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Production, optimization and characterization of extracellular amylase from halophilic *Bacillus lichineformis* AH214

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Twenty one moderately halophilic bacterial strains were isolated from seawater and sediment in Alexandria Eastern Harbour, Egypt. The isolates were screened for the production of four extracellular degradative enzymes. The majority of isolates (57.1%) possessed significant enzyme activities, 43% of them have potentiality to produce amylase enzyme. The most active isolate for the production of amylase enzyme was identified by using a 16S rRNA sequence analysis as *Bacillus lichineformis* AH214. Optimization of the fermentation medium components and environmental factors using One Variable at a Time Approach and Plackett-Burman design was applied to enhance the amylase production by *Bacillus lichineformis* AH214. The maximum microbial amylase production could be achieved using an optimized medium of the following composition (g/l): 1.0 g yeast extract, 0.05 g K_2HPO_4, 0.25 g $FeCl_3$, 15.0 g starch, 30.0 g NaCl, 0.75 g $MgSO_4.7H_2O$ and inoculums size of 1.5 ml/50 ml and incubated at optimum conditions of pH 7, agitation speed 160 rpm, time 30 h and temperature 40°C. On applying optimized medium in the fermentation process, an enzyme productivity of 13.44 U/mg protein was achieved with two fold increase compared to the basal one. The crude amylase produced by *Bacillus lichineformis* was stable up to 40°C, pH 7.5 and 1.5 M NaCl.

Key words: Halophiles, amylase, *Bacillus lichineformis*, Plackett-Burman, optimization.

INTRODUCTION

Enzymes are vitally important to existence of life itself, capable to act as biocatalyst for a wide variety of chemical reactions (Van der Maarel et al., 2002). Salts are required for all life forms and halophiles are distinguished by their requirement of high salinity conditions for growth. Based on definition of Das Sarma et al. (2012), the term of moderate halophiles was applied for microorganisms that grow optimally or very well at 0.85 to 3.4 M (5-20%) NaCl. The ability of halophiles to produce hydrolytic extremozymes has been much studied

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for its possible applications in industries (Moreno et al., 2013; Ali et al., 2014). Among bacteria, the use of halophiles in comparison to the extensive use of extremozymes from thermophiles and alkaliphiles is very low. Halophilic enzymes have thus far found applications in industries and biotechnology (Abdu Al-ZaZaee et al., 2011; Moreno et al., 2013). Mostly halophilic hydrolases such as amylases, cellulases, lipases, xylanases, and proteases have been reported from halophilic bacteria (Abdu Al-ZaZaee et al., 2011; Moreno et al., 2013; Ali et al., 2014). Amylases are among the most important industrial enzymes and also have great significance in biotechnological studies (Van der Maarel et al., 2002).

Amylases are enzymes which hydrolyze starch molecules to give diverse products including dextrins and progressively smaller polymers composed of glucose units (Abdel-Fattah et al., 2013). Three kinds of amylase were divided by Ekunsaumi (2002) based on their ability to breakdown amylum. They were alpha amylase, beta amylase and amyloglucosidase. Amylase can be simply classified in two groups. (1) Endo–acting or endo–hydrolases e.g. α-amylase: -α-Amylases (1, 4-α-glucan-glucanohydrolases) are extracellular enzymes which hydrolyze α-1, 4-glycosidic bonds. These enzymes are endoenzymes, splitting the substrate in the interiors of the molecule. (2) Exo-amylase or exo-hydrolases e.g., β-amylases, glucosidase and α-glucosidase. Glucoamylases (α-1, 4-glucan-glucohydrolases) act on starch by splitting glucose units from the non-reducing end. β–glucosidase is usually of plant origin, but some microbes are also known to produce it (Parmar and Pandya, 2012).

Although amylases can be derived from several sources, including plants, animals, and microorganisms, microbial enzymes generally meet industrial demands (Abdel-Fattah et al., 2013; Alariya et al., 2013; Panneerselvam and Elavarasi, 2015). These enzymes were isolated from many microbial sources including bacteria, fungi and some actinomycetes, that are resistant to high salt concentration, temperature and pH (Abdu Al-ZaZaee et al., 2011).

Totay, α-amylases of microbial origin have replaced the chemical hydrolysis of starch in starch processing industry (Pandey et al., 2000). The major advantages of using microorganisms for the production of amylases are the economical bulk production capacity and, secondly, the fact that microbes are easy to manipulate to obtain enzymes of desired characteristics (Abdel-Fattah et al., 2012; Panneerselvam and Elavarasi, 2015). The α-amylase is an important class of amylases which constitutes approximately 25 to 30% of its share in total enzyme market (Abdu Al-ZaZaee et al., 2011; Ali et al., 2014).

Bacillus sp.*, Halobacillus* sp.*, Haloferax mediterranei* and *Halothermothrix sp.* are known to be good producer of α-amylase. However, the production of α- amylases depends on the strain, composition of media, metal ions, pH, temperature, moisture and oxidative stress. Due to extensive industrial uses which withstand the high load of salt concentration about 10 to 25% and other industrial harsh conditions, enzymes from halophilic bacteria are frequently preferred (Abdu Al-ZaZaee et al., 2011).

Amylases have extensive applications in textile industries, detergent manufacturing processes pharmaceutical, and food industries. The microbial amylases could be potentially useful in various pharmaceutical, fine-chemical industries etc. With the event of new frontiers in biotechnology, the use of amylase has widened in clinical research, medical chemistry and starch analytical chemistry (Asgher et al., 2007; Liu and Xu, 2008; Shafiei et al., 2010; Abdel-Fattah et al., 2013; Ali et al., 2014).

Each application of amylase requires unique properties with respect to specificity, stability, and temperature and pH values dependence. Screening of microorganisms with higher α-amylase activities could therefore, facilitate the discovery of novel amylases suitable to new industrial applications (Abdel-Fattah et al., 2013).

Enzyme overproduction can be achieved by both genetic manipulations and media engineering. As excretion of metabolism products is a part of survival strategy of microbes in certain environments, overproduction of enzymes by media manipulation may be considered a better strategy (Abdel-Fattah et al., 2013).

The classical method for medium optimization involves changing one independent variable, keeping the other factors constant OVAT (one-variable-at-a-time). This method is time-consuming and incapable of detecting the true optimum, due to the interactions among the factors and this limitation of a single factor optimization process can be eliminated by different techniques (Abdel-Fattah et al., 2013).

Unlike conventional optimization, statistical optimization methods present a more balanced alternative to the OVAT approach, since it takes into account the interaction of variables in generating the process response. Statistical experimental designs have been used for many decades and can be adopted on several steps of an optimization strategy, such as for screening experiments or searching for the optimal conditions of a targeted response. Recently, the results analyzed by a statistical planned experiment are better acknowledged than those carried out by the traditional OVAT method. Some of the popular choices, applying statistical designs to bioprocessing, include the Plackett-Burman design (El-Sharouny et al., 2015).

