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Isolation, Identification and Characterization of Thermostable Amylase Producing Bacteria from Budamada Hot Spring, Misrak Badewacho, Hadiya Zone, Southern Ethiopia

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Authors' contributions

This work was carried out in collaboration among all authors. Author DSW designing study, performing analysis, writing the first draft of the manuscript; reading and approving the final manuscript. Author TDM follow up the research, managing the analysis and searching all possible literatures, reading and approving the final manuscript. Author SSA managing and analyzing data using SPSS software. All authors read and approved the final manuscript.

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ABSTRACT

Thermophilic microorganisms are microorganisms that are stable over a wide range of harsh environmental conditions. Thermophillic amylase enzyme producing bacteria live at 45°C–80°C. Thermostable microbial amylases are more simplified and economical than other sources. The present research was aimed to isolate, identify and characterize thermostable amylase producing bacterial isolated from Budamada hot spring of Misrak Badawacho woreda. Thirty samples were collected from the source. Totally 18 isolates were isolated and 10 isolates were screened based on their ability to hydrolyze starch. Different biochemical tests were done to identify isolates based on Bergey's Manual of systematic bacteriology. The results revealed that three enzymes from Bacillus spp. (SED2), Pseudomonas spp. (SED5) and Bacillus spp. (SED7) have maximum activity at 4% starch, 65˚C and pH of 8.5. Lactobacillus spp. (W20-0) and Bacillus cereus (SED12-1) at 8% starch, 75 and 65˚C, 8.5 and 7.5 pH respectively. Bacillus subtilis (W26-2) Bacillus licheniformis (W20-1), Lactobacillus spp. (SED10) at 6%starch; 35, 65, 45˚C; 6.5, 4.5, 4.5 PH., Bacillus spp.(SED8-3)&Bacillus spp. (W9) at 4% & 2%, 55˚C, 8.5 &4.5 pH and have maximum activity at 4% starch, 65˚C and pH of 8.5 and at 8% starch, 75 and 65˚C, 8.5 and 7.5 pH respectively., Lactobacillus spp. (SED10) at 6% starch; 35, 65, 45˚C; 6.5, 4.5, 4.5 PH. Bacillus spp. (SED8-3) and Bacillus spp.(W9) at 4% and 2%, 55˚C, 8.5 &4.5 PH. The amylase produced by bacterial isolates from Budamada hot spring was thermostable and performed from 65oC up to 75˚C. For most of the enzymes the optimum temperature was observed to be 65˚C; whereas optimum substrate concentration and pH for most of them were 4% starch and 8.5pH respectively. They were active at a various starch conditions (2%-10%), pH of 4.5-8.5, and temperature of 35-75°C.Generally, the bacteria isolated were thermophilic and produced thermostable amylase. Therefore, Budamada hot spring could be the source of thermophilic microorganisms and thermozymes as well.

Keywords: Hot spring; optimization; thermostable amylase; thermophilic microorganisms.

1. INTRODUCTION

Thermophilic microorganisms have the characteristics of adapting to survive in harsh environmental conditions [1]. Existence of life at high temperatures is quiet fascinating. At elevated temperatures, only thermophilic microorganisms are capable of growth and survival. Thermophilic bacteria are microbes that mostly inhabit hot springs, live and survive in temperatures above 42°C [2]. The discovery of thermophilic bacteria capable of carrying out life processes in the boiling hot springs of Yellowstone National Park has become a foundation of developments in medicine and biotechnology. Then, thermophiles have been isolated in geothermal features of all over the world [3]. Thermophiles have been isolated from different ecological zones (e.g., hot springs and deep sea) of the earth. Thermophiles can be categorized into moderate thermophiles (growth optimum, 50–60°C), extreme thermophiles (growth optimum, 60–80°C), and hyperthermophiles (growth optimum, 80–110°C) [4]. Thermophilic microorganisms can be classified as Gram-positive or Gram-negative, they can exist under aerobic or anaerobic conditions, and some of them can form spores. The microorganisms with the highest growth temperatures (103–110°C) are members of the genera *Pyrobaculum, Pyrodictium, Pyrococcus, and Melanopyrus* belonging to *Archaea*; within Fungi, the Ascomycetes and Zygomycetes classes have high growth temperatures [5,6].
Due to their increased importance, potential applications, and roles in different fields, scientists have concentrated their studies to discover new genus and species across the world [7,8,9].

The production of microbial thermophilic amylase from bacteria is dependent on the type of strain, composition of medium, method of cultivation, cell growth, nutrients requirements, incubation period, pH, temperature, metal ions and thermostability. Thermostability is a desired characteristic of most of the industrial enzymes. Thermostable enzymes are isolated from thermophilic organisms had found a number of commercial applications because of their stability are at high temperature (100-110°C) where enzymatic liquefaction and gelatinization of starch have been performed [10]. Thermophilic amylase enzymes are biological catalysts which are an indispensable component of biological reaction [11]. These enzymes are now being used in various sectors of industry. They are used in detergents; paper industry, textile industry, food industry and many others industrial applications. Thermophilic amylase enzymes

have been in use since ancient times and they have been used in saccharification of starch, production of beverages like beer, treatment of digestive disorders and production of cheese from milk [12]. Thermostable amylolytic enzymes have been currently investigated to improve industrial process of starch degradation and were of great interest for the production of valuable products like glucose, crystalline dextrose, dextrose syrup, maltose and maltodextrins [13].

