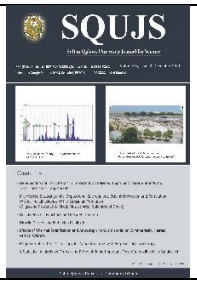
DOI:10.53539/squjs.vol29iss1pp28-43

Sultan Qaboos University Journal for Science

Journal page: www.squ.edu.om/index.php/squjs/index



Production and Optimization of Extracellular α-amylase From Halophilic Bacteria *Cytobacillus oceanisediminis* Isolated From Wadi-El-Natrun, Egypt

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ARTICLE HISTORY

Received 24 June 2013
Received revised 16 August 2023
Accepted 4 September 2023

ABSTRACT

The current study aimed to maximize α-amylase enzyme production by halophilic bacteria *Cytobacillus oceanisediminis* isolated from soil of Wadi-El-Natrun, Egypt. The results showed that of 21 halophilic bacterial isolates recovered from saline soil samples, eight isolates were high amylase producers. Interestingly, the bacterial isolate AHB6 exhibited the highest extracellular amylase production and was selected for further studies. This isolate was molecularly identified based on the 16srRNA gene sequencing as *Cytobacillus oceanisediminis* and has the accession number OM759999**.** The optimum temperature for α-amylase enzyme was 45 °C and exhibited thermostability at 45 °C for 1 h and retained more than 80% of its original activity. The α-amylase enzyme was active in almost all tested pHs and reached its highest activity at pH 9. Also, the optimum culture conditions for α-amylase enzyme production were wheat bran as carbon source, 2 g/l K_2HPO_4 as potassium source, 6 g/l $MgSO_4$ as magnesium source and 0.5 g/l KNO_3 as nitrogen source. These findings suggest the applicability of bacterial isolate *Cytobacillus oceanisediminis* as a potent producer of α-amylase for industrial purposes.

Keywords: Amylase; Optimization; *Cytobacillus oceanisediminis;* Starch hydrolysis; Thermo-halophilic bacteria.

إنتاج وضبط العوامل المزرعية المؤثرة على إنزيم ألفا أميليز المنتج خارجيا بواسطة سايتوباسيلس أو شينسدمينيز والمعزولة من وادي النظرون ، مصر

ندا عادل هاشم[1]، نعيمة محمد همام يوسف[2]، رشا محمود الشاذلي[1]

الملخص: هدفت الدراسة الحالية إلى تحسين الظروف المزرعية لإنتاج انزيم ألفا أميليز بواسطة سايتوباسيلس أوشينسدمينيز المحبة للملوحة والمعزولة من وادي النظرون،مصر. أظهرت النتائج انه من بين 21 عزلة بكتيرية تم الحصول عليها من عينات التربة المالحة، تم أختيار 8 عزلات الأكثر انتاجية للانزيم. ومن المثير للأهتمام اختيار العزلة AHB6كأفضل عزلة منتجة لأنزيم الاميليز من بين ال8 عزلات الاعلي انتاجا للانزيم وقد أستخدمت في أجراء تجارب تكميلية. وقد تم تعريف هذه العزلة على أساس الخصائص الوراثية على انها سايتوباسيلس أوشينسدمينيز وتم تسجيلها في قاعدة بيانات بنك الجينات برقم OM759999. تم الوصول للظروف المثلي لانتاج انزيم الاميليز عند درجة حرارة 45 درجة مئوية والتي ظل محتفظا عندها بحوالي 80% من نشاطة لمدة ساعة. لوحظ نشاط جيد لانزيم الاميليز عند مدي واسع من الرقم الهيدروجيني وتم تسجيل أعلي نشاط عند الرقم الهيدروجيني 9. وقد أتضح أيضا ان نخالة القمح كانت افضل وسط كربوني، 2جرام /لترفوسفات البوتاسيوم افضل تركيز كمصدر لعنصر البوتاسيوم، 6جرام/لتر كبريتات ماغنيسيوم أفضل تركيز كمصدر لعنصر الماغنيسيوم، و 0.5جرام/لتر نترات البوتاسيوم كأفضل تركيز كمصدر لعنصر النتروجين. وتشير هذه النتائج إلى إمكانية استخدام العزلة البكتيرية سايتوباسيلس أوشينسدمينيز كعزلة قوية لإنتاج انزيم ألفا أميليز للأغراض الصناعية.

الكلمات المفتاحية: انزيم الأميليز، ضبط الظروف المزرعية، سايتوباسيلس أوشينسدمينيز، التحلل المائي للنشا، البكتيريا المحبة للملوحة والحرارة.

1. Introduction

High salinity represents an extreme environment in which relatively few organisms have been able to adapt and survive. Hypersaline environments are those with salt concentrations 9–10 times higher (30–35% of NaCl) than sea water (3.5% of NaCl). These sites are widely distributed around the world and can harbor microorganisms from three different life domains (archaea, bacteria, and eukaryota); together, these microorganisms are known as halophiles, which survive or even thrive in saline environments [1].

Archaea and bacteria are the most widely distributed organisms in hypersaline environments, [2,3], especially in those in which salinities exceed 1.5 M (about 10%). In recent years, halophilic organisms are mainly isolated from saline environments, such as salt lakes, marine solar salterns, saline soils, and marine sediments [4].

Halophilic enzymes are extremozymes produced by halophilic microorganisms; they have similar characteristics to regular enzymes but different properties, mainly structural. Among these properties is a high requirement of salt for biological functions.

In recent years, different studies have focused on the detection of halophiles in saline environments in order to isolate and characterize new enzymatic activities. This resulted in several halophile hydrolases being described, including amylases, lipases, and proteases.

Nowadays, investigation on the production of extremozymes from different bacterial genera of halophilic bacteria has intensified. This interest is due to their capacity to efficiently catalyse a process and show optimal activities at different salt concentrations.

Most of the evaluation studies on the enzymatic capacities of halophiles begin with the isolation of these microorganisms from environments considered extreme due to specific characteristics such as high salt concentrations, high pH values, and extreme temperature conditions.

Amylases enzymes hydrolyze the starch molecules into polymers composed of glucose units. α-Amylases (E.C.3.2.1.1) are enzymes that catalyses the hydrolysis of internal α-1,4-glycosidic linkages in starch in low molecular weight products, such glucose, maltose and maltotriose units [5]. Three categories of amylases, denoted alpha, beta, and gamma, differ in the way they attack the bonds of the starch molecules.

Amylases have potential application potential in a wide number of industrial processes such as food, fermentation and pharmaceutical industries. α-Amylases can be obtained from plants, animals and microorganisms. However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors such as food, textile, paper and detergent industries [6].

α-amylase production in the fermentation medium is reported to be growth associated. Therefore, optimization of the culture conditions, such as the physical and chemical parameters, is important due to their impact on the bacterial growth and enzyme production [7]. The most important factors are the fermentation medium constituents, carbon source, nitrogen source, pH of the medium, and incubation [8].

The main aim of this work was to optimize the medium components and cultural conditions for extracellular amylase production by the halophilic *Cytobacillus oceanisediminis* isolate (AHB6) from Wadi-El-Natrun, Egypt. This optimization plays a significant role in enhancing amylase production by various microorganisms.

2. Materials and methods

2.1. Collection of samples

Eight saline soil samples were collected from different locations of Wadi El-Natrun lakes, Egypt. that kindly provided from Assiut University Moubasher Mycological Center (AUMMC). The physiochemical properties of these collected samples such as pH, electrical conductivity and total soluble salts were determined.

