Multi-Knowledge Electronic Comprehensive Journal For Education And Science Publications (MECSJ)
ISSUE (16), Jan (2019)

www.mescj.com

Isolation and Screening of Thermophilic α-amylase Producing Bacteria from Hot Springs in Southern Region of Saudi Arabia

Randa Yahya Akour (Master Student)
Department of Biology, King Khalid University.

Abstract

Hot springs have been recognized as an important and untapped resource for isolation of bacteria produced bioactive compounds. The aim of this investigation is isolation of thermophilic bacteria from hot springs in southern region of KSA and evaluates their potential for α-amylase production with industrial value. A 64 bacterial isolates were obtained from three sites; Al-Majardah (24 isolates) and Al-Khubah (25 isolates) and Al-Ardah (15 isolates) using two different media. The clearance zone forming ability on starch agar plates was used for the primary selection of amylolytic isolates. A total of ١٧ bacterial isolates were produced clear zone around the colonies; only four isolates were produced high clearance zone around the colonies. The highest amylase activity was produced by isolates; MA-19 (2.28 mm), MA-24 (3.2 mm) from Al-Majardah and KH-5 (2.75 mm) and KH-25 (3.6 mm) from Al-Khubah regions. The promising strain KH-25 isolate was tested for enzyme production using broth starch medium. Maximum α-amylase production was achieved at 36 h in the end of the exponential growth phase. From this study, it was concluded that the hot springs are the promising source for isolation of thermophilic bacteria producing α-amylase with industrial importance.

Keywords: Bacteria, α-amylase, thermophilic bacteria, Spring.

2-Introduction

Geothermal areas are the main permanent hot places of the world, and these areas are the main habitats of thermophilic bacteria. These springs have variable chemical compositions and pH values, generally containing sufficient levels of nutrients to support populations of bacterial growth (Rawana, 2007; Sen et al., 2010). With respect to, geothermal resources in Saudi Arabia, majority of the geothermal activity encountered along the western and southwestern coast of Saudi Arabia. There are about 10 hot springs with varying deep temperatures of 50 to 120°C, located in Gizan and Al-Lith regions (Lashin et al., 2015). Unfortunately, it not extensively exploited for electricity generation, heating purposes, tourism and therapeutic benefits. In general, few reports published about biological activities in hot springs in Saudi Arabia ((Lashin, 2013). Extremophiles are organisms that have evolved to grow in variety of extreme environments and fall into a number of different classes such as thermophiles, psychrophiles and halophiles. Because of their harsh environment, they produce unique enzymes that function under conditions in which their mesophilic counterparts cannot survive. Thermophiles are organisms that have evolved to grow in extreme environments, it grow best at temperatures between 45 and 70°C. The major challenge for thermophilic bacteria is their survival and production of thermo-stable enzymes and other bioactive molecules at high temperatures. One of the attractive characters of these enzymes is the thermostability; this thermal stability is not due to any specific characteristic but as consequences of various attributes, which contribute to the whole stability of the protein. These enzymes are not only thermostable, but also more resistant to chemical agents than their mesophilic counterparts, which make them extremely interesting for industrial processes (Lasa and Berenguer, 1993). Enzymes from thermophilic organisms have mainly found their practical commercial use due to their overall inherent stability (Demirjian et al., 2001). Because of their harsh environment, thermophilic bacteria largely remain a reservoir for biodiversity, molecular phylogeny, production of industrial useful unique enzymes and other compounds. Amylases are one of the most important industrial enzymes and account for nearly 30 % of the world's enzyme market (Rajagopalan and Krishnan 2008). These enzymes have a great significance with extensive biotechnological applications including starch degradation, detergent, food, pharmaceutical, textile, and paper manufacturing (Acourene and Ammouche, 2010). Each application of α-amylase requires unique properties with respect to specificity and thermal stability (Konsula and liakopoulou-Kyriakides, 2006). The α-amylase is produced by a wide variety of microorganisms, but on account of its industrial applications the best commercial producers are Bacillus licheniformis, Bacillus stearothermophilus, and Bacillus amyloliquefaciens (Goyal et al., 2005). The main expected outputs of this study will recommend that the hot springs exhibit diverse thermophilic bacteria, which serve as potential reservoirs for production of thermostable α-amylase importance with diverse facet of activities. The present study focuses on the (i) isolation of thermophilic bacteria from hot springs, of southern region of KSA (ii) screening of the isolates for industrially important α-amylase production.

