Screening for Hydrolytic Enzyme Production by Thermophilic Microbes Isolated from Egyptian Hot Spring

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Abstract: Extreme environments like thermal springs are of considerable value as a source of thermophilic microbes, enzymes and biotechnological substances. The hot spring of Ras-Sedr is one of the Egyptian hot springs which not discovered yet. This research was carried out to isolate and screen thermophilic microbes that have the capability to produce thermostable enzymes. The sources for microbial isolation were sediment and water samples, collected from Ras-Sedr's hot spring, Ras-Sedr, Egypt. Three different medium types were used for microbial isolation. The isolates obtained were subjected for enzymatical activity. Three different enzymes, cellulase, lipase and ά-amylase, were selected to be screened by isolated strains. For cellulase activity testing, carboxy methylcellulose (CMC) agar was used as growth medium whereas tributyrin agar medium was used for lipase enzyme, while, starch agar plates were used for amylase enzyme screening. Our results showed that sediment sample harbored higher count of microbes than the water sample. Similarly, ATCC medium recorded higher thermophilic microbial count compared to the other two media used. The isolates obtained also reported a positive activity for all enzymes tested. Therefore, these promising isolates could be a source for pharmaceuticals and industrial applications.

Keywords: Hot spring, Thermophiles, Cellulase, Lipase, Amylase

INTRODUCTION

Hot springs are of great interest to the general public and to scientists because of their unusual and extreme conditions. Microbiologists around the globe are searching for hot springs interested in their chemical properties, thus creating a strong selective pressure on their microbial inhabitants (Wilkins *et al*., 2019). Microorganisms are essential enzyme resources, and are preferable to plant and animal sources. Currently, more than fifty percent of the enzymes used in industrial applications are from microbial origin. From the time of their discovery, extremophiles gives an excellent system for scientists. Including development under harsh conditions, researchers have been impressed by their capacity to produce industrially important substances including enzymes (Turner *et al*., 2007).

Enzymes from thermophilic microorganisms attracted considerable interest from industry due to their specific characteristics, such as elevated stability to pH changes. Reasons for targeting these enzymes include their suitability as models for investigating the thermo-stability of thermoenzymes and their potential as biocatalysts (Andrade *et al*., 1999). For decades, microorganisms have been used in the manufacturing of beer, yogurt and cheese, but the amount of prospective and realized products continues to expand in other sectors, including food industries, pharmaceuticals, paper, textile, etc. Already when they operate, many enzymes have very specific pH and temperature specifications. Currently, it is obvious that certain microorganisms, such as extremophiles, can generate enzymes that can survive and operate under extreme circumstances that are usually necessary for these processes (Sujatha *et al*., 2005). Therefore, this study was intended to isolate thermophilic microbes that have the ability to produce industrially important thermo-stable enzymes including cellulase, lipase and ά-amylase.

MATERIALS AND METHODS

Source of Microorganisms:

Water and sediment samples were collected from a geothermal hot spring located in Abo Swira, El Mahager road, Ras-Sedr, Egypt (Map 1). Samples were collected during summer season (July - August) in 1000 ml bottles of sterile Pyrex. Water temperature, pH, and EC were measured during the sampling using a portable combined pH/ EC/Temperature tester (HANNA HI98129/HI98130). Samples were maintained on ice until processing and then stored at 4°C.Water and sediment samples were subjected to physical and chemical characterization.

Isolation and purification of strains:

Water and sediment samples were inoculated to three distinct growth media using pour plate technique. These media are ATCC, TSA and Nutrient agar. The ingredient (per 1 liter) of each medium is as follow:

ATCC medium: 697 (Thermus medium): Yeast extract 4.0 g, Polypeptone Peptone, 8.0 g NaCl 2.0 g, Agar 30.0 g. Final pH was adjusted to 7.5 (Khalil, 2011).

TSA medium: Casein peptone (pancreatic digest) 15.0 g, Soya peptone (papain) 5.0 g, Sodium chloride 5.0 g, Agar 15.0 g. Final pH was adjusted to 7.5 (Wells-Bennik *et al*., 2019).