Halophiles have been perceived as a potential source of industrially useful enzymes endowed with exceptional stabilities. The present study focuses on the (i) isolation of moderate halophilic bacteria from seawater and sediment samples collected along Alexandria Eastern Harbour, Egypt, (ii) screening for industrially important enzymes (especially amylase, lipase, cellulase and

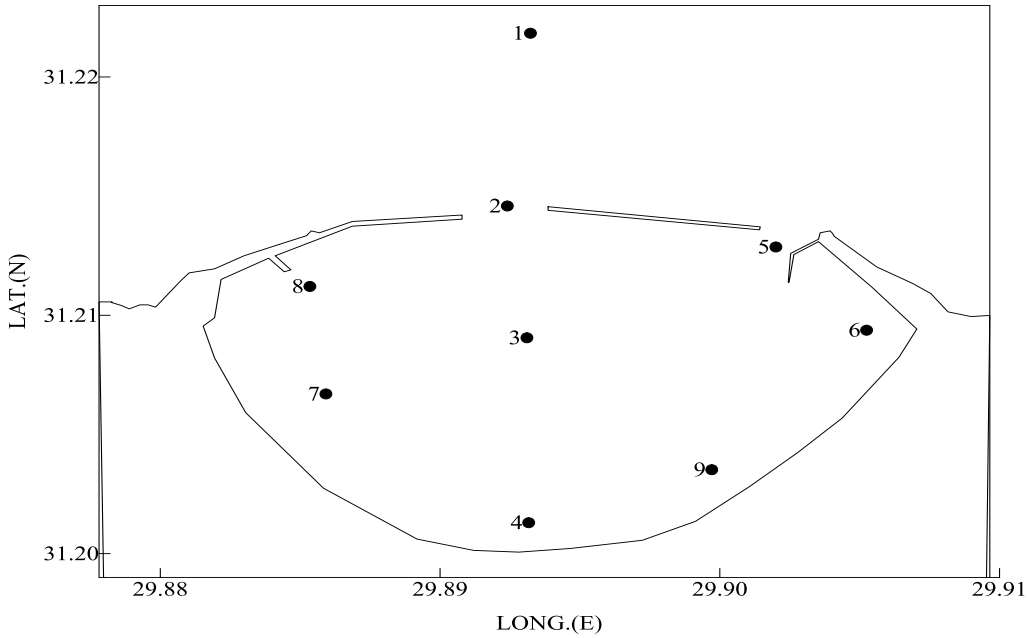

Figure 1. Location of sampling sits along Alexandria Eastern Harbour, Egypt.

protease) and (iii) studying the optimization of fermentation conditions and properties for amylase enzyme.

MATERIALS AND METHODS

Sampling

Sediment sample were collected from nine sites along Alexandria Eastern Harbour, Egypt (Figure 1), during spring 2012. Sampling was performed according to the World Health Organization manual for recreational water and beach quality monitoring and assessment (Clesceri et al., 2012).

Isolation of bacteria

Total heterotrophic marine bacteria (THB) were counted using standard pour plate method into Marine agar 2216 (MA), (Oxoid LTD, England). Plates were incubated at 30°C and final counts of colony forming units (CFU) taken after 24 to 48 h (Clesceri et al., 2012).

Halophilic bacteria were isolated using standard pour plate method into MH medium. The composition of the isolation medium was as follows: Yeast extract, 1% (w/v); peptone 0.5% (w/v); glucose, 0.1% (w/v); and Bacto Agar, 2% (w/v). This medium was supplemented with a balanced mixture of sea salts to give final concentrations of 10% (w/v). The pH was adjusted to 7.2 with 1 M KOH. The final counts of colony forming units (CFU) were taken after incubation at 30°C for 5 days in sealed plastic (Quesada et al., 1984).

Enzymatic profile of bacterial isolates

The ability of halophilic bacterial isolates to produce extracellular degradative enzymes was examined on MH medium agar plates (Quesada et al., 1984). The plates were amended with starch, olive oil, skimmed milk, or carboxymethylcellulose at 4 g/l to detect the production of amylase, lipase, protease, or cellulase, respectively. Formation of hydrolytic zone (clear zone, mm) around the colonies, resulting from polymer hydrolysis, was taken as evidence of hydrolytic activity (Moreno et al., 2013).

Selection of amylase producing bacteria

For selection of amylase- producing bacteria from marine isolates, qualitative determination of α-amylase was carried out using well cut or cup assay with some modifications (Sudharhsan et al., 2007). The agar plates were amended with 1% of starch and 1.5% of agar for well-cut assay. After agar solidification, around 10 mm diameter of well was cut out aseptically using cork borer. The well was filled with the culture filtrate (100 μl), incubated overnight at 35°C. The plates were flooded with Lugol solution (1% iodine in 2% potassium iodide w/v), for 1 min until the entire medium became colored in blue, then the hydrolytic zone around the well (clear zone) is measured. The negative control is maintained by adding sterile water in a separate well (Amoozegar et al., 2003).

Culture condition

Sterilized nutrient broth (20 ml) was inoculated with bacterial suspension of 24 h bacterial slant and incubated in orbital shaker at 150 rpm (35°C) until the absorbance at A_{600} nm reached to 0.15 (cell density about 1×10^6 CFU/ml).

Production of amylase

The production medium for amylase consisted (g/l): 3.0 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4.7H$ O, 0.1 g K_2HPO_4, 0.5 mg $FeCl_3$, 20 g NaCl, 10.0 g starch and pH 7.5. The media was inoculated with 1% ($1x\ 10^6$ CFU/ml) and incubated with shaking (150 rpm) at 35°C for different time intervals in an orbital shaker incubator.

Enzyme assay for amylase

Amylase was determined by spectrophotometric method as described by Fisher and Stein (1961). According to procedure 1.0 ml of culture broth was taken in test tube in duplicate and 1.0 ml of substrate (starch) was added in test tube. The test tubes were incubated at 35°C for 15 min in water bath. Then 2.0 ml dinitro-salicylic acid reagent was added in each tube to stop the reaction and kept in boiling water bath for 15 min. After cooling at room temperature, the absorbance was read at 540 nm by spectrophotometer. A unit of amylase activity was defined as the amount of amylase required to catalyze the liberation of reducing sugar equivalent to one ì mol of D-glucose per minute under the assay conditions (Miller, 1959).

Estimation of protein content

The total protein was estimated using Lowry's method (Lowry et al., 1951). Bovine serum albumin (BSA) was used as a standard.

Bacterial identification

Genomic DNAs of the selected isolate was extracted with the genomic DNA extraction protocol of Gene Jet genomic DNA purification Kit (Fermentas). Polymerase chain reaction (PCR) using Maxima Hot Start PCR Master Mix (Fermentas).The amplifications were carried out in a thermal cycler (Multigene Optimax, Labnet international, Inc). The PCR thermocycler was programmed as follow: 95°C for 5 min for initial denaturation, 30 cycles at 95°C for 1 min, 55°C for 1 min, 72°C for 2 min and a final extension at 72°C for 10 min. The PCR mixture contained 25 pmol of each primer, 10 ngchromosomal DNA, 200 mmol/LdNTPs and 2.5 U of *Taq* Polymerase in 50 µl of Taq polymerase buffer 10X Standard Taq Reaction Buffer.

