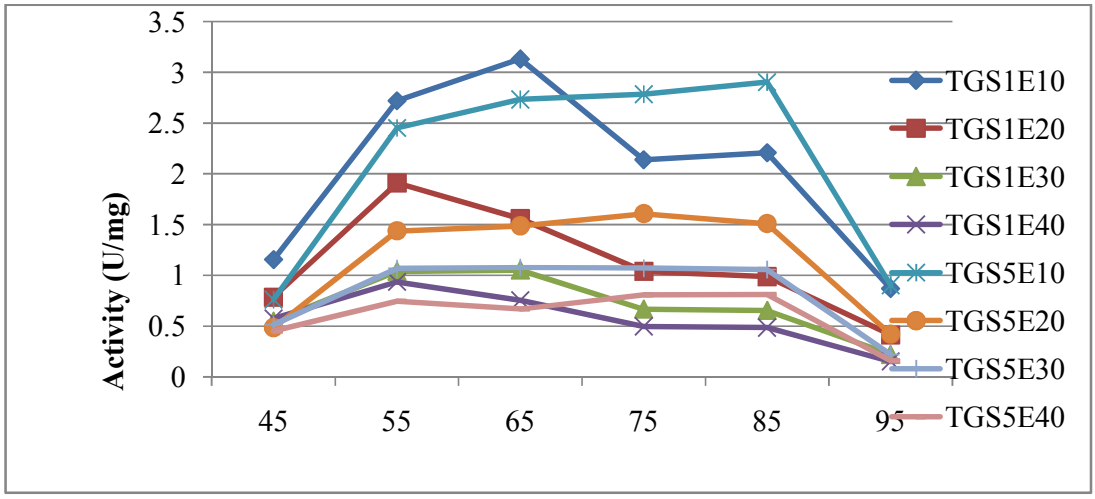

Fig. 4. Effect of incubation temperature on the activity of crude bacterial alpha amylase

Table 3. Amount of enzyme produced at different incubation of temperatures and times

CODE	Activity (U/mg)			
	U at 50ºC	U at 55ºC	U at 60ºC	Time of incubation
TGS1	16.3443±0.1060^{e}	34.7878±0.1060^{a}	38.7989±0.5832^{a}	6
TGS1	14.6968±0.0362^{f}	20.3139±0.0725^{d}	25.9054±0.3627^{c}	9
TGS1	12.7290±0.0275^{h}	13.7817±0.0275^{f}	18.4600±0.0276^{ed}	12
TGS1	12.3876±0.0444^{i}	13.5666±0.0222^{g}	17.3552±0.1779^{f}	15
TGS1	16.4770±0.1676^{d}	10.3920±0.0186^{j}	18.8215±0.0931^{d}	18
TGS1	8.6359±0.0320^{mn}	8.6813±0.0320^{l}	8.7719±0.0641^{g}	21
TGS1	8.6924±0.0562^{ml}	6.0767±0.0422^{n}	7.2005±0.7595^{h}	24
TGS1	8.5327±0.0125^{n}	4.8999±0.0375^{o}	4.5986±0.0376^{j}	27
TGS1	9.2193±0.0226^{k}	4.2661±0.0100^{p}	-	30
TGS1	5.6443±0.0205^{q}	3.6295±0.0102^{q}	-	33
TGS1	5.1336±0.0094^{r}	2.8372±0.0094^{r}	-	36
TGS5	23.3918±0.1060^{b}	33.0634±0.2120^{b}	35.2002±0.1590^{b}	6
TGS5	25.8798±0.0362^{a}	22.9045±0.0362^{c}	25.9054±0.3627^{c}	9
TGS5	13.1774±0.0551^{g}	17.8168±0.0551^{e}	18.4405±0.0551^{ed}	12
TGS5	12.2776±0.1111^{i}	13.4409±0.0222^{h}	17.4653±0.0222^{f}	15
TGS5	18.4527±0.0186^{c}	12.7101±0.0186^{i}	17.9522±0.0931^{ef}	18
TGS5	8.7606±0.0160^{l}	9.5879±0.0320^{k}	8.6473±0.2404^{g}	21
TGS5	8.6426±0.0140^{mln}	8.0459±0.0422^{m}	6.6436±0.0281^{h}	24
TGS5	11.4833±0.0250^{j}	5.963±0.0125^{n}	5.7593±0.0251^{i}	27
TGS5	7.7733±0.0113^{o}	2.5006±0.0113^{s}	-	30
TGS5	6.0807±0.0205^{p}	2.2184±0.0102^{t}	-	33
TGS5	3.9320±0.0094^{s}	1.9360±0.0188^{u}	-	36

