African J. Biol. Sci., 16 (1): 197-206 (2020)
ISSN 1687-4870 e- ISSN 2314-5501 (online)
www.ajbs.journals.ekb.eg E.mail: aasdjournal@yahoo.com

Potential enzyme activity of thermophilic bacteria from hot spring in Egypt

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Received: November 2, 2020; Accepted: November 30, 2020; Available Online December 7, 2020

ABSTRACT

This study aims to isolate and identify thermophilic hydrolytic bacteria from hot spring in South Sinai, Egypt for several industrial applications. In this work, 29 bacterial isolates from hot spring in Egypt were isolated and screened for the production of three thermozymes (amylase, cellulase and protease) at different high temperatures. Fifteen isolates were amylase producer, twenty-one were cellulase producer and twelve isolates were protease producer at different high temperatures. Ten bacterial isolates (34.5%) produced the three extracellular enzymes. All bacterial isolates were identified phenotypically as *Bacillus* spp. This study concludes that hot springs in Egypt is a source for the isolation of thermophilic bacteria producing thermostable enzymes.

Keywords: *Bacillus* spp- hot spring -thermophilic bacteria- thermozymes.

INTRODUCTION

Extremophiles are microorganisms thriving extreme ecosystems (Aanniz *et al*., 2015). Extremophilic microorganisms are classified according to the type of extreme condition which they prefer to grow into seven families, thermophiles, psychrophiles, halophiles, acidophiles, alkaliphiles, metalophiles and piezophiles (Gerday, 2002; Gupta *et al*., 2014). Thermophiles are microorganisms optimally grow at high temperatures more than 45°C, while hyperthermophiles grow above 80°C (Aanniz *et al*., 2015; Kumar *et al*., 2019).

Thermophiles are studied due to their potential to produce thermostable enzymes (amylases, proteases, cellulases, xylanases and lipases) and exopolysaccharides (Pinzón-Martínez *et al*., 2010; Singh *et al*., 2010; Al-awsy *et al*., 2017; Suleiman *et al*., 2020). These thermo-enzymes are not only stable at high temperature but also active under other extreme conditions such as high or low pH, presence of salts and high pressure (Gomes and Steiner, 2004; Kumar *et al*., 2019).

Thermophilic bacteria are used in bioremediation, improvement the quality of petroleum oil, composting (Rawat *et al*., 2005; Poli *et al*., 2009; Lin *et al*., 2014). Microbial thermozymes have been extensively used in waste management, biofuel, food, paper, detergent, medicinal and pharmaceutical industries, pulp, feeds, starch, textile and used as biocatalysis, biotransformation and biodegradation due to their extreme stability in elevated high temperatures (Kumar *et al*., 2019). Bacterial α-amylase, protease and cellulase enzymes are the most enzymes used in biotechnology processes (Baltaci *et al*., 2017).

The aim of this study was to isolate thermophilic bacteria, characterize them phenotypically and evaluate their ability to produce thermostable enzymes. Further work needs to be done to study the possible

use of the two isolates in production of thermostable hydrolytic enzymes which have industrial applications.

MATERIALS AND METHODS

Sampling site

Ten soil samples were collected in sterile plastic containers from Pharaoh bath hot spring, South Sinai, Egypt in March 2018, then transferred to the laboratory for analysis. Samples were pooled together, and air dried then stored in -20 °C.

Isolation of thermophilic bacteria

The soil samples were diluted and inoculated onto Petri plates containing nutrient agar medium (Techno pharmchem, India). The inoculated plates were incubated at 60°Cfor 24-48 h(Osman *et al*., 2018). The bacterial colonies developed on the plates were purified by subculturing on nutrient agar media and maintained in 15% (v/v) glycerol(Sazakli *et al*., 2005).