3- Literature Review

3.1. Hot springs in Saudi Arabia:

Ten hot springs have explored in Saudi Arabia and scattered in southern region. Fifteen isolates of thermo-aerobic bacteria were found. Bacillus cereus, B. licheniformis, B.thermoamylovorans, Pseudomonas sp., Pseudomonas aeruginosa and Enterobacter sp (Khiyami et al, 2012).

The thermal waters of hot springs reach the surface through a complex grid of structural elements, which follow the main tectonic elements and activities that influence the entire Red Sea region. Preliminary assessment was made on the geothermal potential of the hot springs encountered at Al-Lith and Jizan sites (Ain Al Harrah, Al Khouba, Al-Ardah and Bani Malik) using integrated geological, geochemical and geophysical techniques. Wide ranges of reservoir temperatures (130-220ºC) and heat flows (120-210 μW/m2) are estimated from geothermometers (Lashin, 2015).

The water chemistry of two natural hot springs in the Gizan area was studied .These hot springs contain of Sodium at 350 mgl1, calcium 159 mgl1, chloride 346 mgl1, potassium 347 mgl1, ammonia and nitrate (below the detection limits of 0.001 mgl1). Iron, manganese, magnesium, and zinc were present in Iow quantities. Blue-green, green algae and filamentous green algae present at vary temperatures. The maximum temperature registered were 50ºC in Ain AI-Harra and 57ºC for Ain AI-Khoba, and the PH values were 8.1 and 7.8 respectively (Basahy, 1994).

The water of hot springs in Gizan slightly to moderately alkaline,and orifice contained hydrogen sulfide (>5mg\l) (Arif, 1997).

3.2. Thermophilic bacteria:

The term thermophilic was first used by Miquel (1879) to describe the organisms that can grow at so high temperature, unlike the most microorganisms. There were many tries to define this term to more understanding for these organisms. Giltner designated as thermophilic those that have a nimum temperature of 45ºC., optimum, 55°C. and maximum, 70°C. Muir and Ritchie in 1913, define thermophilic bacteria as organisms that grow best at a temperature of from 60 to 70ºC. Hiss and Zinsser 1920) say that thermophilic bacteria are high temperature bacteria obtained from hot springs and from the upper layers of the soil. Rahn in 1917, in Marshall's Microbiology describes thermophilic bacteria as extraordinary organisms having their maximum between 70º and 80ºC a temperature which coagulates albumin; corresponding to the high maximum the thermophiles have a very high optimum, and the minimum lies with most species above 30ºC. According to Hewlett in 1902, there is a group of so-called thermophilic bacteria, which thrive best at a temperature of 60º to 70ºC. Those bacteria whose optimum temperature is above 40ºC, and which are spoken of as the "thermophile" bacteria, is the definition given for them by Morrey in 1921. Buchanan in 1915 does not mention thermophiles in his book but speaks of the organisms which produce large quantities of heat as thermogenic bacteria. In his book Chester in 1901, places thermophilic bacteria in a class that does not grow at room temperatures or below 22º to 25ºC (Morrison and Tanner, 1921).

Temperature is one of the most important factor controlling the activity and evolution of microorganisms. Microorganisms that have been found to be growing in extremely hot environments i.e. at a temperature range of 55 to 121 °C are known as thermophiles. The cellular components of thermophiles are extremely thermostable and these together with their unique metabolic capabilities, offer considerable promise for biotechnological applications. Such environments are thus of great interest to microbiologists and biotechnologists, as the organisms isolated from these environments are a good source of thermostable enzymes (Sharma, 2013).

Thermophiles are stable to such high temperature only because they are structurally adapted and have thermostable biomolecules. They have several modifications in their structural components and biomolecules. The biomolecules such as proteins,lipids, enzymes, ribosome, RNA and DNA have higher intrinsic stability(Rekadwad, 2015).

3.3. Isolation of thermophilic bacteria:

Thermophiles have been documented as a very good source of industrial enzymes which are thermostable. Therefore, in the present investigation, cultivable diversity of 101 thermophilic microbial strains isolated from hot springs of northern Himalayan region of Himachal Pradesh has been studied. These isolates have been found to be aerobic and sustaining a temperature of or above 50 ºC. All the isolates were checked with respect to their industrial enzyme production potential and were found to be a good source of amylase, cellulase and xylanase. Maximum enzyme producing strains were selected based on the enzyme units of amylase; cellulase and xylanase produced and were identified on the basis of 16S ribosomal RNA gene sequence analysis. (Sharma et al, 2013).

