Hot Spring Derived Bacterial Hydrolytic Thermozymes for Biotechnological Application

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Abstract

Bacterial thermozymes based on hot spring research, with a wide range of applications across different industrial, biomedical and agricultural sectors, have grown tremendously in the last few years. However, there are few reports in this field concerning the Ethiopian context, although the country abounds in hot springs. The aim of this project is to assess the potential for isolation, screening and characterization of prospective thermophilic bacteria from local hot springs in the Koye Gore farmland of Addis Ababa City for the production of thermo tolerant hydrolytic enzymes such as amylase and protease. Various characterization tools and techniques have been used to elucidate the biochemical characteristics of the thermo tolerant microbe and the use of their enzymes in their crude state. Three bacterial isolates were found to be positive for gram staining, catalase, starch and casein hydrolysis tests. Producers of potential amylase and protease enzymes for prospective biotechnological applications of extracted enzymes in the production of glucose from wheat flour hydrolysis and protein (albumin) as detergent-inclusion assessed. This study examined the thermo tolerant bacteria with multi-enzyme producers and confirmed their biotechnological application with their respective substrate.

Keywords: Hot springs, Thermophiles, Thermozymes, Amylase, Protease

Introduction

A wide range of biocatalysts such as amylase, lipase, keratinase and others in their free and immobilized states drive a number of industrial, agricultural and biomedical sectors. Constant research in the field of enzyme biotechnology has led to the search for enzymes, which are functional across a wide range of physicochemical parameters and, in particular, functional even under high temperature conditions [1, 16]. At this juncture, it is important to note that the demand for thermostable enzymes is constantly increasing, requiring a large-scale investigation and exploitation of thermophilic microorganisms. Compared to plant and animal sources, enzymes extracted from thermophilic microbes are expected to have advantages of intrinsic thermo stability and a comparatively higher resistance to modulation in physical and chemical factors [2, 17]. In this context, the researchers have focused considerable attention on the isolation and characterization of various hot spring thermophiles for the production of a myriad of industrially relevant enzymes in the various geographical pockets of the world. Hot springs are distinctive hydrothermal features formed as a result of underwater heating due to geothermal energy [3]. As a matter of fact, hot springs are characterized by a moderate to high-temperature environment that shelters a plethora of industrially important thermophiles (class of extremophiles, thriving at relatively high temperature, ranging from 41^0C to 121^0C) belonging to various species, including Thermus and Geobacillus, to name a few. Considerable attention has been paid to industrially important enzymes, amylase, protease and cellulase [4, 16]. For example, thermostable amylase, accounting for almost 30% of the enzyme market, is used in various industries such as food, detergent, paper, textile, beverage, pharmaceutical and fine chemical industries and, as a result, understanding its structure and function attributes under extreme physicochemical conditions has been active research – area. Protease derived from thermophilic bacteria is also widely used in bio-industrial food, pharmaceutical and various biological applications. Similarly, cellulase is used in the biostoning of denim and lignocellulosic biomass conversion for the production of biofuel, among others [5]. Literature repositories such as Scopus and Google Scholar host a number of reports and review articles on thermozymes isolated from various microbial sources from different hot springs in the world. However, as far as the Ethiopian context is concerned, not many works have been projected. It goes without

saying that Ethiopia is trying its best to boost its economy by setting up its own food, textiles, pharmaceutical industries etc. [6].

There is a tremendous prospect of exploring the microbial diversity of various hot springs in our country. Under the growing global pressure to 'go green' in various manufacturing units (as mandated under the UN's sustainable objectives), the use of biocatalysts harnessed from our country (instead of importing) for different industrial units could lead to a positive trend in terms of self-reliance and resource savings[7, 17]. In this regard, this project is streamlining the use of hydrolytic thermozymes extracted from local hot spring bacterial isolates in the Koye-Goro area of Addis Ababa (a few kilometers away from AASTU) for potential biotechnological applications. During our pre-investigation, temperatures between 45-50^{o}C and 7.2-7.5 pH were detected in the hot spring. We therefore envisage that this could be a potential site for isolating thermophilic microbes for the extraction of commercially relevant biocatalysts [8].