The PCR Clean-Up of the PCR product was performed by using Gene JET™ PCR Purification Kit (Fermentas) at Sigma Scientific Services Company, Egypt, 2013. The sequencing of the PCR product was made by the GATC Company by using ABI 3730xl DNA sequencer with universal primers (16S 27F and 16S 1492R), (5′-AGAGTTTGATCCTGGCTCAG-3′ and 5′-GGTTACCTTGTTACGACTT-3′). Genotypic characterization was performed using 16S sequence analysis. Multiple alignments with sequences of the most closely related members and calculations of levels of sequence similarity were carried out using BioEdit (software version 7) (Hall, 1999). Sequences of rRNA genes, for comparison, were obtained from the National Center for Biotechnology Information (NCBI) database.

Effect of different carbon and nitrogen sources on amylase production

This was studied by adjusting different carbon sources as starchy substrates (starch, rice kerench, wheat bran, potato cubes and potato peels), different sugars (maltose, glucose, sucrose, fructose, dextrose and lactose) and different nitrogen sources ($(NH4)_2SO_4$, NH_4Cl, $NaNO_3$, KNO_3, urea, peptone yeast extract and casein) in the production medium (Dharani, 2004; Ashwini et al., 2011).

Effect of incubation time, agitation, temperature and pH on amylase production

The bacterial isolate was grown on the production medium at different incubation time (6, 12, 18, 24, 30, 36 and 48 h), different speed (80, 120, 160, 180, 200 and 240 rpm), different temperatures (20, 25, 30, 35, 40, 45 and 50°C) and different pH (4, 5, 6, 6.5, 7, 7.5, 8, 9 and 10) (Ashwini et al., 2011).

Table 1. Factors examined as independent variables affecting amylase enzyme production and their levels in the Plackett-Burman experimental design.

Factors	Symbol	Levels		
		-1	0	1
Yeast extract	Ye	1	2	3
K_2HPO_4	K2	0.05	0.1	1.05
Starch	St	5	10	15
NaCl	Na	10	20	30
$MgSO_4.7\ H_2O$	Mg	0.25	0.5	0.75
$FeCl_3$	Fe	0.25	0.5	0.75
Size of inoculums*	IS	0.5	1	1.5

*cell density about 1×10^6 CFU/ml.

Optimization of growth medium using Plackett-Burman experimental design

The Plackett-Burman design was proved to be a powerful tool to rapidly determine the effects of medium constituents on amylase production. The Plackett-Burman experimental design (Plackett and Burman, 1946), a fractional factorial design was used in this research to reflect the relative importance of various growth media component factors on amylase activity in liquid cultures. In amylase assay experiment, seven independent variables were screened in eight combinations organized according to the Plackett-Burman design matrix (Tables 1 and 3). For each variable, the high (+1) and low (-1) levels were tested. Medium components are given in g/l and inoculum size was added in ml with culture (A_{600} = 0.15). All trials were performed in duplicates and the average of amylase activity results were treated as the responses. The main effect of each variable was determined by the following equation: $E_{xi} = (\Sigma M_{i+} - \Sigma M_{i-}) / N$. Where Exi is the variable main effect, Mi+ and Mi- are the amylase production in trials where the independent variable (xi) was present in high and low concentrations, respectively, and N is the number of trials divided by 2. A main effect with a positive sign indicates that the high concentration of this variable is nearer to optimum and a negative sign indicates that the low concentration of this variable is nearer to optimum. Using Microsoft Excel, statistical t-values for equal unpaired samples were calculated for determination of variable significance.

Verification experiments

A verification experiment was carried out in triplicates, the predicted optimum levels of the independent variables were examined and compared to the basal condition setting and the average of enzyme activity was calculated. Moreover, the Plackett-Burman reverse medium was applied.

Characterization of the crude enzyme

The effect of temperature and pH on the enzyme activity was determined. The standard assay conditions were used for 15 min at temperatures between 25 and 60°C. The pH of the reaction mixture was ranged from 4 to 10 using 50 mm buffers (Sodium acetate pH 3.6-6.5, Tris-HCl pH 7 -8.5 and glycine buffer 9-10).

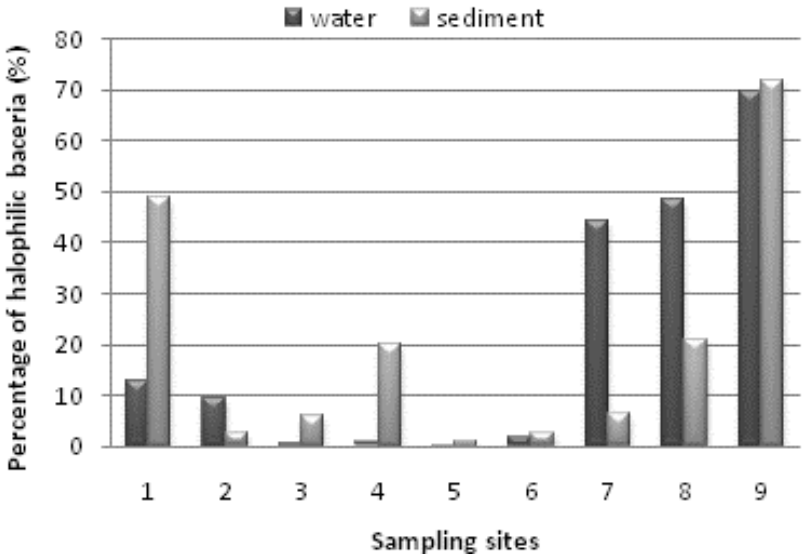

Figure 2. The percentage (%) of moderately halophilic bacteria with respect to the total heterotrophic bacteria isolated from seawater and sediments samples along Alexandria Eastern Harbour, Egypt.

Effect of salt tolerance of amylase

The crude amylase enzyme was incubated in 5 mM phosphate buffer (pH 7.5) containing different NaCl concentrations (0 to 5 M) for 2 and 12 h at 4°C, and enzyme activities were estimated in the same way as described above.

RESULTS

Isolation of halophiles

The distribution of halophiles in seawater and sediment along Alexandria Eastern Harbour of Egypt was investigated (Figure 2). The moderate halophiles bacteria was isolated at 10% NaCl, it ranged from 1.2×10^2 to 8.8×10^2 CFU/ml in seawater and from 7.0×10^3 to 2.8×10^5 CFU/g in sediment. The percentage of moderately halophilic bacteria with respect to the total heterotrophic bacteria ranged from 0.12 to 69.60% in seawater while it ranged from 0.88 to 48.88% in sediment.

Screening of hydrolase producers

Twenty one morphologically different colonies able to grow well at 10% NaCl were obtained from seawater and sediment samples, and tested for production of extracellular hydrolytic enzymes. The majority of moderate halophilic bacterial isolates (57%) expressed significant enzyme activity when applied on substrates like starch, olive oil, carboxy methyl cellulose or skimmed milk. Based on the preliminary screening experiment, twelve bacterial isolates have potentiality to produce one or more of the hydrolase enzymes, while most bacterial isolates (43%, nine out of twenty one isolates) have potentiality to produce amylase enzyme (Table 2).

