Egypt. J. Chem. **Vol. 65,** No. 10 pp. 225 - 238 (2022)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Thermostable Protease, Amylase and Lipase Enzymes of Thermophilic Bacteria Isolated from Egyptian Hot Springs

Eman khaled[1], M. Fayez[2], Mona H. Badawi[2], Wafaa Mohamed Abd El-Rahim[3], Haitham Shawky[3], Hassan Moawad[3].

[1] Cairo University Research Park, Cairo University, Giza, Egypt
[2]Department of Microbiology, Faculty of Agriculture, Cairo University, Giza, Egypt
[3]Department of Microbiology, National Research Centre, Cairo , Egypt

Abstract

Geothermal water samples collected from Dakhla Oasis, Kharga Oasis, Pharaoh Baths and Ras Sedr hot springs in Egypt were explored for the isolation of industrially efficient thermostable amylase-, protease- and lipase- producers. Of 170 enzyme-producing isolates secured from colonies developed on agar media, 12 superior ones were subjected to morphological characteristics and biochemical profiles. Cells appeared cocci and spiral with the majority as *bacilli*. Adopting the Diagnostics GN/GP 24 (Ref. 1001, 1002), the tested candidates successfully utilized various substrates as carbon and nitrogen sources besides their abilities to produce a number of exoenzymes. Six potent amylase-, protease- and lipase-producing thermophiles. two for either, were further identified by 16S rRNA gene sequencing. The amylase-producers were identified as *Aneurinibacillus thermoaerophilus* and *Bacillus licheniformis* with respective similarity percentages of 99.48 and 100. Genetic analysis of protease-producers showed the similarity to *B. licheniformis* and *B. sonorensis* (90.02 and 98.14 % identities). Both lipase-producing isolates described as *B. licheniformis* with similarity percentages of 98.80 and 100). The Plackett-Burman multifactorial Design was implemented to screen the limiting components for growth and subsequent use of Central Composite Design to tailor a suitable medium that supports exponential growth and consequently the protease production of the tested thermophilic bacterium. For optimization of protease production by *Bacillus licheniformis* (isolate DO24), the applied Placket-Burman methodology screened eleven effective factors including skim milk, peptone, yeast extract, $CaCl_2$, $MgSO_4.7H_2O$, NaCl, KH_2PO_4, inoculums size, pH, temperature and incubation time. Among those; skim milk, yeast extract, inoculum size and incubation time deemed the most influential factors. Adopting the Central Composite Design, the optimized protease activity was achieved at the respective component records of 40 ml L^{-1}, 4.0 g L^{-1}, 40 ml L^{-1} and 24 h. This study proves that Egyptian hot springs are beneficial reservoirs for thermostable enzyme-producing microbiota of great importance for various bio-industrial applications.

Key words:
Hot springs, thermophiles, thermostable enzymes, 16S rRNA gene sequencing, Plackett-Burman Design, Central Composite Design

Introduction

Hot springs, the very special niches, are those produced by the immergence of geothermal heated groundwater from the earth's crust and occur in a few widely separated locations of the world and are observed in the areas of active volcanism or those having active volcanoes. Of the popular ones, are Yellowstone national park (U.S.A), Suryakund (Bihar, India), Sohna hot spring (Sohna, Delhi), Atri hot spring (Khordha, Odisha), Manikaran hot spring (Himachal Pradesh, India), Cimanggu hot spring (West Java, Indonesia), hypersaline and heliothermal Ekho Lake (East Antarctica), Garampani hot spring (Assam), Unapdev and Sunapdev hot spring (Maharashtra, India) and Bakerswar hot spring (West Bengal, India) [1]. These particular environments are densely accommodated by a great variety of microorganisms that possessing the capability to withstand the prevailing rigorous conditions. They are developing unique resistance to perform reactions and activities at either acidic or alkaline pH, temperatures falling in the range 45-140 °C, or very close to the freezing point of water, high

*Corresponding author e-mail: renadkhaled4@gmail.com.; (Eman Khaled).
Receive Date: 01 January 2022, Revise Date: 14 February 2022, Accept Date: 27 February 2022
DOI: 10.21608/EJCHEM.2022.113659.5190

pressures or in non-aqueous environments and water/solvent mixtures. Such characteristics represent an excellent biotechnological tool to support and catalyze non-limited reactions in severe conditions [2]. Here, it could be realized that thermophiles, in general, are proved a rich source of extremoenzymes and therefore gained an importance in what so called "white biotechnology" which is defined as the use of several microorganisms and theirs in the industrial processes beside the production of certain materials and chemicals [3, 4]. The whole cells, their macromolecules or metabolites are commonly used in bioremediation, bioenergy, biomining and biosurfactant production [5, 6]. Among the thermophile cell components, extremoenzymes are occupying a non-tiny place on the map of the biotechnological applications of microorganisms in the various industrial processes. Actually, the thermoenzymes are considered among the pillars of industrial processes referring to the fact that higher temperature are necessary to improve the solubility of many reaction components (mainly polymeric substrates) and minimize the hazards of contamination. Of those enzymes; amylases, cellulases, chitinases, esterases, lipases, pectinases, proteases, pullalanases and xylanases are of special concern. Principally; the industrially important amylases, proteases and lipases account the majority of the total global enzyme sale.

Initially, the term amylase was used originally to designate an extracellular enzyme capable of hydrolyzing α-1,4-glucocidic linkages in polysaccharides containing three or more glucose units. This enzyme is indispensible in the various biotechnological approaches, cosmetics, nutrition and pharmacy [7]. Amylase produced by thermophiles might be thermo stable, a characteristic that is necessary for several applications requiring relatively high temperature such as starch industry which involves the processes of gelatinization and liquefaction [8]. [9] Reported that, the *Bacillus* species; B. amyloliquefaciens, B. licheniformis, B. stearothermophilus and B. subtilis are having the ability to synthesize the α- amylase. The results of 16S rRNA sequencing of [10] indicated that the amylase-producing LBKURCC190 isolates had the highest similarity (> 98 %) with *Bacillus*.

Proteases, as some of the cornerstone exoenzymes unavoidable for non-limited industries, hydrolyze the peptide bonds present in proteins and polypeptides. They have a wide biotechnological applications such as leather, pharmaceutical and food industries as well as manufacture of protein hydrolyzate and waste processing industry. A vast array of thermophilic microbiota is of special importance, a phenomenon that attributed to their capabilities to catalyse numerous reactions at high temperatures. This results in increased solubility of the reactant and product and consequently accelerates the reaction rates. This is besides minimizing the microbial contamination risks and alleviation of the mesophilic bacterial activity [11]. [12] Isolated 36 thermophilic bacteria from Gavmesh Goli hot spring in Sareyn, North West of Iran. All the secured isolates were potentially protease producers, among those five were characterized by high enzyme activities. Morphological, biochemical and molecular analyses and the 16 S rRNA gene sequencing indicated that four isolates (DH15, DH16, DH20 and DH29) were assigned as Thermomonas hydrothermalis while one (PA10) was identified as *Bacillus altitudinis*.

Lipases, as well, are universally applied in a great number of industries; they are defined as triacylglycerol acylhydrolases that biocatalyze the hydrolysis of long chain triacylglycerols and are widely present in microorganisms, plants and animals. Of those originated from bacteria seemed stable and easier for cultivation [13]. Several industries like detergents, foods, cosmetics, pharmaceutics and biodiesel production are conspicuously relying on lipases [14, 15]. These particular enzymes are selected for each application according to their specific substrate, position of fatty acid esters and stereospecificity, beside their temperature and pH stability. The phylogenetic analysis of the potentially lipase-producing candidates isolated by [16] belonged to the genus *Geobacillus* with 98 % similarity with G. jurassicus DS1 and G. uzenensis U. In addition, the isolate CHI1 had 99 % identity with G. stearothermophilus IFO12550T.

Indeed, the hot springs in Egypt have not yet been investigated in respect to microbial community composition and biotechnological prospects. Therefore, the present work introduces original information on the dominant thermophilic bacteria prevailing these harsh conditions and possessing extraordinary biotechnological and environmental potential. This is an attempt to guarantee special microbiome store for industrial applications.