Materials and Methods

Sample collection

500ml of samples were collected aseptically from hot springs in Koye Goro ICT village, Addis Ababa, Ethiopia. It was then transported to the Addis Ababa Science and Technology University Microbiology Laboratory and tested for its pH and temperature. The temperature and pH of hot spring water samples were measured using Bench top pH Meters and hot springs were found to have a temperature of 50^{o}C and a pH of 7.5. Sample was then enriched with nutrient broth containing 5 per cent starch and 5 per cent casein in a ratio of 1 to 1 (v/v) and incubated for 48 hours below 50^{o}C.

Isolation of Thermophilic Bacteria

Thermophilic bacteria have been isolated using both temperature and selective media. Primarily serial dilution was performed and the dilution factor from 10^{-1} to 10^{-6} was cultivated on the selective ATCC Thermus medium using a sterile glass spreader and incubated at 50°C for 48 h. After incubation, growth was examined and promising results were selected and stored at 4°C until further processing.

Screening of bacterial isolates and their crude enzymes for hydrolytic enzyme production

Bacterial isolates were screened using hydrolytic plate assay tests on a starch agar plate and Skim milk agar plate for testing α-amylase and protease production, respectively. The bacteria were cultured on both starch and protein containing skim milk media and they made clear zone as a result of extracellular enzymes of the bacteria's α-amylase and protease degrade starch and protein respectively [18]. After analysis of zonal inhibition crude enzyme was extracted using submerged fermentation followed with centrifugation method. Loop of inoculum (containing ~2-3.5x106 cells/mL) of each thermophilic amylolytic and proteolytic isolates was introduced to submerged fermentation of nutrient media (100 mL) containing Nutrient Broth in multiple 250 ml Erlenmeyer flasks. Flasks were incubated at 50 °C on a rotary shaker incubator at 150 rpm for 48 h. [13]. After termination of the fermentation period, 10ml of fermented broths were transferred into sterile screw-capped tubes for each isolate centrifuged at 2500 rpm for 20 min. The supernatant 9 thus obtained from the various flasks after centrifugation were served as a crude enzyme source (Shaikh et al., 2013).

Inoculation of crude Enzyme was done by placing 100 microliters of a sample inside holes prepared by sterile borer on both starch agar plates and skim milk gar followed with incubation under room temperature for 48 hrs. After incubation starch hydrolysis was determined by flooding the plates with iodine solution and whereas zones of clearance on skim milk agar were selected as positive protease producing bacteria [3, 9]. The promising three bacterial isolates name TB1, TB2, and TB6 isolates, based on the hydrolytic capacity (i.e., the ratio of the diameter of the clear zone) was selected for further study of the bacterial colony and Biochemical tests.

Biochemical test

Three Bacterial isolates of Promising Hydrolytic Activity was selected for Colony and morphological characterization [5, 10], Different biochemical methods like Gram staining, catalase test, citrate utilization test, Triple sugar Iron Agar, and Lysine Iron Agar biochemical tests.

Biotechnological application of the hydrolytic enzymes

Prospective use of protease as laundry detergent additive Evaluation of prospective Application of protease and Amylase Crude Enzymes extracted from three thermophilic bacterial isolates

were done through protease hydrolysis of Egg Albumin on cotton wool and Amylolytic Wheat flour starch Hydrolysis respectively. For protease prospective Application, 1 ml of Egg Albumin was added on 5 cm by 5 cm cotton wool placed on a larger dish of a Petri plate for all the three candidates and control as forth. Plates were then exposed to dry at room temperature followed by the addition of 3 ml of crude Enzyme on each cotton wool and water on the control plate and the result was recorded.

