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Isolation and Characterization of Thermostable Amylase Producing Bacteria from Hot Spring at Arba Minch Nech Sar National Park, Southern Ethiopia

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Abstract: **Microorganisms can carry out extremely useful processes under extreme environmental condition and they are the best preferable sources of thermostable enzymes like amylase compared to plant and animal . Thermostable amylases are the most important enzymes in present with potential industrial applications such as detergent, food, pharmaceutical, paper, beverage, textile and fine-chemical industries. The application of enzyme increases more with the discovery of thermostable enzymes because thermophilic process is more stable, faster, needs lower costs. This work aimed to isolate thermostable amylase producing bacteria from hot spring found in Arba Minch Nech Sar national park for use as flour fermentation in bakery industry. Isolation of amylase producer was undertaken using starch containing nutrient agar medium. A total of 72 thermophilic bacteria were isolated from the study area. Out of 72, ten amylase positive isolates were screened on the basis of clear zone formation and from these, four potential isolates (isolate 1-4) were selected based on thermo tolerance ability. All four isolates were found to be positive for Gram reaction, citrate utilization, methyl red, catalase tests; and rod shaped morphology. However, they found to be negative for VP test. The crude enzyme from all potential isolates (isolate-1 -4) were rise 20gm of wheat flour dough from 1cm to maximum of 4.7cm after 22hrs incubation with 3ml crude enzyme. These properties suggest that thermostable amylase enzyme produced in this study could find potential application in bread making process and replace conventional yeast technology which have an economic implication.**

Keywords: **Thermostable Amylase, Thermophilic, Hot Spring, Isolate, Bacteria.**

1. INTRODUCTION

The microbial world is the largest unexplored reservoir of biodiversity on the earth planets **[1]**. Extremophiles are microorganism that survive at extreme environmental condition **[2]**. Among this thermophiles covers the larger portion that show optimum growth at temperatures of 50°C or higher. Hot springs are characterized by moderate to high temperature environment **[3, 4]** which is best source of industrially important thermophiles like *Thermus aquatics*. However, microbial diversity of this hot spring has not yet been fully explored due to difficulties in isolation, maintenance of pure culture and thus, their diversity and biotechnological potential remains to unexplored **[5, 6]**. The cellular components of thermophiles are extremely thermostable and these together with their unique metabolic capabilities, offer considerable promise for biotechnological applications.

Thermophiles are a potent source of thermos enzymes, which show utmost stability and activity under conditions of high temperature. Thus, biocatalysts using thermophiles as well as thermos enzymes are rapidly being transformed from an academic science to an industrially viable technology **[7]**. Among industrially important enzymes, amylases, protease and cellulase are getting more attention and importance because these enzymes have wide applications in many fields. For instance, amylases are used in various industries such as food, detergent, paper, textile, beverage, pharmaceutical and fine-chemical industries **[8, 9, 10, 11, 12]**. The application of enzyme increases more with the discovery of thermostable enzymes because thermophilic process is more stable, faster, needs lower costs **[13]**.

Thermostable enzymes from thermophilic microorganisms have found a number of commercial applications because of their overall inherent stability **[14]**. The most widely utilized thermostable enzymes are amylases in the starch industry **[15]**. These amylases should be active at the high temperatures of gelatinization (100°C-110°C) and liquefaction (80°C-90°C) to economies these processes **[16, 17]**. Therefore, the thermos stability of the amylases must be matched to their application. Based on this demand, there has been a need and continual search for more potent thermophilic microorganisms for production of novel thermostable amylases.

Although amylases can be derived from several sources, such as plants, animals and microorganisms, amylases from microbial sources generally meet the industrial demands. Screening for microorganisms with higher amylase activities could facilitate the discovery of novel amylases that are suitable for industrial application **[18]**. Fermentation using thermophilic microorganisms has many advantages, including a reduction in cooling cost, better solubility of substrates and the reduced risk of microbial contamination.