Screening of amylase producers

In the present study a total of nine bacterial isolates were selected and qualitative determination of amylase activity was performed. Among these only three isolates showed the highest hydrolytic zone around the well, while the hydrolytic zone were 36, 28 and 19 mm for isolates 7W-1, 3S-3 and 2S-1, respectively. Based on the data obtained, isolate 7W-1 was selected for identification and study the production of amylase enzyme.

Identification of amylase producing bacterial isolate

For molecular characterization, DNA sequencing of 16S rDNA of the selected isolate (isolate 7W-1), showed a highest similarity of 99.7% to *Bacillus lichineformis* (Figure 3), isolate 7W-1 was identified as *Bacillus lichineformis* AH214 and the nucleotide sequence was deposited to National Center for Biotechnology Information (NCBI) data bank with the accession number of KT199247.

Pre-optimization experiments by one variable at a time approach

Effect of starchy substrates and incubation period on amylase production

The enzyme production varies with incubation period of

Table 2. Enzymatic profiles of selected bacterial isolates.

Isolate code	Amylase	Lipase	Cellulase	Protease
	Hydrolytic zones (mm)			
1S-1	25	0	0	0
2S-1	20	0	0	0
3S-3	14	0	0	0
7S-1	0	14	0	0
7S-2	19	29	17	0
7S-3	14	0	0	0
8S-2	13	0	0	0
9S-1	24	0	0	0
2W-1	21	24	19	0
3W-1	0	15	0	0
7W-1	28	21	16	0
8W-1	0	18	14	0

S, isolated from sediment samples; W**,** isolated from seawater.

Table 3. The Plackett-Burman experimental design matrix for seven factors.

Trials	ye	K2	st	Na	Mg	Fe	Is	Protein content (mg/ml)	Enzyme activity (U/mg protein)
1	-1	-1	-1	1	1	1	-1	3.14	9.17
2	1	-1	-1	-1	-1	1	1	2.44	9.38
3	-1	1	-1	-1	1	-1	1	2.63	9.47
4	1	1	-1	1	-1	-1	-1	2.43	8.78
5	-1	-1	1	1	-1	-1	1	3.23	12.49
6	1	-1	1	-1	1	-1	-1	3.45	9.90
7	-1	1	1	-1	-1	1	-1	3.02	9.17
8	1	1	1	1	1	1	1	3.45	12.05
9	0	0	0	0	0	0	0	3.55	10.25

the *B. licheniformis* cells. The results (Figure 4) indicated that the production of amylase enzyme increased gradually from 18 h (4.99 U/mg protein) and reached its maximum (6.99 U/mg protein) at 30 h of incubation, thereafter, the enzyme production started decreasing.

The different starchy substrates were used to substitute pure starch in the fermentation medium. The results given in Figure 4 Revealed that the maximum amylase production (6.99 U/mg protein) was recorded in starch supplemented medium and minimum amylase production was recorded in wheat bran (5.34 U/mg protein).

Effect of carbon source on amylase production

The addition of carbon source in the form of either monosaccharide or polysaccharides may influence the production of amylase enzyme. The influence of maltose was more effected (6.29 U/mg protein) than the other sugar sources tested. Glucose was the second best supplementary carbon source (5.23 U/mg protein). Lactose gave the lowest amylase enzyme activity (1.34 U/mg protein) (Figure 5).

Effect of nitrogen source on amylase production

The nitrogen sources are secondary energy sources for the organisms, which play an important role in the growth of the organism and the production. The effect of supplementary nitrogen sources on amylase production by *B. licheniformis* cells showed that yeast extract was the better nitrogen source for this isolate (8.12 U/mg protein) (Figure 6).

Effect of agitation speed, temperature and pH on amylase production

The production of amylase by *B. licheniformis* was

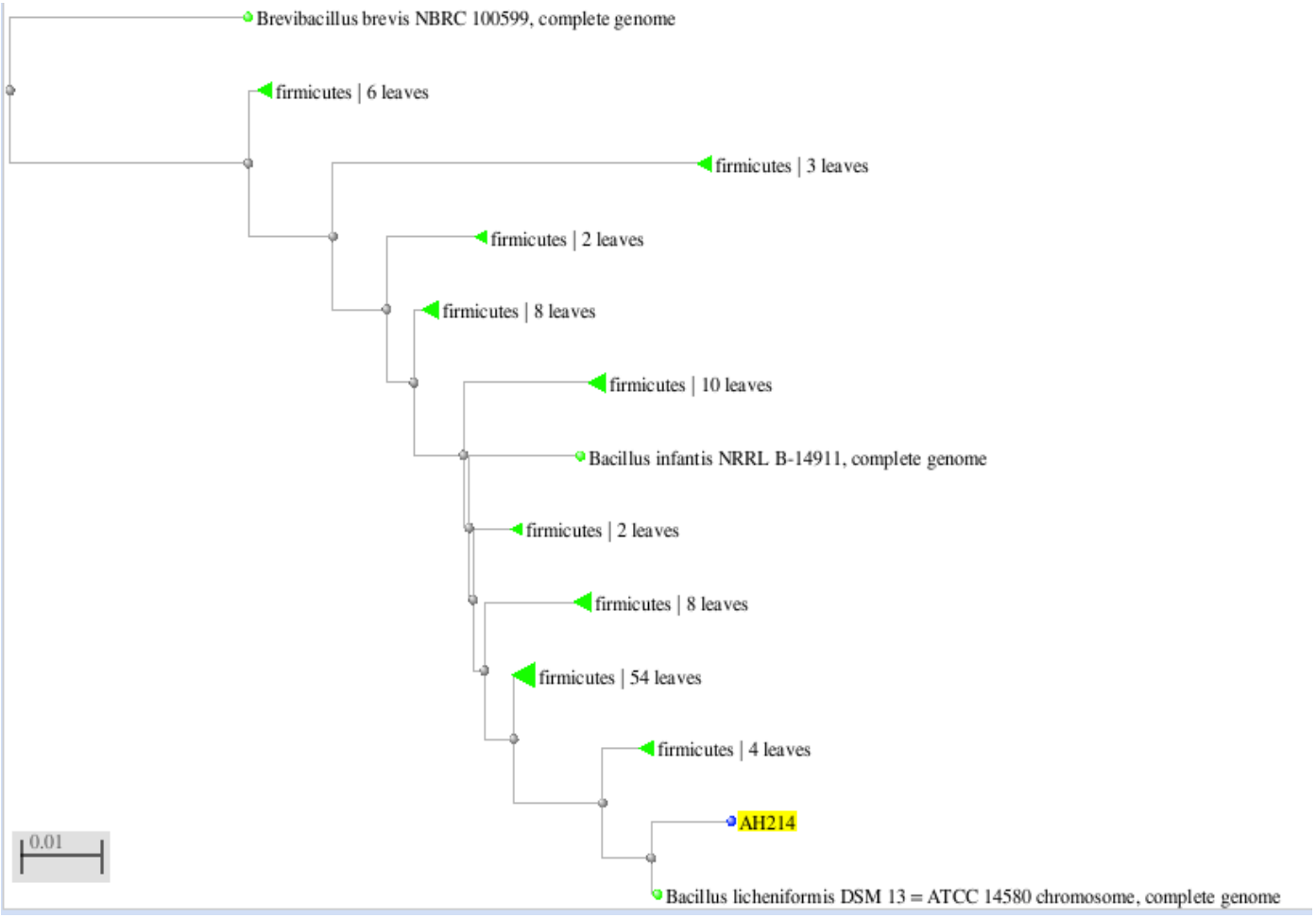

Figure 3. Phylogenetic relationship of strain *B. lichineformis* AH214 (isolate 7W-1) and the most closely related strains presented in the Genbank database.