Prospective Application of crude amylase enzyme was determined through Wheat Flour Hydrolysis followed with benedict solution test for the presence of glucose produced through starch Hydrolysis. The extracted amylase was tested for overnight wheat flour Hydrolysis. Four beakers, each containing 2g of wheat flour were used and 5 mL of crude enzyme candidates (TB1, TB2, and TB3) were added to three of the beakers, and water on the fourth beaker served as a control. It was then shaken for 30 minutes. The supernatant was tested with benedict solution for the presence of glucose produced through enzymatic hydrolysis of starch. 1 ml of supernatant was added to 5 ml benedict solution inside test tubes. It was then placed in a water bath for 3 to 5 minutes expecting a color change from blue solution to greenish-yellow color. The result was then observed.

Result and Discussion

Results of water sampling and isolation of thermophilic bacteria

Water sampling and isolation of thermophilic bacteria began by collecting aseptic samples from hot springs in a 500 mL sterile glass container, which is then examined for its PH and temperature, and our sample was found to have a temperature of 50oC and a pH of 7.5, which makes it a potential source of thermophilia. Pre-enrichment of samples with a nutrient broth containing 5 per cent starch and 5 per cent casein at a ratio of 1 to 1 (v/v) will enrich retired and weakened microbes that may have been affected during transport, maximize the chance of obtaining microbes, and the addition of starch and casein will stimulate bacterial metabolism to produce the prospective enzyme.

For the isolation of thermophilic bacteria, both the temperature selective procedure and the use of selective insulation media were used to increase the insulation of thermophilic bacteria and also to check the growth difference between the two methods. After incubation of the bacterial enrichment media for 48 hours, the mixture was turbid, indicating successful bacterial

enrichment. Serial dilution of 10-1 to 10-6 dilution levels was prepared by inserting 1 ml of the pre-enriched sample into 9 ml of the sterile distilled water triplicate at the third, fourth and fifth dilution levels to obtain pure colony counts.

Using the Temperature Selective Isolation method, bacterial isolates were found grown on Nutrient Agar medium of fourth and fifth dilution level. A total of 3 candidates (2 from 4^{th} and 1 from 5th) were selected for hydrolytic plate assay. Given names TB1, TB2, and TB3. Using Selective Isolation media ATCC Thermus medium 697, bacterial isolates were found grown on ATCC Thermus medium 697 from all 3 candidates (3rd, 4th, and 5th) as shown in figure 2. The result was obtained because of Thermus medium 697 compositions of NaCl (2), peptone (8.0), and yeast extract [4, 11] that help thermophilic microbes grow selectively with a short period under high temperature providing salt for maintaining osmotic balance, peptone as water soluble polypeptide and Amino acid source to synthesize bacterial proteins, and Yeast Extract as a soluble component of yeast cells that microbes utilize as Nutritional source. All three candidates were then named TB4 TB5 and TB6 respectively.

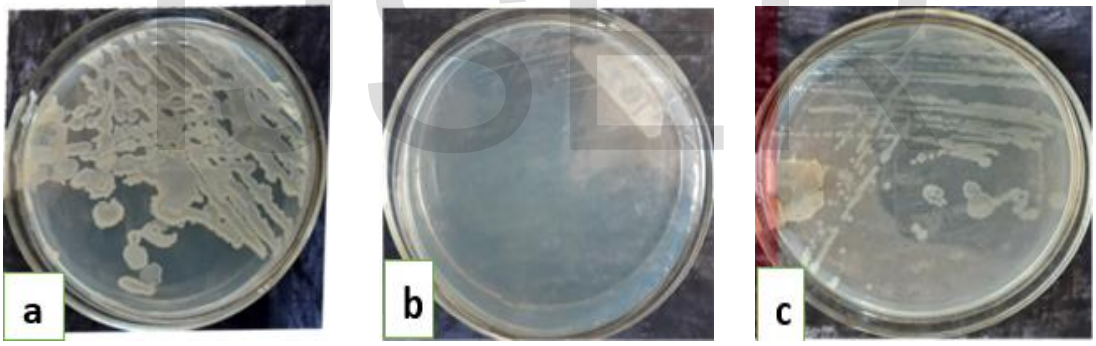

Figure 1: Strick plating for potential microbial isolates. Three bacteria were selected further zonal inhibition tests TB1, TB2, and TB6 for (a), (b) and (c), respectively.