Despite the vast microbial diversity of Ethiopia, thermostable amylase producing bacteria for industrial application have not yet been explored. Therefore a research project has been initiated with the objectives of isolation and characterization of thermostable amylase producing bacteria from hot springs located in Arba Minch Nech Sar national park for application in bread making process.

2. MATERIALS AND METHODS

2.1. Description of the Study Area:

Arba Minch is a city and separate woreda in southern Ethiopia. The first common name for this city was Gnatagro. Located in the Gamo Gofa zone of the southern nations, nationalities and people's region about 500kilometers south of Addis Ababa, at 1285 meters above sea level. It is the largest town in Gamo Gofa zone and the second town in SNNPR next to Hawassa. It is located to the west of lake Abaya and its annual rainfalls and altitude ranges from 623.5 mm to1061mm and from 2200 to 1400meter above sea level respectively, with 32°C temperature. Mango tree, Banana and Apple are the most common vegetation and fruit type grow in Arba Minch. The present study was conducted at the laboratory of industrial Biotechnology, Department of Biology Biotechnology program, Arab Minch University (AMU) from January 2017 to February 2017.

2.2. Sample Collection:

Total of 2 sediments of samples were collected from different sites of Arba Minch Nech Sar national park at the area of hot water springs. Each samples were kept in clean sterile sample bottles sealed and transferred to the microbial and industrial biotechnology laboratory and stored at -20ºC in deep freezer till further processing. The temperature and pH of sediments were recorded at 50°C and 4 during the time of sampling respectively. The overall laboratory activities are summarized in the fig 1.

2.3. Isolation and Screening of Thermophilic Amylase Producing Bacteria:

The sediment samples were suspended in water by vigorous vortexing and serial dilutions were made up to 10^{-6} in sterile distilled water. 0.1 ml of appropriate dilution were spread on Nutrient agar plate. Then, the inoculated plates were incubated at 50°C for 48 hr and the growths of thermophilic bacteria were observed. From the mixed thermophilic

bacteria couture different pure thermophilic bacterial colony were isolated. Isolated pure bacterial colonies are further tested for amylase enzyme production on 1% starch containing nutrient agar medium by incubating at 50°C for 48 hours and then examined by dropping an iodine solution **[19]**. Clear zone forming (amylase producer) bacteria were screened and purified by streaking on starch containing nutrient agar plate. The cultures were subsequently sub-cultured and used regularly. Appearance of clear zone around bacterial colonies indicates hydrolysis of starch due to secretion of amylase by the thermophilic organism.

2.4. Selection of Thermotolerants and Hyperthermophilic Bacterial Isolates:

The potential thermostable amylase producing isolates were further screened by on the basis their thermo tolerance and hyperthermophilic characteristics by incubating the strain at 37-50°C and 70°C for 72h respectively. Selection of potential isolates were undertaken using starch containing nutrient agar medium with incubation in the specified temperature.

2.5. Characterization of Potential Thermophilic Isolates:

2.5.1. Morphological Characterization:

Potential isolate was characterized by colony (shape and colour); cell (Gram stain, cell morphology).

2.5.2. Biochemical Characterization:

Potential isolates were characterized by different biochemical methods like catalase test, citrate utilization test, urease test, Voges Proskauer (VP) test and Methyl Red (MR). Strach hydrolysis test were performed by streaking pure thermophilic bacterial cultures onto nutrient starch agar (NSA) and observing zones of clearance.

2.6. Inoculums Preparation for Production of Thermostable Amylase:

A single bacterial colony from potential thermophilic isolate were inoculated in to 10 ml of nutrient broth under aseptic condition and incubated at 50ºC for 24 hrs. After 1 day of incubation, growth (turbidity) was appeared in inoculated broth and this preparation was directly used as a source of inoculums for production of thermostable amylase **[19]**.