Experimental Procedures

1. Sampling and in situ measurements

Representative samples of geothermal waters were taken from four hot springs (Fig. 1) along the period extended from March to June (2019), those are Dakhla Oasis (Site A), Kharga Oasis (Site B) Pharaoh Baths (Site C) and Ras Sedr (Site D), Site A (25.7719 o N, 28.6265 o E), Site B (25.4390 o N, 30.5586 o E), Site C (29.1972 o N, 32.9562 o E)

and Site D (29.5933 o N, 32.7178 o E). Two samples were collected from Site C representing inside and outside, while one sample from each of the other sites was taken. The autochthonous thermophilic microorganisms are subjected to phase changes with high temperature and mass gradients. For microbiological determinations, samples were aseptically collected in sterile brown bottles (500 ml capacity), transported to laboratory and kept at 4 °C until analyses within 48 h of sampling. Water temperature and pH were in situ measured during sample collection.

Fig. 1. Map of sampling the geothermal waters collected from Dakhla Oasis (A), Kharga Oasis (B), Pharaoh Baths (C) and Ras Sedr (D) hot springs

2. Cultivation of thermophilic bacteria for enzyme activity assessments

The nutrient agar medium (Oxoid, UK) was used for cultivation of water thermophiles; it composed of (g L^{-1}): peptone, 5.0; yeast extract, 2.0; lab-lemco powder, 1.0; NaCl, 5.0; agar, 20.0 with pH 7.0. Cultures of each sampling site (in triplicate) were prepared on NA plates which were inoculated with 0.5 ml of the collected geothermal water samples. Plates were incubated for 12-24 h at 50 °C. Randomly, 170 colonies developed on agar plates were picked up, purified and transferred to nutrient broth medium containing 15 % glycerol and stored at -80 °C for further studies. All the secured bacterial isolates were experimented for amylase, protease and lipase activities adopting the agar disc diffusion technique [17]

2.1. Screening of amylase-producing thermophiles

Amylase activity of the bacterial isolates was assessed based on their ability to degrade starch in the NA medium containing 10 g L^{-1} starch with incubation for 12-24 h at 50 °C. Clear zones around developed colonies indicate amylase positive. The amylolytic index was calculated according to the equation of [18] as follows:

Amylolytic Index (AI) = diameter of clear zone (cm) / diameter of colony (cm), with an assumption that the higher amylolytic index the higher enzyme activity. The superior amylase producers were selected for further investigations.

2.2. Isolation of protease-producing thermophilic bacteria

For screening the extracellular protease producers, isolates were spotted on Thermus Medium Modified Agar (TMMA) of the following composition: skim milk, 2.0 %; peptone, 0.05 %; yeast extract, 0.05 %; $MgSO_4.7H_2O$, 0.01 %; NaCl, 0.1 %; K_2HPO_4, 0.1 % and agar, 2.0 %. Incubation took place for 24-48 h at 50 °C. Clear zones surrounding formed colonies represent positive protease-producers [19]. Isolates of the widest zones were chosen for further studies.

2.3. Primary screening for lipase-producers

Bacterial cultures were examined for lipase activity on NA medium containing 10 ml L^{-1} tween 80. The isolates were spot-inoculated on the agar medium and incubated for 12-24 h at 50 °C. Lipase positivity was indicated by opaque zone formation surrounding the developed colonies [7]. Positive cultures that showed the widest zones of hydrolysis around the colonies were selected for further determinations

3. Cell morphology and biochemical characteristics of enzyme producing bacterial isolate

Among the examined 170 bacterial isolates, the twelve that successfully exhibited exceptional activities for the three enzymes tested were experimented for morphological characteristics and biochemical profiles adopting the Diagnostics GN/GP 24 (Ref. 1001, 1002). Cultures were introduced into tubes containing 3.5 ml NaCl solution (0.85 %). Aliquots of 100 µl were taken and spot-dropped on the Micro Titration Plates using micro pipettes. This analytical strategy allows the growth and activity of both Gram-negative and –positive bacteria. Twenty four hours later at 37 °C, biochemical reactions were monitored.

4. Identification of the pioneer enzyme -producing thermophiles

Apart from sampling site, six bacterial isolates representing two of the potent producers of either tested enzyme were further subjected to 16S rRNA gene sequencing to determine their taxonomic status.For molecular identification, the QIAamp DNA mini kits were applied (Ref 51304) for DNA extraction. PCR amplification of 16S rRNA genes used the forward primer U8–27 (′5-AGAGTTTGATC ACTGGCTCAG-3ˋ) and the reverse primer L14941514 (′5-CTACGG AGTACCTTGTTACGAC-3ˋ) modified by [20]. A total of 50 mL PCR amplification reaction mixture was made from Buffer 5 µl buffer + $MgCl_2$ 5 µl, dNTPs 5 µl, $MgCl_2$ 5 µl, DMSO 2.5 µl, primer U8–27 F 1.5 µl, primer L14941514 R 1.5 µl, enzyme 1.5 µl, bacterial DNA 2 µl, nuclease free H_2O 22.25 µl. The stages of the PCR program conducted were pre-denaturation performed at 94 °C for 5 min, followed by 34 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min, extension at 72 °C for 2 min and the final extraction at 72 °C for 10 min then 4 °C. The success of PCR product amplification was known through 1.2 % agarose gel electrophoresis. The existence of a single band of DNA indicated that the DNA was successfully amplified. The amplified PCR products were visualized using an electrophoresis gel. The emergence of PCR DNA bands marked the success of PCR. Furthermore, the PCR products were sequenced. The similarity of sequence was determined using BLAST (http:// www.ncbi.nlm.nih.Gov/Blast). Multiple sequences alignment used the Neighbour-Joining (N-J) method to determine the closeness of the isolates. The phylogenetic trees based on 16S r RNA sequences were constructed.

5. Optimization of conditions for high protease production and activity

The pioneer protease producer *Bacillus licheniformis* (isolate DO24) was investigated for the proper cultivation conditions that guarantee the extraordinary activity of the enzyme adopting the Plackett-Burman design. Eleven factors; skim milk, peptone, yeast extract, $CaCl_2$, $MgSO_4.7H_2O$, NaCl, KH_2PO_4, inoculum size, pH, temperature and incubation time were chosen for screening of significant factors affecting the activity. The two levels of effective factors, -1 and +1 selected by Plackett-Burman screening were considered (Table S1). Thereafter, four selected significant factors from the previous screening were subjected to the Central Composite Design with 31 runs at the five levels of -2, -1, 0, +1 and +2 (Table S2).

For the assessment of optimized enzyme activity level based on Plackett-Burman design, the following preliminary procedure was carried out: flasks of 50 ml capacity containing the nutrient components indicated in the design were inoculated with the bacterial strain. After incubation, cultures were centrifuged at 5000 rpm for 15 min. The cell-free supernatants were tested for enzyme activities.

The enzyme activity was measured performing the modified procedure of [19]. The cell-free supernatant was mixed in glass tube with 1.0 ml casein 1 % (w/v) in 0.02 M NaOH and 2.0 ml of 0.4 M phosphate buffer (pH 6). After 10 min incubation at 65 °C, 3.0 ml trichloroacetic acid 10 % was added to stop the reaction. The mixture was centrifuged at 12000 xg for 5 min. A volume of 0.5 ml supernatant was incubated with 2.5 ml of 0.1 M NaOH in 2 % (w/v) Na_2CO_3 for 10 min. Then, 0.25 ml of Folin phenolic reagent (commercial solution diluted 1:1 in distilled water) was added, mixed and kept at ambient temperature for 30 min. The absorbance was measured at 750 nm in 1-cm quartz cuvette which was subsequently converted to mg of tyrosine/1 using a tyrosine calibration curve (mg tyrosins/1 vs. absorbance). One unit of the enzyme (EU/ml) is defined as the amount of enzyme that produces an absorbance at 750 nm equivalent to 1 µmol of tyrosine/min under the measurement conditions.

6. Statistical analysis

Results were analyzed and experimental matrixes were designed based on the Minitab® software version 19 (Minitab Inc., State College, PA, USA). One-way ANOVA analysis of variance was carried out and the significant differences were determined based on p-values (0.05).

