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Bacillus subtilis and *Pseudomonas aeruginosa* as potent protease enzyme producers isolated from the aquatic environment

Usama M. Tahoun*; Walaa T. Hamza
National Institute of Oceanography and Fisheries (NIOF), Egypt
***Corresponding Author: usamatahoun@yahoo.com**

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ABSTRACT

Protease-producing bacteria were isolated from freshwater samples collected previously from the Rosetta branch (Nile River, Egypt) and were identified as *Bacillus subtilis* and *Pseudomonas aeruginosa*. The optimization of the culture conditions as pH, incubation time, substrates, carbon, and nitrogen sources were applied for maximizing the production of protease enzyme as well as the application of enzyme partial purification and enzyme characterization to study the effects of pH, temperatures, and different types of metals. The results referred to the best conditions for protease production of *Bacillus subtilis* were found to be gelatin substrate, pH 7.5, within 30 hr. incubation period, and the better source of carbon and nitrogen was lactose and peptone respectively. On the other side, the best conditions for *Pseudomonas aeruginosa* were found to be casein substrate, pH 8, within 48 hr. incubation period, and the better source of carbon and nitrogen was galactose and peptone respectively. The maximal activities at pH 9 and 8 for partially purified enzymes extracted from *Bacillus subtilis* and *Pseudomonas aeruginosa* respectively within incubation temperature at 40°C. Finally, Co^{2+} is the best metal ion that enhanced proteolytic activity for enzymes extracted from *Bacillus subtilis*, while the activity of the enzyme extracted from *Pseudomonas aeruginosa* was enhanced by Zn^{2+}, Ca^{2+}, and Co^{2+}. Among all studies for bacterial isolates, the highest activity of protease enzyme was observed in *Bacillus subtilis*.

INTRODUCTION

Protease enzyme is represented a major industrial enzyme that has been widely used in various industries for several years like detergents, pharmaceuticals, cosmetics, leather, peptide synthesis, photography, textile, agricultural sectors, synthetic biotechnology and foods industries such as baking, meat tenderization, brewing and cheese making (**Das and Prasad, 2010).** In addition, protease enzyme has a wide application in the medical fields for humans and animals such as some medical diagnosis, specific medical therapies of inflammations, malicious wounds and unhairing of sheepskins, as well as it is used for different bioremediation processes. It is acting approximately 60% of world's enzyme sales, and the majority of protease enzymes

production is from a variety of sources like plants, animals, and microorganisms such as fungi and bacteria (**Qureshi *et al*., 2018**).

Nevertheless, several microorganisms are playing a vital role in the biotechnology processes as producing of enzymes used in the industrial scale that promoting maximum yields. Under the optimal conditions where the selected microorganism is grown in big fermenters to produce great amount form enzymes, furthermore can be used to make products like bread, cheese, and wine (**Gaurav *et al*., 2015**). Proteases are considered extracellular enzymes that can hydrolyze the protein molecules into amino acids to be utilized by microorganisms. Moreover, according to great diversities of microorganisms, rapid and high growth levels, an inexpensive, eco-friendly, simplicity of production processes, easy troubleshooting, less area for the cultivation process, and the flexibility of genetic manipulation levels, thus, they have been considered the perfect sources for producing the protease enzymes (**Hamza, 2017**).

Noteworthy, the bacterial proteases are produced in great quantities because of their stability, specificity, and their efficiency in a wide range of different conditions. production of protease enzyme is affected by the different factors such as nutritional status as well as the environmental conditions. Consequently, the possibility of increasing the production of protease enzyme by changing culture conditions to reach optimal conditions for microbe growth. Subsequently, the cost of microbial enzyme production depends on the required nutrients. Therefore, detecting low-cost media as well as using optimization strategies during the enzyme production process would be economically beneficial **(Aishwarya *et al*., 2018)**. Though protease enzymes that produced by several microorganisms are already playing significant and serious roles in several industries, in the future, their useful applications processes are likely to rise. Investors are needed to more attention to enforcing the investment in the production of protease enzymes from natural resources for cleaner production in order to mitigate the hazard to the environment (**Hamza, 2017**). *Bacillus subtilis* and *Pseudomonas aeruginosa* are widely used in biotechnological processes and for the production of some industrial enzymes like amylase and protease (**Das and Prasad, 2010; Krishna *et al*., 2018**).