Using fatty acid profiles, BOX PCR fingerprints, and 16S rDNA sequence data, phenotypic and genotypic characterization of thermophilic -isolated from Pasinler hot spring, Turkey- has been determined. After the data of fatty acid analysed the bacterial strains were classified into 2 phenotypic groups, and these data were confirmed by BOX PCR genomic fingerprint profiles and 16S rDNA sequence analysis. The first group, identified as Bacillus licheniformis, was represented by 4 strains, and the second group, identified as Aeribacillus pallidus, was represented by 5 strains (Adiguzel, 2011).

In northern Himalayan region of Himachal Pradesh, four thermophilic bacterial strains isolated from Manikaran hot water spring. Four isolates were identified as thermophilic bacteria, the optimal temperature for growth of these isolates was 65 º C and the optimal pH was 6-8 (Verma et al, 2014).

Geobacillus kaustrophilus PW11, Geobacillus thermoleovorans PW13 and Geobacillus toebii PS4 were isolated form Tattapani hotspring of Himachal Pradesh, India and characterized for extracellular amylase activity. All the three Geobacillus spp. exhibited thermophilic amylase activity، which was predominantly extracellular. The activity was optimum at 90°C and pH 7.0. Interestingly، no amylase activity was observed at temperature less than 40°C, indicating its thermophilic nature. Moreover, pre incubation of enzyme at 100°C enhanced the amylase activity (Sharma et al., 2015).

3.4.α-Amylase (characterization, production and applications):

Amylases are important hydrolase enzymes which have been widely used since many decades. These enzymes randomly cleave internal glycosidic linkages in starch molecules to hydrolyze them and yield dextrins and oligosaccharides. This report focuses on the microbial production of α-amylase and its applications. (Sundarram, 2014).

The production of extracellular thermostable amylase by Geobacillus was detected on nutrient agar plates containing 2.0% soluble starch at 65 oC. Bacterium was produced 8,578 U/mL amylase under SmF. The thermostable amylase was retained stability and activity in the presence of various denaturing agents such as SDS، Triton X-100, Tween60, Tween-80. Ca+2, Cu+2 and Co+2 ions increased activity, while Na2EDTA, Hg+2, Zn+2, Sn+2 showed inhibitory effects (Rekadwad, 2015).

α- Amylases are one of the main enzymes used in industry. Such enzymes hydrolyze the starch molecules into polymers composed of glucose units. Amylases have potential application in a wide number of industrial processes such as food, fermentation and pharmaceutical industries. Dominated applications in industrial sectors. The production of α-amylase is essential for conversion of starches into oligosaccharides. The properties of each α-amylase such as thermostability, pH profile, pH stability, and Caindependency are important in the development of fermentation process (Souza and Magalhães, 2010).

α-amylases constitute a class of industrial enzyme having approximately 30% of the world enzyme production (Van der Maarel et al., 2002), and represent one of the three largest groups of industrial enzymes and account for approximately 60% of total enzymes sales in the world (Rao et al., 1998) and is an important enzyme, particularly in the process of starch hydrolysis(Alkando, 2011).

α-Amylases can be produced by fungi in large amount but they are usually not heat stable beyond 40ºC. On the other hand, bacterial species such as Bacillus subtilis, B. megaterium, B. amyloliquefaciens and B. licheniformis produce more heat stable enzymes. Bacterial species, which produce α-amylase enzymes that can withstand a temperature of 70ºC have been reported previously. There is often a need to isolate species of microorganisms that can grow at high temperatures and whose enzymes can function at temperature up to 95-100ºC. The purpose of this manuscript was to review the literature on the microorganism associated by the production of α-amylase on using different substrate, thermostability profile and its industrial application (Hussain et al, 2013).

4-Materials and Methods

4.1. Samples collection:

Water samples were collected from hot springs in Southern region of KSA (Al-Khubah, Al-Majardah,, Al-Ardah) using sterile thermal flasks (Ledbetter et al., 2007). All samples were immediately transport to the laboratory and directly inoculated onto Thermus agar medium (Brock and Freeze 1969).

4.2. Isolation of thermophilic Bacteria:

Two protocols were used for isolation of thermophilic bacteria:

4.2.1 Direct isolation:

The collected water samples (without dilution) will spread on plates of Thermus agar media and incubated at 60 °C for 3 days. (Malkawi and Al-Omari, 2010).