Results

1. Physico-chemical profiles of water samples

Due to the unique properties of the geothermal water samples and consequently the prevailing microbiota, the analysis of the samples was made accessible to several researchers exploring the biotechnological potentials of the thermophilic microorganisms. The collected water samples were analysed in the laboratory of Egyptian Microbial Culture Collection Network(EMCCN), NRC branch (http:emccn.eg.net).the analysis revealed a temperature range of 50-90 °C with variable pH and EC estimates (Table 1). Samples of Dakhla Oasis, Pharaoh Baths and Ras Sedr had neutral to slightly alkaline pH records of 7.05-8.16, the situation was different in case of Kharga Oasis water where pH was extremely alkaline (9.66). Fluctuations in EC values among the water samples

were more conspicuous. Kharga Oasis water was the only non-saline (EC, 0.71 $dSm^{-1)}$ while others seemed saline to highly saline (4.28-27.50 dSm^{-1}). In respect to nutrient pool of waters, sodium was the most dominant in quantities up to 268.1 meq L^{-1} followed by calcium (up to 84.0 meq L^{-1}). Potassium rarely detected and ranged between 0.12 and 3.40 meq L^{-1}. As expected, chlorine scored the highest amounts compared to other anions, being as high as 134 meq L^{-1} in average.

2. Enzyme activities of thermophilic isolates

2.1. Screening of thermostable amylase producers

Adopting the agar disc diffusion technique, the 170 thermophilic bacterial isolates secured from water samples differentially showed amylase activity (Table 2). The majority of Kharga Oasis water isolates (89.3 %) successfully produced the enzyme as indicated by clear zones of 0.8-2.3 cm surrounding developed colonies. Dakhla Oasis isolates (77.4 %) as well recorded appropriate enzyme activities, those of outside Pharaoh Baths ranked thereafter (69.8 %). Apart from the number of amylase producers, the diameters of clear zones appeared in culture medium containing starch were falling in the range 0.6-3.0 cm. The maximum amylolytic index obviously varied among the bacterial isolates, while an index of 4.0 was calculated for Ras Sedr isolates, the corresponding for Pharaoh Baths (inside) ones was 2.0.

2.2. Protease activity of thermophilic isolates

Regardless of the isolate source, 36 % of the total examined ones had measurable protease activity on culture medium supplemented with peptone (Table 2). Ras Sedr isolates, in particular, showed remarkable protease activities, a finding that expressed in the widest clear zones around the developing colonies. The enzyme producing candidates represented 72.7 % of total. On the contrary, the inside Pharaoh Baths isolates having the ability to produce the enzyme represented 8.3 % of total. Thermophilic isolates secured from the other hot spring samples allocated 22.6-54.8 % of the assayed ones. Apart from the sampling site, the enzyme activity assay varied from 0.9 to 4.0 cm around the developed colonies. In general, Dakhla Oasis and Ras Sedr thermophiles exhibited the highest activities where the estimated proteolytic indices were 1.1-3.0 for the former and 1.3 -3.0 for the latter.

2.3. Themophiles producing lipase

Among the collected water samples, those of Kharga Oasis accommodated the superior lipase-producing thermophiles, this is expressed in their high abundance percentage of 46.4 of the total 28 examined ones (Table 3). Isolates secured from Dakhla Oasis ranked thereafter with 35.5 % prevalence. Of the 45 bacterial candidates isolated from out-side Pharaoh Baths hot spring, only 8 showed lipase activity representing 15.1 % of total. The range of opaque zones surrounding the developed bacterial colonies speaks well on the enzyme activity. While the widest range of 1.0-3.5 cm measured for Kharga isolates, the narrowest of 0.6-2.0 cm was scored for out-side Pharaoh Baths correspondings. All of all, the active lipase-producers represented 26.5 % of the total 170 examined thermophiles.

The cell morphology and biochemical properties of 4 Gram-negative and 8 Gram-positive bacterial members of high enzyme-producing capabilities were assessed using the Diagnostics GN/GP 24 (Ref. 1001, 1002) system. Based on microscopic observations, cells were either cocci or spiral, with the majority of bacilli. Referring to the biochemical tests of Diagnostic GN kit (Table 4), the amylase-producing isolate PhBo22 secured from outside Pharaoh Baths positively acted with 13 tests (54.2 %) among the 24 examined ones, followed by protease-producing isolate RS3 obtained from Ras Sedr (11 positive tests, 45.8 %). The amylase-producer RS10 of Ras Sedr hot spring was the inferior (3 positives, 12.5 %). In respect to Diagnostic GP kit (Table 4), as high as 62.5 % of the tests were positive for amylase-producing PhBo9 isolate obtained from outside Pharaoh Baths hot spring, followed by the lipase-producer DO11 taken from Dakhla Oasis (58.3 % positives). The protease-producing isolates DO24, DO28, RS1 and RS7 appeared positives to 13 tests among the examined 24 ones.

4. Molecular identification of superior enzyme-producing members

Gene sequencing results of the most active amylase-, protease-and- lipase-producing isolates, two for either enzyme, were analyzed and compared to sequences in the GenBank database. The amylase-active isolates PhBo9 and RS10 corresponded to *Bacillus licheniformis* and *Aneurinibacillus thermoaerophilus* with 100 and 99.80 % identities respectively (Fig. 2). The phylogenetic analyses indicated that protease-producing isolates DO24 and RS7 belonged to *Bacillus licheniformis* and *Bacillus sonorensis* with 90.02 and 98.14 % identities (Fig. 3). Both lipase-producing thermophiles DO31 and KhO24 fitted the description of *Bacillus licheniformis* with respective similarity percentages of 98.80 and 100 (Fig. 4).

Table (1): Physicochemical profiles of water samples collected from the studied hot springs

Hot springs	*In situ* measurements		EC measurement	Cations (meq L^{-1})				Anions (meq L^{-1})		
	Temp(°C)	pH	EC (dSm^{-1})	Ca^{+2}	Mg^{+2}	Na^{+}	K^{+}	SO_4^{-2}	Cl^{-}	HCO_3^{-}
Dakhla Oasis	55	8.16	4.28	8.0	11.5	21.0	2.00	23.7	17.0	1.80
Kharga Oasis	55	9.66	0.71	2.0	2.0	3.5	0.12	2.3	4.0	1.30
Pharaoh Bath(outside)	50	7.07	25.10	62.0	28.0	220.3	3.10	62.5	250.0	0.90
Pharaoh Bath(inside)	55	7.69	27.50	84.0	44.0	268.1	3.40	113.2	285.0	1.30
Ras Sedr	90	7.05	15.37	47.0	16.0	101.0	2.10	51.4	114.0	0.70

Table (2): Amylase and protease producing thermophilic bacterial isolates grown on agar media inoculated with geothermal waters

Hot springs	No.+ve isolates	No.-ve isolates	% positives in total	Ranges of clear zones (cm)	Ranges of colony dia. (cm)	Activity indexes	No. of selected isolates	Isolate Codes
Amylase								
Dakhla Oasis	24	7	77.4	0.7-2.8	0.6-2.0	1.1-2.6	0	DO
Kharga Oasis	25	3	89.3	0.8-2.3	0.6-2.0	1.0-2.1	0	KhO
PharaohBaths (outside)	37	16	69.8	0.6-3.0	0.6-2.0	0.8-3.5	2	PhBo
Pharaoh Baths(inside)	10	26	27.8	0.7-2.4	0.6-2.0	1.0-2.0	0	PhBi
Ras Sedr	15	7	68.2	0.7-2.3	0.6-2.3	1.0-4.0	1	RS
Protease								
Dakhla Oasis	17	14	54.8	1.4-4.0	0.8-4.1	1.1-3.0	2	DO
Kharga Oasis	13	15	46.4	1.1-2.6	1.0-1.4	1.1-2.0	0	KhO
PharaohBaths (outside)	12	41	22.6	0.9-2.7	0.6-2.0	1.1-2.5	0	PhBo
PharaohBaths (inside)	3	33	8.3	1.0-4.0	0.7-1.0	1.1-2.0	0	PhBi
Ras Sedr	16	6	72.7	1.2-4.0	0.6-1.2	1.3-3.0	3	RS