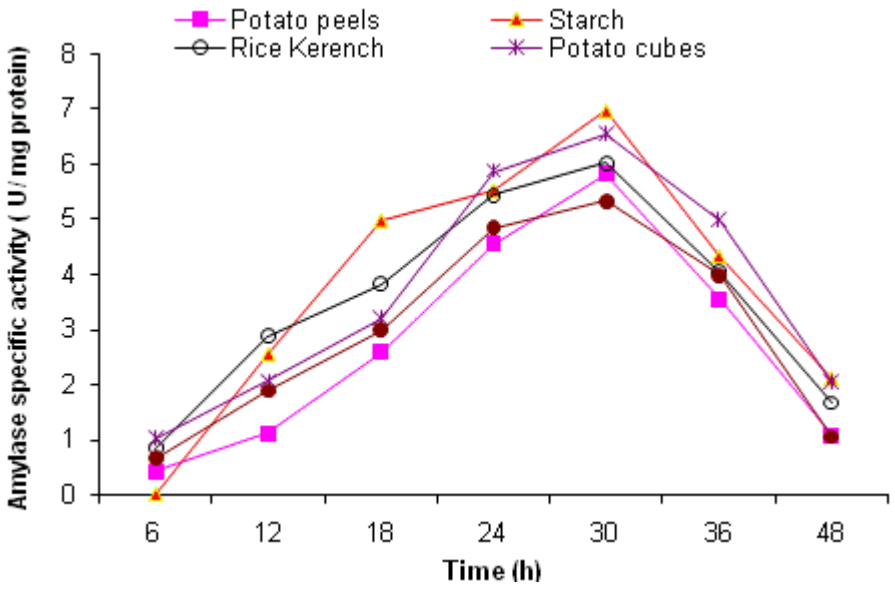

Figure 4. The effect of different starchy substrates and different incubation times on amylase production from *B. licheniformis.*

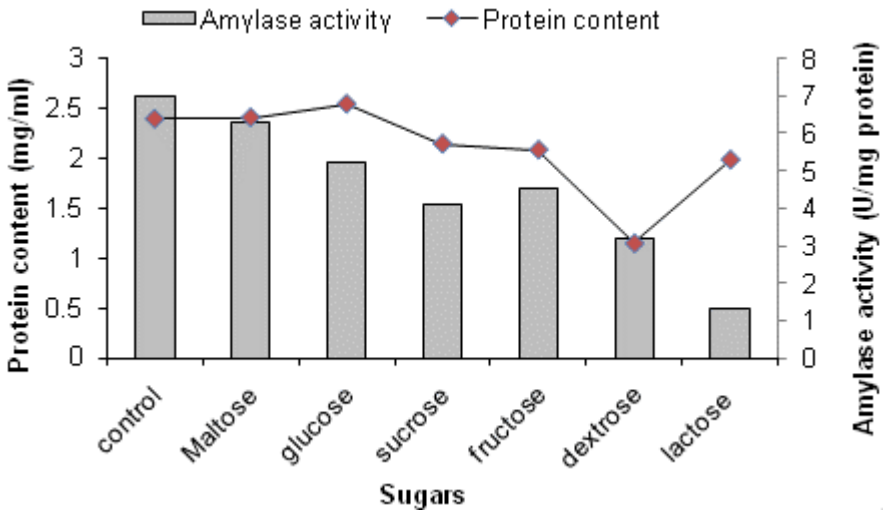

Figure 5. The effect of different carbon sources on amylase production from *B. licheniformis.*

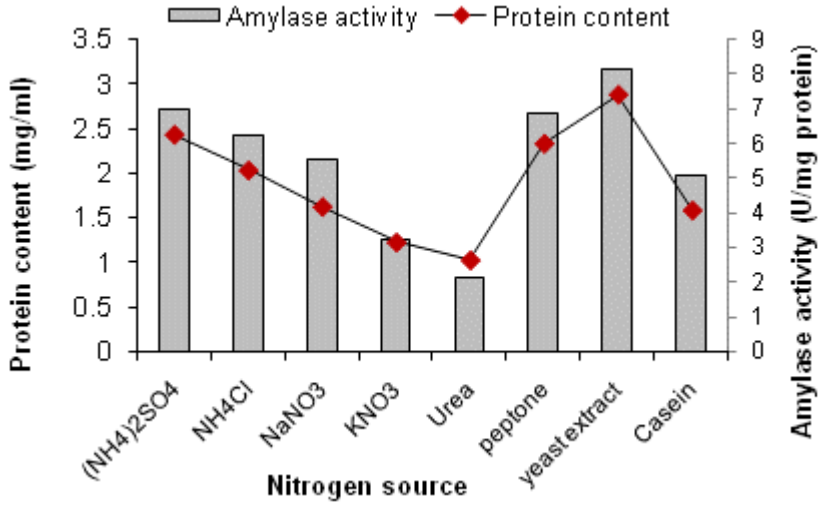

Figure 6. The effect of different nitrogen sources on amylase production from *B. licheniformis.*

investigated at different speed of agitation. The production of amylase enzyme increased gradually by increasing agitation and reached its maximum (8.16 U/mg protein) at 160 rpm of incubation, thereafter, the enzyme production started decreasing, compared by static incubation (6.56 U/mg protein) (Figure 7A)**.**

The results represented in Figure 7B showed that the optimum temperature which yielded the highest enzyme activity (10.4 U/mg protein) was 40°C. Higher or lower temperatures have an adverse effect on the amylase activity; the lowest activity (2.04 U/mg protein) was demonstrated at an incubation temperature of 20°C. Also, the protein content increased gradually by increasing the incubation temperature and reached its maximum value at 40°C.

The production of amylase was investigated at different pH values ranging from 4.0 to 10.0. The results presented in Figure 7C showed that enzyme synthesis of *B. licheniformis* was observed between pH 6.0 and 8.0. The pH 7 was the optimum pH value for amylase production (11.92 U/mg protein). When pH is altered below or above the optimum, the activity is decreased or becomes denatured.