4.2.2 Enrichment isolation:

For enrichment of the amylase producing thermophilic bacteria, 5 gm of sterile starch will added separately to a spring water samples, mixed and incubated for 2 weeks at 55°C. The samples will diluted in sterile distilled water, plated on agar medium and incubated at 55 °C for 1-2 weeks. Two isolation media were used: (1) Thermus medium which has the following composition: Nitrilotriacetic acid (100mg/l), $CaSO_4$ (60 mg/l), $MgSO_4$ (100 mg/l), KNO_3 (103 mg/l), $NaNO_3$ (689 mg/l), Na_2HPO_4 (111mg/l), MnSO (2.2 mg/l), $ZnSO_4$ (0.5 mg/l), $CuSO_4$ (0.016 mg/l), H_3BO_3 (mg/l), Na_2 MoO_4 (0.025 mg/l), $CoCl_2$ (0. 046 mg/l), $FeCl_3$ (0.28 mg/l), 1 gm/l trypton, 1 gm/l yeast extract and agar 20 gm/l. Concentrated H_2SO_4 (0.5ml) was added to dissolve the salts and the pH of the medium was adjusted to 7.0. (2): ATCC medium 697 containing NaCl (0.5 %), yeast extract (0.2 %), beef extract (0.4 %), peptone (0.5 %), and agar (2 %).

4.3. Evaluation of thermophilic isolates for hydrolytic enzymes production

α-amylase producing bacteria among the isolates will screen by plate assay on starch agar plates. Amylolytic activity of the isolates will screen on starch thermus medium plates containing 10.0 gL-1 of starch. After incubation at 55 ºC for 48h, the zone of clearance will be determined by flooding the plates with iodine solution (Mazzucotelli at al., 2013). The potential amylase producers will selected based on ratio of zone of clearance diameter to colony diameter (Kumar et al., 2012). For qualitative screening of enzyme, the zone of hydrolysis was measured and enzyme index was calculated as per formulae. Enzyme index= Zone of hydrolysis in mm / Colony diameter in mm (Perez et al., 2009). The promising cultures showing high potency index were selected for further study.

4.4. Enzyme production:

The inoculum was prepared by growing the bacterial culture, in the nutrient broth medium, at 55 ^{O}C for 24 hours. Erlenmeyer flasks (250 ml) containing 50 ml of production medium were inoculated with 2% inoculum (approximately 2 x 108 CFU/ml) and incubated at 55 ^{O}C in an incubator shaker at 200 rpm for 12 to 96 hours. At regular (12 hour) intervals, the duplicate samples were harvested and the obtained growth was measured as turbidity at 600 nm. Cells were removed by centrifugation (6000 rpm for 20 minutes) and supernatant was used for enzyme assay.

4.5. Enzyme assay: α-amylase was assayed by adding 1 ml of enzyme to 1 ml soluble starch (1%) in acetate buffer pH 5, and incubating at 50 ^{O}C for 15 min. The reaction was stopped by the addition of 2 ml of 3,5-dinitrosalicylic acid reagent (Bernfeld, 1955). The absorbance was measured using a double beam UV/Vis scanning spectrophotometer (Model: Shimadzu, 1601PC) at 550 nm. One enzyme unit (Uml-1) is defined as the amount of enzyme, which releases 1µ mole glucose.

5- Results and discussions

5.1. Isolation of hot spring thermophilic bacteria

A total 64 different thermophilic bacterial isolates were isolated on the basis of colony morphology from three sites; Al-Majardah, Al-Khubah and Al-Ardah using two different media. The results clearly indicate that the highest numbers of hot springs bacteria were isolated from Al-Majardah (24) and Al-Khubah (25) regions while; low numbers of isolates were collected from water samples of Al-Ardah (15). The occurrence of isolates in the different localities may be affected by the prevailing environmental conditions, pollution rate and the chemical properties of water (Medio et al., 2000). Panda et al., (2013) reported that the thermophilic microorganisms are microbes that mostly inhabit hot springs, live and survive in temperatures around 60°C. They less explored due to difficulties in isolation and maintenance of pure culture. Therefore, their diversity and biotechnological potential remains to explored from majority of the thermal habitats. On the other hand, Khalil (2011) reported that three strains of thermophilic bacteria were isolated from two different hot springs in Saudi Arabia. These strains were designated Bacillus sp and Brevibacillus borstelenesis which were isolated from Al-Khoba hot spring and Deinococus geothermals which was isolated from Al-Arida hot spring. Cells are Gram positive-stain, strictly aerobic, grew optimally at pH 7.5 to 8.5 and temperature of 55 to 60°C, and tolerated maximally 10% (w/v) NaCl. A thermophilic bacterium, Geobacillus debilis was isolated from the Tatta Pani hot spring in Azad Kashmir-Pakistan. It grew within pH range of 5.5 to 8.5 with optimum growth at pH 7.0 and showed optimum growth at 65ºC. It produced significant amount of industrially important enzymes i.e. α-amylase, CMCase, Protease and Lipase (Zahoor et al., 2016).