Table (3): lipase producing thermophilic bacterial isolates grown on agar media inoculated with geothermal waters

	Lipase					
Hot springs	**No.+ve isolates**	**No.-ve isolates**	**% positives in total**	**Ranges of colony dia. (cm)**	**No. of selected isolates**	**Isolate Codes**
Dakhla Oasis	11	20	35.4	0.9-2.5	1	DO
Kharga Oasis	13	15	46.4	1.0-3.5	1	KhO
PharaohBaths (outside	8	45	15.09	0.6-2.0	0	PhBo
PharaohBaths (inside	7	29	19.4	1.0-2.8	1	PhBi
Ras Sedr	6	16	27.2	0.8-2.8	1	RS

Table (4): Biochemical characteristics of superior enzyme-producing thermophilic G-negative bacteria using Diagnostic GN24 (Ref.1001) test

Biochemical test code	Biochemical test name	Thermophile codes			
		PhBo22	**RS3**	**RS10**	**RS20**
URE	Urea	+	+	-	+
GLU	Glucose	-	-	-	-
H2S	Hydrosulphide	-	-	-	-
ARG	Arginine	+	+	+	+
ORN	Ornithine	-	-	-	-
LYS	Lysine	-	-	-	+
SCI	Simmons citrate	-	-	-	-
bGL	b- glucoronidase	+	+	-	+
NAG	N-acetyl-glucosaminide	-	-	-	-
SUC	Sucrose	+	+	-	-
TRE	Trehalose	+	+	-	-
MAN	Mannitol	+	+	-	+
LAC	Lactose	-	-	-	-
CEL	Celobiose	+	+	-	-
MAL	Malonate	-	-	-	-
GGT	Gamma glutamyl transferase	+	+	-	+
ESL	Aesculine	+	+	+	+
DUL	Dulcitol	-	-	-	-
ADO	Adonitol	-	-	-	-
SOR	Sorbitol	+	-	-	+
RHA	Rhamnose	+	-	-	-
RAF	Raffinose	-	-	-	-
INO	Inos(z)ito	+	+	-	-
bGA	b-galactosidase	+	+	+	+

Isolates PhBo22 and RS10 are amylase producers; isolate RS3 is protease producer; isolate RS20 is lipase producer.

5. Optimization of significant factors affecting protease activity using Plackett-Burman and Central Composite Design

The promising results obtained with *Bacillus licheniformis* strain as superior producers of protease were the bases for selection of this strain for further optimization to ensure higher thermophilic protease production. The procedure for designing various assemblies in the Plackett-Burman Design represented by -1 and +1 in an assembly indicates the lower and higher levels of the corresponding components. Effects of the 11 variables on protease activity of *Bacillus licheniformis* (isolate DO24) using Plackett-Burman Design were monitored. According to the model; skim milk, yeast extract, inoculum size and incubation time were the most significant (p, 0.05) in relation to the enzyme activity (Table 6). Other experimental variables had no apparent influence on the enzyme behaviour. The Central Composite Design was further applied to detect the optimum cultivation component estimates for the maximum enzyme production; those were 40 ml L-1 skim milk, 4.0 g L-1 yeast extract, 24 h incubation time with inoculum size of 40 ml L-1 (Fig. 5). The response surface curves illustrated in Figure (6) speak well on the interactions among the different factors particularly yeast extract and inoculum size.

Table (5): Biochemical characteristics of superior enzyme-producing thermophilic G-positive bacteria using Diagnostic GP24 (Ref.1002) test

Biochemical test code	Biochemical test name	Thermophile codes							
		DO24	DO28	DO31	KhO24	PhBo9	PhBi14	RS1	RS7
URE	Urea	-	-	+	-	+	+	+	+
MLT	Maltose	+	+	+	+	+	+	+	+
SOR	Sorbitol	+	+	+	+	+	+	+	+
LAC	Lactose	-	-	-	-	-	-	-	-
FRU	Fructose	+	+	+	+	+	-	-	-
ARA	Arabinose	+	+	+	+	+	+	+	+
RAF	Arabinose	-	-	-	-	-	-	-	-
bGA	β-galactosidase	+	+	+	+	+	+	+	+
ARG	Arginine	+	+	+	+	+	+	+	+
MAN	Manitol	+	+	+	+	+	+	+	-
TRE	Trehaloze	+	+	+	-	+	+	+	+
CEL	Cellibiose	-	-	+	+	+	+	-	+
MNS	Manos	+	+	-	-	+	+	+	+
RIB	Ribose	-	-	-	+	-	-	-	-
MLZ	Melezitóza	-	-	-	-	-	-	-	-
GLR	b-glucuronidase	-	-	-	-	-	-	-	-
NIT	Nitrate	-	-	-	-	-	-	-	-
ESL	Eskulin	+	+	+	+	+	+	+	+
MLB	Mellibioze	-	-	-	-	-	-	-	-
SUC	Saccharose	+	+	+	+	+	+	+	+
GAL	Galactose	-	-	-	-	-	-	-	-
XYL	Xylose	-	-	-	-	-	-	-	-
NAG	N- acetyl glucosamine	+	+	+	+	+	+	+	+
bGL	b-glucosidase	+	+	+	+	+	+	+	+

Isolate PhBo9 is amylase producer; DO24, DO28, RS7, RS1 are protease producers; PhBi14, DO31, KhO24 are lipase producers.

Table (6): ANOVA test for thermophilic protease of *Bacillus licheniformis* (isolate DO24)

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	6	0.24051	0.040085	12.39	0.007
Linear	6	0.24051	0.040085	12.39	0.007
Incubation time	1	0.03121	0.031212	9.65	0.027
Inoculum size	1	0.02881	0.028812	8.91	0.031
KH_2PO_4	1	0.01599	0.015987	4.94	0.077
Yeast extract	1	0.03499	0.034992	10.82	0.022
Peptone	1	0.01229	0.012288	3.80	0.109
Skim milk	1	0.11722	0.117216	36.24	0.002
Error	5	0.01617	0.003234		
Total	11	0.25668			

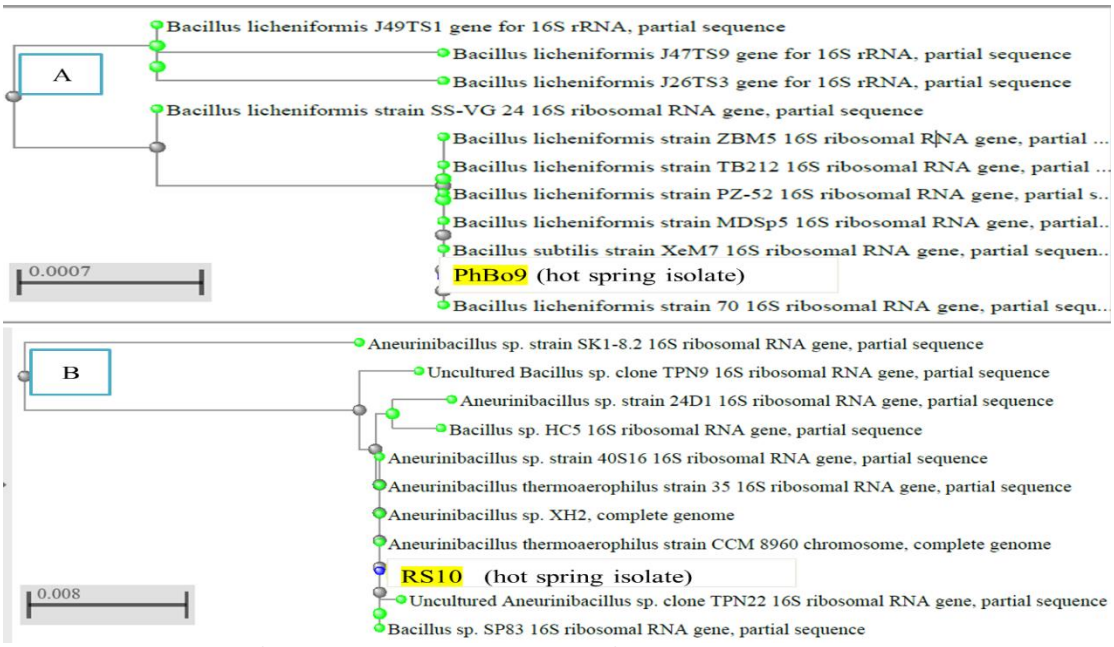

Fig. (2): Phylogenetic trees for amylase-producing *Bacillus licheniformis* isolate PhBo9 (A) and *Aneurinibacillus thermoaerophilus* isolate RS10 (B) obtained from Pharaoh Baths outside and Ras Sedr hot springs respectively.