2.2. Determination of pH of soil samples

To determine the pH in mud samples, sample extracts were prepared by shaking a known weight of the sample in a known volume of distilled water in a ratio 1:10 (w/v) for about 30 min and the mixture was left overnight to settle. The electrode of the pH meter (Model pH -206 Lutron) was immersed directly in the mud suspension [9].

2.3. Determination of electrical conductivity (EC) and total soluble salts (TSS%) of mud samples

Total soluble salts (TSS%): A linear relationship exists between the electrical conductivity (EC) in soil samples and the concentration of soluble salts. The specific electrical conductance in samples were measured by means of conductivity meter (model 4310 JENWAY). The total soluble salts were then calculated as described by Jackson [9] according to following equation: TSS= 0.94 × EC mS/cm.

2.4. Determination of Sodium and Potassium of soil samples

Sodium and potassium ions were determined by the flame emission technique Carl-Zeiss DR LANGE M 7 D flame photometer was used and determined according to [10].

2.5. Bacterial counts

Five grams of each soil sample were suspended in 45 ml sterilized saline solution (0.8 % NaCl). Appropriate dilutions of the samples were prepared, and then plated on nutrient agar containing 5% NaCl Medium. Three replicate plates for each sample were used. After incubation for 2-4 days at 30 °C, the developing bacterial colonies were counted, isolated & indicated.

The colony forming unites (CFU) was calculated by using the following equation:

$$\text{CFU/ml} = \frac{\text{A colonies (average)}}{\text{B volume of plates (ml)}} \text{ x dilution factor (DF)}$$

3. Isolation of amylolytic-halophilic bacteria

Starch degrading bacteria were isolated from soil samples using serial dilution. All samples were cultured in a saline DSM broth medium containing 10% NaCl. All

media were sterilized by autoclaving at 121°C for 20 min and the pH was adjusted before sterilization. The following culture media (g/L) were employed throughout the work:

3.1. DSM 371 medium specific for isolation of haloalkaliphilies

The DSM 371 modified medium with composition (g/L): peptone 10, yeast extract 5,NaCL 200, $MgSO_4$ 10,KCl 2, $Na_3C_6H_5O_7$ 2, Mn 0.001 , Fe 0.001; yeast extract, 5.0; casamino acids, 1; agar, if necessary, 20. The medium pH was adjusted to 8 before autoclaving. Agar was heated and dissolved before the addition of sodium chloride.

3.2. Modified nutrient agar medium

This medium contained the following: Peptone, 10; yeast extract, 5; NaCl, 100.0; $MgSO_4$, 10; and agar, **20 (pH 8 ±0.2)**. The constituents were suspended in 1000 ml distilled water. Heated, if necessary to dissolve the constituents completely, then dispensed as desired and sterilized by autoclaving at 121ºC for 15 minutes. This medium was used for isolation and propagation of the bacterial isolates [11].

3.3. Preparation of nutrient agar media

As in above medium but with the addition of 15 g agar. This medium was used for culturing and propagation of bacteria. Nutrient agar is a general purpose medium supporting growth of a wide range of non-fastidious organisms. It typically contains (mass/volume):1% peptone, 0.5% yeast extract, 1.5% agar, 0.5% sodium chloride and 1 liter distilled water, pH adjusted to 8 at 25°C. Modified media for halophilic bacteria uses 10% NaCl instead of 0.5 % [11].

These ingredients are combined and boiled for approximately one minute to ensure they are mixed and then sterilized by autoclaving, typically at 121°C for 20 minutes. Then they are cooled to around 50°C and poured into sterilized Petri dishes which are covered immediately. Once the dishes hold solidified agar, they are stored upside down and are often refrigerated until used. Inoculation takes place on warm dishes rather than cool ones: if refrigerated for storage, the dishes must be rewarmed to room temperature prior to inoculation. Inoculate bacterial suspension on sterilized Petri dishes which contain nutrient agar media.

3.4. Medium for production of alkaline amylase

The alkaline amylase production medium contained the following: starch, 10; K_2HPO_4, 1; $MgSO_4$, 0.2; KNO_3, 0.5; $CaCl_2$ 1; $FeCl_3$ 1000µ and NaCl, 40. Medium constituents were autoclaved separately and mixed after cooling [12], with few modifications.

3.5. Preparation of soil extract

Weight 1g of soil for each of 8 soil samples were added to test tubes which contain 10 ml of sterilized distilled water and then put on shaker for 30 minute, then use the supernatant part, take 1 ml of this part and add them to sterilized test tube (which contain 10 ml of sterilized nutrient broth media with 5% NaCl) and another 1 ml of supernatant and add to test tube with same media but with 10% NaCl.

3.6. Isolation, preservation and maintenance of bacterial isolates

Bacterial isolates were isolated from the soil samples collected from Wadi El-Natrun, Egypt. Soil extracts were directly inoculated into flasks containing liquid DSM 371 medium for culture enrichment. This was followed by plating aliquot of 1 mL of enriched liquid medium of samples on DSM 371 and modified nutrient agar media. Bacterial isolates were purified by streak plate technique on DSM 371 solid medium to obtain pure colonies. Isolates were preserved by freezing at -8°C in DSM 371 broth medium containing 10% glycerol [13]. Bacterial isolates were maintained, for up to two weeks, by continuous sub-culturing on DSM 371 medium and keeping in a refrigerator.

3.7. Test of starch degradation by isolated amylolytic-halophilic bacteria

After obtaining halophilic bacteria in pure form, the following media (Minimal media) was used to test starch degradation by amylase enzymes produced from halophilic bacteria. The medium was supplemented with 10g/L of soluble starch as a sole carbon source. The pH of culture media was adjusted to 8.5 then autoclave at 121ºC for 20 min.

Cultures were incubated at 37°C in an orbital shaker, at 140 rpm for 3days. The bacterial growth showing turbidity was purified for isolation of single bacterial colony.

4. Amylolytic-halophilic bacteria

4.1. Screening of enzyme production

All isolates were curried using the starch plate [14]. Bacteria were inoculated on starch plates to test for α-amylase secretion, incubated at 37 °C for 3 days, and stained with an iodine solution (0.5%). Amylase-positive stains were determined by the presence of a clear zone of starch hydrolysis around the colony on the starch plates, while presence of blue color around the growth indicated negative result [15]. The bacterial isolates which produced a clear zone of hydrolysis in starch agar were selected as α-amylase producers for subsequent investigation. Selected colonies were maintained on nutrient agar slants at 4 °C and sub-cultured monthly.

5. Molecular identification of the amylolytic bacterial isolate

The bacterial isolates that showed the highest amylase enzyme production was selected for molecular identification by partial sequencing of the 16s rRNA gene. The PCR product was sequenced in both directions using 27F and 1500R primers. A PCR product for sequencing was conducted by using the following two primers, 27F (5'-GAG TTT GAT CCT GGC TCA G-3', positions 9-27 on 16S rDNA) and 1500R (5'-GTT ACC TTG TTA CGA CTT-3', position 1509-1492 on 16S rDNA). The obtained 16S rRNA gene sequences were aligned with known 16S rDNA

sequences in the GenBank database using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information. The sequence of the strain has been deposited in the GenBank nucleotide sequence database.

6. Detection of extracellular enzymes produced by the bacterial isolates

Qualitative detection of enzymes was carried out by determination of protein concentration in the cell free supernatant described by [16].