2.7. Amylase Production Medium:

The production medium contained 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) at pH 4 was prepared for amylase enzyme production under submerged condition. A loop full of thermophilic amylase positive bacterial isolate maintained in nutrient broth was inoculated with 1ml of inoculums into 250 ml Erlenmeyer flasks containing 100 ml production medium followed by incubation at 50°C for 48 hours in water bathe shaker at 100 revolutions per minute (rpm). Culture filtrates were separated by centrifugation at 5000 revolution per minute (rpm) for 15 min and the supernatants were used as crude enzyme source for further application test **[19]**.

2.8. Wheat Flour Fermentation using Crude Thermostable Amylase Enzyme:

The activity crude amylase enzyme produced from all the isolates were tested for wheat flower fermentation. Four beakers were taken and 20g of wheat flour was added in to each beakers. Then 3ml of crude amylase enzyme was added in to each beakers which contain 20g of wheat flour. Beaker with 20g of wheat flour and 3ml of water were used as control and beaker with 20g of wheat flour and yeast were also used for comparison.

2.9. Data Analysis:

The data were analyzed using basic statistical parameters like table, percentage. In addition to this, Microsoft office Excel worksheet 2010 was used to construct tables and data presentation.

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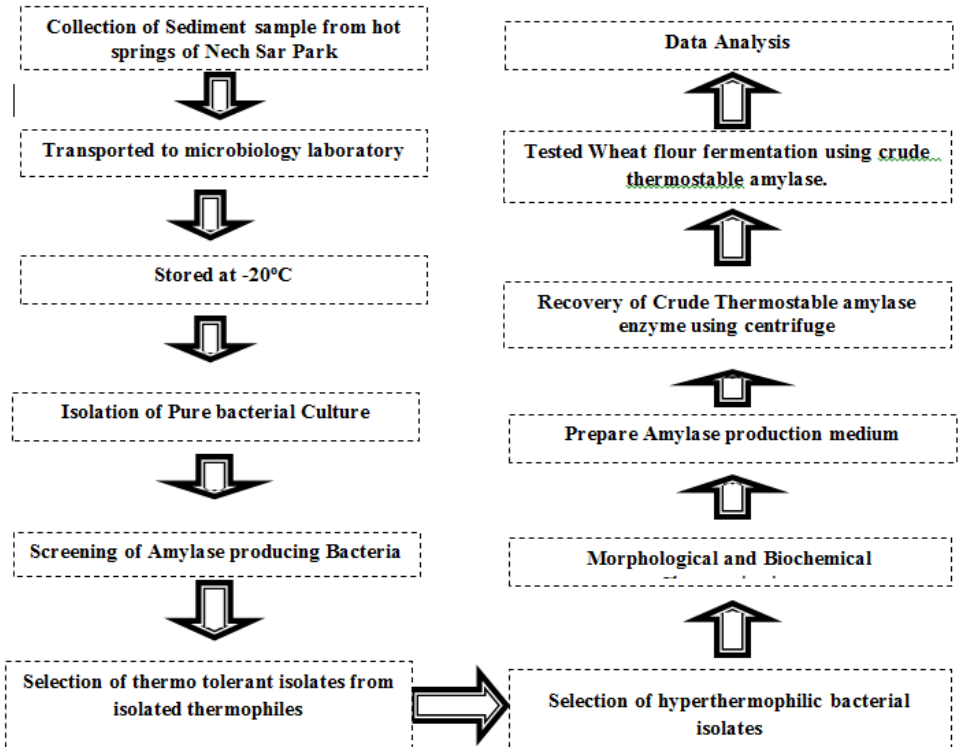

Figure 1. Flow chart summering the overall laboratory activities in this study

3. RESULTS

3.1. Isolation and Screening of Thermophilic Amylase Producing Bacteria:

The original hot-spring habitat parameters were used for isolation of thermophilic bacterial for production of thermostable amylase. Spread plate followed by streak plate method were used in starch containing nutrient agar medium for isolation and screening of potential amylase producing thermophilic bacteria. Accordingly, seventy two (72) thermophilic bacterial isolates were obtained from hot spring sediment samples (fig 2). Among 72 thermophilic isolates, 10 Potential amylase producer were screened based on the observation of clear zore around the colony after addition of drop of iodine solution in starch containing nutrient agar medium after 48hrs incubation. These 10 amylase positive bacterial isolates with having clear zone were further examined.