Characterization of thermophilic bacterial isolates

Morphological characteristics of the isolates were studied on nutrient agar.The colony morphology, i.e., color, size, margin, elevation and Gram stain according to Sandle (2004). Cell morphology and Gram reactions of the isolates were examined by light microscopy (LABOMED, USA).The optimum temperature of growth for each isolate was determined by inoculation onto nutrient broth medium and incubation at 37, 45, 50, 55, 60, 65, 70 and 75°C for 24 h. The turbidity obtained after incubation was measured spectrophotometrically at OD600 nm (Unico UV – 2000) (Abu Bakar *et al*., 2015).

Screening for enzyme activity of the isolates

Bacterial isolates were screened for amylolytic, proteolytic and cellulolytic activity.Tests were carried out at four different elevated temperatures of 50, 55, 60 and 65°C. Amylase was tested by using starch agar mediumas described by Vaidya and Rathore (2015). The pure isolated colonies were inoculated on starch agar plates and incubated for 24-48 h. After incubation, the plates were flooded with iodine solution, a clear zone around the growth indicated the hydrolysis of starch.

Protease was tested on skimmed milk agar medium as described by Carrim *et al*. (2006).The pure colonies were inoculated on skimmed milk agar plates and incubated for 24-48 h. After incubation, 2.0 ml of HCl 0.1 mol l^{-1} was added to the plates, clear halos around the growth indicated the hydrolysis of casein.

Cellulase was tested on carboxy methyl cellulose medium as described by Amaresan *et al*. (2014). The pure colonies were inoculated on CMC agar plates and incubated for 24-48 h. After incubation, the plates were flooded by iodine solution, a clear zone around the growth indicated the hydrolysis of cellulose.

Quantitative assay of amylase activity

Two bacterial isolates were selected for amylase production at 55°C for 24 h. The pure isolated colonies were inoculated in amylase production broth medium as described by Kanimozhi *et al*. (2014). After incubation, the broth was centrifuged at 8.000 rpm for 20 min and the cell free supernatant was used as crude enzyme for the amylase activity assay. The amylase activity was measured by the glucose released from the starch hydrolysis by DNSA method as described by Karnwal and Nigam (2013). The amylase activity was determined by incubating 0.5 ml of crude enzyme with 1 ml of 1% soluble starch in 0.1 M of sodium phosphate buffer (pH 7.0) at 50ºC for 30 min. After incubation, 2 ml of 3,5 dinitro salicylic acid was added and the

mixture was boiled for 10 min. The mixture was measured spectrophotometrically at 540 nm. One unit of enzyme activity was defined as the amount of enzyme which releases one µmol of reducing sugar as glucose per min under the assay condition (Ezeji and Bahl, 2006). Amylase activity was determined by the formula of Karnwal and Nigam (2013). All the experiments were performed in triplicates.

RESULTS

Isolation of thermophilic bacteria

A total of 29 isolatesof thermophilic bacteria were recovered from hot spring soil in Egypt. The bacterial isolates were characterized by cultural characteristics and Gram staining. All isolates were Gram positive bacilli, endospore forming rods. Representative Gram stain of two isolates (Ge 1 and Ge 2) is shown in Figure (1), The endospores position is terminal for isolate Ge 1 and central for isolate Ge 2. The color of the colonies was off white and the size of colonies varied from pinpoint to large colonies. The configuration of the isolates was round or concentric. Representative culture characteristics of the bacterial isolates are shown in Figure (2). All isolates were moderate thermophiles, 27 isolates have optimum temperatures of 55°C and the two isolates (Ge 1 and Ge 2) have optimum temperatures of 60°C. All bacterial isolates were identified as *Bacillus* spp according to morphological characteristics and Gram stain.