The present study aimed to select suitable bacterial isolates as potent producers of protease enzymes and investigate the optimization procedures of the production processes from isolated bacteria.

MATERIALS AND METHODS

Isolation and screening of protease producers

Eight freshwater samples were collected along the Rosetta branch, Nile River, Egypt, during the spring of 2018, as a source of freshwater bacterial isolates. samples were used for isolation and screening of bacteria that can produce protease enzyme through the modified method described by **Hamza (2017)** on a skim milk agar plate (peptone 0.1%, NaCl 0.5%, Agar 2% and skimmed milk 10%), then incubated at 37°C for 24-48 hrs. A clear zone formed colonies were picked then purified by streaking plate

method on skim milk agar. Two bacterial strains were selected on the basis of the clear zone size, then two microorganisms was identified according to the standard methods based on morphological and physiological properties with the keys of (**Barrow and Feltham, 1993; Hensyl, 1994; Holt *et al.*, 1994**). Then, Followed by using (Analytical profile index) API system identification (**BioMerieux, France**), tests in the API 20NE strip , API 20E strip, and API 50 CHB strip were used.

Inoculum preparation

A loopful of 24 hour culture was transferred into sterilized broth medium (containing peptone 0.5%; NaCl 0.5%; pH 7), then incubated (at 37^{o}C for 24 hr.) for inoculum preparation. This preparation was used directly to inoculate the protease production medium.

Production of protease enzyme

Producing the crude enzyme by fermentation 50 ml of nutrient broth was supplemented with a protein source (as casein or gelatin), autoclaved (120ºC for 20min), and inoculated by bacterial isolates incubated for 24 hr. at 37 ºC before enzyme assay.

Extraction of protease enzyme

Bacterial isolates were inoculated into 100 ml of nutrient broth was supplemented with a protein source followed by incubation in rotary shaker at 30°C and 150 rpm for 48 hr. Then using centrifugation for 15 min at 5000 rpm, the supernatants were used as crude enzyme source for the quantitative assay.

Protein estimation

For estimation of the total concentration of protein, the Lowry method (**Lowry *et al.*, 195**1**)** was selected through colorimetric techniques using U.V spectrophotometer (absorbance at 750 nm).

Qualitative Enzyme assay

Gelatin clearing zone technique

The enzyme activity was measured as described by **El- Safey and Ammar (2002)**. Soluble gelatin (1% w/v) was emulsified, supplemented with agar (1.5% w/v), and adjusted pH at 7, then cups were made in each plate. The extracted enzyme (0.1 ml) to be assayed was introduced into each cup. plates were incubated at 35 ºC for 24 hr. Then, plates were flooded with Mercuric chloride solution (15g of HgCl, 20 ml of 6N HCL, and complete to 100 ml distilled water), and the mean diameters of clearing zones were measured.

Quantitative protease assay and enzyme unit (U/ml)

Proteolytic activity was measured using casein solution as a substrate with some modification of the method described by **Gaur *et al***. (**2010)**. The absorbance (*A*) of the filtrate was measured using a UV–Visible light spectrophotometer. The standard graph for calculating protease concentration was prepared with 10-50 µg/mL of tyrosine solutions. One unit of the enzyme was defined as the amount of enzyme that releases 1

µmol of tyrosine per ml of crude extract per minute under standard conditions. The enzyme activity is in formula (U/ml).

Optimization of enzyme production

a- Study the effect of different substrates of production media

Three substrates were examined (casein, gelatin, and bovine serum albumin) to study the effect on enzyme production, and then incubated for 24 h at 37 ºC. The supernatants obtained was used to assay protease.

b- Study the effect of different incubation time

The optimized media were prepared in six conical flasks (100 ml), and strain culture broth (1%) was inoculated, and kept for shaker fermentation. Every 12, 18, 24, 30, 36, 48 hr, flasks were filtered, followed by centrifugation (at 5000 rpm) for 15 min. The supernatants obtained was used to assay protease.

c- Study the effect of different carbon sources

The optimized media were supplemented with various carbon sources such as glucose, starch, dextrose, fructose, sucrose, galactose, maltose and lactose (1% w/v) then incubated and the supernatants obtained was used to assay protease.

d- Study the effect of different Nitrogen sources

The optimized media were supplemented with various nitrogen sources such as peptone, potassium nitrate, urea, sodium nitrate, ammonium chloride, ammonium carbonate, and ammonium nitrate (1% w/v), then incubated and the supernatants obtained was used to assay protease.

e- Study the effect of different pH

The optimized media at different pH values were applied. Media was adjusting using a standard and calibrated pH meter. Other conditions were taken into consideration. After incubation, the supernatants obtained were used to assay protease.