Ethiopia have different ecological areas having from highest altitude to lowest altitudinal variation which gives chance of getting different ecologies including extreme environments like hot springs live volcano etc. Budamada hot spring is one of the extreme environments where thermophiles will be found which is located at Misrak Badewacho Woreda and surrounding area is one of the hot springs in Southern region of Ethiopia. Budamada hot spring is one of the potential sources of thermotolerant amylase producing microorganisms. The microbial lode of the hot spring has been not yet fully explored.

1.1 Statement of Problem

For industrial applications, enzymes must be stable under process conditions. However, amylases that are derived from plants and animals are not sufficient enough to be used at industrial scale. In addition to their production in less quantity, enzymes from animal and plants are not thermostable. These make them less stable for industrial applications. Hence, thermostable microbial enzymes play an important role in different industries due to their stability at harsh environmental conditions, such as extreme temperatures. Even though, its uses at different areas of industries hold a great position, the thermostable amylase produced by thermophilic bacteria for industrial application in Ethiopia has not yet been fully explored. Several researchers have reported thermophilic bacteria from diverse environmental habitats such as geothermal sites and hot springs around the world. Here in our country we have diversified ecological areas having extreme conditions. From those Budamada hot spring is one of the area having this future. However, the thermophilic microbes of this site and their enzymes have not been reported till date. So, isolation and characterization of microbes from this site is important to know the diversity of thermophilic bacteria that can produce thermostable amylase.

1.2 Objective of the Study

1.2.1 General objective

- ✓ The main objective of study is to isolate, identify and characterize thermostable amylase producing bacteria from Budamada hot spring.

1.2.2 Specific objective

- ✓ To isolate and characterize the thermophilic bacteria from Budamada hot spring.
- ✓ To extract crude thermostable amylase from bacteria by submerged fermentation.
- ✓ To assess the enzymatic activity and to determine optimum conditions of crude thermostable amylase for enzymatic starch hydrolysis.

1.3 Significance of the Study

Nowadays industry has been minimizing the use of chemicals or replaced by enzymes to solve environmental problems associated with those chemicals. But most of industries have operated at high temperature. Consequently, thermostable enzymes are most significantly applicable because thermophilic process is more stable, faster, needs lower costs. They have higher stability to organic solvents, acidic and alkaline pH and detergents. As a result, thermostable amylases are of great significance in industrially viable technology and have a number of commercial applications due to their overall inherent stability. So, this research will increase thermostable amylase enzymes for industrial use as amylase is one of the most important industrial enzymes, having applications in different industrial processes such as brewing, baking, textiles, pharmaceuticals, starch processing, and detergents. Therefore, in this regard searching enzymes from such environment will fill the gap to feed different industries.

2. MATERIALS AND METHODS

2.1 Sample Collection and Description of the Study Sites

The study was conducted in the Misrak Badawacho district, Hadiya zone, Southern Nations Nationalities and People Regional State (SNNPRS), Ethiopia. Geographically, the location of the district is between 70 9’ 00" to 8 0 15’00" North latitude and 370 5’00" to 400 00’ 00"East longitude. The average annual rainfall of

the district is 1000 mm with bi-modal distribution of rainfall with a short rainy season starting from March to May and the long rainy season covering from June to October. The mean temperature within the district is 21^0c. The area is known for having annually flowing rivers (Bilate, Bishanguracha and Chelekeleka), used for irrigating of land. The experimental work was conducted from February to June 2023.Total of 30 samples (i.e. 15 water samples and 15 sediment samples) were collected from Budamada hot spring aseptically in falcon tubes and the samples were transported in icebox aseptically to Wachemo University, Post Graduate Microbiology laboratory, for processing and microbial isolation. Sample processing, laboratory isolation, identification and preservation of bacteria were carried out. The temperature and pH of sample was recorded during the time of sampling.

2.2 Isolation of Pure Culture

After the samples were transported to laboratory, Isolation of thermophilic bacteria from the culture were done by serial dilution up to 10^{-5}or 10^{-6}dilution. 0.1 ml of each dilution was inoculated by spread plate technique into the prepared Starch Agar Medium (which consists of nutrient agar-yeast extract, peptic digest of animal tissue, meat extract, peptone, agar and soluble starch. Then, the inoculated plates were incubated at 53^{o}C for 48hr and the growths of thermophilic bacteria were observed. Representative 3-5 colonies of bacterial isolates were randomly picked from countable starch agar plates. Each bacterial isolate was purified by repeated streak-plating on SAM agar for three times. The pure isolates were maintained on nutrient broth containing 10%glycerol at -4^{o}C and sub-cultured every four weeks until required for characterization and kept at -80^{o}C for long term preservation [14,15].

2.3 Biochemical Characterization of Thermophilic Isolates

The morphological characterization of bacterial isolates was determined according to their cultural characteristics using microscope (colony size, colony color, colony texture) and Biochemical characterizations will be conducted. These were oxidase test, indole test, H_2S test, VP test, citrate test, catalase test and methyl red test based on Bergey's Manual of systematic bacteriology. The preliminary selection test to be used for characterization of the bacterial isolates will be graham staining [16]

2.3.1 Oxidase test

Oxidase test were undergone by soaking a filter paper in a solution of 1% *kovac*'s oxidase reagent, followed by smearing colonies on the filter paper with a clean loop [17].

