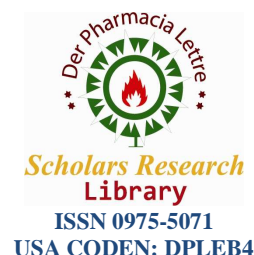




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Optimization of medium compositions and cultural conditions for increasing of extracellular protease production from *Bacillus cereus*- S6-3

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ABSTRACT

Microbial proteases are important group of enzymes that are widely used in pharmaceutical, food agriculture industries and bioremediation process. Present study aimed to isolate a new source of protease producing *Bacillus* and increase the enzyme produced by optimization of some culture conditions. Obtained results revealed that out of 153 bacterial isolates, only 30 of them were preliminary identified as *Bacillus* sp.. *Bacillus* S6-3 was the most potent isolate in protease production, which identified as a strain of *Bacillus cereus* according to its cultural and morphological characteristics, 16S rRNA gene sequences also confirmed the identification. To increase the protease yield from *Bacillus cereus* S6-3, some nutritional requirements (skim milk concentration, type of sugar and K_2HPO_4 concentration) and some environmental conditions (pH level, incubation temperature degree and inoculum size) were investigated. Obtained results showed that the optimum parameters for maximum protease production (327.32 U/ml) by *Bacillus cereus* S6-3 were 6.0 g/L skim milk, 10.0 g/L fructose, 0.5 g/L K_2HPO_4 , 1.0 g/L yeast extract, 1.0 g/L $MgSO_4 \cdot 7H_2O$, 5.0 g/L KCl, 0.2 g/L $CaCl_2 \cdot 2H_2O$, and 5.0 g/L NaCl, initial pH level 6.0, incubation temperature 37 °C, inoculum size (0.5%) and incubation period 48h.

Key words: *Bacillus*, protease, optimization, nutritional requirements, environmental conditions.

INTRODUCTION

Proteases are a group of enzymes, whose catalytic function is to hydrolyze peptide bonds of proteins and break them down into polypeptides or free amino acids. They constitute 59% of the global market of industrial enzymes [1]. Among the various proteases, microbial proteases are important group of enzymes that widely used in pharmaceutical, food, agriculture, detergent, leather, textile industries and bioremediation process [2, 3, 4, 5]

Protease is produced by a wide range of microorganisms including bacteria, molds and yeasts. In bacteria, this enzyme is produced mainly by many members belonging to genus *Bacillus* especially, *B. licheniformis*, *B. horikoshii*, *B. sphaericus*, *B. furmis*, *B. alcalophilus*, *B. cereus* and *B. subtilis* [6, 7]. Production of protease by different microorganisms is influenced by many parameters such as; type and concentration of carbon, nitrogen & phosphorus, pH level, incubation temperature degree, inoculum size and incubation period [8, 9, 10, 11, 12, 13, 14]. Current study was designed to isolate a new source of protease producing *Bacillus* and increase the enzyme produced by investigation of some nutritional requirements and environmental conditions.

MATERIALS AND METHODS

Isolation and screening of protease producing *Bacillus*

Soil samples were collected in sterile polyethylene bags from different agricultural areas of Sharkia governorate, Egypt. Serial dilution of the samples in sterile saline solution (0.9% NaCl) were made up to 1×10^{-5} g/ml, then 1 ml of each diluent was spread on the surface sterile nutrient agar plates and incubated for 48 h at 37°C. After incubation, *Bacillus* isolates were initially identified by Gram and Malachite green staining, then, picked up and purified by striking on sterile nutrient agar plates and incubated for 48 h at 37°C. *Bacillus* isolates with different morphological characteristics were selected, cultivated again on sterile skim milk agar plates and incubated under the same previous conditions. After incubation, the most potent *Bacillus* isolates in proteolytic activity (based on zone diameter around the colony) were selected for the subsequent studies.

Production of protease in different broth media

Preparation of *Bacillus* inoculum

A loopful from fresh culture of each tested *Bacillus* isolates was separately transferred into 20ml test tube containing 5ml sterile nutrient broth medium and incubated at 37 °C for 24h. After growth, each tested culture was centrifuged at 5000rpm for 15 min, then, the supernatant discarded and the bacterial precipitate was suspended with 5 ml of sterile saline solution and used as an inoculum. Production of protease by different *Bacillus* isolates growing in three different broth media (suggested by [15, 16, 17] was studied to select the most potent *Bacillus* isolate and most suitable medium for maximum protease production. The study was carried out for each tested *Bacillus* by using 1ml inoculum /100ml of sterile tested medium and incubated at 37 °C for 48h. After incubation, cultures of each tested strain were centrifuged at 5000 rpm for 15 min at 4°C. The amount of extracellular protease was determined in cell-free supernatant of tested *Bacillus* culture according to [18].

Identification of the most potent *Bacillus* isolate

The most potent *Bacillus* isolate on protease production was identified according to the method described by [19]. The Basic Local Alignment Search Tool (BLAST) data base [20] of National Center for Biotechnology (NCBI) Information was used to compare the sequence of 16S rDNA of the most resistant strains with known 16S rDNA sequences of bacteria, then, obtained alignments were constructed using phylogeny.fr program [21].

Effect of nutrients and conditions on protease production

The effect of some nutrients such as skim milk concentrations (4, 6, 8, 10 or 12 g/L); addition of 10.0 g/L sugar (fructose, glucose, rhamnose, sucrose or starch), K_2HPO_4 concentrations (0.25, 0.5, 1.0, 2.0 or 4.0 g/L) on extracellular protease production was investigated by the most potent *Bacillus* strain and most suitable medium. Under the optimal nutrients concentrations, the effect of some conditions including initial pH levels (5, 6, 7, 8 or 9), incubation temperature degrees (20, 28, 37 or 45 °C) and inoculum sizes (0.5, 1.0, 2.0 or 4.0 %) on protease production was investigated by selected strain.