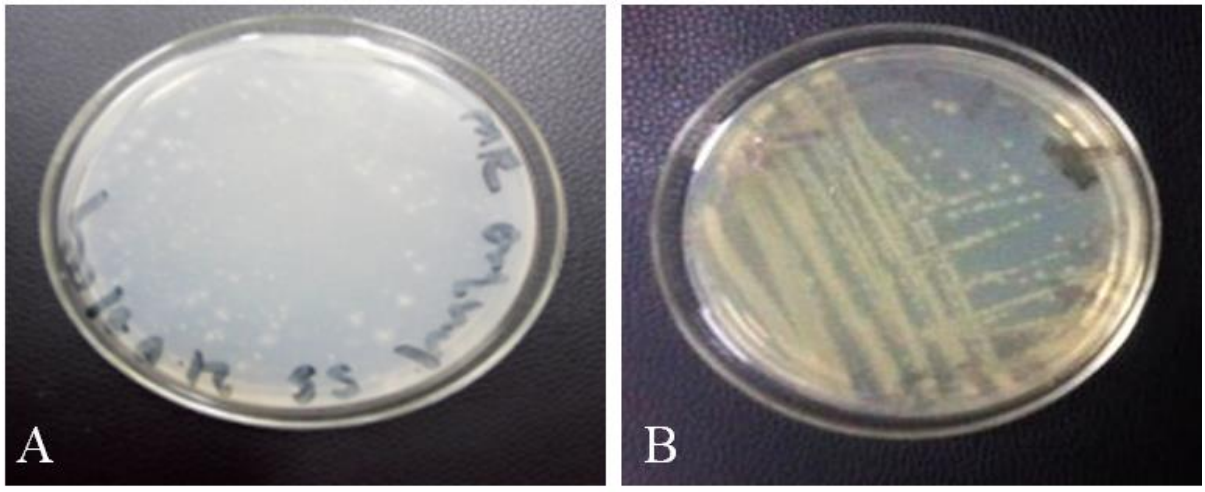

Figure 2. Growth and isolation of thermophilic bacteria on starch nutrient agar medium at 50°C after 48 hours; (A) Spread plate and (B) Streak plate.

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3.2. Selection of Thermotolerants and Hyperthermophilic Bacterial Isolates from Thermophilic Isolates:

Ten potential thermophilic and amylase producing bacterial isolates were compared for thermo tolerance and hyperthermophilic characteristic by incubating at 37°C and 70°C respectively. Four potential isolates have thermo tolerance ability have been selected, however none of the isolates shows hyperthermophilic characteristics since could not grown at 70°C and above.

3.3. Morphological and Biochemical Characteristics of Potential Isolates:

Table 1, table 2 and fig 3 shows the result of morphological and biochemical tests of the selected potential isolates. Rod shaped cells were observed under compound light microscope**.** The selected potential isolates were characterized on the basis Gram stain, Simon citrate tests, starch hydrolysis, Urease, catalase and methyl red tests.

Table 1. Morphological Characteristics of Potential Isolates

Isolates	Cell Shape	Colony Characteristic	Growth on NB	pH	Tolerance Range
Isolate-1	Rod	White	Moderate	4	37-60°C
Isolate-2	Rod	Yellow	Fast	4	37-60°C
Isolate-3	Rod	Off white	Fast	4	37-60°C
Isolate-4	Rod	Yellow	Moderate	4	37-60°C