5.2. Screening of isolates for hydrolytic enzymes production

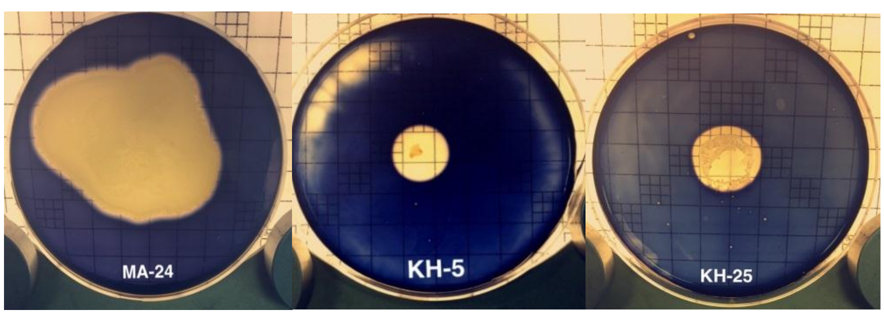
All different isolates were screened for their ability to produce α-amylae using iodine test. The clearance zone forming ability on starch agar plates was used for the primary selection of amylolytic isolates. Screening and measurement of zone of clearance by bacterial isolates are shown in Table (1) and Photo (1). Among the 64 isolates, 17 isolates hydrolyzed the starch on starch agar. The highest amylase activity was produced by isolates; MA-19 (2.28 mm) and MA-24 (3.2 mm) from Al-Majardah and KH-5 (2.75 mm) and KH-25 (3.6 mm) from Al-Khubah regions.The findings of Khalil, (2011) supported the present study; they reported that thirteen strains of the thermophilic bacteria were successful isolated two hot springs in Saudi Arabia. All isolates were lipase positive, 11 isolates showed different amylase activity, while only 3 isolates showed different cellulase activity. On the other hand, Ghati et al., (2013) reported that three thermophilic bacteria were successful isolated from the Bakreshwar hot spring, West Bengal, India and designated as Acinetobacter lwofii strain 412; Bacillus cereus subsp. and Geobacillus stearothermophilus strain G1017-C12. Among the three bacteria, Geobacillus sp. showed maximum lipase production with comparison to other isolates. Many heterotrophic bacteria are known to carry genetic and metabolic potentials to synthesize and control extracellular enzymes, which can degrade and modify a large variety of natural polymers in water basins (Mudryk and Skorczewski, 2004). According to Boetius (1995), production and activity of bacterial hydrolytic enzymes depends on the availability, distribution and concentration of organic substrates. For this reason, according to many investigators, enzyme assays can provide powerful tools for studying organic matter degradation and nutrient cycling in aquatic ecosystems (Mudryk and Skorczewski 2004). On the other hand, Sen et al., (2014) isolates, characterizes, and demonstrates the novel heat-adapted amylase-producing bacteria *Bacillus barbaricus*, *Aeromonas veroni*, and *Stenotrophomonas maltophilia* from Taptapani hot spring, indicating its potentiality and stability under acidic conditions. Sena et al., (2016) study the isolation and application of novel Hot spring bacterial thermostable α-amylase from a newly hot spring isolate, Exiguobacterium sp. In addition, Geobacillus kaustrophilus PW11, Geobacillus thermoleovorans PW13 and Geobacillus toebii PS4 were isolated form Tattapani hotspring of Himachal Pradesh, India and characterized for extracellular amylase activity (Sharma et al., 2015).