Statistical analyses

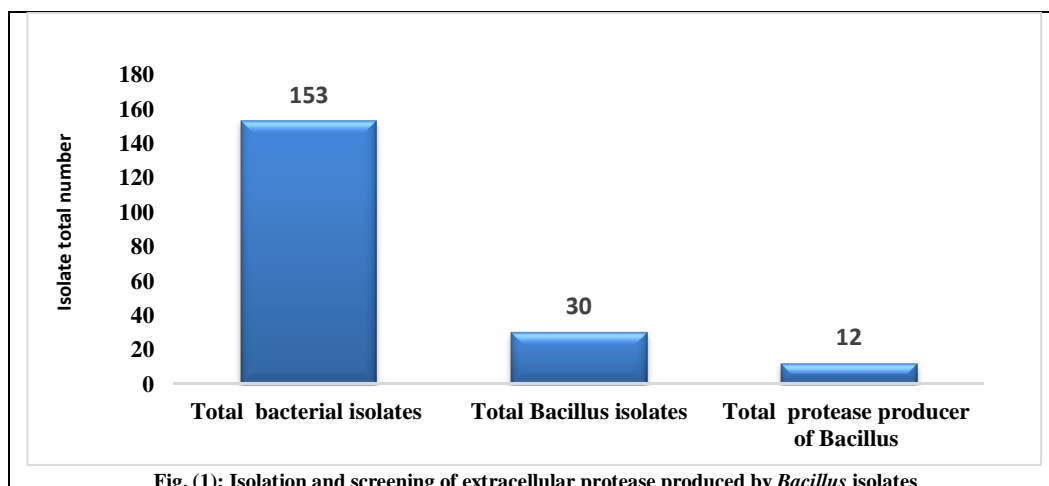
The tests were performed in 3 replications. Standard division calculated using the statistical software SPSS version 18.0. All analyses performed at $p \leq 0.05$.

RESULTS

Fermentation conditions play an essential role in the growth and metabolic production of microbial population. Considering the above-mentioned facts, the present study focused on the isolation of new protease producing *Bacillus* spp. and optimization of enzyme production by investigation of nutritional requirements and environmental conditions.

Isolation and screening of protease producing *Bacillus*

Data illustrated in Fig. (1) revealed that out of 153 bacterial isolates, only 30 of them were preliminary identified as *Bacillus* sp. because they were Gram positive aerobic endospore forming bacilli, while 12 (7.84%) *Bacillus* isolates showed their efficiency on protease production. Data recorded in Table (1) showed that all tested *Bacillus* isolates can grow and produce extracellular protease in various tested media. The maximum protease yield by different *Bacillus* isolates carried out in M2 followed by M1 and M3, which ranged from 15.19 to 112.09; from 0.28 to 92.93 and from 3.09 to 55.34 U/ml, respectively (Table, 1).



Obtained results revealed that the maximum proteolytic activity in M2 was carried out by *Bacillus* S6-3, which produced 112.09 U/ml followed by S6-10; S6-7; S6-1; S9-2; S6-1; S6-9; S9-5; S6-6; S6-8; S9-3 and S6-5, which attained their proteolytic activities to 89.08; 86.88; 71.48; 69.83; 69.28; 56.17; 51.49; 48.19; 17.85; 16.48 and 15.19 U/ml, respectively. Also, the maximum proteolytic activity in M1 was achieved by *Bacillus* S6-7, which produced 92.39 U/ml, followed by S9-5; S6-3; S6-5; S6-6; S6-10; S6-1; S6-9; S9-2; S6-8; S9-3 and S6-2, which attained their proteolytic activities to 88.25; 76.98; 69.01; 63.69; 62.77; 47.64; 45.90; 26.93; 10.15; 6.58 and 0.28 U/ml, respectively. However, the maximum proteolytic activity in M3 was carried out by *Bacillus* S6-10, which produced 55.34 U/ml, followed by S6-9; S9-3; S6-6; S6-5; S6-7; S6-8; S6-3; S9-5; S6-1; S9-2 and S6-2, which attained their proteolytic activities to 36.46; 34.91; 28.58; 20.97; 20.79; 18.59; 5.29; 4.11; 3.92; 3.83 and 3.09 U/ml, respectively (Table, 1).

Table (1): Effect of different media compositions on extracellular protease¹ production from *Bacillus* isolates

1: Assayed after 2 days of incubation at 37 °C according to (Anson ,1938); M1: Irfan et al., (2010); M2: Yossana et al., (2006); M3: Nascimento and Martins (2004); Values in the same column followed by the same capital letter are not significantly different, while values in the same raw followed by the same small letter are not significantly different according to ANOVA (L.S.D. $p \leq 0.5$), S.D.: Standard deviation.

<i>Bacillus</i> isolates	Production media		
	M1	M2	M3
	Enzyme unit mean \pm S.D. (U/ml)		
S6-1	47.64 ^{Aa} \pm 0.32	69.83 ^{Ab} \pm 0.42	3.92 ^{Ac} \pm 0.42
S6-2	0.28 ^{Ba} \pm 0.03	71.48 ^{Bb} \pm 0.16	3.09 ^{Ac} \pm 0.69
S6-3	76.98 ^{Ca} \pm 0.16	112.09 ^{Cb} \pm 0.00	5.29 ^{Bc} \pm 0.42
S6-5	69.01 ^{Da} \pm 0.16	15.19 ^{Db} \pm 0.16	20.97 ^{Cc} \pm 0.42
S6-6	63.69 ^{Ea} \pm 0.16	48.19 ^{Fb} \pm 0.16	28.58 ^{Dc} \pm 0.16
S6-7	92.39 ^{Fa} \pm 0.28	86.88 ^{Gb} \pm 0.16	20.79 ^{Cc} \pm 0.16
S6-8	10.15 ^{Ga} \pm 0.16	17.85 ^{Hb} \pm 0.16	18.59 ^{Cb} \pm 0.28
S6-9	45.90 ^{Ha} \pm 0.16	56.17 ^{Ib} \pm 0.16	36.46 ^{Dc} \pm 0.00
S6-10	62.77 ^{Ia} \pm 0.16	89.08 ^{Jb} \pm 0.16	55.34 ^{Ac} \pm 0.27
S9-2	26.93 ^{Ja} \pm 0.16	69.28 ^{Ab} \pm 0.16	3.83 ^{Ac} \pm 0.16
S9-3	6.58 ^{Ka} \pm 0.16	16.84 ^{Kb} \pm 0.16	34.91 ^{Dc} \pm 0.16
S9-5	88.25 ^{La} \pm 0.16	51.49 ^{Lb} \pm 0.16	4.11 ^{Bc} \pm 0.16

From previous data, it could be concluded that protease production is influenced by the medium composition and tested *Bacillus* isolate. In addition, M2 and *Bacillus* S6-3 were the most suitable medium and most potent strain for maximum protease production. Thus, they were selected for subsequent investigations.

