Potential enzyme activity of thermophilic bacteria from hot spring in Egypt

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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, alkaliophiles, 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°C for 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 medium as 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 carboxymethyl 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 50°C 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 isolates of 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 2 thermophilic 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 |
|--------------|---------|-----------|
|              | 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 the 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. 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).

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Conflict of Interest

There is no conflict of interest

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النشاط الپظلی للبکتریا المحبة للحرارة من ينبوع ساخن في مصر

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المستخلص

تهدف هذه الدراسة إلى عزل وتعريف بکتریا محبة للحرارة والتي لها القدرة على إنتاج انزیم محلة من ينبوع ساخن في مصر للاستفادة منها في العديد من التطبيقات الصناعیة. في هذا العمل تم عزل 49 عزلة بکتریة من مصر واختبار قدرتها على إنتاج 3 انزیمات أی المخلیز، السیلیلیز، البروتیز وذلك عند درجات الحرارة العالیة المختلفة. ووجد أن 15 عزلة من اجمالі العزلات لها القدرة على إنتاج انزیم أمیلیز، 21 عزلة أنتجت انزیم السیلیلیز، 12 عزلة أنتجت البروتیز عند درجات حرارة عالیة مختلفة. وأظهرت النتایج أن 10 عزلات بکتریة (68.3%) لها القدرة على إنتاج 3 انزیمات. كل العزلات البکتریة تم تعريفها وذلك على أساس النمط الظاهري لها. وستنتج هذه الدراسة أن الینابيع الساخنة في مصر تعتبر المحبة للحرارة المنتجة للانزیمات التي تعمل عند درجات حرارة عالیة Bacillus spp مصدر لعزل البکتریا

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