Nutrient Agar medium: Beef Extract 3.0 g, Peptone 5.0 g, Agar15.0 g. Final pH was adjusted to 6.8 ± 0.2 (Wells-Bennik *et al*., 2019). The plates were wrapped with parafilm and kept for 24-48 h of incubation at 55°C in clean plastic bags. At the end of the incubation period, total viable count was recorded for all samples. Distinctive colonies from the culture plates were picked up and streaked on fresh medium plates and incubated at 55°C for 24h. This step is repeated for getting purified isolates.

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Map (1): Location of the sampling site

Hydrolytic Enzymes Assessment:

Lipase detection:

Thermophilic isolates were screened for Lipolytic activity on Tributyrin agar contained all common components used in NA with 1% tributyrin and incubated at 55°C for 24-48 h. Positive lipase-producing bacteria will be indicated by clear or 'halo' zones surround the colonies on tested agar following the method of Heravi *et al*. (2008).

ά- Amylase detection:

ά-Amylase assay was carried by culturing the thermophilic isolates on starch agar plates followed by incubation at 55°C for 24-48h. After incubation, 1% iodine solution (freshly prepared) was flooded on the starch agar plate. The presence of blue color around the growth was determined as a negative result; however, a clear zone of around the growth colonies reflected the ability of the isolates to hydrolyze starch and recorded a positive result (Hamilton *et al.*, 1999).

Cellulase detection:

For testing the cellulolytic activity, actinobacteria were screened on carboxy methyl cellulose (CMC) agar according to the method of Ibrahim and El-diwany (2007). Cultivation medium containing: 0.1 g of $CaCl_2.2H_2O$, 5.0 g of NaCl, 10.0 g of peptone, 2.0 g of CMC and 20.0 g of agar. The pH of the medium was adjusted to pH 7.0 using 1M of NaOH. The streaked plates were then incubated at 55°C and checked after 48 h. The plates were flooded with iodine and the development of halo zones suggested positive activity of cellulose hydrolysis.

RESULTS AND DISCUSSION

Sediment and water samples were collected from hot spring located in Abo Swira, El Mahager road, Ras Sedr, Egypt. The physiochemical characterization of the specimen sites (Table 1) revealed that the average temperature was ranged between 80-85°C. The pH of the sediment sample recorded 7.09 and 6.51 for sediment and water samples respectively. Sediments texture analysis observed to be sandy loam and organic matter content was 2.615%. However, the presence of different anions, cations and heavy metals composition was measured and recorded (Table 1).

Three different culture media was used to isolate thermophilic bacteria from water and sediments samples (Table 2). Sediment sample showed more bacterial and actinobacterial counts for all three media while fewer isolates from water sample were collected. ATCC medium was the optimum for the growth of thermophilic microbiota and showed the highest number of bacterial and actinobacterial count. Sikdar *et al*. (2015) and Khalil (2011), also successfully used ATCC medium for thermophiles isolation. Aerial mycelia and sporulation give the colony of actinobacteria a white powdery appearance. It should be mentioned that actinobacteria isolates were fast growing, as aerial mycelium observed after only 48h. Kurapova *et al*. (2012) and Carrillo *et al*. (2009) also observed such rapidly growing thermophilic actinobacteria. To determine their capacity to produce hydrolytic enzymes, bacterial and actinobacterial isolates were screened to evaluate lipase, amylase and cellulase activity (Figure 2).

A total twenty four isolates were subjected to enzyme screening, the majority of the isolates showed activity for cellulase. According to Krzyśko-Łupicka *et al.* (2016) cellulose is the principal mass of organic matter that is degraded by soil microorganisms completely or partially. Another factor can affect the activity of cellulase is pH of the sample. In this study, the pH of sediment was 7.09; Sethi *et al.* (2013) detected high cellulase activity and stability in the range of neutral and alkaline pHs. On the other hand, fifteen isolates of bacteria and actinobacteria showed activity for lipase and only actinobacterial isolates showed activity for amylase.