2.3.2 Voges-proskauer (VP) test

Suspended colony from pure culture were investigated in VP medium and incubated at 53^{o}c for 48 hr., then added 0.2ml of 40% of KOH and 0.5 ml of alpha naphtol solution. VP Positive result will show Pink or red color at the surface of the medium and VP Negative result has shown yellow or copper color at the surface of the medium [18].

2.3.3 Citrate test

Bacteria were inoculated in citrate medium (sodium citrate, an ammonium salt and the indicator bromothymol blue) and incubated at53^{o}C for 48hr. The positive result has developed color change from green to blue and the negative result shown no color change on citrate medium [18].

2.3.4 Catalase test

The bacteria were spread on starch agar plate and it was incubated for 48hr under appropriate condition. Then bacteria were collected from colony and applied on microscopic slide. After that one drop of hydrogen peroxide was added. Then the positive result was formation of oxygen in the form of bubble and in negative result no bubble was formed [19].

2.3.5 Methyl red test

The methyl red test was used to detect the ability of bacteria to produce and maintain acid end products from glucose fermentation. 3-5 drops of MR reagent were added to bacterial isolates grown on starch agar medium. The Positive result was pink or red in color and the negative was yellowish-orange [20]

2.3.6 Indole test

The indole test screens for the ability of bacteria to degrade the amino acid tryptophan and produce indole. Tryptophan was an essential amino acid, which was oxidized by some bacteria resulting in the formation of indole, pynivic acid and ammonia. The indole which was produced was detected by adding KOVAC's reagent which produced cherry red colored ring [17].

2.3.7 Hydrogen sulfide test

The ability of the microorganisms to produce H_2S was tested by using H_2S production test. SIM medium can be used for the test. Colonies were grown on the SIM medium at 53^0c. If H_2S production is present, black color was appeared [17].

2.4 Screening Thermotolerant Amylase-Producing Bacteria

Amylase producing bacteria were screened based on their ability to degrade starch in the medium. Single bacterial isolate was grown on starch containing nutrient agar medium at 53°C for 48hr., and then was examined by flooding,1% iodine solution on starch agar plate. The presence of blue color around the growth media indicated negative result, and formation of clear zone around the bacterial colony indicated a positive amylase-producing bacterium [21].

2.4.1 Salt tolerance test

Salt tolerances of the bacterial isolates were tested by inoculating each isolate into nutrient broth in a test tube with varying NaCl concentrations (0.5, 1.5, 2.5, 3.5, and 4.5). The nutrient broth without NaCl was used as negative control and bacterial growth was assessed by measuring its OD [22]

2.4.2 Crude thermostable amylase production

Positive amylase producer isolates were transferred in to production medium for production of thermostable amylase. The production medium contains soluble starch (10 g/L), peptone (5 g/L), $(NH_4)_2\ SO_4$ (2 g/L), KH_2PO_4 (1 g/L), K_2HPO_4, (2 g/L), $MgCl_2$ (0.01 g/L) prepared at 7 PH. 2ml of inoculum was taken in to 250 ml Erlenmeyer flask containing 40ml of production medium. After two days of incubation at 53^0c and agitation with shaker at 100 rpm. After incubation, fermented broths were centrifuged at 4000 rpm for 25 minutes in a centrifuge. As amylase is extracellular enzyme, it is released to the medium as a supernatant [23]. Hence, the supernatant was taken and served as a crude amylase for further enzyme activity. This crude enzyme extract was used in amylase enzyme activity measurement and characterization [14,24].

2.5 Enzyme Activity Assay

The activity of the amylase was determined by using starch as the substrate. The reducing sugar released as an enzymatic reaction product was measured. A sample tube containing 1 ml of 1% starch was incubated for 5 minutes at 5°C. After five minutes 1 ml of amylase and 1ml of 0.85% NaCl was added and the incubation was continued for 30 minutes. The enzyme activity was stopped by adding 1 ml of 0.7% NH_2SO_4 and by putting in water bath at 100^oC. A control tube was also prepared using the same procedure in the absence of amylase. The absorbance of the samples was measured at 660 nm and enzyme activity was determined [25,26].

3. ENZYMATIC STARCH HYDROLYSIS CONDITION OPTIMIZATION

3.1 Substrate Concentration Optimization

Starch was used as a substrate. Substrate solutions with varying concentration of starch that include 2%, 4%, 6%, 8%, 10%(w/v) were produced. The starch solution was then dissolved with each enzyme and incubated in oven at 53°C for 45 minutes. The variation in enzymatic activity due to substrate concentration was measured using spectrophotometer (Subra et al. 2013), [27]

3.2 Optimum pH Determination

Each enzyme isolates and substrate at the optimum concentration was dissolved in 5 ml of various buffers (pH 4.5 to 8.5). For pH adjustment Tris-HCl and borax NaOH buffer were used. Then after incubation for 45 minutes, the enzyme activity variation at different pH level was measured using spectrophotometer [28,22,27].