Plackett-Burman design for optimization of *α*-amylase production by *Bacillus licheniformis*

Plackett-Burman design was employed to identify significant variables that enhance amylase production

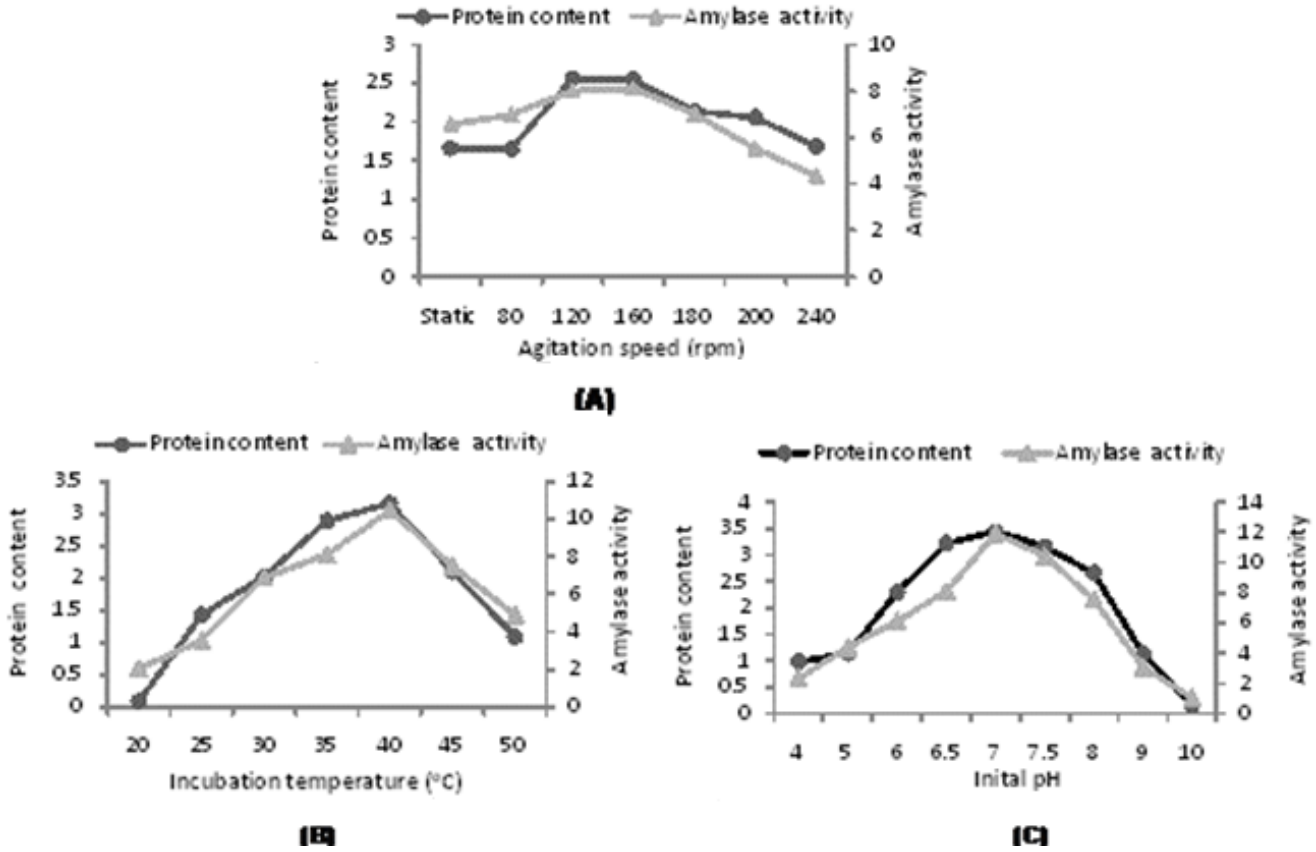

Figure 7. The effect of different (A) agitation speed, (B) incubation temperature and (C) pH on protein content (mg/ml) and amylase activity production (U/ mg protein) by *B. licheniformis.*

and to find out their probable optimal levels in a limited number of experiments. In this study, seven variables were analyzed with regard to their effects on enzyme production using a Plackett-Burman design. The independent variables were examined and their settings are shown in (Table 1).

The seven nutritional factors were yeast extract, K_2HPO_4, starch, NaCl, $MgSO_4.7\ H_2O$, $FeCl_3$ and inoculum size. The environmental factors were kept at optimum conditions of pH 7, agitation speed 160 rpm, incubation time 30 h and temperature 40°C. The seven variables were carried out by eight trials according to a design matrix (Table 3), which was based on the number of variables to be investigated.

The protein content and enzyme activity were measured (Table 3). For determination of variable significance, statistical t-values for equal unpaired samples were calculated with respect to observations. The necessary statistical analyses of this experiment are shown in Table 4. The main effect of each variable upon amylase activity was estimated and presented graphically in Figure 8. The production of amylase by *B. licheniformis* was positively affected by starch, NaCl, $MgSO_4.7H_2O$ and inoculums size, and negatively affected by yeast extract**,** K_2HPO_4 and $FeCl_3$, that is, the high concentration of yeast extract had the most significant negative effect on amylase production and the high concentration of starch had the most significant positive effect on amylase production by *B. licheniformis*. Therefore, decreasing the yeast extract concentration and increasing the starch concentration in the culture medium will enhance the extracellular amylase production.

According to the data obtained from the Plackett-Burman experimental results and all calculations related

Table 4. Statistical analysis of the Plackett-Burman experimental result for *B. licheniformis.*

Variable	Main effect	T-test
yeast extract	-0.046	-0.043
K_2HPO_4	-0.36	-0.344
Starch	1.701	**2.065**
NaCl	1.142	1.176
$MgSO_4.7H_2O$	0.191	0.178
$Fecl_3$	-0.219	-0.204
Size of inoculum	1.596	1.859

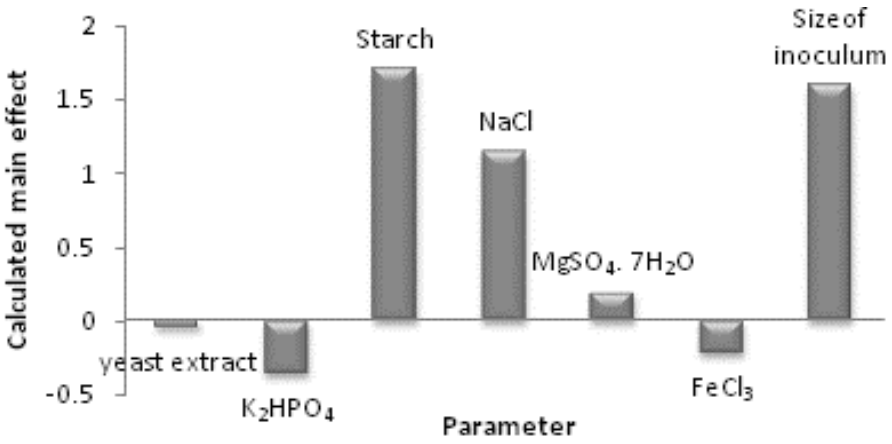

Figure 8. Elucidation of cultivation factors affecting amylase production by *B. licheniformis* using Plackett-Burman experimental design.