Identification of the most potent strain

The gene analysis of 16S rRNA of *Bacillus* S6-3 revealed that the tested strain belongs to family bacillaceae, displayed 98% sequence similarity with *Bacillus cereus*, *B. anthracis* and *B. thuringiensis*. The tested strain was motile, showed β - hemolysis on blood agar, produced un-swollen sporangium, was resistant to penicillin but did not

produce a crystal protein. These characteristics are completely closer to *Bacillus cereus* than other suggested strains. Thus, *Bacillus S6-3* was identified as a strain of *Bacillus cereus*. Figs (2 and 3).

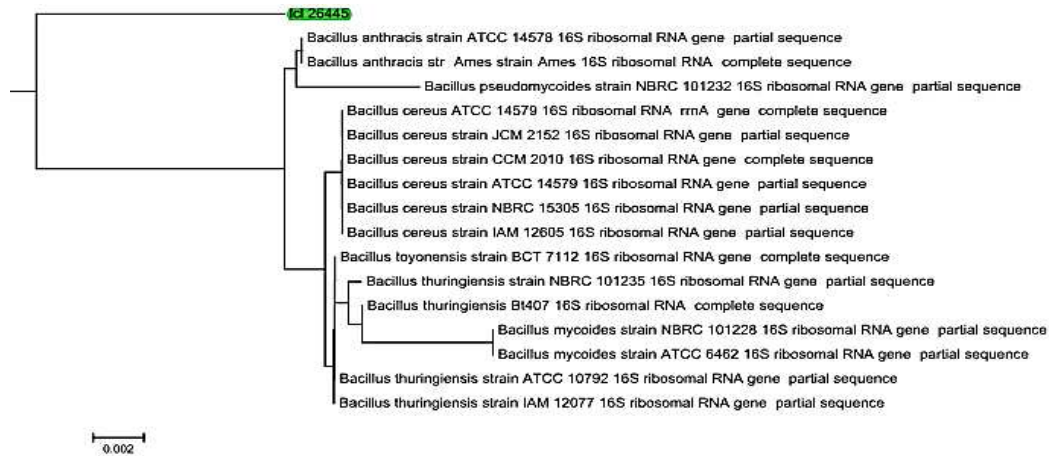


Fig. (2): Phylogenetic tree of *Bacillus S6-3* (ICI26445)

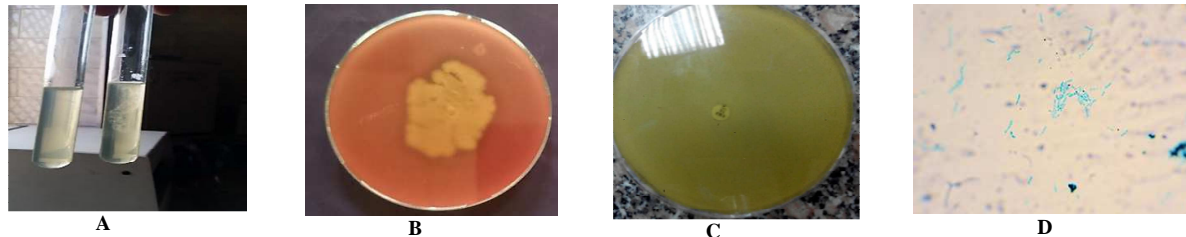


Fig. (3): Some cultural characteristics of *Bacillus S6-3*
 (A: Motility test (+), B: β -hemolysis on Blood agar (+), C: Resistance to ampicillin antibiotic disk. D. Unswollen spores)

Effect of some nutritional requirements on protease production

Effect of different concentrations of skim milk

The effect of skim milk concentrations and incubation period on extracellular protease by tested strain was evaluated in M2. Data illustrated in Fig. (4) revealed that production of protease by *Bacillus cereus*- S6-3 was affected by incubation period and skim milk concentration. In addition, protease production was gradually increased by increasing of skim milk concentration until 6 g/L, further, increase of skim milk concentration in production medium resulted a decrease in protease production.

Effect of incubation period on protease production by tested strain was variable based on the medium content of skim milk. In case of production medium contained 4g/L skim milk, the amount of protease produced was significantly stable after 24 and 48 h of incubation, then it decreased after 72 h. While, the amount of protease produced in tested medium which contained 6 or 8 g/L skim milk was significantly decreased by increasing of incubation period. Other impact was found with 10 and 12 g/L skim milk, the amount of protease produced was significantly increased by increasing of incubation period until 48h, then, it decreased with increasing of incubation period (Fig., 4).