Table 2. Biochemical Characteristics of Potential Thermophilic Isolate

Potential Isolates	Catalase Reaction	Gram Staining	Methyl Red test	VP test	Urease test	Citrate test
Isolate 1	+	+	+	-	+	+
Isolate 2	+++	+	+	-	+	+
Isolate 3	++	+	+	-	+	+
Isolate 4	+	+	+	-	+	+

(Key: +++ = strong positive; ++ = medium positive; += weekly positive; -= negative)

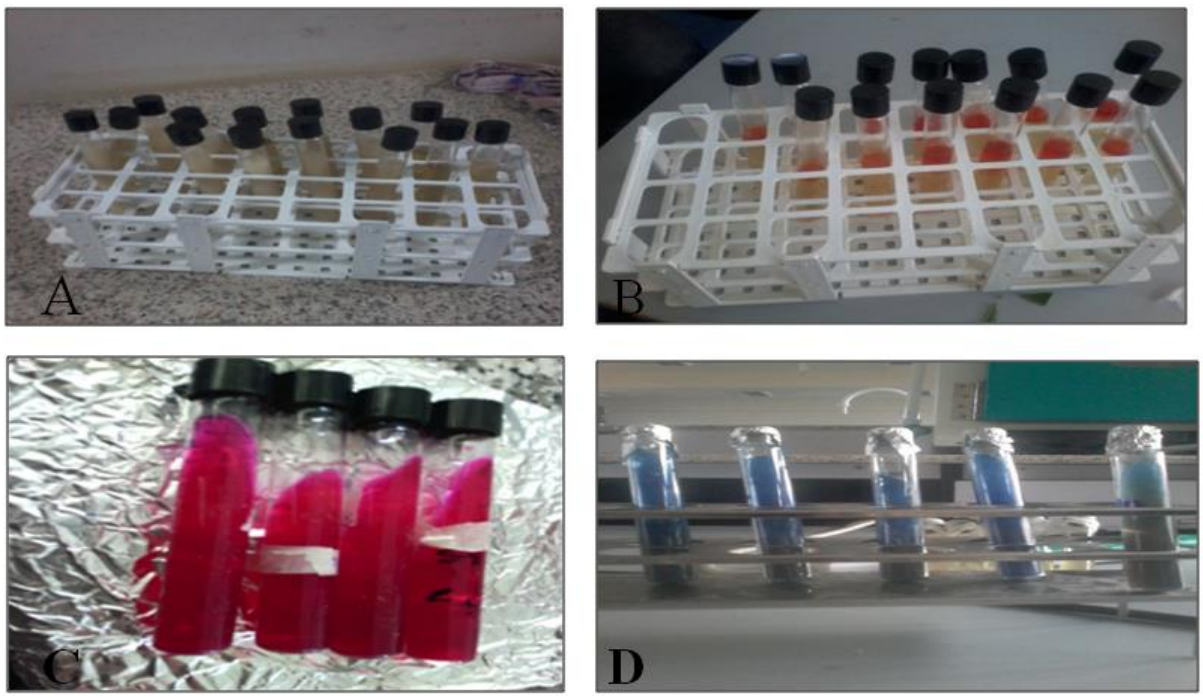

Figure 3. Result of Biochemical tests of potential isolates at 50°C: (A) Negative VP test result; (B) Positive result for methyl red test; (C) Positive result for urease test and (D) Positive citrate utilization test.

3.4. Recovery of Crude Thermostable Amylase Enzyme after Fermentation:

The crude enzymes were produced from four potential thermophilic bacterial isolates under submerged condition. Culture filtrates were separated by centrifugation at 5000 rpm for 15 min and the supernatants containing cell free extract were used as crude enzyme source for testing the activity on wheat flour fermentation (fig 4).