Six Bacterial isolates were transferred to a fresh medium of 10 ml Nutrient broth for submerged fermentation. Since submerged fermentation allows the cultivation of microbes in liquid nutrient broth medium making nutrients easily accessible to microbes and secret metabolic enzymes under appropriate physicochemical conditions.

Screening of Bacterial isolates was determined by flooding plates with iodine solution, and clearance zones on skim milk agar were selected as positive bacteria-producing protease [3,14] whereas skim milk agar plate were used for protease testing. The result was promising as similar

three isolates were found to hydrolyze skim milk agar with a different clearance zone (1, 0.1, and 0.5cm) radius. Skim milk powder and casein enzymatic hydrolysate of composition (28 g and 5g) which are used by microbes to check the proteolysis activity of several bacteria, including Bacillaceae and Enterobacteriaceae

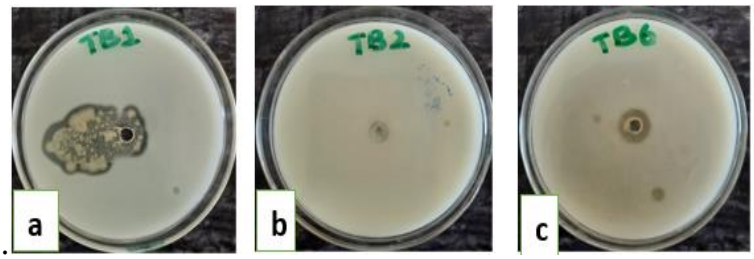

Figure 2: Skin Milk Agar hydrolytic assay tests for protease activity in different thermophile isolates (a) TB1 (Large), (b) TB1(Less), and (c) TB6 (Small).

Screenings using hydrolytic plate assay tests on the starch agar plate have been identified as potential for three bacterial isolates. Since starch agar plates contain 6 grams of gar per 100 ml of nutrient agar, the bacterial isolates used starch as a carbon source from the central hole to the maximum halo hydrolysis zone. The bacterial clear starches found in the media in diameter of 4cm, 2cm, and 3cm as presented on picture a, b, and c respectively. Our result indicates our isolates have high hydrolytic potential when compared to previous work reported from Ethiopia by Aynadis et al (2013) 0.6-1.4mm diameter of inhibition zone [15].

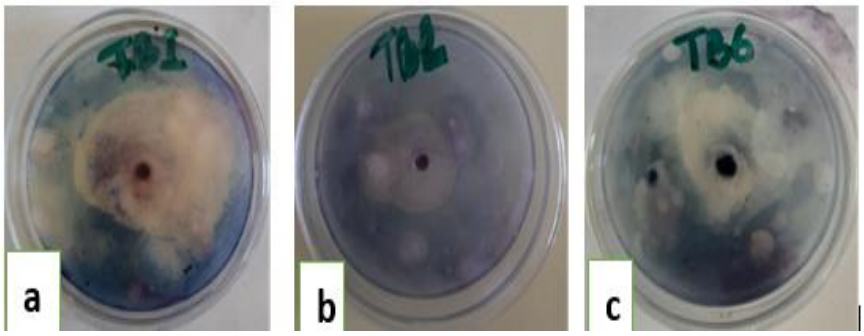

Figure 3: Starch agar hydrolysis for different isolates shows to different size of hydrolysis observed after addition of iodine solution. (a) TB1 wide size of hydrolysis, (b) TB2 medium size of hydrolysis, and (c) TB6 some Part is hydrolysis.

Iodine solution was used to stain the unhydrolyzed part of the media by flooding the plates with an iodine solution expecting a blue-black color while the hydrolyzed part remains white. Potential isolates for starch and protein hydrolysis have been identified, depending on the results of TB1, TB2 and TB6, so that they have been selected for further biochemical tests.