Table (1): Analysis of sediment and water samples

Sample	T.N. %	T.P. %	T.K. %	O.C. %	O.M. %	Sand %	Silt %	Clay %	Tex.
Sediment	0.156	0.0764	0.030	1.5165	2.615	66.0	24.0	10.0	Sandy Loam

Sample	EC dSm^{-1}	pH	Cations meql-1				Anions meql-1			
			Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}	Cl^{-}	HCO_3^{-}	SO_4^{2-}	CO_3^{2-}
Water	7.16	6.51	34.6	12.4	31.3	1.70	48.5	3.00	28.5	0.00
Sediment	5.15	7.09	24.4	8.60	18.5	1.50	33.5	1.50	18.0	0.00

Sample	Cu mg/l	Zn mg/l	Mn mg/l	Co mg/l	Pb mg/l	Fe mg/l	Cd mg/l
Water	ND	0.0466	0.414	ND	ND	0.442	ND
Sediment	ND	11.360	90.260	ND	ND	379.6	ND

Sample	D.O (mg/l)		B.O.D. (mg/l)	C.O.D. (mg/l)
	Before incubation	After incubation		
Water	13.4	12.0	1.40	2.26

Table (2): Total actinobacterial and bacterial counts represented in mean ± SE of colony forming unit (CFU) in gram or milliliter of sediment and water samples, respectively

Samples	Total microbial count (Mean ± SE)					
	Medium Used					
	ATCC		NA		TSA	
	Actinobacteria*	Bacteria†	Actinobacteria*	Bacteria†	Actinobacteria*	Bacteria†
Sediment CFU/g	59.5 ± 3.5^{a}	110 ± 1.53^{a}	10 ± 0.58^{c}	30 ± 1.53^{c}	20 ± 1.52^{b}	50 ± 1.15^{b}
Water CFU/ml	3.0 ± 0.58	6.0 ± 0.58	0.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	2.0 ± 0.58

*Columns with different Letter are significantly different for actinobacterial count per sediment sample
†Columns with different Letter are significantly different for bacterial count per sediment sample

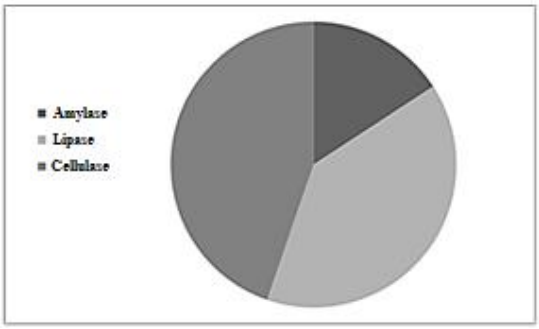

Figure (2): Enzymes activity evaluation of the isolated thermophiles

The ability of isolates for cellulase production was indicated by producing a clear zone in the presence of iodine solution staining (Figure 3). These isolates showed the ability to use carboxy methyl cellulase (CMC) as their primary source of carbon. Maki *et al*. (2011) performed a similar experiment for screening and identification of cellulase-producing bacteria.

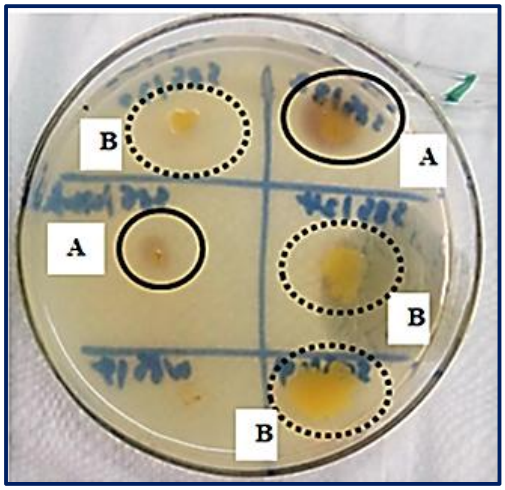

Figure (3): Cellulatic activity by isolated strains; A, represents a negative activity of cellulose enzyme by one of the isolate; B, represents cellulase-producing isolates which showing hallo zone around the screened colonies

In their study, they found that iodine solution was quick and effective in detecting many positive cellulase producing bacteria from broad range of samples. In similar study done by Norashirene *et al.* (2014) also used CMC agar plates and iodine solution staining to evaluate cellulase producing thermophiles isolated from hot spring. In general, nineteen of our isolates obtained; in this study, showed a powerful cellulase activity.

Meanwhile, for screening thermostable ά-amylase, actinobacterial isolates obtained showed activity at 55°C in which the clearance of halos zones around the growth indicated the enzymatic activities (Figure 4). The halos zones were different in sizes; which revealed the different intensity of enzyme produced by the screened isolates. Selection of the powerful isolates will be promising for industrial application. Similar result was reported by (Sen *et al.,* 2010) in which they reported different enzyme activity by different isolates.