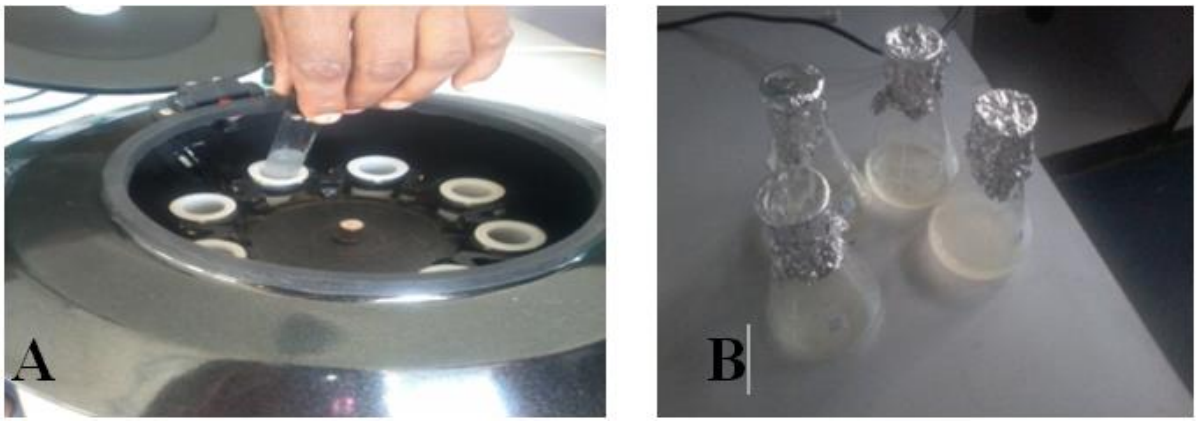

Figure 4. Recovery and isolation of crude thermostable amylase enzyme. (A) during centrifugation and (B) cell free extract after centrifugation

3.5. Wheat Flour Fermentation using Crude Thermostable Amylase Enzyme:

The activity test of crude amylase produced from potential isolates were done on wheat flower fermentation process. 20gm of the wheat flower measured and added in to four different beakers and two beakers for control and comparison. 3ml of crude enzymes were added to each beaker containing 20gm of wheat flour except the control and yeast containing beaker. The amylase activities of all the isolates (Isolate-1, Isolate-2, Isolate-3 and Isolate-4) were sharply recorded in different time interval. The result of wheat flour fermentation using crude thermostable amylase enzymes were shown in table 3 and fig 5.

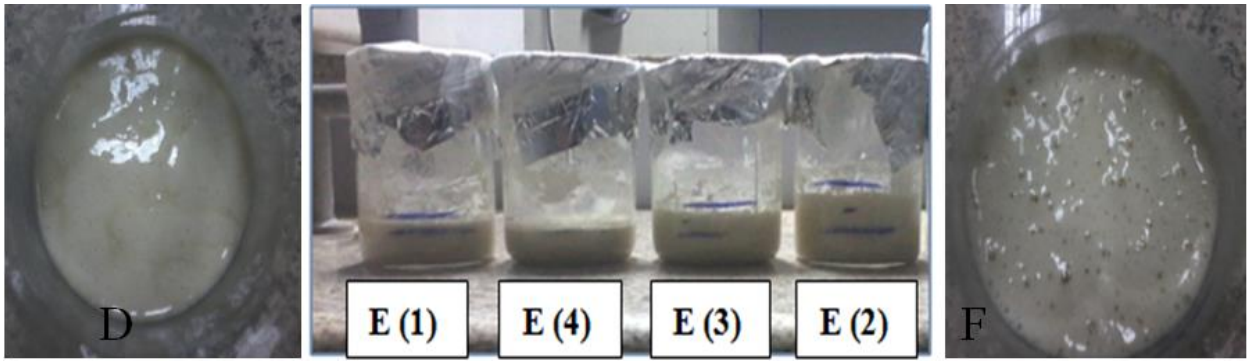

Figure 5: Wheat flour fermentation by thermostable crude enzyme produced from potential isolates. Sample pictures: (D) control (20gm wheat flour + 3ml water only) after fermentation; (E1-E4) fermentation of wheat flour using crude enzyme from potential isolates after 22 hrs incubation at 40^{o}C and; (C) fermented flour dough using crude enzyme.