Biochemical tests

Several biochemical tests have been conducted to identify isolate bacterial candidates depending on their Biochemical response towards the Biochemical test. Our bacterial isolates were found to be gram-positive, observing blue colonies under the microscope. For further checking, Lysine Iron Agar, and Triple sugar iron agar test were conducted as confirmatory tests. Triple sugar iron agar test is conducted to check for the ability of bacteria to utilize multiple sugars and produce hydrogen sulfide. It has pH sensitive die (phenol red) that form color change when bacterial fermentation produces an acidic environment and black color for the production of Hydrogen sulfide. Lysine Iron Agar contains Lysine, peptone, glucose, sodium thiosulphate and Ferric Ammonium citrate, and Sodium thiosulphate. When glucose is the fermented bottom of the media becomes yellowish color due to Acidic environment pH < 5.2 and purple color above pH >6.8. Citrate utilization test was also negative but yellow colonies were observed. Since the basic purpose of the citrate utilization test is to differentiate among various Gram-Negative enteric bacteria based upon their ability to utilize citrate. Color change from green (Neutral) to blue (Alkaline). As shown in the figure below our result was green (no change), which indicates a negative result. We have used this test as a confirmatory test for gram staining to check for isolates that are stained gram-positive. The catalase test is used to identify organisms that produce the Enzyme Catalase that detoxify hydrogen peroxide by breaking it down into water and oxygen gas. Our samples were Catalase positive due to the presence of bubbles after a drop of 3% Hydrogen peroxide has been added to fresh slant nutrient agar.

Table 1: list of biochemical tests for characterization of thermophiles

	Citrate	Lysine iron agar	Triple sugar iron agar	Staining	Colony character	Starch hydrolysis	Casein hydrolysis	Catalase
TB 1	-	-	-	+	Wide	+	+	+
TB 2	-	-	-	+	Small	+	+	+
TB 3	-	-	-	+	Intermediate	+	+	+

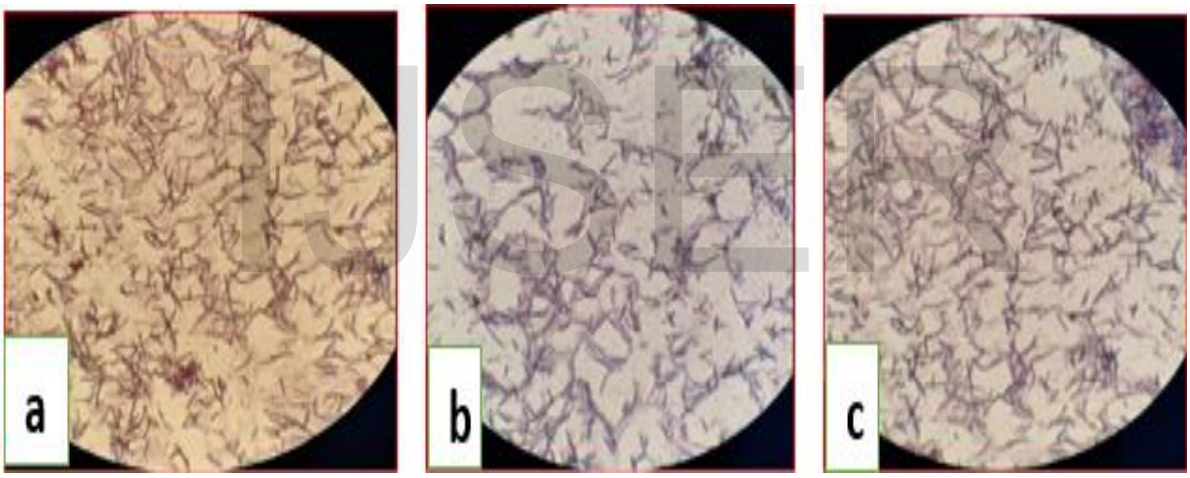

Figure 4: Gram staining for three selected isolates is positive. (a) TB1, (b) TB2, (c) TB6 observed under Light Microscope.

Finally, the test results were analyzed in ABIS online software which is used to analyze the genus of the organism. Based on the morphology characteristics, Biochemical test and ABIS software the following results were predicted as *Bacillus vietnamensis* ~similarity 88.1%; or *Bacillus siamensis* ~similarity 88.1%.