Partial Purification of Protease

The crude enzyme was exposed to the precipitation processes by ammonium sulfate $(NH_4)_2SO_4$ as mentioned by **Maruthiah *et al.* (2015)** and the culture was harvested, then filtration by filter paper (125 mm of pore size) had occurred, and centrifugation processes at (5000 rpm, 30 minutes and 4°C) via centrifuge (**Model, Sigma 2-16 P**) were done. by slow addition of ammonium sulfate $(NH_4)_2SO_4$ and continuous shaking at saturation point to precipitate the protein. the precipitated protein was exposed to separation processes via centrifugation at (5000 rpm and 30 minutes) and was dissolved in phosphate buffer, pH 7, dialysed, and stored, at each step, the enzyme activities and total protein contents were measured in the U-V spectrophotometer. This will be a source of the crude enzyme that was taken for further enzyme characterization.

Characterization of protease Enzyme

Effects of pH on the activity of partially purified protease enzyme were studied via incubation of the protease enzyme in different buffers that covered a wide range of pH values viz; 6, 7, 8, 9, and 10, then enzyme activity assay was carried out.

Evaluating the effects of various temperatures on protease enzyme activities were tested by incubating the enzyme at various temperatures viz.: 20, 25, 30, 35, 40, 50, and 60ºC respectively.

The effects of various metal ions on the activity of proteases were evaluated by incubating the proteases along with 1 mM concentrations of various metal ions for 30 minutes followed by the protease activity assay. Salts used were NaCl, $CaCl_2$, $MgSo_4$, $FeCl_3$, $MnCl_2$, $NiCl_2$, $BaCl_2$, $CdSo_4$, $ZnSo_4$, $CuSo_4$, $CoCl_2$, and $Al_2(So_4)_3$ which give the metal ions Na^+, Ca^{2+}, Mg^{2+}, Fe^{3+}, Mn^{2+}, Ni^{2+}, Ba^{2+}, Cd^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, and Al^{3+} respectively.

Application of protease enzyme

The stability of partial purified protease enzyme extracted from microorganisms was examined using the commercial detergents; where enzymes already present in the detergent were firstly exposure to heating inactivated by boiling at 100°C for one hour to achieving denaturation for all enzymes that found previously in the detergents. The test enzyme solution was mixed with the detergent solution for one hour at 40ºC, then the enzyme assay was done.

The wash performance studies

Wash performance of the partial purified protease enzyme extracted from isolated microorganisms was examined by the method described by **Pathak and Deshmukh (2012)** with a little modification. The wash performance analysis of protease was studied on white cotton cloth piece (5cm×5cm) previously stained with animal blood and dried for 12hr. then put in conical flasks then subjected to the following washing treatments study.

Flask 1: 100 mL distilled water +stained cloth piece

Flask 2: 100 mL detergent solution+stained cloth piece

Flask 3: 100 mL detergent solution+1mL of protease enzyme solution+stained cloth piece

Incubation occurred in a water bath shaker for 30 minutes at 40ºC, after that, the cloth pieces were rinsed and dried, then the results were taken by the visual examination to inspect the efficiency of the stain removal.

Immobilization of protease

The partial purified protease enzyme was immobilized on the suitable carrier by the simple method of immobilization including physical adsorption, protease enzyme immobilized by physical adsorption on sodium alginate and chitosan. Beads of the immobilized enzyme are easy and safe to store; also save stabilities of the free enzyme were significantly improved by the immobilization process.

RESULTS AND DISCUSSION

Bacterial strains that have proteolytic activity were evaluated for their ability to utilize casein found in the skimmed milk, and two bacterial strains that produced a large clear zone were selected. Sixteen bacterial isolates from samples collected from the Rosetta branch were protease positive (Table 1, Fig. 1.). Highly hydrolysis occurred by bacterial isolate No.2 which was recorded (24 mm clear zone) and bacterial isolate No. 10 which was recorded (22 mm clear zone).