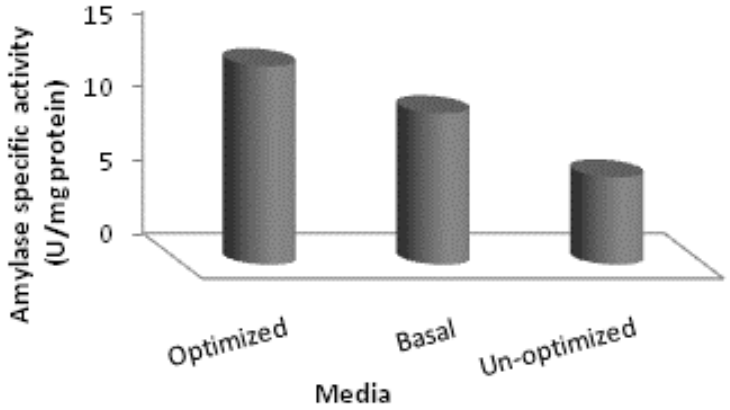

Figure 9. Verification experiment for amylase activity produced by *B. licheniformis.*

to this experimental design, it can be predicted that high microbial amylase production could be obtained using a medium formula of the following composition (g/l): 1.0 g yeast extract, 0.05 g K_2HPO_4, 0.25 g $FeCl_3$, 15.0 g starch, 30.0 g NaCl, 0.75 g $MgSO_4.7H_2O$ and inoculums size of 1.5 ml / 50 ml.

Verification experiment

In order to validate the obtained data and to evaluate the accuracy of the applied Plackett-Burman statistical design, a verification experiment was carried out in triplicates to predict the near optimum levels of independent variables. The data were examined and compared to the basal and anti-optimized medium. Data was revealed that the amylase production (13.44 U/mg protein) raised by 1.3 fold for *B. licheniformis* when growing in optimized medium (Figure 9).

Some properties of the crude amylase produced by *B. lichineformis*

Effect of enzyme and substrate concentration

This part deals with the study of some properties of crude enzyme included the effect of enzyme and substrate concentrations. From the proceeding experiments, an enzyme protein concentration of 1.28 mg/ml and substrate concentration of 7.5 mg/ml reaction mixture was the most favorable for maximum activity of the crude amylase enzyme produced by *B. lichineformis*

Effect of temperature and pH of the reaction mixture

To test the effect of temperature, the reaction was carried out for 15 min at 25, 30, 35, 40, 45, 50, 55 and 60°C. The results (Figure 10A) showed that the enzyme activity (36.11 U/mg protein) was stable up to 40°C, above this

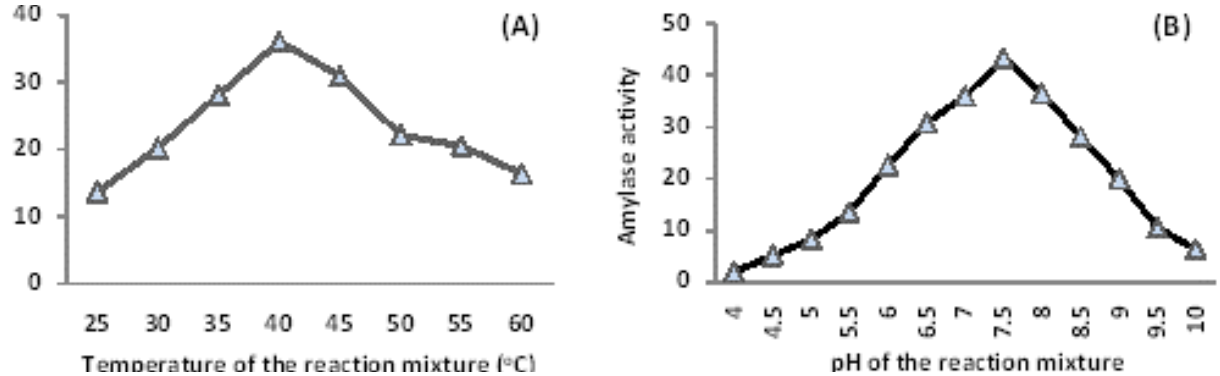

Figure 10. The effect of (A) reaction temperature and (B) reaction pH on amylase activity (U/ mg protein) by *B. licheniformis.*

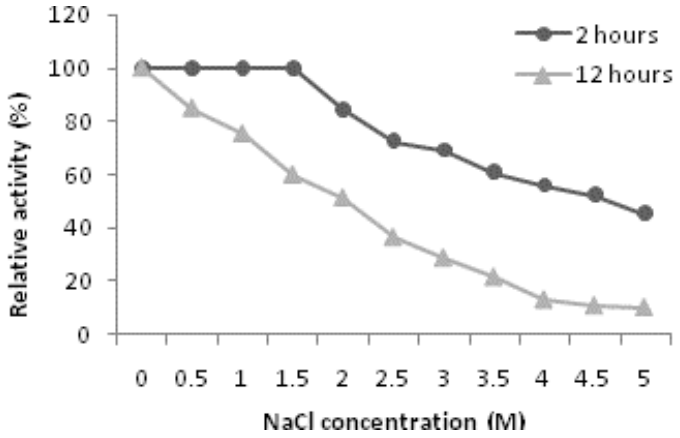

Figure 11. The stability of amylase enzyme activity by *B. licheniformis* at different NaCl concentration.

temperature, a rapid decrease in the enzyme activity was observed where only 45% of the activity was remained at 60°C. The effect of the pH of the reaction on the activity of crude amylase was studied using different buffers with different pH varied from 4 to 10. The data given in Figure 10B indicated that the optimum pH value of the reaction mixture was 7.5. At this value, the enzyme reached its maximal specific activity (43.42 U/mg protein). Above or below this value the activity of the tested crude enzyme decreased gradually.

Stability to NaCl

Effect of NaCl concentration on amylase activity and stability exhibited that the crude enzyme retained 100 and 60% of its activity for up 2 and 12 h, respectively, at 1.5 M (8.7%) NaCl. Also, 60% of relative activity retained for 2 h at 3.5 M of NaCl (Figure 11).

DISCUSSION

Halophilic bacteria are very abundant in the coastal regions, due to its potentiality in producing the extracellular hydrolytic enzymes; it has gained importance in industries for their commercial usage (Moreno et al., 2013). The variation of enzymatic profile was dependent on bacterial species and nature of substrate (El-Sharouny, 2015). The majority of moderate halophilic bacteria (57%) isolated from Alexandria Eastern Harbour of Egypt expressed significant enzyme activity when applied on substrates like starch, olive oil or carboxy methyl cellulose. Moreno et al. (2013) reported that some isolated strains harbored all the extracellular hydrolytic activities screened, except for the chitinase activity. In the present study, most bacterial isolates (43%) have potentiality to produce amylase enzyme. Generally, the strains producing amylolytic enzymes were the most diverse and abundant physiological group among the hydrolytic producers (Moreno et al., 2013).

In the present study the most active isolate for the production of amylase enzyme from the local marine environment was identified by using a 16S rRNA sequence analysis as *B. lichineformis* AH214. Most environmental isolates able to produce hydrolytic enzymes were Gram-positive bacteria, although the isolates were assigned to the family Bacillaceae, comprising species of the genera *Bacillus, Halobacillus* and *Thalassobacillus* (Moreno et al., 2013). Several researchers (Panneerselvam and Elavarasi, 2015; Paul et al., 2015; Khunt et al., 2011) produce amylase enzyme using *Bacillus* sp. isolated from moderate halophiles. This genus is well known as an extracellular enzyme producer and many industrial processes use species of this genus for commercial production of enzymes (Schallmey et al., 2004; Moreno et al., 2013).