7. Optimizing of enzyme production

7.1. Quantitative assay of amylase production

The isolates were assayed for starch-degrading potentials in a modified basal salt medium. The bacterial isolates subjected to amylase production in 250 mL Erlenmeyer flasks using basal medium with selected carbon source for 48h of fermentation period at 35°C with agitation speed of 140rpm. Bacterial growth was monitored by turbidity at OD_{600}. The quantitative assay method was used to determine the amylase activity of the selected bacterial isolate in liquid medium. The amylase activity of each culture was measured by determining the amount of reducing sugars by using a dinitro salicylic acid method [17]. A bacterial isolate with the highest activity was selected for optimization of amylase production.

7.2. Optimization of culture conditions for amylase production

The optimization factors for maximum enzyme production were investigated. Inocula were prepared from the bacterial grown on starch medium at 37°C, for 3 days.

7.3. Shaking and static condition

The effect of shaking and static conditions on the amylase production was assessed. The bacterial culture was allowed to grow under shaking at 100 rpm or in static condition.

7.4. Salt concentrations

The effect of salt concentrations on the amylase production was assessed. The bacterial strain was allowed to grow in media at different NaCl concentrations ranging from (1% to 10%). Definite amount of salt (1 to 10 g of NaCl) was added in 100 mL of minimal media in 250mL Erlenmeyer flask, then sterilized for 30 min at 121ºC.After that 20 mL of bacterial suspension were inoculated at 35ºC for 3-5 days under shaking condition. Cultures were centrifuged at 5000 rpm for 10 min. amylase activity was assayed in the clear supernatants [18]. Three replicates were prepared for each treatment.

7.5. Carbon sources

The effect of various carbon sources such as maltose, soluble starch, starch, potatoes and wheat bran was examined in the amylase production medium. One gram of soluble starch was dissolved in 100 mL of minimal media in 250 ml Erlenmeyer flasks, then sterilized in an autoclave for 20 min at 121ºC. After that 20 mL of bacterial suspension were inoculated at 35ºC for 3 days under shaking condition. Cultures were filtered and then centrifuged at 5000 rpm for 10 min (three replicates were prepared for each treatment). Amylase production was assayed in the clear supernatants) [18].

7.6. Temperatures

Selected bacterial strains were inoculated in amylase production broth medium at pH 8 overnight. Then, the inoculated broth medium was incubated at different temperatures (25, 30, 35, 40 and 45ºC) for 48 h. At the end of incubation period, the cell-free culture filtrate was obtained and used to determine the amylase production.

7.7. pH values

To determine the optimum pH value for enzyme production, the isolate was grown on basal broth medium containing 20 g per litre soluble starch as a sole carbon source at different pH values (5, 6, 7, 8, 9, 10 and 11 pH) and enzyme production was tested after 3 days of incubation. The initial pH was adjusted by 0.1 HCl or 0.1 NaOH.

7.8. Substrate concentrations

Different concentrations of starch (5, 10,15, 20,25 and 30 g per litre) were incorporated into basal salt broth medium in 100 mL flasks. Media were inoculated with the bacterial isolate under investigation at 30ºC for 48 h after that, enzyme production was tested.

7.9. Calcium Chloride

The effect of $CaCl_2$ concentrations on the amylase production was assessed. The bacterial strain was allowed to grow in media at different $CaCl_2$ concentrations ranging from (0.01% to 0.35%).

7.10. Magnesium sulphate

The effect of $MgSO_4$ concentrations on the amylase production was assessed. The bacterial strain was allowed to grow in media at different $MgSO_4$ concentrations ranging from (0.02% to 0.8%).

7.11. Potassium phosphate

The effect of K_2HPO_4 concentrations on the amylase production was assessed. The bacterial strain was allowed to grow in media at different K_2HPO_4 concentrations ranging from (0.1% to 0.8%).

7.12. Potassium Nitrate

The effect of KNO_3 concentrations on the amylase production was assessed. The bacterial strain was allowed to grow in media at different KNO_3 concentrations ranging from (0.01% to 0.25%).

Specific amounts of ($CaCl_2$, $MgSO_4$, K_2HPO_4 and KNO_3) were added in 100 mL of minimal media in 250 mL Erlenmeyer flask, then sterilized for 30 min at 121ºC.After that 20 mL of bacterial suspension were inoculated at 35ºC for 3-5 days under shaking condition. Cultures were

centrifuged at 5000 rpm for 10 min. amylase activity was assayed in the clear supernatants [18]. Three replicates were prepared for each treatment.

8. Statistical analysis

Analysis of variance (ANOVA) was performed on the data with three replicates of six measurements from two independent experiments. Analysis of variance (ANOVA) was performed using the SPSS statistical 11.0 package. For comparison of the means, a post-hoc test (Duncan‟s multiple range tests) ($p < 0.05$) were used for significant differences. Visualization of corrplot, packages, integrated into the R software using RStudio.

Results

1. Physiochemical and biological properties of collected samples

The collected samples were represented by eight samples from Wadi-El-Natrun. The physiochemical properties of these collected samples such as pH, electrical conductivity (EC) and total soluble salts (TSS) are summarized in table 1.

1.1. Collective data of soil samples of Wadi-El-Natrun

The **pH** of soil samples were alkaline and gave the highest value (10.7) in sample 7 lowest was 8.6 in sample 5. Total Soluble Salt (TSS%) of the soil of sample 6 registered the highest TSS value (39.29 %) while lowest (6.90%) was registered in sample 4.

Sodium cations (Na^+mg/g dry wt.) content was highest in mud samples giving with a maximum of 595 mg/g dry wt in sample 6 while the lowest was 356 mg/g dry wt was recorded in sample 4. For potassium cations (K^+mg g^{-1}dry wt.) the highest was 24.3 mg/g dry wt in sample 3 and lowest 3.87 mg/g dry wt in sample 7.

2. Isolation and screening of amylase production

Amylolytic halophilic bacteria were enriched and isolated by inoculating the diluted soil samples in basal broth medium containing 1% soluble starch as a sole carbon source. The results showed that the medium turned cloudy indicating bacterial growth. The bacterial isolate was tested to detect its capacity for amylase degradation. The results revealed that eight bacterial isolates produced 1-16 mm of clear zone. The obtained results showed also that clear zone and HC value ranged between 0.1to 1.6 (Figure 1). The results in Figure 2 revealed that the bacterial isolates AHB6 exhibited the highest extracellular amylase compared to other isolates.

3. Amylase production by different bacterial isolates

Eight halophilic bacteria were tested for amylase production. The result revealed that the bacterial isolate 6 showed high production of amylase.

Table 1. Physiochemical and biological properties of soil samples.

Soil sample	Na^+ mg g^{-1}	K^+ mg g^{-1}	PH	EC (mS/cm)	TSS%	NO. of bacterial species	acterial counts $\times 10^3$
1	522	20.1	10.5	18.79	17.66	1	540
2	455	8.31	10.25	19.58	18.40	1	420
3	500	24.3	10.55	17.41	16.36	1	324
4	356	12.7	8.73	7.35	6.90	1	234
5	411	6.62	8.64	7.47	7.02	1	280
6	595	12.1	10.15	41.8	39.29	1	200
7	527	3.87	10.70	16.68	15.67	1	320
8	467	9.60	10.56	15.63	14.69	1	264

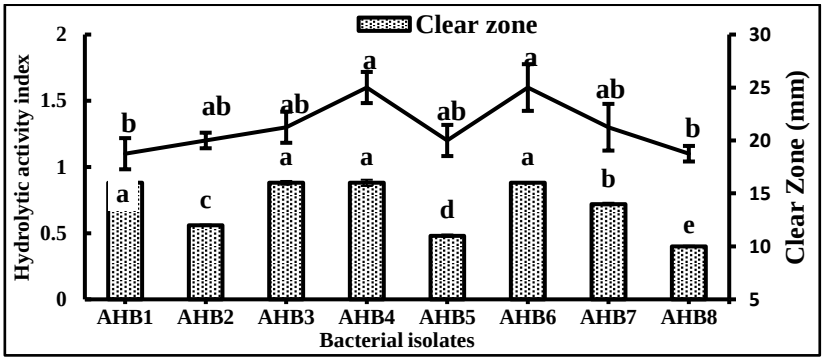

Figure1. Hydrolytic activity and clear zone of the bacterial isolates on starch agar plates.