Table 3. Summary of thermostable amylase enzyme activities during fermentation process at different time interval

Time	Enzyme-1 (cm)	Enzyme-2 (cm)	Enzyme-3 (cm)	Enzyme-4 (cm)	Yeast(cm)	Control (cm)
Initial	1	1	1	1	1	1
After 8 hr	1.5	1	2.2	1	1.6	1
After13 hr	2.5	1.2	3.5	2.2	1.6	1
After 22hr	4.7	4.6	4.6	4	3.5	1

(Key: cm=centimeter, used to measure the level beaker during fermentation)

4. DISCUSSION

One of the main concerns of this study was to isolate and characterize thermostable amylase producing bacteria from hot spring found in Arba Minch Nech Sar National Park, Southern Ethiopia for application of bread making process. This was performed by serial dilution spread plate technique. Similar method has been used for isolation of thermophilic microorganism by some researcher **[1, 20]**. Accordingly, 72 thermophilic bacterial strains were isolated from the study area. Ten thermophilic isolates had formed clear zone are screened as amylase producers. Formation of clear zone around the bacterial colony after addition of drop of iodine solution indicated the amylase positive thermophilic strains hydrolysed the starch present in the media. The use of starch containing nutrient agar medium for the isolation of amylase producing bacteria has earlier been reported by some workers **[20, 21].** As shown in the present investigation, hot spring found in Arba Minch Nech Sar National park is rich in thermophilic amylase producing bacteria. This indicates that hot springs have potential for screening of thermophilic bacterial strain which produce industrially important thermo enzymes, especially for industries operating under extreme condition.

Out of ten potential thermophilic isolates, four isolates, Isoltes-1-4 were selected which exhibited high thermo tolerance capacity for further examination. These all imply that this thermophilic strains have potential for extraction of industrial important products like thermostable amylase used for industrial application. Similar studies were reported from Iran **[13]**.

Biochemical and morphological characterization of the selected bacterial isolates was done; all the thermophilic amylase producing isolates (isolates 1-4) were displayed positive result for methyl red, citrate utilization, catalase, urease and starch hydrolysis tests. While all four potential isolates were found to be Gram positive, rod shaped cell and negative for Voges Proskauer test (table 1, 2 and fig 3). Results are almost similar with that investigated by some researchers **[19, 20].**

The crude enzyme activities were demonstrated on wheat flour and the rate of flour fermentation recorded at different time interval. Then after 22 hr fermentation, the dough sample contain crude enzymes from isolate-1, 2, 3 and 4 were rise from 1cm to 4.7, 4.6, 4.6 and 4cm respectively, but in the case of yeast the dough sample rise from 1cm to 3.5cm and the control beaker remained as it is 1cm (table 3 and fig 5). All beakers were treated at the same condition. This study is consistent with the previous reports **[19, 21, 22, 23]**. The present study revealed that thermostable amylase isolated in this study have applicable in bread baking process and replace the conventional yeasts with better performance. This causes the starch to hydrolyze into small dextrin's and be fermented the wheat flour without the presence of yeast by increases the rate of fermentation.

Microorganisms are the most important sources for production of industrially important enzymes. Selection of the right organism plays a key steps toward enzyme production beside enhancing the yield of desirable products. The present finding highlights, crude thermostable enzymes produced from thermophilic bacteria have potential application for wheat flour fermentation for making bread and undergoes the process without any addition of yeast or some other fermentative chemicals.

5. CONCLUSIONS

The method described in this investigation for wheat flour fermentation by thermostable amylase enzymes produced from thermophilic bacteria have economic implication by replacing conventional yeast technology in bakery industry. The present study revealed that thermostable amylase produced from Arba Minch hot spring is applicable in bread making process instead of yeasts which save time and money with better performance. This enzyme have potential of hydrolyzing starch into small dextrin's and ferment the flour by increasing the rate of fermentation.

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