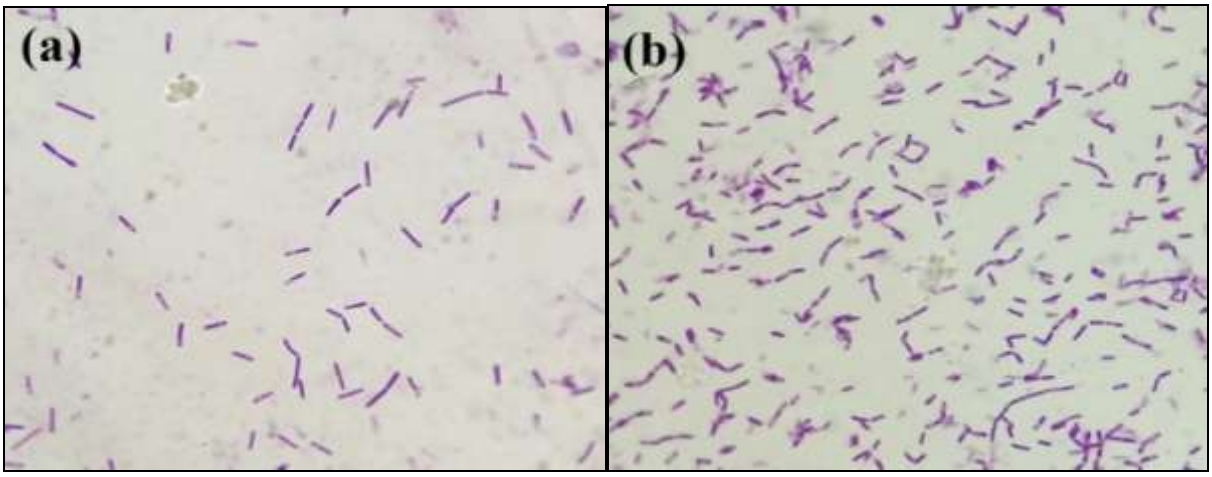

Fig. 1.Gram stain smears of 2thermophilic isolates under light microscope. (a) Ge 1. (b) Ge 2.

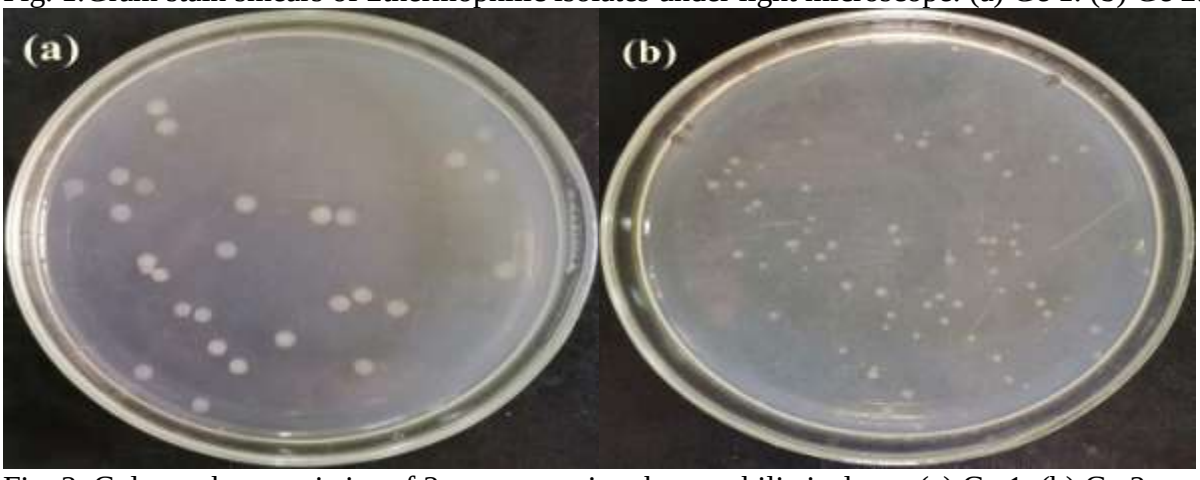

Fig. 2. Culture characteristics of 2 representative thermophilic isolates. (a) Ge 1. (b) Ge 2.