4. Molecular identification of the selected bacterial isolate

The selected bacterial strains with the ability to degrade amylase were subjected to molecular identification by partial sequencing of the 16s rRNA gene and the result demonstrate 99% homology *Cytobacillus oceanisediminis*. Furthermore, its nucleotide sequence was deposited

in the GenBank in NCBI under the accession number OM759999. To confirm the position of the isolate AHB6 in the phylogeny, numbers of sequences of other bacterial species were chosen from Genebank database for establishment of the phylogenetic tree. The tree showed that AHB and *Cytobacillus oceanisediminis* (OL875278.1) shared a one cladE cluster (Figure 3). Therefore, the strain AHB6 was identified as *Cytobacillus oceanisediminis.*

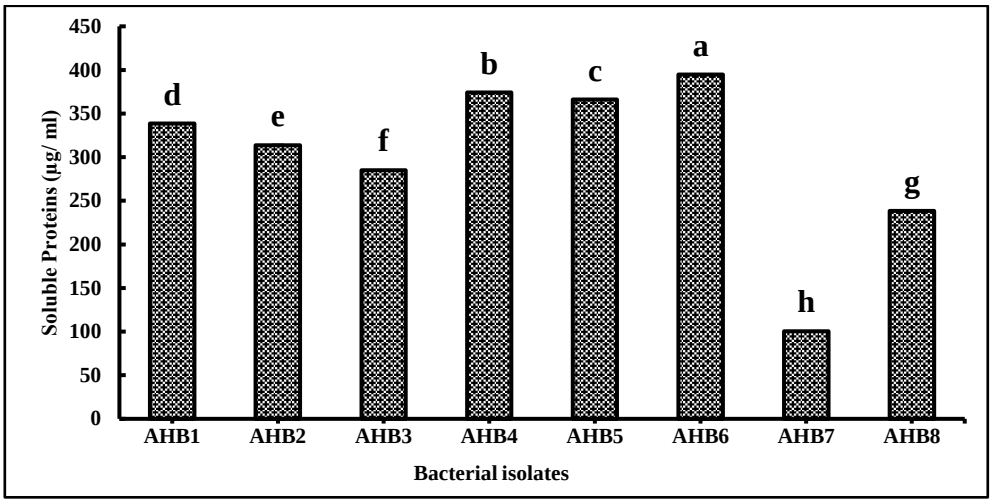

Figure 2. Amylase production by different bacterial isolates.

5. Optimization of amylase production by selected bacterial isolate

The optimum parameters of starch degrading enzyme by bacterial strain by using starch as a sole carbon source were investigated. The bacterial isolate (AHB6) was grown under different conditions; temperature, pH, salt concentration, substrate concentration, shaking and static condition and inocula concentration. For all these parameters protein content were determined.

5.1 Effect of shaking and static conditions

The effect of shaking on amylase production of AHB6 is presented in (Figure 4). The obtained results revealed that incubation of AHB6 under shaking conditions gave more amylase production than static incubation.

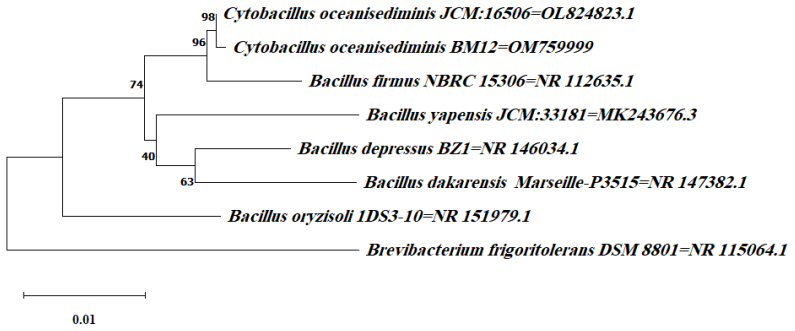

Figure 3. Phylogenetic relationship between the strain AHB6 and other 16S rDNA sequences of published strains belonging to *Cytobacillus oceanisediminis*. In the phylogenetic tree, AHB6 and *Cytobacillus oceanisediminis* were clustered together as one clade.

5.2 Effect of salt concentrations

The selected bacterial isolate (AHB6) was allowed to grow at different salt concentrations ranging from 2% to 10% (Figure5). Amylase production by *Cytobacillus oceanisediminis* was dependent on the NaCl concentration. The bacterium was able to grow up to 10% of salt concentration with maximum growth at 4%. Bacterial growth was observed even at 2% NaCl, showing that non dependency on salt for its moderate growth, thus indicating the halotolerant nature of the bacterium.

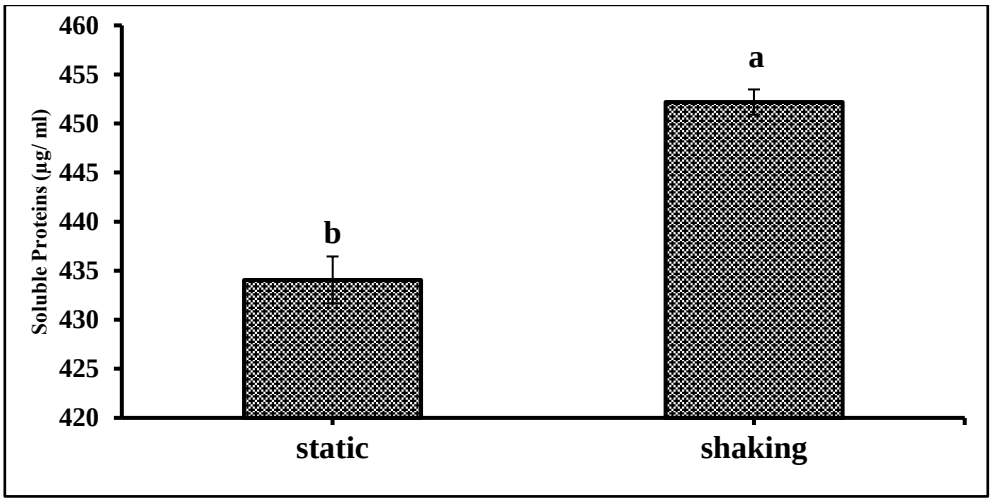

Figure 4. Effect of shaking and static conditions on amylase production.

5.3. Effect of different carbon sources

Ten gram/L from each of maltose, soluble starch and starch powder were separately inoculated with bacterial isolates and incubated at 30°C for 7 days. The results showed the high bacterial growth and amylase production in wheat bran substrate followed by potatoe (Figure 6).