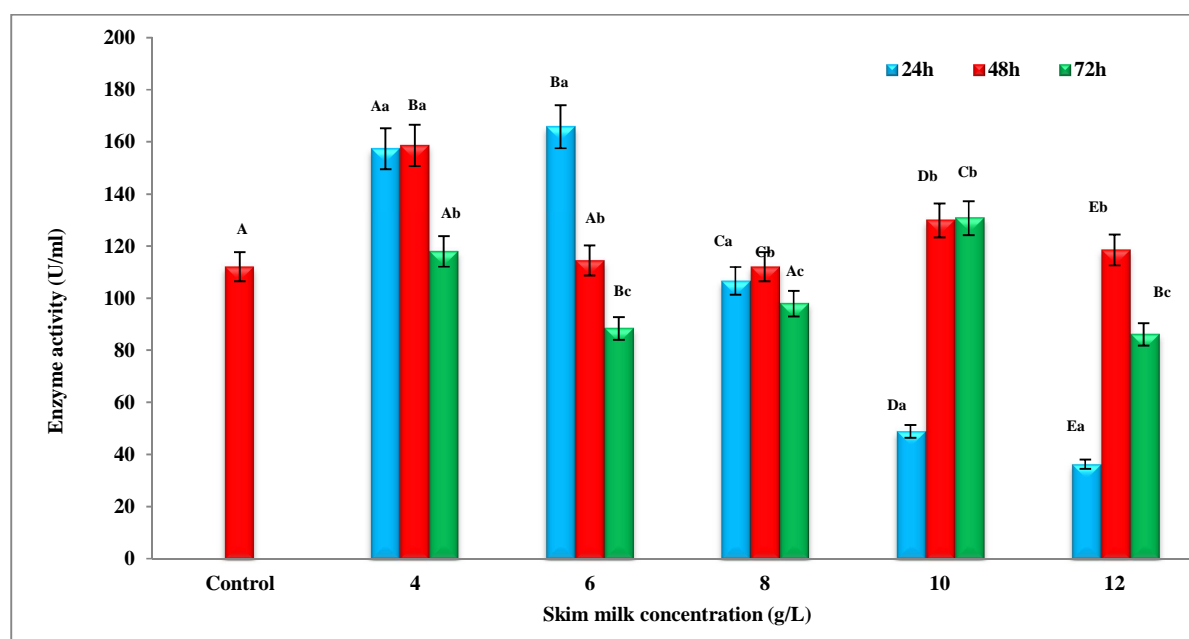


Fig. (4): Effect of skim milk concentration on extracellular protease production from *Bacillus cereus*- S6-3

* Values in the same column's color followed by the same capital letter are not significantly different, while values in the different column's color followed by the same small letter are not significantly different according to ANOVA (L.S.D. $p \leq 0.5$). Control: 8g/L skim milk.

Data illustrated in Fig.(4) showed that the maximum protease production by tested strain was found in M2 contained 6 g/L skim milk after 24h of incubation at 37 °C, which reached to 165. 81 U/ml, followed by 4, 10, 12 and 8g/L skim milk after 48, 72, 48 and 24 h of incubation at 37 °C, which reached to 158.66, 130.70, 118.51 and 106.68 U/ml, respectively. Thus, the most suitable concentration of skim milk for maximum protease production by *Bacillus cereus*- S6-3 was 6 g/L, which was mentioned for subsequent studies.

Effect of different sugars on protease production

The influence of different sugars (10.0 g/L) and incubation at different periods on extracellular protease production by *Bacillus cereus*- S6-3 was evaluated under the optimum previous conditions. Obtained results revealed that the addition of sugar in production medium had different effects on extracellular protease produced by tested strain. In case of monosaccharides sugar, such as fructose or rhamnose, the proteolytic activity of tested strain reached the maximum after 24h of incubation, then, it was gradually increased by increasing of incubation period with the first sugar, while, it had decreased after 48h of incubation and arisen again after 72h with second sugar (Fig.,5). Presence of glucose in production medium had led to increase the proteolytic activity of the tested strain, which attained to the maximum protease production after 48h of incubation and the yield of enzyme remained unchanged until 72h later.

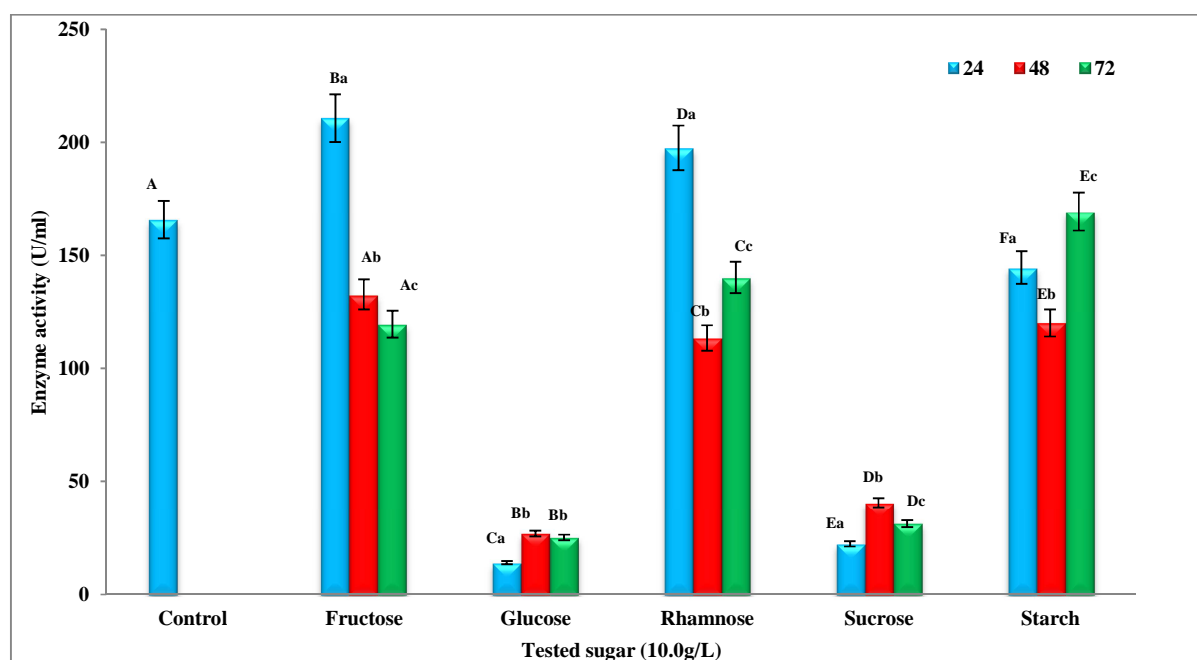


Fig. (5): Effect of type of sugar on protease production from *Bacillus cereus*- S6-3

* Values in the same column's color followed by the same capital letter are not significantly different, while values in the different column's color followed by the same small letter are not significantly different according to ANOVA (L.S.D. $p \leq 0.5$). Control: Sugar-free medium

Disaccharide, such as sucrose, had a different impact on protease produced by tested strain compared to other tested sugar, the proteolytic activity of tested strain was gradually increased by increasing of incubation period until 48h of incubation, then, the yield of enzyme had dropped with increasing of incubation period (Fig.,5). Polysaccharides, such as starch, had a different impacts on protease production from tested strain compared to both monosaccharides and disaccharides, the proteolytic activity of tested strain was relatively high after 24h of incubation compared to 48h, while it had arisen again after 72h of incubation (Fig., 5).