The enzyme production by *B. licheniformis* cells reached its maximum (6.99 U/mg protein) at 30 h of incubation. Lowest enzyme activity on prolonged incubation could be due to inhibition and denaturation of the enzyme (Gautam et al., 2002). Short incubation time offers potential for inexpensive production of enzymes. In another study the optimum amylase enzyme activity produced by *B. licheniformis* 44 MB 82-G was recorded

after 96 h using glucose as carbon source (Tonkova et al., 1993). *Bacillus* sp. VS04 produced highest amylase enzyme after 72 h incubation (Vishnu et al., 2014).

Rao and Sathyanarayana (2003) reported that the different carbon sources have varied influence on the extracellular enzymes especially amylase strains. Also Bajpai and Bajpai (1989) found that the different carbon sources can greatly influence the production of amylase. Starch is generally accepted as nutritional component for induction of amylolytic enzymes. The maximum amylase production by *B. licheniformis* AH214 cells was achieved in the presence of starch and maltose as carbon source. Adeyanju et al. (2007) and Ashwini et al. (2011) used starch as a carbon source for amylase production from *B. licheniforms* and *B. mairini*. There are also reports on maltose as a best carbon source for amylase production from *Bacillus* sp. (Gurudeeban et al., 2011; Sivakumar et al. (2011). It was mentioned that induction of amylase requires starch and maltose as substrate, whereas the minimum enzyme activity and protein content were observed in the presence of dextrose (Ashwini et al., 2011). These results are similar to the findings of Haseltine et al. (1996) who observed that glucose represses the production of amylase in the hyper thermophilic archaeon *Sulfolobus solfataricus.* According to them glucose prevented amylase gene expression and not merely secretion of performed enzyme.

The present result explained that the yeast extract was the better nitrogen source for the highest yield of starch hydrolyzing enzyme by *B. licheniformis* AH214. Yeast extract is the best nitrogen source for amylas production, probably due to its high content in minerals vitamins, coenzymes and nitrogen components (Guerra and Pastrana, 2002; Roses and Guerra, 2009). The amylase production by *A. oryzae* was also reported as high in yeast extract and casein (Pederson and Neilson, 2000). Ramachandran et al. (2004) reported that peptone gave an increase in enzyme yield. Yeast extract and peptone is favored for the growth and synthesis of amylase by *Bacillus sp*. (Teodoro and Martins, 2000) also Vishnu et al. (2014) used yeast extract as a nitrogen source for *Bacillus* sp. VS 04.

Physical factors are important in any fermentation for optimization of biochemical production. The important physical factors that determine the bioprocess are pH, temperature, aeration and agitation (Kunamneni et al., 2005). In the present study, *B. licheniformis* yielded the maximum amylase production at 40°C, 160 rpm, and pH 7. The present results are matched with the results obtained by other investigation on the α-amylase enzyme from *B. licheniformis* BT5.9 (Ibrahim et al., 2013). Alariya et al., (2013) reported that the all the four bacteria yielded maximum amylase production at 35 to 40°C. Another study by Shafiei et al. (2012) found the optimal temperature for the amylase activity was at 45°C.

According to Vidyalakshmi et al. (2009), the growth temperature plays an important role not only in the growth of bacteria but also in enzyme production. A cultivation temperature beyond the optimal one caused a reduction in the catalytic rate of amylase, as either the enzyme or substrate became denatured and inactive. Ibrahim et al., 2013 reported that the maximum production of the enzyme was obtained when the bacteria was agitated at 100 rpm. The increase in enzyme production could be attributed by increased oxygen transfer rate, increased surface area of contact with the media components and better dispersability of the substrate during fermentation under agitated condition (Elibol and Ozer, 2001).

The pH of the growth medium plays an important role in terms of inducing enzyme production and morphological changes in the microbes (Pederson and Nielson, 2000; Kathiresan and Manivannan, 2006). Different organisms have different pH optima and decrease or increase in pH on either side of the optimum value results in poor microbial growth and reduction in enzyme production (Gangadharan et al., 2006; UL-Haq et al., 2002). Gangadharan et al. (2006) reported that pH 4 was the best for the production of amylase by *Bacillus amyloliquefaciens*. UL-Haq et al. (2002) reported pH=7.5-8.0 to be the best for the production of alpha amylase by *Bacillus subitlis.* Plackett-Burman designed an efficient technique for medium component optimization (El-Sharouny et al., 2015). Results obtained in this study are in accordance with others findings where it reported that soluble starch and yeast extract play an important role in enhancing the α-amylase activity (Abdel-Fattah et al., 2013). Also, inoculum size has significant effect on the enzyme production. Therefore, a suitable inoculum size is needed to have the highest enzyme production as lower inoculum size was able to slow down the biomass proliferation. Thus, the degradation of the substrates by the microbes is slower and affects the metabolite production (El-Sharouny et al., 2015). Data in the present results revealed that the amylase production raised by 1.3 fold for *B. licheniformis* AH214 when growing in optimized medium. These results agreed with those of Abdel-Fattah et al. (2013) who reported that the optimal value of the enzyme activity by thermotolerant *B. licheniformis* AI20 is more than two folds of the basal medium of optimization process.

The enzyme activity by *B. licheniformis* (43.42 U/mg protein) was stable up to 40°C, and pH 7.5, above or below this values the activity of the tested crude enzyme decreased gradually. Also the crude enzyme retained 100 and 60% of its activity for up 2 and 12 h, respectively, at 1.5M (8.7%) NaCl. In the last years, several extracellular halophilic α-amylases have been purified from moderate halophiles. This enzyme being relatively stable at pH 6.5-7.5 and temperature 45°C. The purified enzyme was highly active in a broad range of NaCl concentrations (0-4 M) with optimal activity at 0.25 M NaCl. This amylase was highly stable in the presence of 3-4 M NaCl (Shafiei et al., 2012). The α-amylase by a

thermotolerant *B. licheniformis* AI20 isolate had an optimal temperature and pH of 60-80°C and 6-7.5, respectively (Abdel-Fattah et al., 2013). The α-amylase was highly stable over a broad range of temperatures (30-90°C), pH (6.0-12.0), and NaCl concentrations (0-20%) (Moreno et al., 2013). Many other amylases from moderately halophilic *Bacillus* showed to exhibited higher optimal salinity with more NaCl stability than that obtained in our study.

Conclusion

The marine environment is a good source for valued microflora hat need to be explored. The present study explored our natural environments, searching for halophilic marine bacteria producing extracellular degradative enzymes. The findings of the present study suggested that the halophilic *B. licheniformis* AH214 isolated from the local marine environment could be used for amylase production. Optimization of the fermentation medium components, environmental conditions and inoculums size for the isolate was applied using One Variable at a Time approach and Plackett-Burman design, leading two fold increased in enzyme activity**.** The produced crude amylase enzyme was stable up to 40°C, pH 7.5 and 1.5 M NaCl.

Conflict of interest

The authors have not declared any conflict of interest.

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