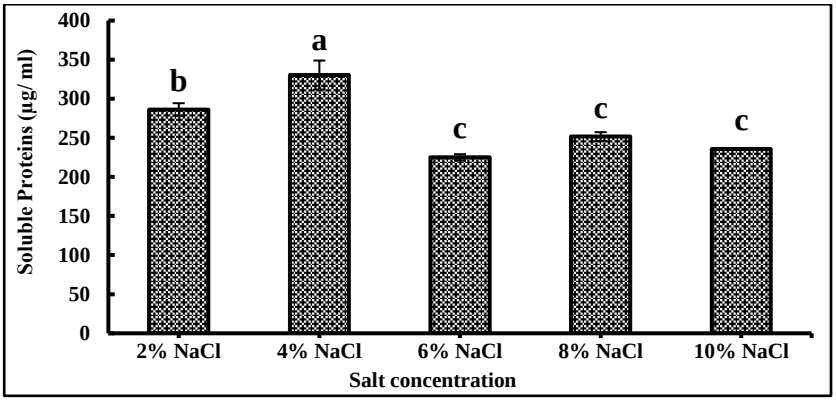

Figure 5. Effect of salt concentrations on amylase production.

5.4. Effect of temperature

The incubation of bacterial isolate (AHB6) at different temperatures showed that the optimum temperature for maximum bacterial growth and maximum enzyme production was achieved at 45°C (Figure 7).

5.5. Effect of pH values

The results showed that the bacterial isolate (AHB6) could grow and produce extracellular amylase in a wide range of pH (5.0 -11.0). The results showed that pH 9 was the optimum pH for the maximum yield of amylase production (Figure 8).

5.6. Effect of substrate concentrations

Soluble starch was used as a carbon source by the bacterial isolate (AHB6). It was prepared in serial concentration (5, 10, 15, 20, 25, 30g/L) at pH8. It was found that the maximum product of amylase was obtained at concentration 25g starch per litter (2.5%) (Figure 9). For following experiments, 25g/L was used.

5.7. Effect of $CaCl_2$ concentrations

$CaCl_2$ was prepared in serial concentration (0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5g/L) at pH8. It was found that the maximum product of amylase was obtained at concentration 2.5g calcium chloride per litter (Figure 10).

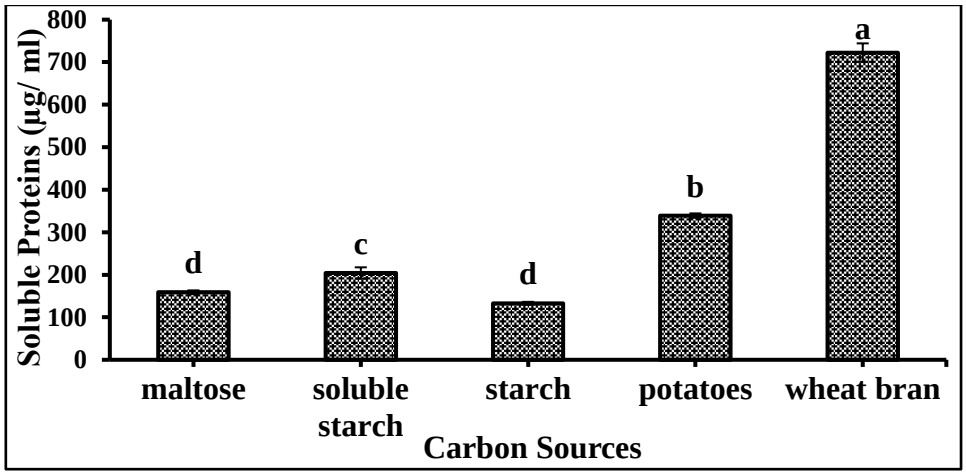

Figure 6. Effect of different carbon sources on amylase production.

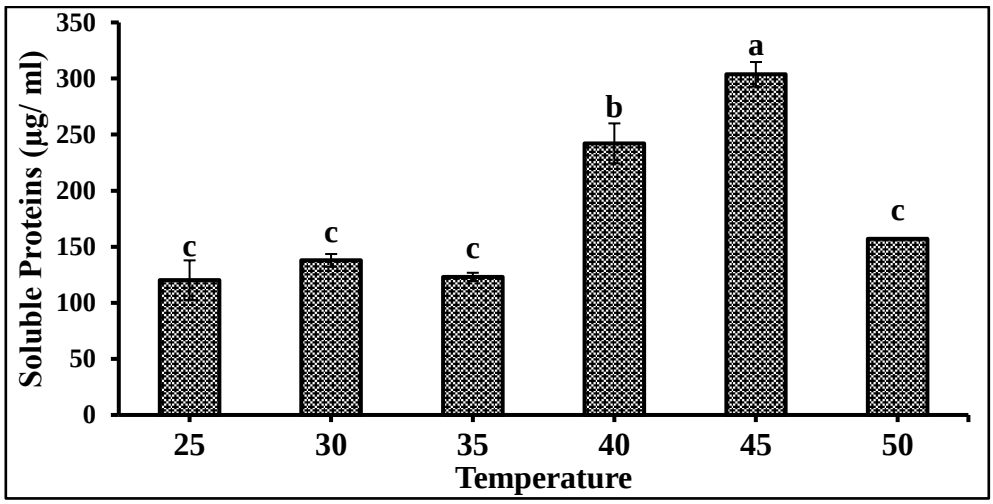

Figure 7. Effect of temperature on amylase production.

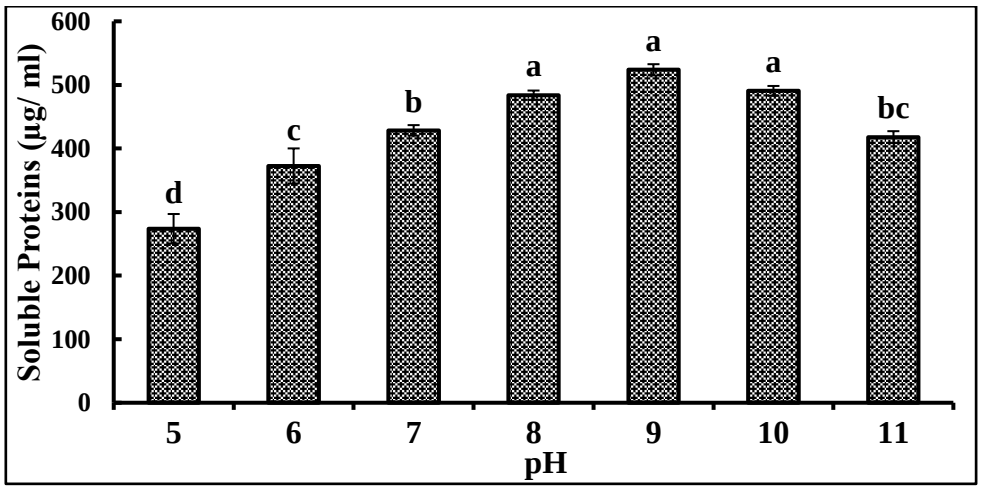

Figure 8. Effect of pH conditions on bacterial amylase production.

5.8. Effect of Potassium concentrations

K_2HPO_4 was prepared in serial concentration (1, 2, 4, 6, 8 g/L) at pH 8. It was found that the maximum product of amylase was obtained at concentration 2 g potassium per litter (Figure 11).

5.9. Effect of magnesium concentrations

$MgSO_4$ was prepared in serial concentration (0.2, 1, 2, 4, 6, 8g/L) at pH 8. It was found that the maximum production of amylase was obtained at concentration 6g $MgSO_4$ per liter (Figure 12).

5.10. Effect of nitrate concentrations

KNO_3 was prepared in serial concentration (0.1, 0.5, 1, 15, 20, 25g/L) at pH 8. It was found that the maximum product of amylase was obtained at concentration 0.5g nitrate per litre (Figure 13).