Values are mean ±standard deviation. Values followed by different letters with in a column indicate significant difference (P < 0.05)

3.5.2 Effect of metal ions on the activity and stability of bacterial α-amylase

The effect of metal ions on α-amylase activity was measured in the presence of various metal ions at a concentration of 1 mM, 5 mM and 10 mM (Table 5). The 1 mM concentration was more effective than the 5mM. Activities of both enzymes were decreased in the presence of Ca^{2+}, Zn^{2+} and Mg^{2+} ions in all concentrations except in 5mM of Mg^{2+} where the activity of TGS1E was neither inhibited nor activated. Similarly, Adeyanju et al. [24,25] have reported that Zn^{2+} and Cu^{2+} had inhibitory effect on amylase activity. The amylase produced in this study did not require any specific ion for catalytic activity.

Table 4. Activity of bacterial alpha amylase incubated for 10-40 minutes

Temperature(°C)	Activity(U/mg)							
	TGS1E				TGS5E			
	10	20	30	40	10	20	30	40
45	1.156	0.786	0.550	0.576	0.760	0.486	0.515	0.451
55	2.722	1.909	1.041	0.935	2.452	1.439	1.069	0.748
65	3.134	1.558	1.053	0.755	2.734	1.489	1.079	0.670
75	2.140	1.040	0.667	0.497	2.786	1.608	1.072	0.809
85	2.106	0.988	0.655	0.486	2.904	1.510	1.058	0.812
95	0.870	0.415	0.220	0.152	0.904	0.423	0.220	0.158

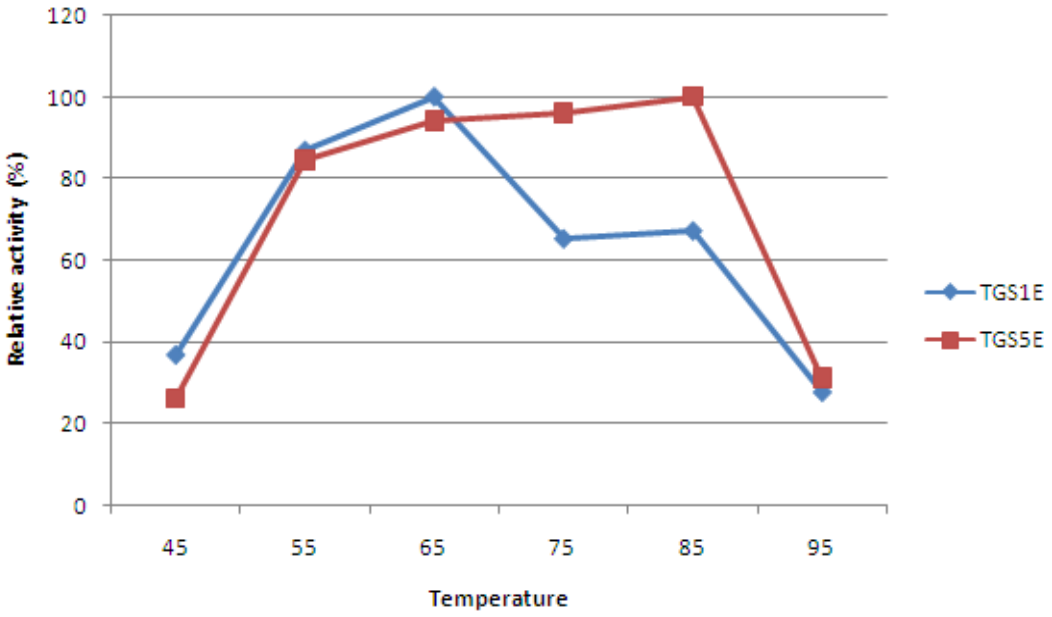

Fig. 5. Relative enzyme activity at different temperatures incubated for 10 m

Table 5. Activity of bacterial alpha amylase after characterization with metal ions incubated for 10 minutes

Metal ion	Concentration (mM)	Activity(U/mg)	
		TGS3E	TGS5E
Ca	1	0.770	0.768
Ca	5	1.012	0.864
Ca	10	0.652	1.442
Mg	1	0.778	0.608
Mg	5	1.052	0.964
Mg	10	0.666	0.882
Zn	1	0.934	1.178
Zn	5	0.912	0.650
Zn	10	0.998	0.620
Standard (without ion)		1.042	1.502

4. CONCLUSION

The thermostable microorganisms screened in this study seem to have a desirable characteristic for large-scale starch hydrolysis and further characterization is underway in our laboratory.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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