3.3 Optimum Temperature Determination

This procedure was performed using spectrophotometer at various temperatures of 35, 45, 55, 65 and 75°C for 45 minutes, under optimum pH and substrate concentration. The temperature that resulted in the highest reducing sugar was selected as the optimum temperature for enzymatic reaction [26,27].

3.4 Data Analysis

The data in this study were analyzed using SPSS version 26. Means and standard deviations were calculated using analysis of variance (ANOVA) to analyze the significant differences between the

means using Duncan's multiple range test ($p < 0.05$). The triplicates mean values of tests were analyzed. Significant difference was defined as $p < 0.05$ [29].

4. RESULTS AND DISCUSSION

4.1 Physical Characteristics Sample

At the source, different parameters of the samples (such as temperature, pH, and salt concentration) were measured and recorded. The pH range was between 8.02 up to 8.68, temperature range was between 47^0c up to 57^0c, and the amount of salt was recorded as 0.017g. Then, the samples were transported to Wachemo University Post Graduate Microbiology Laboratory.

4.2 Isolation of Pure Culture

From 30 samples collected and processed a total of 18 bacterial isolates were isolated and purified as indicated in sample plate of (Fig.1).

Purified isolates that are found to be Gram-positive and Gram-negative with diverse cultural characteristics with regard to color (white, gray pink or whitish), shape (circular or irregular), edge (Irregular or smooth) and elevation (flat or raised). Furthermore, the sizes of the colonies ranged from medium to large and motility motile and nonmotile as summarized in Table 1.

4.3 Biochemical Characterization

Among 10 isolates, 8 isolates were positive for gram test which was done using 3%KOH showing that they were gram positive, whereas the rest 2 isolates were gram negative. The mechanism was based on the sticky and viscous formation. Negative isolates had formed viscous. For catalase reaction, four isolates were positive. This result depicted that the isolates were aerobic. The remaining 6 isolates were negative indicating they were not aerobic. The positive isolates had formed bubble, while the negatives had not. Nine isolates were positive for methyl red, forming red and pink color. This confirmed that the isolates have the ability to form acid up on fermentation. All were shown negative reaction to VP test. They were not turned to red. For citrate test, all isolates except one were developed blue color. They were recorded as a positive citrate reaction. This made sure that the isolates able to utilize citrate as a carbon source and ammonium as nitrogen source as presented in Table 2.

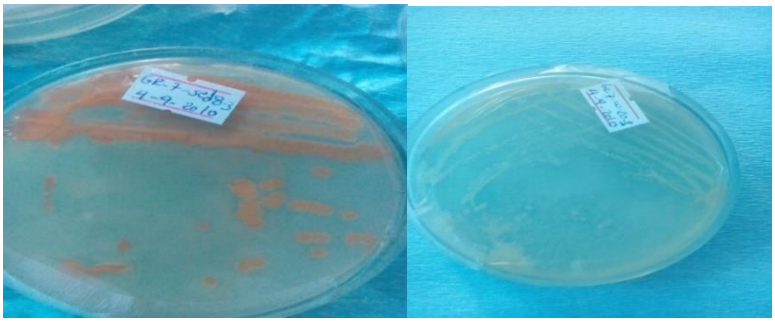

Fig. 1. Isolation of pure culture

Table 1. Morphological characteristics of bacterial isolates from water and Sediment samples of Budamada hot water spring

Colony code	Bacterial isolates	Colony Morphology					
		Shape	Color	Texture	Size	Motility	Elevation
Sed-2	*Bacillus spp.*		Grey	Smooth	Medium	None	
W-26-2	*Bacillus subtilis*		Grey	Rough	Large	Motile	Rise
Sed-8-3	*Bacillus spp.*	Regular	Grey	Smooth	Small	Motile	
W-20-0	*Lactobacillus spp.*		Grey	Smooth	Large	None	Rise
Sed-12-1	*Bacillus cereus*		White	Smooth	Large	Motile	Flat
Sed-7-1	*Bacillus licheniformis*		Grey	Rough	Medium	Motile	Rise
Sed-7	*Bacillus spp.*		Grey	Smooth	Small	Motile	Flat
Sed-5	*Pseudomonas spp.*		Grey	Rough	Small	Motile	Flat
Sed-10	*Lactobacillus spp.*		Grey	Rough	Small	None	Rise
W-9	*Bacillus spp.*		Whitish	Smooth	Small	None	

Table 2. Biochemical characteristics of the bacterial isolates isolated from Budamada hot spring

Sample code	Bacterial Isolates	Biochemical tests								
		Gram test	Starch hydrolysis	Citrate	Methyl red test	VP test	Catalase test	H_2S test	Oxidase test	Indole test
Sed-2	*Bacillus spp.*	+	+	+	+(pink)	-	+	-	+	+
W-26-2	*Bacillus subtilis*	+	++	+	+(red)	-	+	-	+	+
Sed-8-3	*Bacillus spp.*	+	+	+	+(pink)	-	-	-	-	+
W-20-1	*Lactobacillus spp.*	+	+	+	+(red)	-	-	-	+	-
W-20-0	*Bacillus cereus*	+	+	+	+(pink)	-	-	-	+	-
Sed-12-1	*Bacillus licheniformis*	+	+	+	+(pink)	-	-	-	+	+
Sed-7	*Bacillus spp.*	+	+++	-	-(yellow)	-	+	-	+	+
Sed-5	*Pseudomonas spp.*	-	++	+	+(red)	-	-	-	+	+
Sed10	*Lactobacillus spp.*	+	+++	+	+(pink)	-	-	-	Delayed +	-
W-9	*Bacillus spp.*	+	++	+	+ (red)	-	+	-	Delayed +	-