Screening for enzyme activity

The twenty-nine bacterial isolates were screened for amylolytic, cellulolytic and proteolytic activity at different incubation temperatures as shown in

Fig. 3 and 4) and Table (1). Data revealed that 15 isolates (51.7%) produced amylase at different incubation temperatures. Twenty-two isolates (75.9%) produced cellulase. Twelve isolates (41.4%) produced protease. Fifteen isolates (51.7%) co-produced amylase and cellulase, ten isolates (34.5%) co-produced the three tested extracellular enzymes.

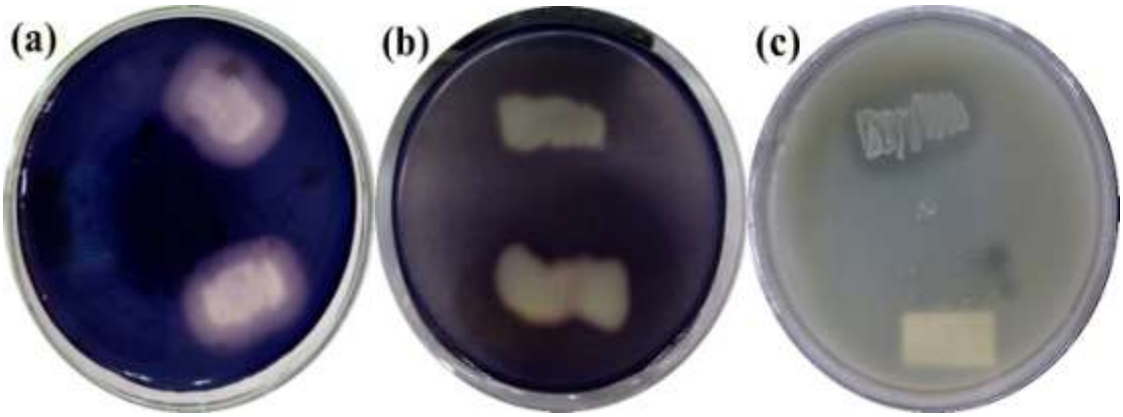

Fig. 3. Production of amylase, cellulase and protease enzymes by the two bacterial isolates. (a) Zone clearance around two isolates for amylase production at 50°C. (b) Zone clearance around two isolates for cellulase production at 50°C. (c) Zone clearance around Ge 2 isolate and negative for Ge 1 at 60°C.

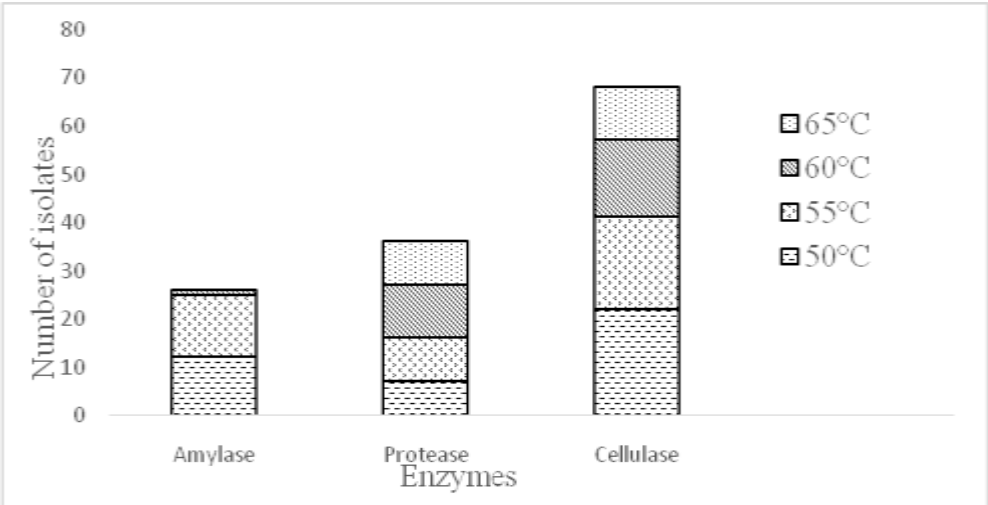

Fig. 4. Production of amylase, cellulase and protease enzymes by the twenty-nine bacterial isolates at 4 different temperatures (50, 55, 60 and 65°C).