6. Correlation analysis

A visual plot of correlation analysis was used to find positive and negative correlations visually among multiple parameters under different treatments (Figure 14). Strong negative correlations were observed between potassium and nitrogen source. On the other hand, a strong positive correlation could be noticed between carbon source and magnesium concentrations. A positive correlation was seen between pH and temperature, calcium and potassium sources.

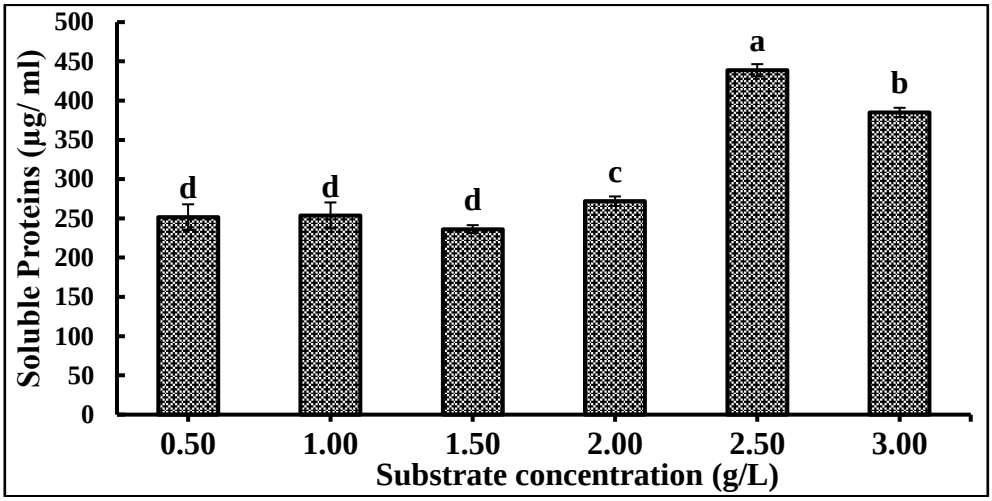

Figure 9. Effect of substrate concentrations amylase production.

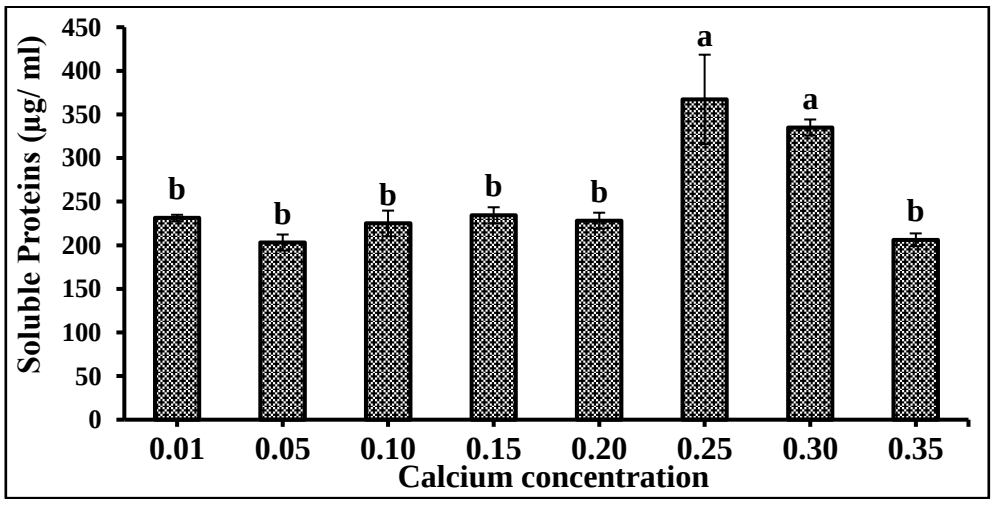

Figure10. Effect of $CaCl_2$ concentrations on amylase production.

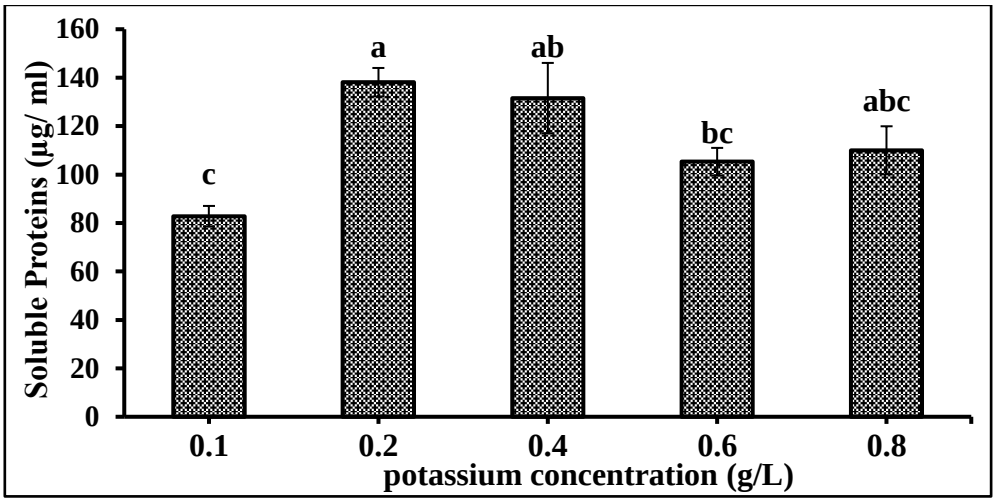

Figure 11. Effect of potassium concentrations on amylase production.

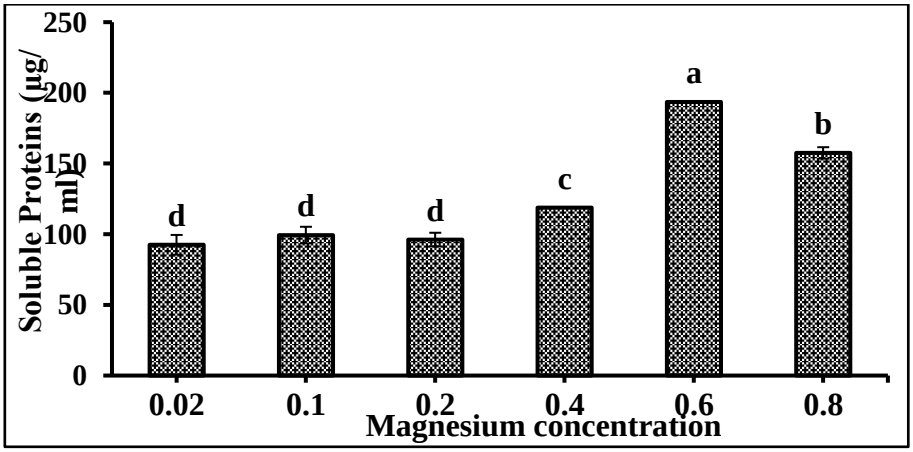

Figure 12. Effect of magnesium concentrations on amylase production.