Key: +++-strong positive, ++-very good, +-good, - (negative)

Table 3. Effect of salt concentration on bacterial isolates

Sample code	Bacterial Isolates	Salt Concentration					p
		0.5	1.5	2.5	3.5	4.5	
Sed-10	*Lactobacillus spp.*	2.02±0.95	1.59±0.30	1.85±0.45	2.02±0.02*	1.73±0.45	0.146
W-9	*Bacillus spp.*	1.91±1.22	1.66±0.47	2.07±0.13*	2.04±0.19	1.67±0.18	0.174
Sed-12-1	*Bacillus cereus.*	2.09±0.91*	1.72±0.54	1.75±0.23	1.70±0.44	1.61±0.34	0.149
W-20-1	*Bacillus licheniformis.*	1.06±0.07	2.03±0.29*	1.77±0.17	1.96±0.03	1.57±0.10	0.000
Sed-7	*Bacillus spp.*	2.30±0.05*	1.74±0.25	1.82±0.45	2.00±0.18	1.65±0.09	0.000
W-20-0	*Lactobacillus spp.*	2.10±0.06*	1.44±0.22	1.83±0.23	1.98±0.17	1.59±0.06	0.000
W-26-2	*Bacillus Subtilis.*	1.92±0.44	1.55±0.16	2.01±0.09	2.05±0.24*	1.71±0.08	0.000
Sed-2	*Bacillus spp.*	2.26±0.25*	1.71±0.22	1.70±0.21	1.64±0.20	1.59±0.28	0.000
Sed-8-3	*Bacillus spp.*	2.24±0.46*	1.59±0.11	1.72±0.07	1.94±0.28	1.69±0.16	0.000
Sed-5	*Pseudomonas spp.*	2.21±0.02*	1.87±0.11	1.88±0.13	2.09±0.04	1.56±0.28	0.000

*Key: * maximum salt concentration tolerance of the isolates.*

According to Bergey"s Manual [30], the 10 bacterial isolates were identified to genus and species level by using the previous biochemical tests. So bacterial isolates obtained from Budamada hot spring were identified under genus Bacillus, lactobacillus and pseudomonas. From the identified bacterial isolates 70% were under genus Bacillus, 20% were *Lactobacillus spp.* And the rest one isolate was *Pseudomonas Spp*. The bacterial isolates were identified as *Bacillus Spp., Bacillus subtilis, Lactobacillus Spp., Bacillus cereus, Bacillus licheniformis, Pseudomonas Spp., Lactobacillus spp.*

4.3.1 Salt Tolerance Test

Selected isolates have different salt tolerance for different sate concentration some have low salt tolerance capacity and others have high salt tolerance capacity as it is indicated in Table 3.

The isolates were tested for salt tolerance at different NaCl concentrations (0.5, 1.5, 2.5, 3.5, and 4.5 %). Above 50% of them were shown maximum performance at 0.5% and 40% and 3.5% and 20% respectively performed well at all concentrations. Most were performed less at 1.5% and the growth of three isolates was decreased at 4.5%. Generally, the result revealed that the isolates could be survived at saline environments.

4.3.2 Starch hydrolysis test

After the plates were flooded with IKI solution (iodine, 1 g; potassium iodide, 2 g; distilled water, 100 ml). A clear zone around a colony was recorded as positive reaction. The clear zone indicates the level of starch hydrolysis capacity of isolates. Potential isolates can produce large clear zone and indicates complete hydrolysis of starch.

A clear zone around a colony on starch (after addition of iodine) gave an indication amylase producing bacteria. The isolate showing the maximum zone of hydrolysis was selected for amylase production because it is an indication for the bacteria to produce amylase based on its hydrolysis potential [31,28], Similarly, in our study, from 18 bacterial isolates 10 isolates were selected as a strong positive for the reaction. This procedure showed a positive result for the Aspergillus strain as Singh *et al.,* 2016 indicates. The formation of clear zone observed was due to the fact that the amylase produced during the growth of the organisms has hydrolyzed the starch around the colony. The unhydrolyzed part was appeared as blue black indicating negative activity of amylase producer bacteria.

4.3.3 Crude thermostable amylase production

Following the incubation of the isolates with production medium at 53^0C with shaker, after two days, crude amylase was produced. By centrifugation at 4000rpm for 25 minutes, 60ml of crude enzyme was extracted.