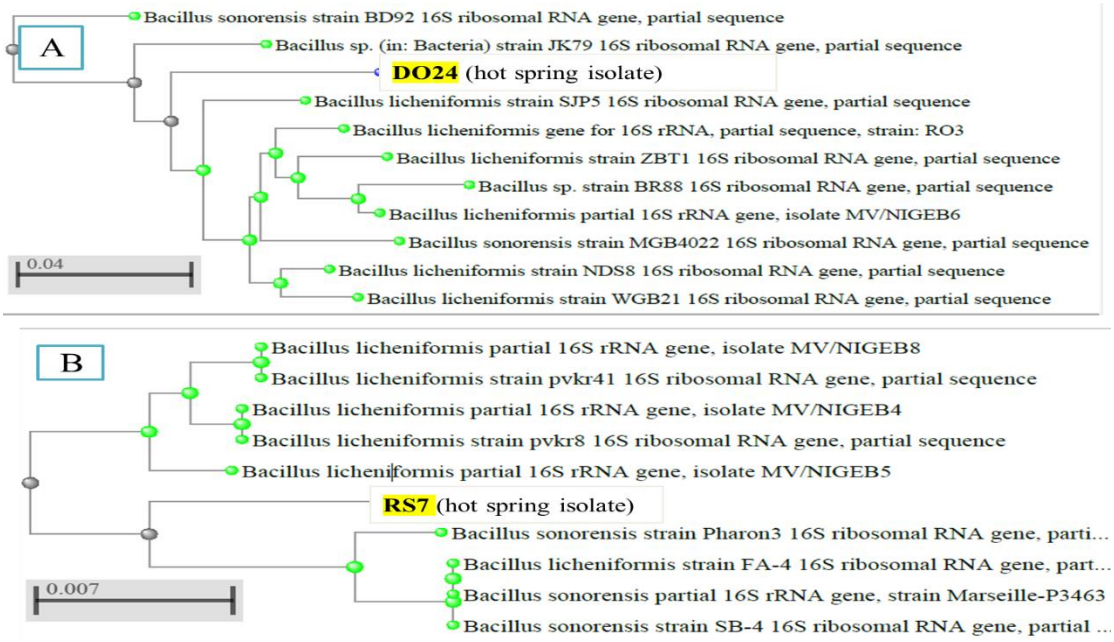

Fig (3): Phylogenetic trees for protease producing isolates DO24 (*Bacillus licheniformis*) and RS7 (*Bacillus sonorensis*) obtained from Dakhla Oasis and Ras Sedr hot springs respectively.

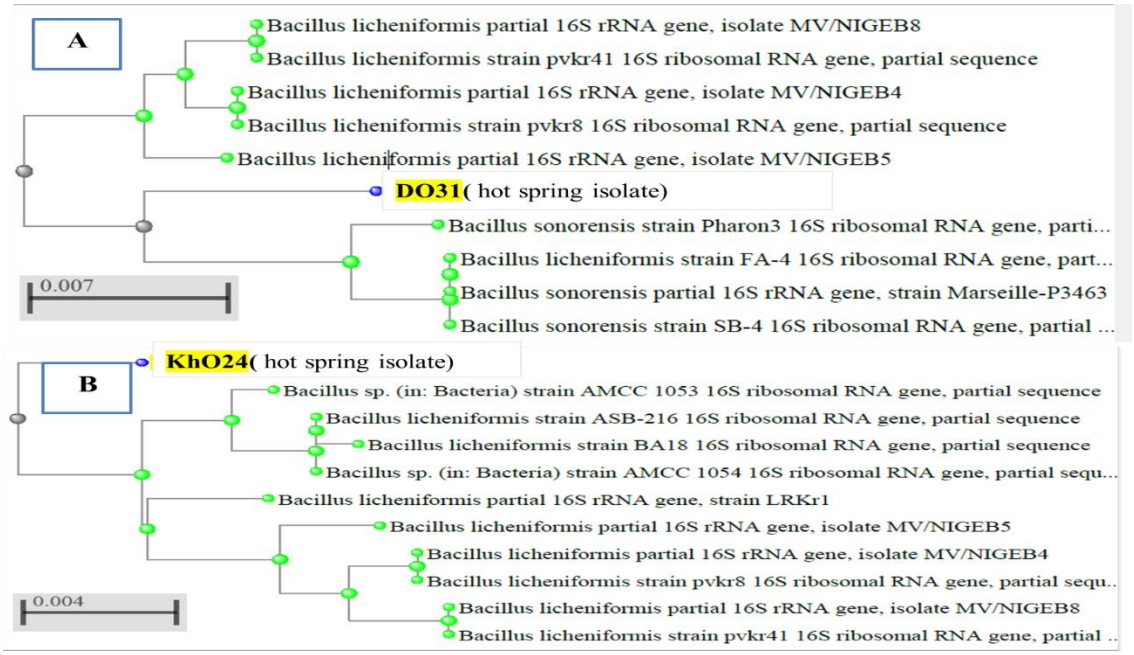

Fig. (4):Phylogenetic trees for lipase-producing *Bacillus licheniformis* isolates DO31 and KhO24 obtained from Dakhla Oasis (A) and Kharga Oasis (B) hot springs respectively.

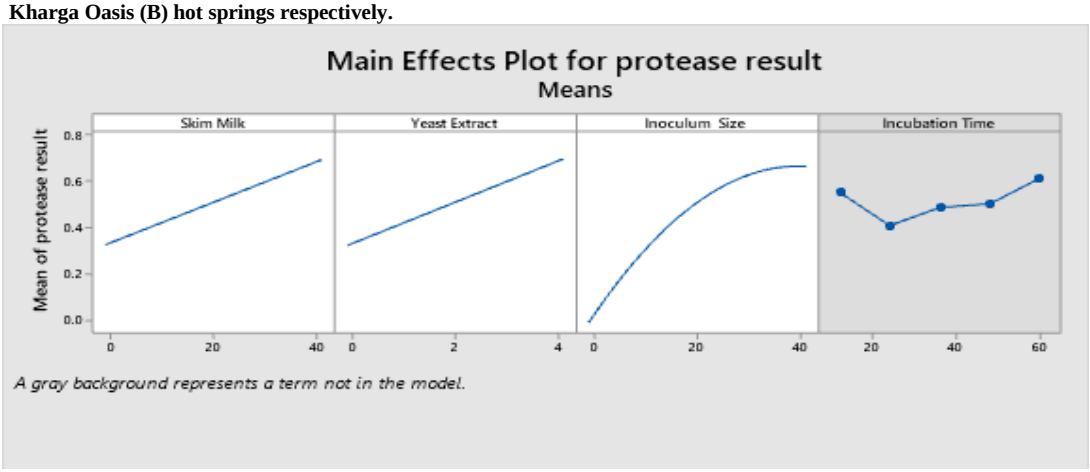

Fig (5): Central Composite Design results for the variables significantly supporting protease activity of *Bacillus licheniformis* (DO24).