Data illustrated in Fig. (5) revealed that fructose (10.0g/L) was the most suitable sugar for maximum extracellular protease production by tested strain, which reached to 210.72 U/ml after 24h of incubation, followed by rhamnose, starch, sucrose and glucose, which reached to 197.52, 169.38, 40.50 and 26.93U/ml after 24, 72, 48 and 48h of incubation, respectively (Fig.,5). Thus, fructose (10.0 g/L) was selected for subsequent experiments.

Effect of K_2HPO_4 concentration on protease production

Proteolytic activity of *Bacillus cereus*- S6-3 using different concentrations of K_2HPO_4 was evaluated under the optimum previous conditions. Data illustrated in Fig. (6) revealed that supplementation of production medium with K_2HPO_4 caused different impacts on protease production by the tested strain. The proteolytic activity of tested strain was gradually increased by increasing of K_2HPO_4 concentration until 0.5 g/L, then, it decreased by increasing of K_2HPO_4 concentration, thereafter, it started to increase again with 4.0 g/L K_2HPO_4 . In addition, 48h was the optimum incubation period for maximum proteolytic activity by tested strain using various K_2HPO_4 concentrations, except, 4.0 g/L K_2HPO_4 gave the maximum proteolytic activity by tested strain after 24h of incubation.

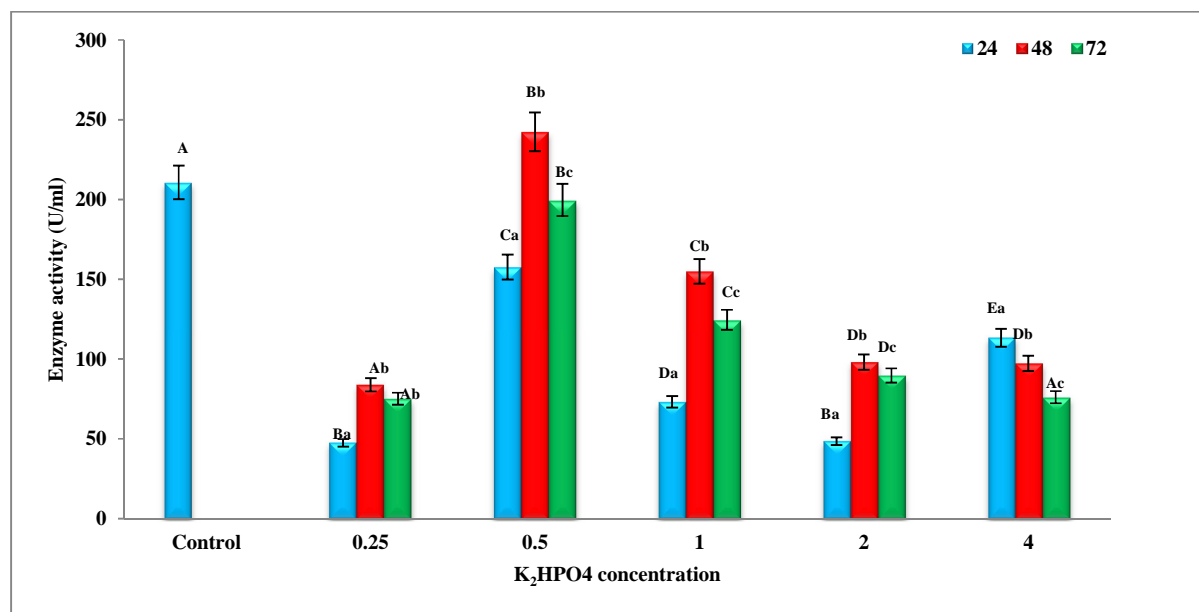


Fig. (6): Effect of K₂HPO₄ concentrations on protease production from *Bacillus cereus*- S6-3

* Values in the same column's color followed by the same capital letter are not significantly different, while values in the different column's color followed by the same small letter are not significantly different according to ANOVA (L.S.D. $p \leq 0.5$). Control: K₂HPO₄-free medium.

Data illustrated in Fig. (6) revealed that 0.5 g/L K₂HPO₄ was optimum concentration for maximum extracellular protease production by tested strain, which reached to 242.3 U/ml after 48h of incubation, followed by 1, 4, 2 and 0.25 g/L K₂HPO₄, which reached to 113.28, 154.99, 97.97 and 83.86 U/ml after 24, 48, 24, 48 and 48h of incubation, respectively. Thus, 0.5 g/L K₂HPO₄ was selected for subsequent studies.

Effect of some environmental conditions on protease production

Effect of initial pH levels

Proteolytic activity of *Bacillus cereus*- S6-3 under different pH levels and incubation periods were evaluated under the optimum previous conditions. pH level play an essential role in production and stability of protease produced by tested strain. Under different tested pH levels, 48h was the optimum incubation period for maximum yields of protease produced by tested strain. The proteolytic activity of tested strain was relatively high in neutral and acidic initial pHs compared to alkaline pH, while it was more stable in pH 7.0 compared to other tested initial pH level (Fig., 7).