4.3.3.1 Enzyme activity assay

The amylase activity assessed by measuring OD value and the observed result was as the basis of level of productivity of the amylase, an isolate producing a maximum of amylase activity was Sed-5 (*Pseudomonas spp.)* and W-20-0 (*Lactobacillus spp.)* used for detailed investigation as indicated to Fig. 3. Screening of the amylase producing organisms was carried out on the starch agar. Based on the enzyme activity assay, all were positive. However, SED5 *(Pseudomonas spp*.) involved the highest activity and W20-0 *(Lactobacillus spp*.) shown the second maximum enzyme activity next to SED5 (*Pseudomonas spp.).*

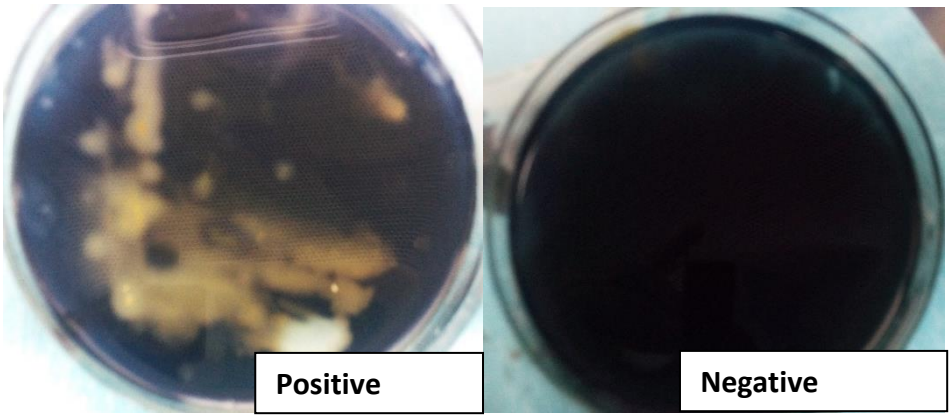

Fig. 2. Clear zone observed around amylase-producing bacteria

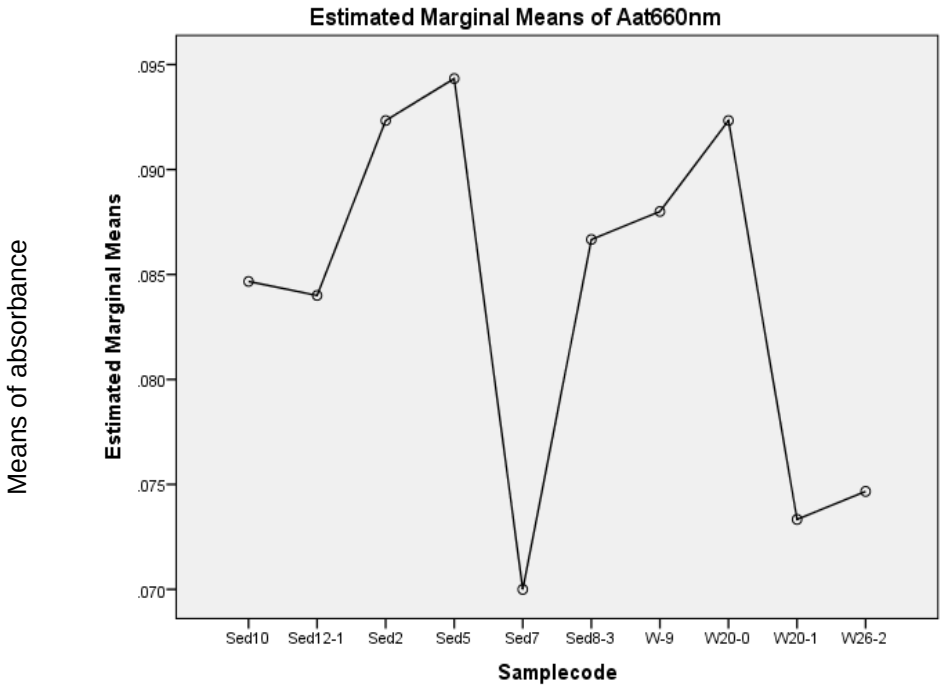

Fig. 3. Profile plot showing estimated marginal plot

Key: Sed-10 (Lactobacillus spp.), W-9 (Bacillus spp.), Sed-12-1 (Bacillus cereus), W-20-1 (Bacillus licheniformis), Sed-7 (Bacillus spp.), W-20-0 (Lactobacillus spp), W-26-2 (Bacillus Subtilis), Sed-2 (Bacillus spp.), SED5 (Pseudomonas spp.), Sed-8-3 (Bacillus spp.)

4.3.3.2 Enzymatic starch hydrolysis condition optimization

A. Substrate concentration optimization

Starch which was used as a substrate was prepared with concentrations; 2%, 4%, 6%, 8% and 10%, (w/v) at pH7. Substrate concentration variation was measured using spectrophotometer summarized in the Table 4.

Following the positive activity of the amylase, the enzymatic hydrolysis process parameters such as optimum substrate concentration, pH, and temperature optimization were determined. For substrate optimization, starch at different concentration (2%, 4%, 6%, 8% and 10%) was used. Out of 10 enzymes, 40% of them had utilized 4% starch; 30% had used 6% starch for their maximum growth. 20% were seen to have optimum activity at 8% starch, where as 10% had used 2% starch as the optimum substrate.

B. Optimum pH determination

Substrate at the optimum concentration was prepared at various pH ranges (i.e., at pH of 4.5, 5.5, 6.5, 7.5 and 8.5). The pH was adjusted using NaOH and Tris-HCl. The enzyme activity variation at different pH level was measured as in Table 5.