Table 1: Screening of hot springs bacterial isolates for α-amylase production

Isolates NO.	Potent α-amylase produced		
	Colony diameter (mm)	Zone of Clearance (mm)	Unitage (AU)
MA-4	8	15	1.87
MA-19	7	16	2.28
MA-24	5	16	3.2
KH-4	5	10	2
KH-5	4	11	2.75

KH-9	8	10	1.25
KH-11	8	10	1.25
KH-15	6	7	1.66
KH-16	6	8	1.33
KH-24	7	12	1.71
KH-25	5	18	3.6
AR-1	8	10	1.25
AR-2	4	8	2
AR-3	4	10	2.5
AR-9	8	14	1.75
AR-10	7	14	2
AR-15	6	8	1.33

Al-Khubah (KH), Al-Majardah) (MA), Al-Ardah (AR)



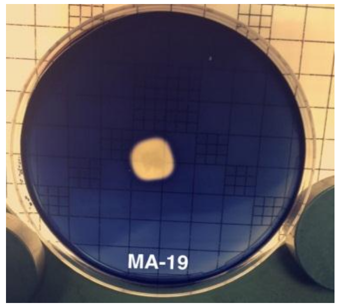


Photo 1: Screening of hot spring thermophilic isolates for amylase production by starch hydrolysis.

5.3. Production of α-amylase using starch broth medium:

The production of α-amylase in starch medium reached maximum activity of 16.01 U/ml at 36 h (Fig.1). The enzyme was secreted early in the active growth phase and reached maximum toward the end of the exponential growth phase, then, the enzyme activity was reduced. It might be due to denaturation and/or decomposition of α-amylase as a result of interaction with other compounds in the fermented medium (Krishna and Chandrasekaran, 1996). These results were in accordance with Sen et al.,(2014) and Fooladi and Saijadian (2014).

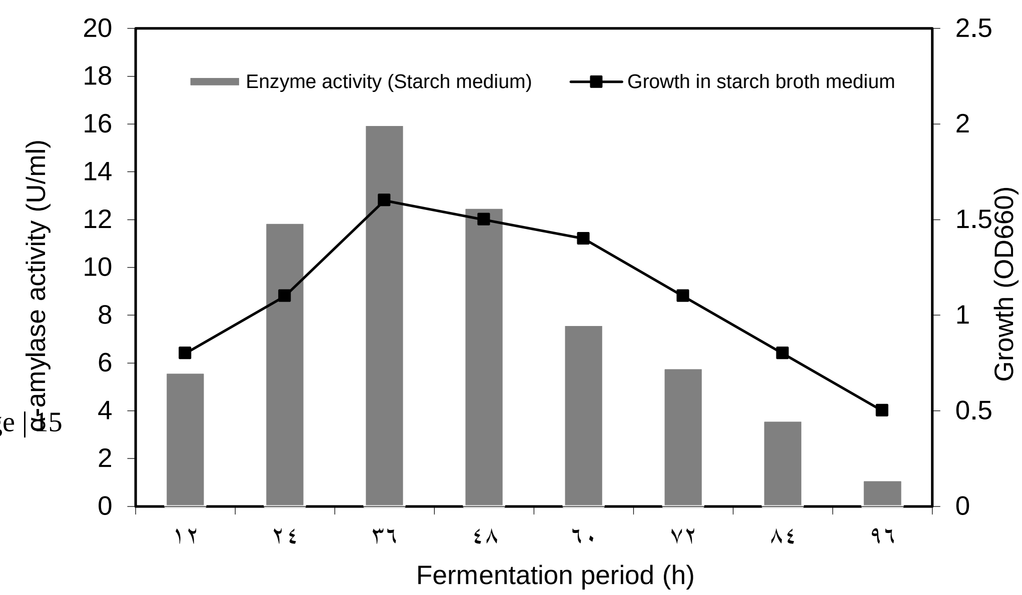


Figure 1: α- amylase production using starch medium

Conclusions:

1- Hot springs in southern region of KSA have been recognized as an important and untapped resource for isolation of thermophilic bacteria produced α-amylase.

2- A 64 bacterial isolates were obtained from three sites; Al-Majardah, Al-Khubah and Al-Ardah.

3- A total of ١٧ bacterial isolates were able to produce the enzyme. Only four isolates were produced high clearance zone around the colonies.

4- The highest amylase activity was produced by isolates; MA-19, MA-24 from Al-Majardah and KH-5 and KH-25 from Al-Khubah regions.

5- Maximum α-amylase production by promising strain KH-25 was achieved at 36 h in the end of the exponential growth phase.

6- Finally, it was concluded that the hot springs are the promising source for isolation of thermophilic bacteria producing α-amylase with industrial importance.

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