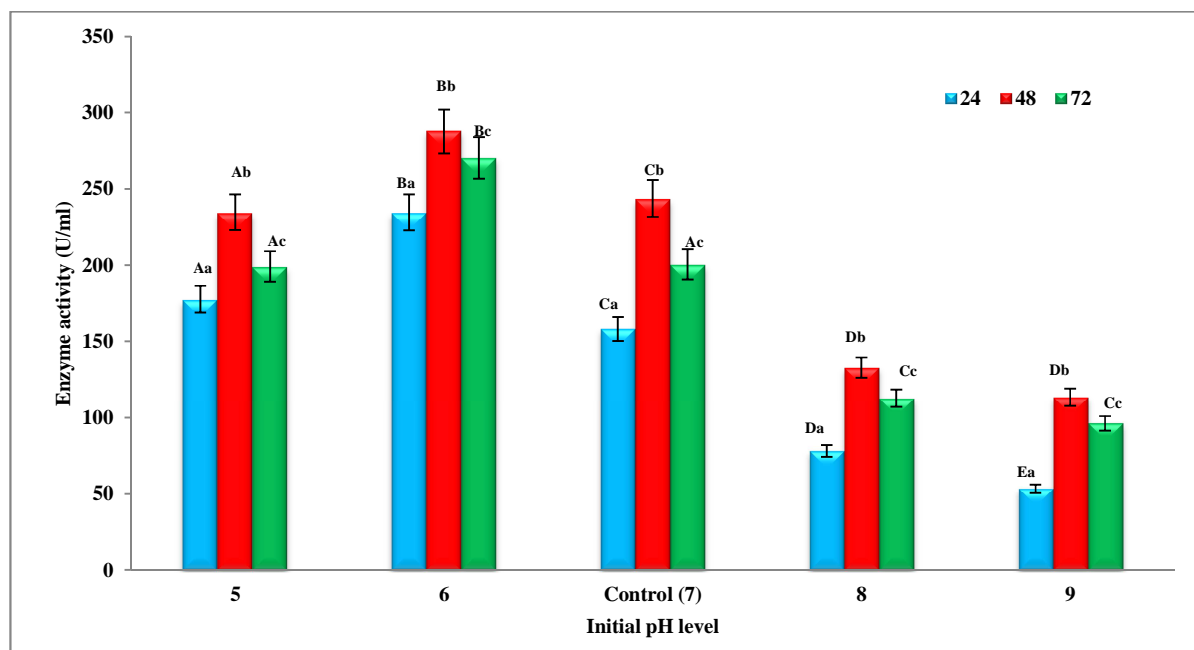


Fig.(7): Effect of initial pH levels on protease production by *Bacillus cereus- S6-3*

* Values in the same column's color followed by the same capital letter are not significantly different, while values in the different column's color followed by the same small letter are not significantly different according to ANOVA (L.S.D. $p \leq 0.5$).

Data illustrated in Fig. (7) revealed that pH 6.0 was the most suitable initial pH level for maximum extracellular protease produced by tested strain, which reached to 287.72 U/ml after 48h of incubation, followed by 7.0, 5.0, 8.0 and 9.0, which reached to 243.82, 234.65, 132.81 and 113.37 U/ml after 24, 72, 48 and 48h of incubation, respectively (Fig.,7). Thus, initial pH 6.0 was selected in subsequent tests.

Effect of incubation temperature degrees

Incubation temperature is a critical parameter affecting the bacterial growth and enzyme production by various microorganisms. Therefore, the proteolytic activity of tested strain under different incubation temperature degrees was investigated under the optimum previous conditions. Data illustrated in Fig. (8) revealed that *Bacillus cereus- S6-3* could grow and produce extracellular protease under different incubation temperature degrees. Under different tested temperatures, the proteolytic activity of tested strain was gradually increased by increasing of incubation period until 48h, then it reduced with incubation period increasing, except 20 °C, the proteolytic activity of tested strain was gradually increased by increasing of incubation period. The yield of extracellular protease produced by tested strain was relatively high at 37 and 45 °C of incubation compared to 20 and 28 °C.

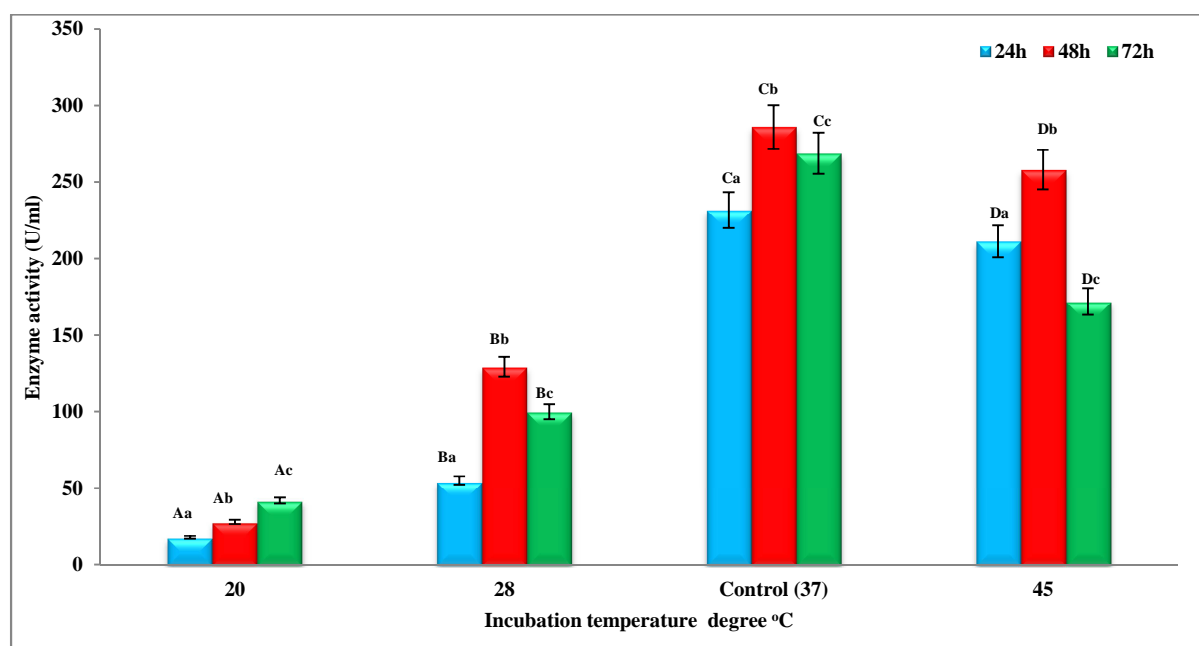


Fig. (8): Effect of incubation temperature degree on protease production by *Bacillus cereus*- S6-3

* Values in the same column's color followed by the same capital letter are not significantly different, while values in the different column's color followed by the same small letter are not significantly different according to ANOVA (L.S.D. $p \leq 0.5$).