Two bacterial isolates (positive protease producers) were picked to further investigation. Finally, colonial morphology, cellular morphology, and biochemical characteristics were undertaken (Tables 2, 3, 4). The identification of the selected bacterial isolates was done according to the keys of (**Barrow and Feltham, 1993; Hensyl, 1994; Holt *et al.*, 1994**). who revealed that isolate No. 2 was *Bacillus subtilis* and isolate No. 10 was *Pseudomonas aeruginosa.*

There are a variety of enzymes secreted by various strains of *P. aeruginosa* and *B. subtilis* including proteases (**Manstala and Zalkin. 1979; Michael *et al.*, 1983; Oh *et al.*, 2000; El-Safey and Abdul-Raouf 2004; Hala and Samaa 2016; Krishna *et al.*, 2018).**

Table (1): Screening of isolated bacteria for protease production

No. of isolates	Diameter of hydrolysis zone (mm)	No. of isolates	Diameter of hydrolysis zone (mm)
1	**12**	**9**	**18**
2	**24**	**10**	**22**
3	**11.5**	**11**	**9**
4	**17**	**12**	**14**
5	**8.5**	**13**	**15.6**
6	**13**	**14**	**17.8**
7	**12**	**15**	**9**
8	**10**	**16**	**9.8**

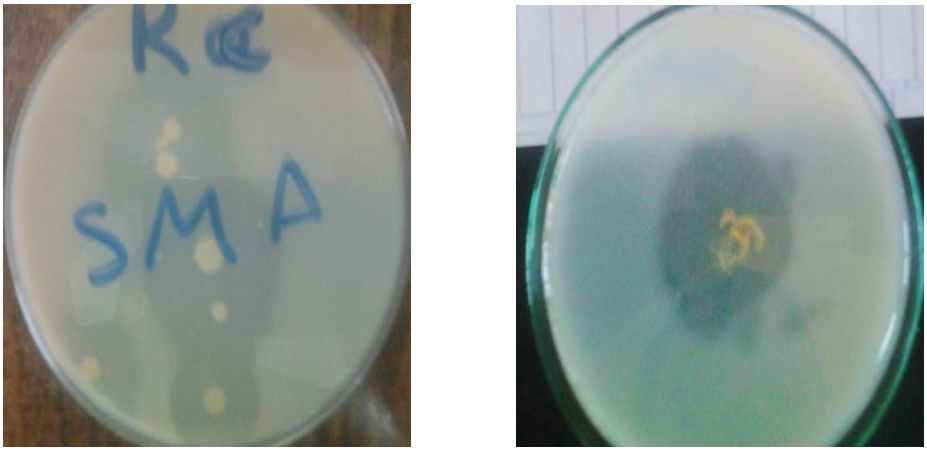


Fig. 1. Screening of proteolytic activity on skimmed milk agar media

Table 2. Morphological and biochemical characterization of isolates 2 and 10.

Test No.	Characteristics	Isolate 2	Isolate 10	Test No.	Characteristics	Isolate 2	Isolate 10
1	Gram stain	-	-	17	KCN resistance	-	-
2	Cell shape	B.	B.	18	O/F test	+/+	+/+
3	Motility	+	+	19	Lactose fermentation	+	-
4	Spore formation	+	-	20	Maltose fermentation	+	-
5	Catalase	+	+	21	Raffinose fermentation	+	-
6	Oxidase	-	+	22	Mannitol fermentation	+	+
7	Indole production	-	-	23	Starch fermentation	+	-
8	MR test	+	+	24	Fructose fermentation	+	+
9	VP test	+	-	25	Glucose fermentation	+AG	+
10	Citrate utilization	-	-	26	Sucrose fermentation	+AG	-
11	KOH (3%)	-	+	27	Dextrine fermentation	-	-
12	H_2S production	-	-	28	Mannose fermentation	+	-
13	Levan formation	-	-	29	Arabinose fermentation	+	-
14	Nitrate reduction(NO3)	-	+	30	Galactose fermentation	+	D.
15	Nitrite reduction(NO_2)	-	+	31	Rhamnose fermentation	+	-
16	H_2S production	-	-				

D.= Doubtful; AG= gas production, B.= Bacilli (Rod shape).