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Table 1. Production of amylase, cellulase and protease enzymes by the twenty-nine bacterial isolates at 4 different temperatures (50, 55, 60 and 65°C).

Isolate code	Amylase				Cellulase				Protease			
	50°C	55°C	60°C	65°C	50°C	55°C	60°C	65°C	50°C	55°C	60°C	65°C
Ge 1	+	+	+	-	+	+	+	-	-	-	-	-
Ge 2	+	+	-	-	+	-	-	-	-	-	+	-
PBG-31	-	+	-	-	+	+	+	-	-	-	-	-
PBG-32	-	-	-	-	-	-	-	-	-	-	-	-
PBG-33	+	-	-	-	+	+	+	+	-	-	-	-
PBG-34	-	+	-	-	+	+	+	+	-	-	-	-
PBG-35	-	-	-	-	-	-	-	-	-	-	-	-
PBG-36	+	+	-	-	+	+	+	+	-	-	+	+
PBG-37	-	-	-	-	+	+	+	+	-	-	-	-
PBG-38	+	+	-	-	+	+	+	+	+	+	+	-
PBG-39	-	+	-	-	+	+	+	+	+	+	+	+
PBG-40	+	+	-	-	+	-	-	-	-	-	-	-
PBG-41	+	+	-	-	+	+	+	+	+	+	+	+
PBG-42	+	+	-	-	+	+	+	+	-	+	+	+
PBG-43	+	+	-	-	+	+	+	+	+	+	+	+
PBG-44	+	+	-	-	+	+	+	+	+	+	+	+
PBG-45	-	-	-	-	+	+	+	+	-	-	-	-
PBG-46	-	-	-	-	+	+	+	-	-	-	-	-
PBG-47	-	-	-	-	+	+	-	-	-	-	-	+
PBG-48	-	-	-	-	+	+	-	-	-	-	-	-
PBG-49	-	-	-	-	-	-	-	-	-	+	+	+
PBG-50	-	-	-	-	-	-	-	-	-	-	-	-
PBG-51	-	-	-	-	+	+	-	-	-	-	-	-
PBG-52	-	-	-	-	-	-	-	-	-	-	-	-
PBG-53	-	-	-	-	-	-	-	-	-	-	-	-
PBG-54	-	-	-	-	+	-	-	-	-	-	-	-
PBG-55	-	-	-	-	-	-	-	-	-	-	-	-
PBG-56	+	-	-	-	+	+	+	-	+	+	+	+
PBG-57	+	+	-	-	+	+	+	-	+	+	+	-

Quantitative assay of amylase activity

Two bacterial isolates (Ge 1 and Ge 2) were selected for rhe amylase activity assay. It was observed that the amylase activity of the isolates was 1.690 and 2.425 IU /ml for Ge 1 and Ge 2 isolates, respectively.

DISCUSSION

Hot springs are considered to be the natural habitat of thermophilic bacteria with optimal growth temperatures >45 °C.Thermophiles represent an important source of biotechnological richness for elevated temperature bioprocesses by their capability of producing a large variety of novel bioactive compounds of biotechnological importance in agriculture, mining, nanotechnology, and other industrial fields.

In this work, bacteria from hot spring in Egypt that produced hydrolytic enzymes

have been successfully isolated and identified. successfully Twenty-nine thermophilic bacterial isolates were obtained. All isolates are aerobic Gram positive, spore forming rod shaped and have off white color. The twenty-seven isolates out of 29 isolates have 55°C optimum temperature, two isolates have 60°C optimum temperature and they are considered moderate thermophilic bacteria according to Rothschild and Mancinelli (2001), and this observation was compatible with the studies about thermophilic microorganisms conducted by Baltaci *et al*.(2017).The presence of endospore forming bacteria in hot springs is related to their capability to adapt and survive extreme environments (Kambura *et al.*, 2016).