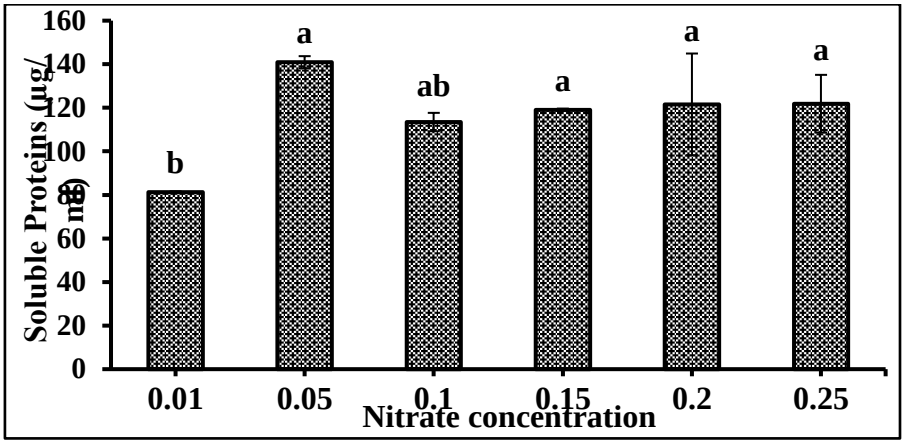

Figure 13. Effect of nitrate concentrations on amylase production.

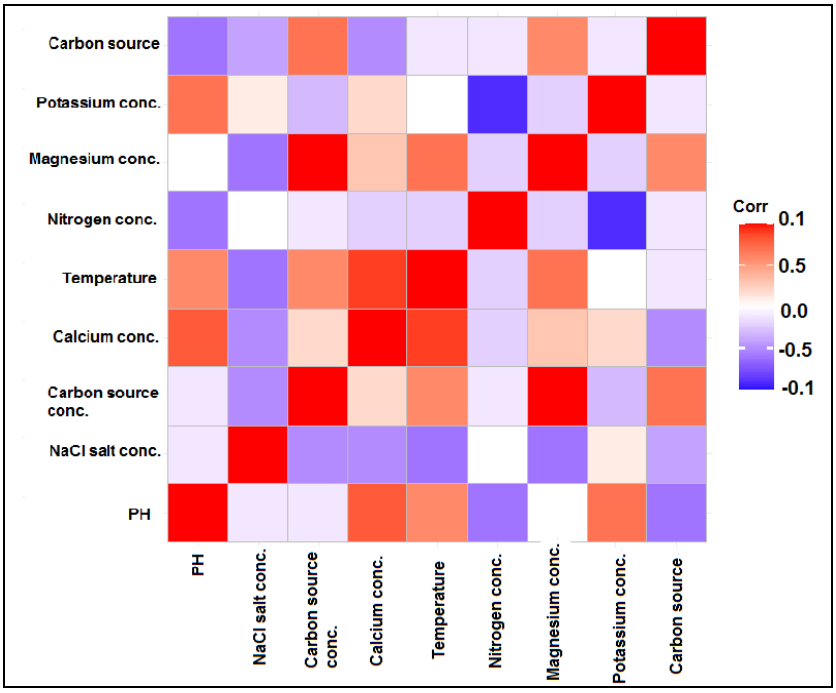

Figure14: Correlation matrix loading plot of *some physical and chemical* optimizations in medium components and cultural conditions for extracellular amylase production by the halophilic *Cytobacillus oceanisediminis* isolate (AHB6).

7. Discussion

Recent decades have seen a progress in studies on microorganisms occurring in extreme habitats, including hypersaline ecosystems. Both molecular and microbiological studies have revealed the existence of moderate to extremely halophilic micro- and some macro-organisms in a wide range of these saline environments [19]. These extremophilic microorganisms have a major advantage in production of valuable metabolites that can be industrially applied in extreme conditions. Bacterial amylases constitute a group of interesting enzymes from the biotechnological point of view [20]. Most interesting applications are in clinical and analytical chemistry, as well as their widespread applications in starch saccharification, textile, food, brewing and distilling industries) [20], where halophilic amylases are useful due to their stability and versatility.

The requirements and the modus operandi of the abovementioned industries suggest a need to explore other sources of enzymes, among the extremophile microorganisms. Recently, the halophilic bacteria gained interest for their capacity to adapt to very high salt concentrations. In the present study, eight bacterial strains were isolated from saline soil of Wadi-El-Natrun, Egypt. Clear zones of hydrolysis were shown by all the eight strains and the comparison of zone diameters shows that halophilic *Cytobacillus oceanisediminis* isolate (AHB6) was the best amylase producer among these strains. The optimization of the physical and chemical parameters plays a significant role in enhancing amylase production by various microorganisms. Thus, the aim of this work was to optimize the medium components and cultural conditions for extracellular amylase production by the halophilic *Cytobacillus oceanisediminis* isolate AHB6 fromWadi-El-Natrun, Egypt. Optimization of culture

conditions is very important for maximum microbial growth and enzyme production by microorganisms (Kathiresan & Manivannan 2006). Among the physical and chemical parameters, optimum temperature, pH range, carbon and nitrogen sources are the most important for enzyme production by microbes) [21].

In the present study, fermentation under shaking conditions induced amylase production in *Cytobacillus oceanisediminis*(AHB6) more than static method. Maximum amylase production was exhibited under shaking conditions, thus shaking accelerates reaching maximum production. Productivity increased because shaking provides sufficient supply of dissolved oxygen in the medium, consequently, nutrient uptake by the strain increased and subsequently the enzyme production increased [22, 23]. Many researchers reported that amylase production decreased when shaking was above 150 rpm; this may be because, the higher shaking rates could increase the oxygen supply to the medium but did not bring about the increase in enzyme production, probably because at high shaking rates, the structure of the enzyme would be altered [24]. It was reported that the maximum amylase production by the halophilic *Micrococcus 39alobios, Halomonas meridiana, Halobacillus* sp.strain MA-2, *Nesterenkonia* sp. Strain F and *Halomonas* sp. AAD21 was exhibited at agitation rates of 140, 200, 200, 220 and 180 rpm, respectively [25, 26, 27, 28, 29].

Amylase production occurred within a broad range of NaCl concentrations, i.e. between 2 and 10 (%, w/v). Amylase production rate by *Cytobacillus oceanisediminis*was based on NaCl concentration. The bacterium was able to grow up to 10% of salt concentration with maximum growth at 4%. At 6 to 10% NaCl w/v, the production rate was stable. These corresponded to NaCl concentrations required for the strain's growth [30, 31]. In contrast to our results, the maximum amylase production by the extremely halophilic archaea *Haloferax mediterranei* and *Haloarcula* sp. Strain S-1 was observed at 25 (%, w/v) NaCl, and 18.5 (%, w/v) for *Haloarcula hispanica* [32, 33, 34]. This may be because *Cytobacillus oceanisediminis*isolate (AHB6) is adapted for growth at lower NaCl concentrations than most other extremely halophilic bacteria which require more than 12 (%, w/v) to grow [30, 31].

It was found that α-amylase production was maximum when using starch as a sole carbon source [35, 36, 37].

Biosynthesis of the enzyme took place not only in the presence of starch but also with other carbon sources. Our study showed that the production of amylase was the highest when wheat bran used as carbon source in the basal media. Potatoes showed moderate effects on enzyme synthesis, followed by soluble starch. These natural substrates may be useful as cheaper alternative sources for halophilic amylase production. The other tested sources, maltose and starch powder did not enhance the amylase production by the strain. Earlier studies reported that, complex substrates induce higher amylase production [27, 35] investigated moderate amylase production by halophilic *Halobacillus* sp. Strain MA-2 in the presence of soluble starch, dextrin, maltose, sucrose, lactose, and glucose, exhibiting its maximum productivity with dextrin followed by starch. [38] reported that certain substrates, i.e. maltose and soluble starch induced amylase production by the halotolerant *eubacterium Chromohalobacter* TVSP 101. *Halomonas sp*. AAD21 produced amylase using soluble starch, sucrose, lactose, and potato starch as carbon sources [29].