Table (3) Identification of isolate 2 using API system

API 20 E	Result	API 50 CHB	Result	API 50 CHB	Result	API 50 CHB	Result	API 50 CHB	Result
ONPG	+	GLY	+	MNE	+	SAL	+	GEN	+
ADH	-	ERY	-	SBE	+	CEL	+	TUR	+
LDC	-	DARA	-	RHA	+	MAL	+	LYX	-
ODC	-	LARA	+	DUL	-	LAC	+	TAG	-
CIT	+	RIB	+	INO	+	MEL	+	DFUC	-
H_2S	-	DXYL	+	MAN	+	SAC	+	LFUC	-
URE	-	LXYL	-	SOR	+	TRE	+	DARL	-
TDA	-	ADO	-	MDM	-	INU	+	LARL	-
IND	-	MDX	-	MDG	+	MLZ	-	GNT	-
V-P	+	GAL	+	NAG	+	RAF	+	2KG	-
GEL	+	GLU	+	AMY	+	AMD	+	5KG	Doutful
NO_2	+	FRU	+	ARB	+	GLYG	+		
				ESC	+	XLT	+		

Table (4) Identification of isolate 10 using API system

API 20 NE	Result	API 20 NE	Result	API 20 NE	Result
NO3	+	PNPG	-	MAL	+
TRP	-	GLU	+	CAP	+
GLU	-	ARA	-	ADI	+
ADH	+	MNE	-	MLT	+
URE	-	MAN	+	CIT	+
ESC	-	NAG	+	PAC	-
GEL	+	MAL	-	OX	+

Protease production

Production processes of an extracellular protease enzyme by *P. aeruginosa* and *B. subtilis* isolated previously from water samples were collected from the Rosetta branch were carried out for selection the optimal culture conditions and maximum levels of protease productivity by two microorganisms (**Fig.2)** . The results revealed to the best medium used for enzyme production that was supplemented with gelatin and casein for *B. subtilis* and *P. aeruginosa* respectively. *B. subtilis* and *P. aeruginosa* can be used for the production of protease enzymes on a large scale to meet today's needs in the industry. In the present study, casein and gelatin was added to the media to stimulate the microorganisms for maximal production of enzyme by strains of *P. aeruginosa* and *B. subtilis*.

Gaurav *et al*. (2015) used a gelatin substrate on the nutrient agar medium for screening and showed the maximum zone of activity (22 mm). On the other hand, **Gaur *et al*. (2010)** mentioned that, the highest production of the protease enzyme occurred in basal medium supplemented with casein for *P.thermaerum*.

Also, protease showed broad substrate activities with various substrates such as casein, gelatin, chicken albumin, and ovalbumin, except hemoglobin **(Ghafoor and Hasnain, 2010)**. Furthermore, gelatin substrate with concentration (0.1% w/v) was the best for thermostable protease produced by *B. brevis geltinoamylolyticus* (**Ammar *et al*., 2003**).

Results indicated that *B. subtilis* and *P. aeruginosa* exhibited their maximum ability to biosynthesize enzyme and the highest protease activity within 30 h and 48 h respectively incubation period (**Fig**.**2**). These results are in complete accordance with many authors (**Michael *et al*., 1983; El-Safey and Abdul-Raouf, 2004).**

Carbon source is significant factor for production of protease enzyme in culture medium. Of. eight carbon sources tested by *B. subtilis* utilized lactose as the best carbon source. while *P. aeruginosa* utilized galactose was the best carbon source. The same results was mentioned by **Yang *et al*. (1999)** who decided the protease produced by *B. subtilis* was mostly enhanced by the addition of lactose into the production media. In addition, **Nigam *et al*. (1981)** was reported that, the strain of *P. aeruginosa* isolated from the soil produced huge quantities of protease enzyme and could utilize sundry organic substances as carbon and nitrogen sources.

However, nitrogen source is also a valuable factor for enzyme production. Different nitrogen sources were tested and the most effective inducer for enzyme production from two bacterial isolates was peptone. **Nigam *et al.* (1981)**, reported that the complex nitrogenous substances supported enzyme production more efficiently in the case of *P. aeruginosa*. A higher concentration of casamino acids suppressed the proteinase synthesis. Microorganisms are sensitive to the changes in the hydrogen ion concentration of their environment when used for the production of enzymes. The results

show that pH 8 and 7.5 were the optimum for the production of protease from *P. aeruginosa* and *B. subtilis* respectively.