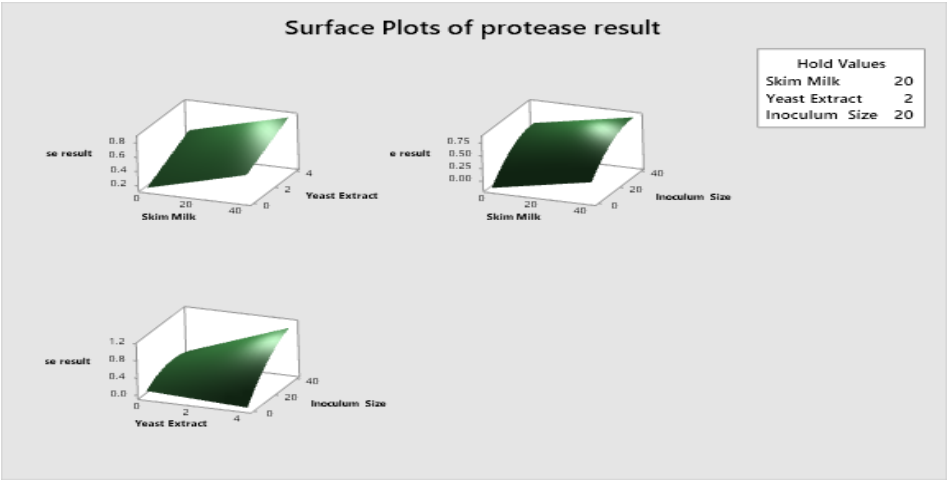

Fig (6). Response surface curves for the interacted factors in relation to proteolytic activity of *Bacillus licheniformis* (isolate DO24).

Table (S1). Plackett-Burman Design for screening the significant factors influencing protease activity levels

Runs	Skim Milk (g/l)	Peptone (g/l)	NaCl (g/l)	Yeast Extract (g/l)	KH_2PO_4 (g/l)	$MgSO_4.7H_2O$ (g/l)	$Cacl_2$ (g/l)	Tem. (°C)	pH	Inoculum size (ml/l)	Incubation time (h)
1	1	1	1	-1	1	1	-1	1	-1	-1	-1
2	1	1	-1	1	1	-1	1	-1	-1	-1	1
3	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
4	-1	1	1	-1	1	-1	-1	-1	1	1	1
5	1	-1	-1	-1	1	1	1	-1	1	1	-1
6	-1	1	-1	-1	-1	1	1	1	-1	1	1
7	1	1	-1	1	-1	-1	-1	1	1	1	-1
8	1	-1	1	1	-1	1	-1	-1	-1	1	1
9	-1	-1	-1	1	1	1	-1	1	1	-1	1
10	-1	-1	1	1	1	-1	1	1	-1	1	-1
11	1	-1	1	-1	-1	-1	1	1	1	-1	1
12	-1	1	1	1	-1	1	1	-1	1	-1	-1

Table (S2). Central Composite Design for the most protease supporting factors

Run	Skim milk	Yeast extract	Inoculum size	Incubation time	Run	Skim milk	Yeast extract	Inoculum size	Incubation time
1	1	1	1	-1	**17**	1	0	-2	0
2	1	0	0	0	**18**	1	1	1	-1
3	1	-2	0	0	**19**	1	1	1	1
4	1	0	2	0	**20**	1	1	1	1
5	1	1	-1	1	**21**	1	0	0	0
6	1	1	-1	-1	**22**	1	0	0	0
7	1	-1	-1	-1	**23**	1	1	-1	-1
8	1	-1	1	1	**24**	1	0	0	0
9	1	0	0	0	**25**	1	0	0	2
10	1	-1	-1	1	**26**	1	0	0	0
11	1	-1	1	-1	**27**	1	-1	-1	1
12	1	-1	-1	-1	**28**	1	0	0	0
13	1	0	0	0	**29**	1	0	0	-2
14	1	2	0	0	**30**	1	0	0	0
15	1	-1	1	1	**31**	1	1	-1	1
16	1	-1	1	-1					

Discussion

Hot springs are unique habitats for a vast array of thermophilic microorganisms. These particular environments are often nutrient-poor with relatively high temperatures. Here, microbiota residing such areas have to develop special strategies to cope and overcome the prevailing abiotic stresses. Exoenzyme production by these creatures is one of the prominent strategies necessary for utilization of organic substances that might be available in these harsh niches [21, 10, 22]. This authenticates that they represent an excellent source of a great number of enzymes for industrial purposes. In fact, Egypt is particularly gifted with a number of extremophilic environments such as hot springs, salt mines and deserts. There are several hot springs renowned for their medicinal properties, however, their microbial community structure and biotechnological applications are hardly monitored. In the present study, five water samples were collected from four well known hot springs in the country. Since the physico-chemical characteristics of a water supply reflect on the growth and activity of its microresident community, a partial profile of these properties was assessed. The average values of temperatures and pH of water samples were falling in the respective ranges of 51-90 °C and 7.05-9.66. The majority of waters seemed saline to highly saline with EC up to 27.5 dSm^{-1}. The nutrient pool

was exceptionally high particularly Ca (40.6 meq L^{-1}, in average) and Na (122.8 meq L^{-1}), with Cl at the highest average level of 134.0 meq L^{-1}.

It is indispensable to screen for geothermal microorganisms for their capabilities to produce exothermal enzymes, which could be an unavoidable and significant source for industrial consistency and cost-effectiveness. The major target of the present study was to screen and isolate thermophilic bacteria, from the selected hot springs, that having the ability to produce high amounts of thermo-stable enzymes. Representative water samples were introduced into various selective media for isolation of thermophiles using plate assay technique. After 24-48 h incubation at 50 °C, different colonies appeared on agar plates of the different culture media, from those a number of 170 isolates was assessed for amylase, protease and lipase activities. This is in an attempt to explore, for the first time, these particular enzymes from the rarely explored hot springs surveyed. Among the tested isolates, 37 secured from outside Pharaoh Baths hot spring showed the highest amylase activity that indicated by the widest clear zones of 0.6-3.0 cm. A number of 17 isolates successfully produced protease extracellularly on skim milk-containing medium. These isolates were obtained from Dakhla Oasis hot spring. Thirteen isolates were potentially lipase producers, those are dominating Kharga Oasis hot spring.

The pioneer 12 enzyme producing candidates representing Gram- negatives and -positives were subjected to morphological and biochemical characteristics. Microscopic examinations revealed that the majority of isolates are spore-forming bacilli. The Diagnostics GN/GP 24 tests indicated that the isolates are having the ability to utilize several substrates as carbon and nitrogen sources beside production of a number of exoenzyms.

Actually, morphological properties and biochemical tests, however, could only identify up to the genus level and perform a relatively low level of accuracy, therefore, the most active six isolates were further identified based on 16S rRNA gene sequencing. The majority were classified into *Bacillus* genus; the isolates DO24, DO31, KhO24 and PhBo9 fitted the description of *B. licheniformis* (89.90-100 % identity); isolate RS7 identified as *B. sonorensis* (98.18 %) and isolate RS10 as *Aneurinibacillus thermoaerophilus* (99.48 %). Interestingly, the 5 former strains have never been isolated from the Egyptian hot springs. In conformity with these findings, previous studies across the world reported *Bacillus spp*. as the dominant strains in hot springs. For example, [23] mentioned that 97.5 % of bacterial isolates recovered from Moroccan hot springs were belonging to *Bacillus spp*. The majority of thermostable enzymes reported so far were produced by *Bacillus* members exemplified by *B. licheniformis* [24], *B. stearothermophilus* [25], *B. subtilis* [26], *B. cereus* [27] and *B. mojavensis* [28]. [19] Isolated three proteolytic thermophiles from Moinit Coastal hot spring, North Sulawesi, Indonesia, one was very closely related to *Bacillus cereus* with similarity of 99 %, while the others showed the highest level of sequence homology with *Bacillus halotolerans* with 100 % identity. The ecological conditions of hot springs are well established as the moderate to high temperatures besides being nutrient-poor. Therefore, bacilli that well adapted to the hot environments and oligotrophic conditions are able to colonize and grow nicely in rigorous conditions such as hot springs, salt marches and desert soils. Besides, they are of great biotechnological interest due to their capabilities to restrict/minimize microbial contamination during industrial processes and working for long periods [29]. In addition, candidates within *Bacillus* have been extensively used for the production of alkaline proteases, as an example, mainly due to their chemoorganotrophic characteristics [30].