The optimum incubation temperature for maximum production of extracellular protease by tested strain was 37 °C, which reached to 285.89 U/ml after 48h of incubation, followed by 45, 28 and 20 °C, which reached to 258.12, 129.32 and 41.97 U/ml after 48, 48 and 72h of incubation, respectively. Thus, incubation at 37 °C was selected for subsequent investigation.

Effect of different inoculum sizes

Proteolytic activity of *Bacillus cereus*- S6-3 using different inoculum sizes and different incubation periods were evaluated under the optimum previous conditions. Data illustrated in Fig. (9) revealed that the proteolytic activity of the tested strain was gradually increased by increasing of inoculum size until 0.5%, then, it decreased with inoculum size increasing. The optimum incubation period for maximum proteolytic activity by tested is stable (48h) for all tested inoculum size.

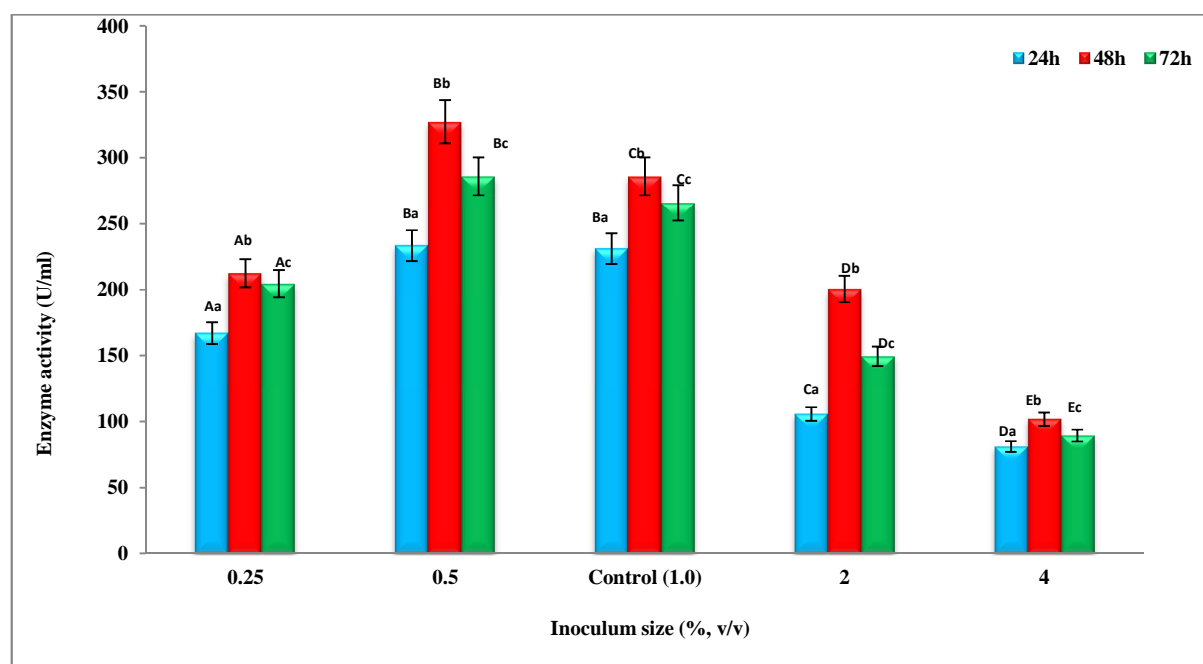


Fig. (9): Effect of inoculum size on protease production by *Bacillus cereus*- S6-3

* Values in the same column's color followed by the same capital letter are not significantly different, while values in the different column's color followed by the same small letter are not significantly different according to ANOVA (L.S.D. $p \leq 0.5$).

The optimum inoculum size for maximum production of extracellular protease by tested strain was 0.5%, which reached to 327.32 U/ml after 48h of incubation, followed by 1.0, 0.25, 2.0 and 4.0, which reached to 285.89, 212.47, 200.53 and 27.85 U/ml after 48 h of incubation, respectively.

Overall results, it could be concluded that nutritional requirements play an essential role in increasing of extracellular protease production by *Bacillus cereus*- S6-3 compared to tested environmental conditions. The most effective tested parameter in protease production by tested strain was skim milk, which increased the amount of enzyme into 32.4% compared to control, followed by fructose, initial pH level, inoculum size and K_2HPO_4 , which increased the amount of enzyme into 21.31, 15.79, 13.03 and 12.1%, respectively. The temperature degree (37°C) was an essential factor in protease production by tested strain because it contributed other optimum level of tested parameter in an increasing of protease produced.

DISCUSSION

There is no general medium for protease production by different *Bacillus* strains, each strain has its specific requirements for maximum protease production. Production of protease by different microorganisms is influenced by the medium compositions and environmental conditions. Therefore, it is necessary to investigate the influencing factors to obtain the highest yield of protease with commercial cost. Current study aimed to increase the protease production from a new *Bacillus* isolate by investigating some nutritional requirements and environmental conditions.

Present study showed that the proteolytic activity of each tested *Bacillus* isolate was different according to the composition of tested fermentation medium. The most suitable medium and the most potent strain for maximum proteolytic activity in the present study were M2 and *Bacillus* S6-3, which identified as a strain of *Bacillus cereus* according to the genes sequences of 16S rRNA. Obtained results revealed that the yield of protease production was affected by the contents of fermentation medium. Similar observations had also been found by previous studies, which revealed that protease production was highly affected by media composition including; variation in C/N ratio, and the presence of sugars, ions, and salts [5, 22, 23].