Using casein substrate under the standard assay conditions recorded the highest activity at pH 8 with *Bacillus* sp. **(Karadag, 2009).** Moreover, **Rao *et al*. (1998)** mention that, the optimum pH range of alkaline proteases is generally between pH 9 and 11, with a few exceptions **.**

Ferrero *et al*. (1996) recorded highly activities of the alkaline protease extracted from *B. licheniformis* at 60°C and it is stable for 10 minutes between 30 °C and 60°C. In addition, the highly activities of serine-protease produced by *B. mojavensis* A21 was recorded a broad range of pH from (7 to 13), as well as the optimal pH was 8.5 and maximum activities at 60ºC (**Haddar *et al*., 2009).**

Moreover, The alkaline protease produced by *B. pumilus* was recorded the optimal activity at temperature of (55 °C to 60°C) and pH 11.5 (**Kumar, 2002**). Likewise, the serine proteases AS and HS from alkalophilic *Bacillus* sp. had optimum activity over a wide range of pH from 8 to 12 and at 65°C (**Durham *et al*., 1987**). Also, The alkaline protease produced by *B. clausii* I-52 had the optimum pH 11 at 60°C (**Joo *et al*., 2003**).

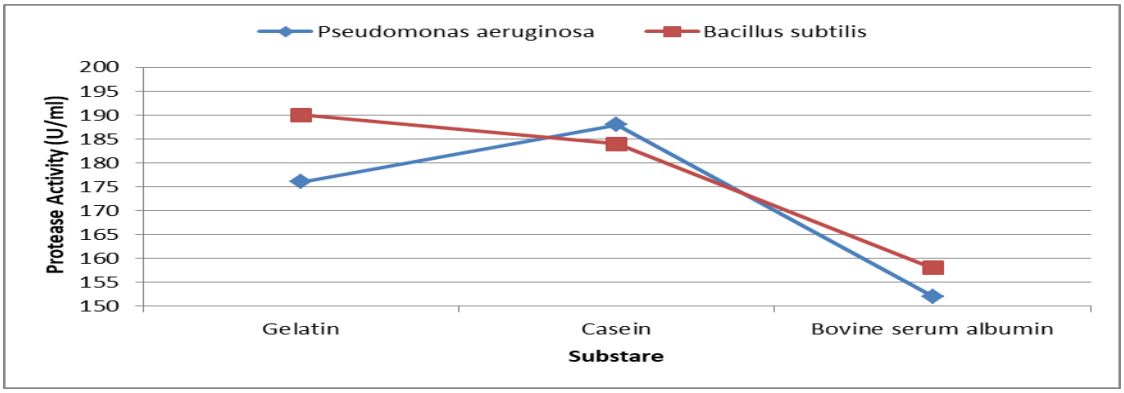
Whereas, protease (EAG-2) produced by *B. subtilis* recorded an optimal activity at pH 8.5 and 65°C (**Ghafoor and Hasnain, 2010**). Furthermore, **Johnvesly *et al.* (2002),** stated that, the high levels of thermostable protease enzyme activities have appeared after 24 hr. of the incubation period.