Biotechnological Application

Submerged fermentation following a biochemical test, 100 ml of fermented broths were transferred to sterile screw-coated tubes for each of the isolates centrifuged at 2500 rpm for 20

min. The supernatant thus obtained from the various flasks after centrifugation was used as expected to serve as a raw enzyme source for biotechnological applications. [13].

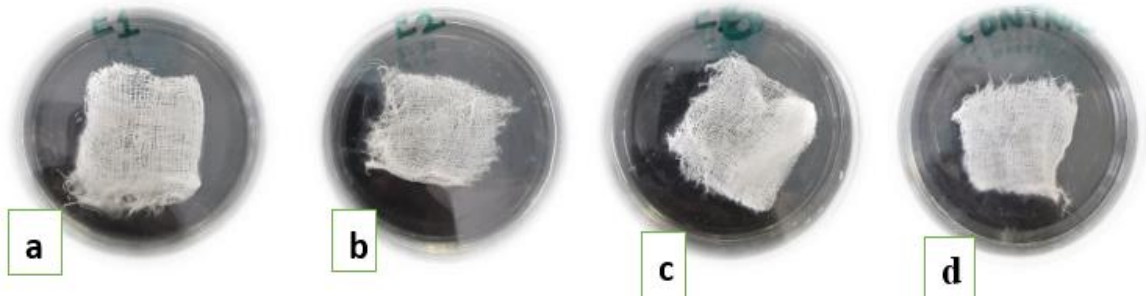

Figure 5: Egg albumin presents in (a) - TB1, (b) - TB1, and (c) - TB6 were successfully hydrolyzed by protease enzyme. (d) -Control that water was added not hydrolyzed.

To evaluate the feasibility of using the extracted protease as a laundry detergent additive, its compatibility and stability towards some commercial laundry detergents, procured from the local market shall be assessed [10]. The residual protease activity was determined by Egg albumin hydrolysis on cotton wool (as mentioned above) and compared with the control (enzyme 1:1 tap water). As a result, shows in figure 7, dried egg albumin (assuming protein dirt on a cloth) was washed out by our crude enzyme within less than 10 seconds while our control lasted for so long and didn't wash the dirt out.

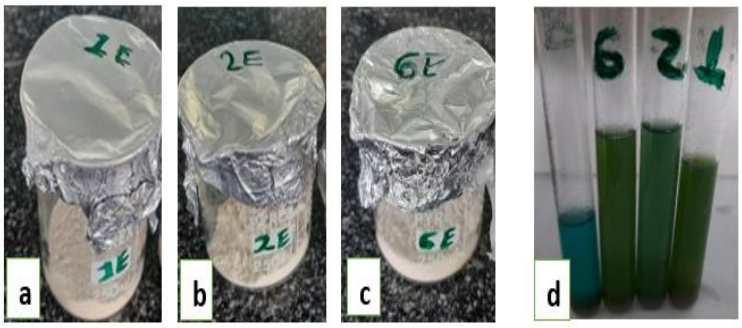

Figure 6: Wheat flour mixed well with crude enzyme to check whether amylase hydrolyses starch (a) - TB1, (b) - TB1, and (c) - TB6 envisaged. (d) Shows after adding Benedicts solution color change observed which means starch converted into Glucose and Maltose.

Crude extract tested for overnight wheat flour Hydrolysis at its optimum temperature followed with positive benedict test (change from blue solution to greenish yellow color) showed a successful result of the production of crude amylase Enzyme. Despite our isolates positively

correlated with Alemayehu and Temam et al, (2017) that reports the bacterial wheat flour fermentation from southern Ethiopia, the new isolate have multi potential capacity of hydrolyzing protein too [14].

Conclusion

In this study, we conclude that three thermophilic bacterial isolates were successfully isolated and characterized on the basis of their biochemical tests. Biochemical tests show that bacterial isolates are most likely bacillus species (Bacillus vietnamensis ~ similarity 88.1 per cent, Bacillus siamensis ~ similarity 88.1 per cent). Use of ABIS (Advanced Bacterial Identification Software). Multipotent enzyme production was also found for these isolates (produce both amylase and protease).

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