Isolates are belonging to Bacillales according to their Phenotypic characters. Morphological and microscopic characteristics for the bacterial isolates were similar to the characteristics of the genus *Bacillus* as was described by De Souza and Martins (2001); Mohammad *et al*. (2017); Gomri *et al*. (2018). Strains of *Bacillus* were the most studied bacteria, Maugeri *et al*. (2001) isolated 87 aerobic,thermophilic and spore-forming bacteria from Eolian Islands (Italy). Moreover, 97.5% of strains recovered by Aanniz *et al*. (2015) from Moroccan hot springs were belonging to genus *Bacillus*. In addition, thermophilic *Bacillus* was reported by Malkawi and Al-omari (2010) from Jordanian hot springs.

Hydrolytic enzymes are not only essential for biochemical reactions within an organism, but their high specificity and catalytic characteristics have enabled them to be used in various industrial sectors for the production of a wide range of products.

Amylase, cellulase and protease are important enzymes in terms of industrial value and they have a wide area of usage in detergent, textile, leather, cosmetics, food, animal feed, pulp and paper industries (Laxman *et al*., 2005; Kuhad *et al*., 2011; El-Fallal *et al*., 2012).

In this study, twenty-nine bacterial isolates were screened for hydrolytic enzymes, amylase, cellulase and protease enzymes. Various extracellular enzymes from thermophilic bacteria isolated from hot springs have also reported by Al-awsy *et al*. (2017), Megahati *et al*.(2017) and Alrumman *et al*.(2018). Twenty-two isolates produced at least one extracellular enzyme at different high temperature. Amylase, cellulase and protease enzymes were produced by 15 isolates (51.7%), 22 isolates (75.9%) and 12 isolates (41.4%), respectively. Fifteen bacterial isolates out of 29 isolates produced amylase and cellulase. Ten isolates out of 29 isolates produced the three tested enzymes.

Amylase activity assay of the two isolates revealed that *Bacillus* spp Ge 2 gave the maximum amylase of 2.425 IU/ml, while the lowest amylase activity of 1.690 IU/ml was obtained for *Bacillus* sp Ge 1. The production of amylase enzyme from *Bacillus* sp were reported by Bukhari and Rehman (2015) and Salem *et al*. (2016).

Acknowledgments

The authors are so grateful to postgraduate studies and research affairs, Faculty of Science, Ain Shams University, Cairo, Egypt for their keen support and providing of possible research grant for obtaining valuable materials and reagents to carry out this work.

Conflict of Interest

There is no conflict of interest

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النشاط الانزيمي للبكتيريا المحبة للحرارة من ينبوع ساخن في مصر

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المستخلص

تهدف هذه الدراسة الى عزل وتعريف بكتيريا محبة للحرارة والتي لها القدرة على إنتاج إنزيمات محللة من ينبوع ساخن في مصر للاستفادة منها في العديد من التطبيقات الصناعية. في هذا العمل تم عزل ٢٩ عزلة بكتيرية من مصر وإختبار قدرتها على إنتاج ٣ إنزيمات ؛ الأميليز، السيليليز، البروتياز وذلك عند درجات الحرارة العالية المختلفة. ووجد أن ١٥ عزلة من اجمالى العزلات لها القدرة على إنتاج انزيم الأميليز، ٢١ عزلة أنتجو إنزيم السيليليز، ١٢ عزلة أنتجو إنزيم البروتياز عند درجات حرارة عالية مختلفة. وأظهرت النتائج أن ١٠ عزلات بكتيرية (٣٨.٥%) لها القدرة على إنتاج ٣ إنزيمات. كل العزلات البكتيرية تم تعريفها وذلك على أساس النمط الظاهري لها. وتستنتج هذه الدراسة أن الينابيع الساخنة في مصر تعتبر مصدر لعزل البكتيريا *Bacillus* spp المحبة للحرارة المنتجة للإنزيمات التي تعمل عند درجات حرارة عالية.