Indeed, the impacts of the prevailing nutritional and environmental conditions on production and activity of extracellular enzymes represent the cornerstone in the induction or repression of the enzyme by specific constituents. The availability of both carbon and nitrogen sources in the cultivation media are exerting regulatory effects on enzyme biosynthesis. Since the enzyme production costs are getting very high due to the increasing costs of growth substances and microbiological media as well as techniques, the development of inexpensive and novel procedures is highly appreciable from a commercial point of view. Therefore, the enzyme-producing industries and agencies are always searching for new and cheaper strategies to guarantee high enzyme production together with decreasing the market prices. In this respect, great efforts are directed at present for alternative means to minimize the enzyme production costs.

It is well established that, harnessing microbiota for their metabolic activities is certainly dependent upon the culture media composition. The approach for optimization strategy includes one variable at a given time and the media structure factors encompass; nutritional factors (carbon and nitrogen sources, in particular) together with physicochemical ones (enzyme-inducing substance, cofactors, incubation time, inoculum size, temperature, pH, inhibitors). Therefore, different media are having various stimulatory effects on

enzyme production. A part of this work seeks to evaluate enzyme-producing potentials of strains secured from different sources to examine the optimal cultivation conditions for scale-up production. Optimization of culture media components by the traditional "one-variable at-a-time" strategy is the most universally applied technique in biotechnology [31]. But, this method is time consuming and expensive when numerous variables are needed to be considered. In addition, this strategy doesn't detect the interactions among different production factors true and then it is difficult to define the optimal production conditions. Recently, the adoption of statistical approaches including Plackett-Burman multifactorial design [32] has gained momentum in culture media optimization. Besides, this system permits the screening of the major factors from a great number of experimental variables, and then to detect the most significant independent ones [33]. In the present study, the four significant variables; skim milk, yeast extract, incubation time and inoculum size for protease of *Bacillus licheniformis*, resulted in the greatest effects on the enzyme production. Although the mineral components, *i.e*. $CaCl_2$, $MgSO_4.7H_2O$, NaCl and KH_2PO_4 seemed to have no significant impacts on the production of tested enzymes, they are necessary for the growth of the producing bacteria themselves. Apart from skim milk, peptone and yeast extract which represent the major and indispensable constituents for bacterial growth and biomass production, other investigated elements exhibited significant effects on enzyme production. For the optimization of protease production by *B. licheniformis*, the inoculum size has shown significant influence, a finding that contradicting the results of [22] with alpha-amylase production by *Aspergillus oryzae*. Owing to the depletion of nutrients in culture medium and possible secretion of toxic substances by the growing bacterium [34], the suitable incubation time is among the prominent factors to maximize the production of a given enzyme. Based on the obtained results, incubation time significantly increased protease yield, a finding that disagreed with the results of [22]. These contradictions emphasize the importance of short or long incubation period effect on enzyme production that depending upon the bacterial strain. The effect of incubation temperature, as a physical factor, on protease activity was variable, whereas the activity increased up to 50 °C and decreased thereafter. [35] Reported a significant decrease in alkaline protease activity by *Bacillus mojavensis* as the fermentation temperature increased.

Several crucial salts are required to support the growth and multiplication of microbiota such as Ca^{++}, Mg^{++} and K^{+}, this might subsequently affect their enzyme activities. [36] Reported significant increase in alpha-amylase production in *Bacillus amyloliquefaciens* culture medium supplemented with $CaCl_2$. Addition of $MgSO_4.7H_2O$ to *Bacillus* sp. Ps-7 growth medium supported alpha-amylase production [37]. Effects of K_2HPO_4 on microbial growth are attributed to K^{+} and $HPO_4^{- -}$, the latter is a component of nucleic acids, nucleotides and phospholipids [38]. These results confirm the importance of such types of salts on the activity of metal-containing enzymes, although these salts showed no significant impacts on protease activities in the present study.

InS spite of media formulations applied in the present investigation and others that used synthetic components for enzyme production optimization were of great success, some attempts have been dealt with a variety of cost-effective natural materials, an approach that is extremely important in industrial-scale enzyme production on the economic point of view. In this respect, [28] Obtained 1783 U/ml protease by *Bacillus mojavensis* A21using the low cost wheat bran flour. [35] Reported significant increases in protease production by the same bacterial strain using chickpea and faba bean. They recorded as high as 9127 U/ml at the optimized cultivation conditions of 40 g L^{-1} chickpea, 30.0 g L^{-1} faba bean, 2.0 g L^{-1} NaCl, 1.0 g L^{-1} KH_2PO_4, 1.0 g L^{-1} K_2HPO_4, 0.5 g L^{-1} $CaCl_2$, 0.1 g L^{-1} $MgSO_4.7H_2O$ and 2 % inoculum size.

Conclusion

In total, 170 amylase-, protease- and lipase-producing thermophilic bacterial isolates were secured from Dakhla Oasis, Kharga Oasis, Pharaoh Baths and Ras Sedr hot springs. The six pioneers were identified as *Bacillus licheniformis* (4), *Bacillus sonorensis* (1) and *Aneurinbacillus thermoaerophilus* (1). This is the first report on the isolation of these novel bacteria that surprisingly revealed high activities for the tested thermostable enzymes of bio-industrial applications. Based on the findings, the quantities of the enzymes produced were dependent on several effective factors and enzyme-producing bacterial potential. In particular, their temperature and pH stability allow a great possibility of using for biotechnological purposes. However, further work encompassing purification is highly required for understanding characters of the enzymes more properly. All in all, this study highlights Egyptian hot springs as important reservoir of thermophilic microorganisms harboring industrially important enzymes.

Acknowledgements

I would like to deep thanks to the Egyptian microbial culture collection network (EMCCN) and eco-friendly products lab at National Research Centre, all of those who did help me in research work and provided all facilities and important information to complete the work in my research .And gratefully recognize the help of Cairo university Research Park (CURP) specially Biotechnology and Microbiology lab for supporting me with devices whatever I need.

References

[1] Ulfah, M.; Helianti, I.; Wahyuntari, B. and Nurhayati, N. (2011). Characterization of a new thermoalkalophilic xylanase-producing bacterial strain isolated from Cimanggu hot spring, West Java, Indonesia. Microbiol. Indonesia, 5(2): 139-143.

[2] Elleuche, S.; Schäfers, C.; Blank, S.; Schröder, C. and Antranikian, G. (2015). Exploration of extremophiles for high temperature biotechnological processes. Curr. Opin. Microbiol., 25: 113-119.

[3] Bergquist, P.L.; Morgan, H.W. and Saul, D. (2014). Selected enzymes from extreme thermophiles with applications in biotechnology. Curr. Biotechnol., 3: 45-59.

[4] Elleuche, S.; Schäfers, C.; Sahm, K. and Antranikian, G. (2014). Extremozymes—biocatalysts with unique properties from extremophilic microorganisms. Curr. Opin. Biotechnol., 29: 116-123.

[5] Zerva, I.; Remmas, N. and Ntougias, S. (2019). Diversity and biotechnological potential of xylan-degrading microorganisms from orange juice processing waste. Water, 11: 274.

[6] Kor-Bicakci, G.; Ubay-Cokgor, E. and Eskicioglu, C. (2020). Comparative analysis of bacterial and archaeal community structure in microwave pretreated thermophilic and mesophilic anaerobic digesters utilizing mixed sludge under organic overloading. Water, 12: 887.

[7] Kumar, G. and Srikumar, K. (2013). Biophysical characterization of thermophilic laccase from the xerophytes: *Cereus pterogonus* and *Opuntia vulgaris*. Cellul., 20: 115-125.

[8] Mehta, D. and Satyanarayana, T. (2016). Bacterial and archaeal α-amylases: diversity and amelioration of the desirable characteristics for industrial applications. Front. Microbiol., 7: 11-29.

[9] Dash, B. K.; Rahman, M.M. and Sarker, P.K. (2015). Molecular identification of a newly isolated *Bacillus subtilis* BI19 and optimization of production conditions for enhanced production of extracellular amylase. Biomed. Res. Int., 29(2): 439-444.