In this study, actinobacterial and bacterial isolates were identified as powerful degrader of lipid and showed clear zones on tributyrin agar plate at 55°C (Figure 5). The clearance halo around the colonies was generated by tributyrin hydrolysis by isolate-based extracellular lipase production. This result is in confirmation with the data recorded by Lokre and Kadam (2014).

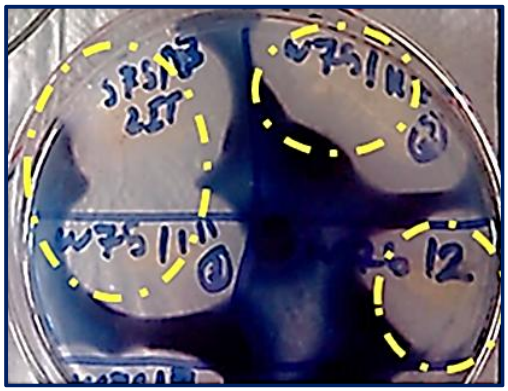

Figure (4): ά-amylase activity of different actinobacterial isolates. The activity was recorded at 55^{o}C and represented by hallo zone around the screened colonies

Despite the utility of enzymes which have thermostability in molecular biological techniques, it is not also surprising that these have also been proposed as powerful tools for industrial and commercial catalysis (Vieille and Zeikus, 2001). Cellulases are important in the fruit, beverage, fiber, paper and pulp industries, along with agriculture and other research purposes (Cavaco-Paulo, 1996; Islam and Roy, 2018). For many industries, amylase and lipase are useful, such as detergent, milk, clothing, pharmacy, and many dairy products (Patnala *et al*., 2016; Hussain *et al*., 2013).

Figure (5): Lipase enzyme activity showing positive strain lipase-producers
Activity represented by hallo zone around the colonies of screened isolates (arrow)

Therefore, our novel actinobacteria isolates and their industrially important enzymes (cellulase, ά-amylase and lipase) will influence the effective productivity and achieve the necessities of industries. However, the specific metabolic pathways in these actinobacteria are in need for further study to optimize these enzyme productions for maximum expanding pharmaceutically, agriculturally, and biotechnologically multifarious applications.

Conclusion & Recommendations:

The thermophiles which studied through the current work have the ability to produce useful thermostable enzymes of industrial significance. Ras-Sedr' shot spring considered a prospective source of economically significant microorganisms which needs more studies to explore more novel isolates with capability for more enzymes production. Consequently, further researches should be carried for such area.

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فحص الميكروبات المعزولة من الينابيع الساخنة المصرية لإنتاج إنزيمات محبة للحرارة

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تعتبر البيئات القاسية مثل الينابيع الحرارية ذات قيمة كبيرة ومصدر للميكروبات المحبة للحرارة والإنزيمات ومواد التكنولوجيا الحيوية. الينابيع الساخنة في رأس سدر هي واحدة من الينابيع الساخنة المصرية التي لم تكتشف بعد. تم إجراء هذا البحث لعزل وفحص الميكروبات المحبة للحرارة التي لديها القدرة على إنتاج إنزيمات مهمة صناعيًا، وتم عزل السلالات من كل من عينات الرواسب والمياه التي تم جمعها من ينبوع راس سدر الحار الموجود في رأس سدر، مصر. تم استخدام ثلاثة أنواع مختلفة من الوسائط للعزل الميكروبي. كما تم فحص نشاط الإنزيمات على الكائنات المعزولة. من أجل اختبار نشاط السليولاز، تم استخدام أجار الكاربوكسي ميثيل سلولوز (كوسيط للنمو في حين تم استخدام وسط أجار ثلاثي الترايبيوترين لفحص إنزيم الليباز وألواح أجار النشا لفحص إنزيم الأميليز. يمكن أن يكون لهذه الكائنات الحية الدقيقة التي تنتج إنزيمات التحلل المائي والصامدة في درجات الحرارة المترفعة تطبيقات واعدة في إنتاج الأدوية والمنظفات والصناعات الأخرى.