Regarding pH optimization, optimum substrate concentrations at 53^0c were adjusted at different pH levels (4.5, 5.5, 6.5, 7.5 and 8.5) using Tris-HCl and NaOH. Reddy *et al.*, 2003 stated amylases are generally stable over a wide range of pH from 4-11. Our study was supported by Reddy et al., 2003 stated; the crude amylase was stable over a pH range of 4.5 to 8.5. However, their optimum pH was varied at acidic, neutral and basic pH. Five (5) of them were developed maximum hydrolysis activity at 8.5 pH. This result indicated that they have maximum activity at alkaline environments than they do at acidic conditions. Three of them were observed to show optimum activity at pH of 4.5 revealing their optimum activity was at acidic environment than basic. For the remaining two neutral pH of about 6.5 and 7.5 were determined as optimum. This investigation depicted that the two enzymes' activity was highest at neutral environments than at alkaline and acidic conditions. Another most important parameter for the enzyme activity was temperature [10].

Table 4. Substrate concentration optimization for amylase activity

Sample code	Bacterial Isolates	Starch concentration					p
		2	4	6	8	10	
Sed-10	*Lactobacillus spp.*	1.99± 0.02	2.62±0.47	2.84±0.18	2.51±0.35	2.48±0.27	0.000
W-9	*Bacillus spp.*	2.63±0.24	2.59±0.28	2.54±0.12	2.40±0.59	2.45±0.54	0.670
Sed-12-1	*Bacillus cereus.*	2.25±0.83	2.75±0.19	2.56±0.41	2.52±0.49	2.53±0.13	0.100
W-20-1	*Bacillus licheniformis.*	2.68±0.15	2.53±0.41	2.72±0.16	2.53±0.33	2.34±0.46	0.038
Sed-7	*Bacillus spp.*	2.59±0.014	2.76±0.03	2.62±0.08	2.6±0.07	2.51±0.14	0.000
W-20-0	*Lactobacillus spp.*	2.44±0.43	2.57±0.39	2.48±0.33	2.66±0.08	2.55±0.22	0.266
W-26-2	*Bacillus Subtilis.*	2.50±0.724	2.55±0.36	2.86±0.33	2.44±0.62	2.44±0.39	0.152
Sed-2	*Bacillus spp.*	2.21±0.03	2.44±0.04	2.56±0.57	2.64±0.31	2.50±0.22	0.015
Sed-8-3	*Bacillus spp.*	2.48±0.63	2.65±0.29	2.59±0.23	2.09±2.17	2.43±1.01	0.613
Sed-5	*Pseudomonas spp.*	2.65±0.03	2.75±0.06	2.71±0.74	2.58±0.08	2.46±0.05	0.152

Table 5. Effect of pH on amylase activity

Sample code	Bacterial isolates	pH					P
		4.5	5.5	6.5	7.5	8.5	
Sed-8-3	*Bacillus subtilis*	2.47±0.163	2.04±0.07	2.16±0.38	2.02±0.01	2.89±0.05	0.000
Sed-7	*Bacillus spp.*	0.94±0.006	0.53±0.009	0.42±0.009	0.22±0.29	1.39±0.03	0.000
Sed-5	*Pseudomonas spp.*	0.52±0.54	1.44±0.11	1.88±0.02	1.50±0.03	2.51±0.01	0.000
Sed-12-1	*Bacillus cereus*	0.23±0.04	0.23±0.10	0.15±0.11	0.46±1.12	0.16±0.01	0.389
W-20-0	*Lactobacillus spp.*	2.48±0.02	2.76±0.23	2.55±1.08	2.75±0.01	2.80±0.01	0.254
W-20-1	*Bacillus licheniformis*	1.66±0.01	1.22±0.02	1.17±0.13	0.98±0.02	1.58±0.01	0.000
Sed-10	*Lactobacillus spp.*	1.76±0.03	1.34±0.6	1.69±0.37	1.18±0.03	1.86±0.17	0.001
W-26-2	*Bacillus subtilis*	1.57±0.09	1.64±0.16	1.96±0.07	1.65±0.03	1.86±0.17	0.000
Sed-2	*Bacillus spp.*	0.87±0.25	0.87±0.005	1.40±0.004	1.21±0.15	2.45±0.08	0.000
W-9	*Bacillus spp.*	2.19±0.005	1.92±0.04	1.86±0.08	1.75±0.03	1.70±0.03	0.000

Table 6. Effect of temperatures on amylase activity

Sample code	Bacterial isolate	Temperature					p
		35	45	55	65	75	
W-26-2	*Bacillus subtilis*	1.08±0.004	1.04±0.007	1.01±0.01	1.01±0.005	0.39±0.0062	0.000
Sed-10	*Lactobacillus spp.*	0.09±0.02	0.89±0.007	0.86±0.01	0.86±0.004	0.30±0.01	0.000
Sed-8-3	*Bacillus spp.*	0.27±0.002	0.37±0.06	0.38±0.01	0.38±0.01	0.31±0.02	0.000
W-9	*Bacillus spp.*	2.45±0.13	2.49±0.01	2.51±0.02	2.45±0.01	2.05±0.01	0.000
Sed-7	*Bacillus spp.*	0.28±0.01	0.33±0.007	0.32±0.01	0.43±0.005	0.18±0.01	0.000
Sed-2	*Bacillus spp.*	0.29±0.004	0.28±0.007	0.32±0.01	0.37±0.01	0.20±0.01	0.000
Sed-5	*Pseudomonas spp.*	0.15±0.02	0.36±0.005	0.37±0.004	0.45±0.01	0.17±0.01	0.000
Sed-12-1	*Bacillus cereus*	0.33±0.005	0.32±0.001	0.41±0.01	0.42±0.01	0.33±0.006	0.000
W-20-0	*Lactobacillus spp.*	0.19±0.006	0.21±0.01	0.20±0.01	0.19±0.01	0.94±2.88	0.367
W-20-1	*Bacillus lichenoformis*	1.05±0.08	1.07±0.01	1.09±0.02	1.08±0.03	1.30±0.009	0.000