Bacterial amylases are produced at a much wider range of temperatures. *Bacillus amyloliquefaciens, B.subtilis, B. licheniformis* and *B. stearothermophilus* are among the most commonly used *Bacillus* species reported to produce α-amylase at temperatures 37-60°C [39, 40, 41, 42]. A wide range of temperatures (35-80°C) has been reported for optimum growth and α-amylase production in bacteria [38,43, 44, 45]. In the present study, for the determination of optimum temperature for enzyme production, the incubation of *Cytobacillus oceanisediminis* (AHB6) was carried out at different temperatures (25 to 50°C). Enzyme production was gradually increased with increasing temperature and maximum enzyme production was observed at 45°C. The optimum range for enzyme production was 40-45°C. Bacterial cells have various mechanisms that allow them strictly to control excretion [46]. Change in the nature of cell envelope can affect the release of extracellular enzymes to the culture medium [47]. Temperature is one of the factors that induces such changes on cell membranes and cell walls [48, 49]. Nusrat and Rahman (2007) reported that, α-amylase production was maximum at temperature 37 °C by the *Bacillusamyloliquefaciens*. Haq *et al.* (2010) reported that, the best activity of α-amylase in stirred fermentor with working volume of 4.5 L was at 37 °C in 48 h by using randomly induced mutant strain of *Bacillus amyloliquefaciens* EMS-6.

Among physical parameters, pH of the growth medium plays an important role in enzyme secretion. The pH range observed during the growth of microbes also affects product stability in the medium [50]. Most of the earlier studies revealed an optimum pH range between 6.0 and 7.0 for the growth of bacterial strains and enzyme production [21, 51, 44]. Previous studies have revealed that fungi required slightly acidic pH and bacteria required neutral pH for optimum growth [52]. So, the effect of initial pH on the production of amylase by *Cytobacillus oceanisediminis* was investigated at different pH (5.0 -11.0). The results showed that pH 9 was the optimum pH for the maximum yield of amylase production. At an acidic pH the enzyme activity was extremely low. It might be due to the fact that the enzyme has low activity in the acidic medium [53]. The composition of cell wall and plasma membrane of microorganisms is known to be affected by the culture pH (Ellwood and Tempest, 1972a and b). According to our data, the change of the initial pH of the medium may lead to change of the nature of the cell membrane and/ or cell wall and hence affecting the α-amylase production and the growth of *Cytobacillus oceanisediminis*. On the other

hand, [46] reported high level of α-amylase at pH 5.0 and 6.0 and when the pH was increased low level of α-amylase was obtained. [54] reported that, α-amylase production by *Bacillus amyloliquefaciens*showed its peak at pH 7.0. Another study [55] showed that α-amylase production by *Bacillusamyloliquefaciens* (267CH) in a fermenter was highest at pH 6.0 [55].

The incorporation of a carbon source in the growth medium is important in the induction of amylase synthesis. Soluble starch was prepared in serial concentration (5, 10, 15, 20, 25, 30g/L) at pH 8 to assess the effect of carbon source concentration on amylase production by *Cytobacillus oceanisediminis* (AHB6). It was found that the maximum production of amylase was obtained at a concentration 25g starch per litre (2.5%). There was a decrease in enzyme production at higher starch concentrations of (30g starch per litre (2.5%)). This might be the high starch concentrations caused the broth culture to be more viscous, thus interfering with O_2 transfer resulting in restriction of dissolved O_2 required for the microbial growth. [56] found that raising the starch concentration increased both growth and α-amylase production by *Bacillus* strain from kitchen wastes and the maximum yield of the enzyme was reached at a starch concentration of 2%. While [57] informed that 4% soluble starch concentration was the best for amylase production by *B. tequilensis* RG-01 and above this concentration enzyme production was slightly decreased.

Most amylases are known to be metal ion-dependent enzymes, requiring divalent ions like Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+} etc. [20]. The effect of calcium ions on amylase production was measured in serial concentrations at pH8. The maximum production of amylase was obtained at 2.5 g $CaCl_2$per litre. [58] stated that, addition of Ca^{2+} ions is often significant for production and stability of amylase of many *Bacillus* spp. According to [21], α-amylase contains at least one Ca^{2+} ion and affinity of Ca is much stronger than that of other metal ions.

It is known that K^+ plays a very important role in the physiology and growth behavior of the extremely halophilic bacteria, consequently enzymes' production. It was observed that, the maximum production of amylase was obtained in a medium containing 0.2% K_2HPO_4. In this respect, halophilic archaea accumulate K^+ intracellularly, for osmoregulation, cells integrity and adaptation of intracellular and extracellular enzymes for maintaining their activity and stability [59, 60, 61]. The effect of K^+ ion on amylase production was observed by Fukushima *et al*., (2005) who reported that the maximum amylase production by the extremely halophilic archaeon *Haloarcula* sp. S-1 occurred at 0.2 (%, w/v) KCl, while Hutcheon *et al*. (2005) found that the optimum KCl concentration required for the maximum amylase production by the extremely halophilic archaeon *Haloarcula hispanica* was 0.5 (%, w/v).

Various systems of halophilic enzymes require divalent ions such as Mg^{2+} for their activity and stability [62,63]. In the present study, the present strain showed maximum amylase production at 6g per litre of MgSO4. Previous studies found that the maximum amylase production was exhibited at 2 and 2.7 %, w/v of $MgSO_4.7H_2O$ for the extremely halophilic archaea *Haloarcula sp*. S-1 and *Haloarcula hispanica*, respectively [33, 34].

Nitrogen source is another essential nutrient that is required by the microorganisms in comparatively larger amounts. The relative concentration of nitrogenous sources in the growth medium are important in the production of amylase [64]. Lower levels of nitrogen and excess nitrogen are equally detrimental causing enzyme inhibition [65]. The influence of nitrogen source concentration on amylase production was determined. Previous studies showed that inorganic nitrogen sources gave comparatively higher yields than organic nitrogen sources. In present study, the enzyme production reached a peak when potassium nitrate used as inorganic nitrogen source at concentration 0.5 g per litre in the culture medium. The nitrogen is metabolized to produce primarily amino acids, nucleic acids, protein, enzymes, and other cellular components that play a vital role in metabolism. The decrease in α-amylase production at excess nitrogen levels could be due to the pH depression or the stimulation of protease enzyme, which repress the amylolytic activity [66].

8. Conclusions

In this study, a combination of physical and chemical parameters was employed to maximize α-amylase synthesis.

A total of 21 amylolytic bacterial isolates were recovered from soil samples. Out of them, 8 isolates were selected as highly amylase producers and the bacterial isolate AHB6 was the most potential one. This isolate was identified phenotypically and genotypically as *Cytobacillus oceanisediminis* AHB6. The fermentation conditions for enzyme production was optimized and the obtained data revealed that the optimum conditions were pH 8.0-10.0, 45°C incubation temperature, wheat bran as carbon source, 2 g/l K_2HPO_4 as potassium source, 6 g /l $MgSO_4$ as magnesium source and 0.5 g/l KNO_3 as nitrogen source. The results obtained in this study illustrated that the optimization of culture conditions played a pivotal role in influencing output through the fermentation bioprocess. Screening of microorganisms with higher α-amylase activities could therefore facilitate the discovery of novel amylases suitable for new industrial applications. Purification and characterization of the enzyme are in progress.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgment

The authors are thankful to New Valley University for the financial and infrastructural support provided for this study.

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