[10] Ardhi, A.; Sidauruk, A.N.; Suraya, N.; Pratiwi, N.W.; Pato, U. and Saryono, (2020). Molecular identification of amylase-producing thermophilic bacteria isolated from Bukit Gadang hot spring, West Sumatra, Indonesia. Biodiv. J. Biol. Div., 21(3): 994-1000.

[11] Abusham, R.A.; Rahman, R.N.Z.R.; Salleh, A.B. and Basri, M. (2009). Optimization of physical factors affecting the production of thermo-stable organic solvent-tolerant protease from a newly isolated halo tolerant *Bacillus subtilis* strain rand. Microbiol. Cell Fact., 8(1): 1-9.

[13] Hasan, F.; Shah, A.A. and Hameed, A. (2006). Industrial applications of microbial lipases. Enz. Microbiol. Technol., 39(2): 235-251.

[12] Abdollahi, P.; Ghane, M. and Babaeekhou, L. (2020). Isolation and characterization of thermophilic bacteria from Gavmesh Goli hot Spring in Sabalan geothermal field, Iran: *Thermomonas hydrothermalis* and *Bacillus altitudinis* isolates as a potential source of thermostable protease. Geomicrobiol. J., 38(1): 87-95.

[14] Tacias-Pascacio, V.G.; Virgen-Ortíz, J.J.; Jiménez-Pérez, M.; Yates, M.; Torrestiana-Sanchez, B.; Rosales-Quintero, A. and Fernandez-Lafuente, R. (2017). Evaluation of die-rent lipase biocatalysts in the production of biodiesel from used cooking oil: critical role of the immobilization support. Fuel, 200: 1-10.

[15] Tacias-Pascacio, V.G.; Ortiz, C.; Rueda, N.; Berenguer-Murcia, Á.; Acosta, N.; Aranaz, I.; Civera, C.; Fernandez-Lafuente, R. and Alcántara, A.R. (2019). Dextran aldehyde in biocatalysis: more than a mere immobilization system. Catalysts, 9(7): 622.

[16] Ovando-Chacon, S.L.; Tacias-Pascacio, V.G.; Ovando-Chacon, G.E.; Rosales-Quintero, A.; Rodriguez-Leon, A.; Ruiz-Valdiviezo, V.M. and Servin-Martinez, A. (2020). Characterization of thermophilic microorganisms in the geothermal water flow of el Chichón volcano Crater Lake. Water, 12(8): 2172.

[17] Al-Dhabi, N.; Esmail, G.; Ghilan, A. and Valan A.M. (2019). Composting of vegetable waste using microbial consortium and biocontrol efficacy of *Streptomyces Sp*. al-dhabi 30 isolated from the Saudi Arabian environment for sustainable agriculture. Sustainability, 11: 6845.

[18] Satrimafitrah, P.; Razak, A. R.; Hardi, J.; Puspitasari, D. J.; Yelenggete, I. and Rafik (2020). Thermostable amylase activity

produced by thermophilic bacteria isolated from Pulu hot spring, Central Sulawesi. J. Physics: Conf. Ser., 1434(1): 1-5.

[19] Ginting, E. L.; Kemer, K.; Wullur, S. and Uria, A. R. (2019). Identification of proteolytic thermophiles from Moinit Coastal hot-spring, North Sulawesi, Indonesia. Geomicrobiol. J., 37(1): 50-58.

[20] Weisburg, W.G.; Barns, S.M.; Pelletier, D.A. and Lane, D.J. (1991). 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol., 173(2): 697-703.

[21] Afridi, S.; Ali, N.; Niaz, M.; Hussain, S. I.; Farman, F. U. and Kim, B. C. (2020). Molecular characterization and production of bacterial amylases from Shahdara spring, Pakistan. Appl. Ecol. Env. Res., 18(2): 2611-2620.

[22] Far, E.B.; Ahmadi, Y.; Khosroshahi, Y. A. and Dilmaghani, A. (2020). Microbial alpha-amylase production: progress, challenges and perspectives. Adv. Pharm. Bull., 10(3): 350-358.

[23] Aanniz, T.; Ouadghiri, M.; Melloul, M.; Swings, J.; Elfahime, E.; Ibijbijen, J.; Ismaili, M. and Amar, M. (2015). Thermophilic bacteria in Moroccan hot springs, salt marshes and desert soils. Braz. J. Microbiol., 46(2): 443-453.

[24] Yilmaz, B.; Baltaci, M.O.; Sisecioglu, M. and Adiguzel, A.(2016). Thermotolerant alkaline protease enzyme from *Bacillus licheniformis* A10: purification, characterization, effects of surfactants and organic solvents. J. Enz. Inhib. Med. Chem., 31(6): 1241-1247.

[25] Abdel-Naby, M.A.; Ahmed, S.A.; Wehaidy, H.R. and Elmahdy, S.A. (2017). Catalytic, kinetic and thermodynamic properties of stabilized *Bacillus stearothermophilus* alkaline protease. Int. J. Biol. Macromol., 96: 265-271.

[26] Marathe, S.K.; Vashistht, M.A.; Prashanth, A.; Parveen, N.; Chakraborty, S. and Nair, S.S. (2018). Isolation, partial purification, biochemical characterization and detergent compatibility of alkaline protease produced by *Bacillus subtilis*, *Alcaligenes faecalis* and *Pseudomonas aeruginosa* obtained from sea water samples. Genet. Eng. Biotechnol. J., 16 (1): 39-46.

[27] Kotlar, C.; Ponce, A. and Roura, S. (2015). Characterization of a novel protease from *Bacillus cereus* and evaluation of an eco-friendly hydrolysis of a brewery byproduct. J. Inst. Brew., 121: 558-565.

[28] Haddar, A.; Agrebi, R.; Bougatef, A.; Hmidet, N.; Sellami-Kamoun, A. and Nasri, M. (2009) Two detergent stable alkaline serineproteases from *Bacillus mojavensis* A21: Purification, characterization and potential application as a laundry detergent additive. Bioresour. Technol., 100: 3366-3373.

[29] Wang, C.; Huang, R.; He, B. and Du, Q. (2012). Improving the thermostability of alpha-amylase by combinatorial coevolving-site saturation mutagenesis. BMC. Bioinfo., 13: 263-270.

[30] Rasmussen, R.S. and Morrissey, M.T. (2007) Marine biotechnology for production of food ingredients. In: Taylor, S.L., Ed., Advances in Food and Nutrition Research, Elsevier, New York, 52: 237-292.

[31] Haaland, P.D. (1989). Experimental design in Biotechnology, Marcel Decker, New York.

[32] Plackett, R. L. and Burman, J. P. (1946). The design of optimum multifactorial experiments. Biometrika, 33(4): 305-325.

[33] Bhunia, B.; Basak, B.; Bhattacharya, P. and Dey, A. (2012). Kinetic studies of alkaline protease from *Bacillus licheniformis* NCIM-2042. J. Microbiol. Biotechnol., 22 (12): 1758-1766.

[34] Hibbing, M. E.; Fuqua, C.; Parsek, M. R. and Peterson, S. B. (2010). Bacterial competition: surviving and thriving in the microbial jungle. Nat. Rev. Microbiol., 8(1): 15–25.

[35] Mhamdi, S.; Haddar, A.; Mnif, I. H.; Frikha, F.; Nasri, M. and Kamoun, A. S. (2014). Optimization of protease production by *Bacillus mojavensis* A21 on chickpea and faba bean. Adv. Biosci. Biotechnol., 5(14): 1049-1060.

[36] Gangadharan, D.; Sivaramakrishna, K.; Nampoothiri, K.M.; Sukumaran, R.K. and Pandey, A. (2008). Response surface methodology for the optimization of alpha amylase production by *Bacillus amyloliquefaciens*. Bioresour. Technol., 99: 4597-4602.

[37] Sodhi, H. K.; Sharma, K.; Gupta, J. K. and Soni, S. K. (2005). Production of a thermostable α-amylase from *Bacillus* sp. PS-7 by solid state fermentation and its synergistic use in the hydrolysis of malt starch for alcohol production. Process Biochem., 40(2): 525-534.

[38] Marison, I. W. and Von stockar, U. (1988). Fermentation control and the use of calorimetry. Biochem. Soc. Trans., 16(6): 1091-1093.