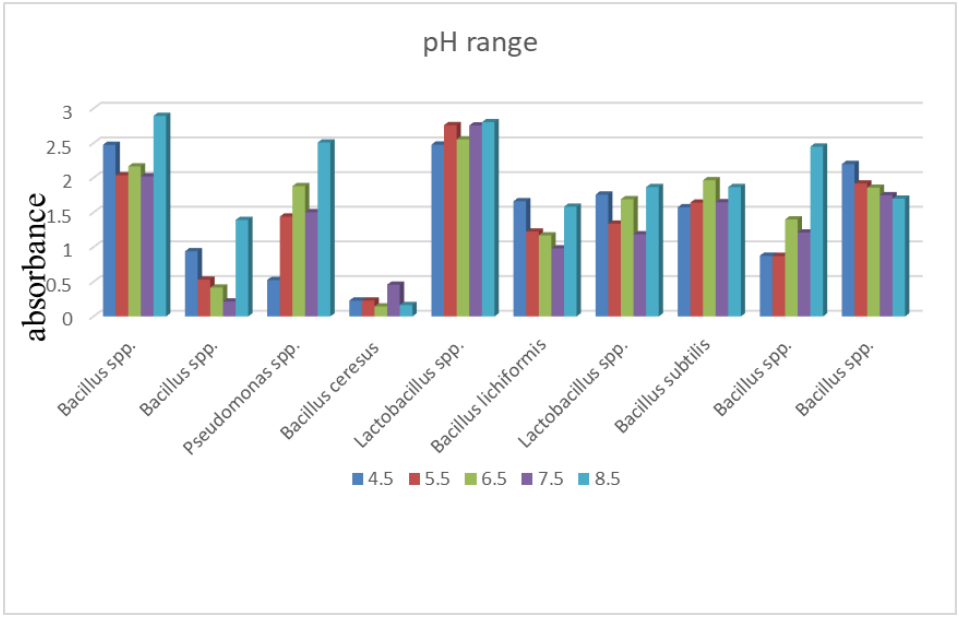

Fig. 4. Optimum pH value for amylase activity

C. Optimum temperature determination

This procedure was performed using spectrophotometer at various temperatures of 35, 45, 55, 65, and 75°C for 45 minutes, under optimum pH and substrate concentration (Table 6). The temperature that resulted in the highest reducing sugar was the optimum temperature for enzymatic reaction.

In this study, to find the optimum temperature for amylase activity, at the optimum pH and substrate concentration, temperature was varied at 35^0c, 45^0c, 55^0c, 65^0c and 75^0c. Even though, Ved Pal *et al*., 2016 reported that thermostable amylase was stable at broad range temperature (50-80^0c) and the same is true for amylase in this work, their optimum temperature was measured as below. 40% were performed best at 65^0c; 20% at 55^0c; 20% at 75^0c; 10% at 35^0c, and 10% at 45^0c. Bacterial species, such as *B. subtilis, B. megaterium, B. amyloliquefaciens and B. licheniformis* which produce α-amylase enzymes that can withstand a temperature of 70°C have been reported previously [32,33]. Likewise, in the present work, *Bacillus licheniformis* produced thermostable amylase that can withstand a temperature of 75^0c. In addition, *Lactobacillus* which was isolated from W-20-0 produced amylase at the same temperature. Generally, this result confirmed that about 90% of the amylases were shown maximum starch hydrolysis activity at a temperature of greater than 45^0c. That means almost they were thermostable and the bacteria isolated from the Budamada hot spring were thermophilic and they had been the source of thermozymes.

5. CONCLUSION

The amylase produced by bacterial isolates from Budamada hot spring was thermostable and performed from 65°C up to 75°C. The optimum hydrolysis conditions required for amylase activity were optimized. For most of the enzymes the optimum temperature was observed to be 65°C; whereas optimum substrate concentration and pH for most of them were 4% starch and 8.5pH respectively. Despite these optimum conditions, the enzymes were active over a wide range of conditions. They were active at a various starch conditions (2%-10%), pH of 4.5-8.5, and temperature of 35-75°C.Generally, the bacteria isolated were thermophilic and produced thermostable amylase. Therefore, Budamada hot spring could be the source of thermophilic microorganisms and thermozymes as well.

6. RECOMMENDATIONS

Since this is one of the biological resources of our country it is necessary to study and explore further microbiological aspects of the Budamada hot spring using molecular technique of characterization and identification of the thermophiles and thermostable amylase producing microorganisms and it is better to go for large scale thermostable amylase production.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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