Our study showed that skim milk (6 g/L) was the most enhanced parameter in protease production from *Bacillus cereus*-S6-3, which increased the enzyme yield to 32.4% compared to control. In addition, the concentrations of skim milk below or above 6 g/L were decreased the protease yield. Protease is an inducible enzyme, which need a substrate to induct the microorganism to excrete it in fermentation medium. Thus, increasing of skim milk concentration in the fermentation medium led to increase of protease produced until the skim milk concentration reduced the dissolved oxygen in fermentation medium or caused a feedback inhibition on protease production [24, 25]. Obtained results supported by [8], who reported that skim milk was the most suitable nitrogen source for maximum protease production by *Bacillus* sp.N-40, which increased the enzyme yield to 43%. In addition, many investigations had highlighted to the importance of skim milk as a nitrogen source for maximum proteolytic activity by *Bacillus* spp. [26, 27, 28]. Other studies found peptone, casoamino acids, beef extract, yeast extract, gelatin, sodium nitrate or ammonium nitrate was the most suitable nitrogen source for maximum protease production by *Bacillus* spp. [12, 13, 29, 30, 31, 32, 33]

Among 5 tested sugars, fructose (10.0 g/L) was the most suitable sugar for maximum proteolytic activity by selected strain, which increase the enzyme yield to 21.31% compared to control, followed by rhamnase, starch, glucose and sucrose. In addition, presence of glucose or sucrose in production medium led to drastic reduce in protease yield up to 83.76% and 79.9% of control medium (sugar-free), respectively. Obtained results were in agreement with many previous studies, which found that fructose was the most suitable carbon source for protease production by *Bacillus* sp. N-40 [8] and *Bacillus* sp. NPST-AK15 [12]. In addition, many investigations noticed the inhibition effect of some sugars in protease production by different *Bacillus* species. [34] found that presence of glucose in production medium of *Bacillus* S-20-9 caused a significant decrease on protease production. Similar results were revealed that the repression effect of glucose in protease production by *Bacillus* spp. [26, 35]. While, other investigations found glucose, galactose, starch, sucrose, lactose, dextrin or xylose was the most suitable carbon source for maximum protease yield from *Bacillus* spp. [10, 13, 30, 31, 36, 37].

Addition of K_2HPO_4 (0.5 - 4.0 g/L) to the production medium had different impacts in protease production by tested strain. While, 0.5 g/L K_2HPO_4 was the ideal concentration for maximum proteolytic activity of the tested strain (242.3 U/ml), which increased protease yield to 13.03 % compared to control. Obtained results were supported by [11], who revealed that 0.5 g/L K_2HPO_4 was the optimum concentration for the highest yield of protease by *Bacillus* sp.S-8. [38] found 1.55 fold higher enzyme activities in the presence of phosphate ions by *Bacillus subtilis*. [39] observed that 39 % increase in enzyme production in the presence of 2mM phosphate ions in the growth medium by *Pseudomonas aeruginosa*.

In current study, the selected strain could grow and produce protease under various initial pH levels (5-9), while, slightly acidic and neutral pH levels were suitable for high yield of protease production compared to alkaline pH levels. In addition, pH 6 was the most suitable initial pH level for maximum proteolytic activity (287.72 U/ml) by selected strain, which increased the enzyme yield to 15.79% compared to control. Obtained results revealed that the yield of protease varied considerably with change of initial pH level. This may be attributed to the strong effects of pH level on many enzymatic processes and the solubility of medium components and their transport across the microbial cell membranes, which in turn support the cell growth and product formation [40].

Similar results had been observed by previous investigations. [7] reported that pH 6.0 was the most suitable pH level for the maximum protease production by *Bacillus cereus*, *Proteus vulgaris*, *P. mirabilis* and *Enterobacter aerogenes*. Subsequently, pH 6 was the most suitable pH for maximum protease production by *Bacillus megaterium* [41]. Other studies found the maximum proteolytic activity of *Bacillus* ssp. at different pH levels, such as; pH 7 [9], pH 7.4 [42], pH 7.5 [43], pH 8 [44], pH 9 [10], pH 10 [13] and pH 11 [45].

Incubation temperature play an essential role in protein synthesis by changing of physical properties of microbial cell membrane [39], influencing of biochemical reaction rate within cell and dissolving of oxygen in fermentation medium [46]. Thus, changing of optimum incubation temperature of microorganism could affect protein synthesis, especially protease production.

Results of the present study indicated that the tested strain could produce protease at different incubation temperature degrees (20-45°C), while 37°C was the optimum temperature for maximum protease production. On the other hand, the proteolytic activity of the tested strain was decreased at incubation temperature above or below 37 °C. Similar studies were found that 37 °C was the optimal temperature for maximum proteolytic activity by different

Bacillus sp. [14, 45, 47]. While, other studies recorded the maximum proteolytic activity of *Bacillus* ssp. at different incubation temperatures, such as; 30 °C [48]; 40 °C [49]; 45 °C [13]; 50°C [50]; 60 °C [51] or 70 °C [52].

In the current study, the most suitable inoculum size for maximum protease production by tested strain was 0.5%, which increased the protease yield to 12.1% compared to control. While, the protease yield was decreased using inoculum size above or below 0.5%. Decreasing of protease yield with below inoculum size may be attributed to the growth of tested strain was not enough to produce a high yield of protease, while decreasing of enzyme yield using of inoculum size above 0.5% may be due to the shortage of nutrients available in the medium and oxygen up take rate. Obtained results were in agreement with many previous investigations, which revealed that the yield of protease enzyme decreased using inoculum size below or above of optimum inoculum of producer microorganism [9, 10, 53]. While, the optimum inoculum for maximum protease production was different from microorganism to another because the total count of each microbial strain was different.

CONCLUSION

A strain of *Bacillus cereus*- S6-3, which isolated from Egyptian soil, had shown a promising future in the field of industrial applications because it could produce a high amount protease in short incubation period using low cost fermentative medium. Our study increased the proteolytic activity of *Bacillus cereus*- S6-3 from 112.09 to 327.32 U/ml by optimizing of the medium contents and environmental conditions. Further studies are required to increase the protease yield by mutagenic treatments and study its different characteristics to find out the potential applications in different industrial aspects.

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