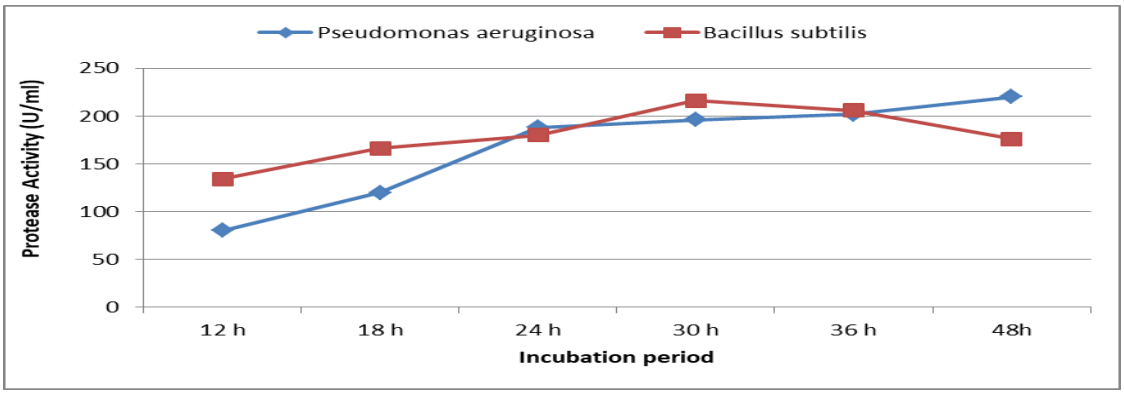
Partial purification of protease

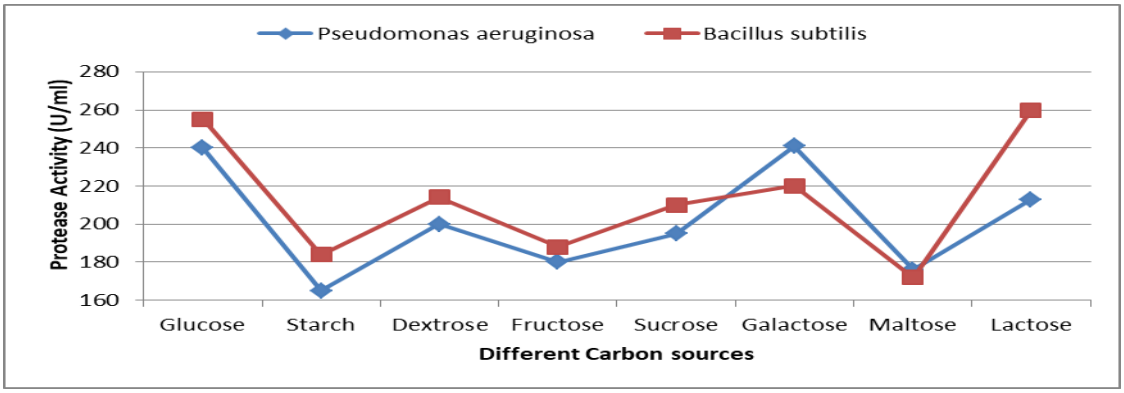
Partial purification of protease enzyme was accomplished relatively easily. The culture supernatant of *P.aeruginosa* and *B. subtilis* containing crude enzymes was concentrated by ammonium sulfate precipitation. The optimal saturation of ammonium sulfate $(NH_4)_2SO_4$ was (75% w/v). Protease was partially purified by ammonium sulfate precipitation and the dialysis was occurred, the partial purification of protease enzyme was done by many authors (**Fitriani and Güven, 2018).**

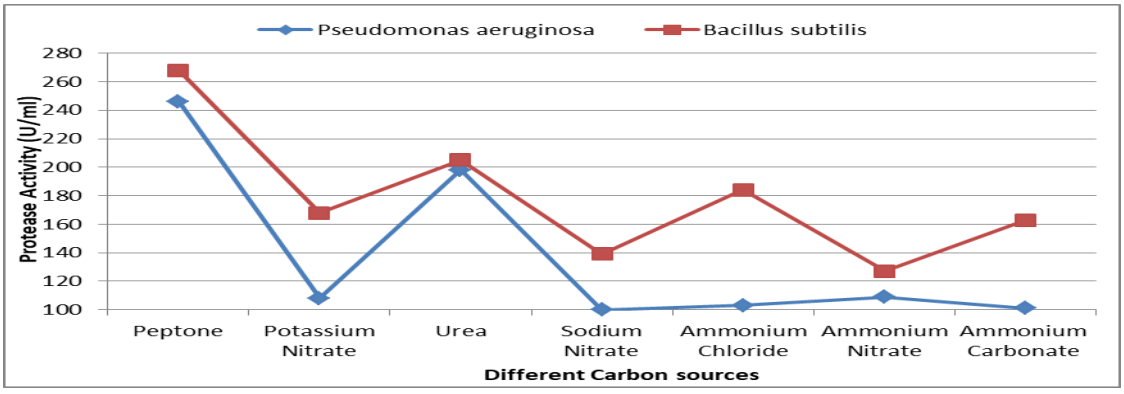
Enzyme characterization

The enzyme activity of partially purified protease enzymes was determined at different pH values **(Fig. 3)** and the results referred to the best pH values for maximal activity were 8 and 9 for enzymes extracted from *P. aeruginosa* and *B. subtilis* respectively. In addition, protease produced from *P. aeruginosa* and *B. subtilis* was found to be active at the range of temperatures between 30 and 60°C whereas the maximum activity of partially purified was recorded at 40°C (**Fig. 3).**


Pseudomonas aeruginosa
Bacillus subtilis
Protease Activity (U/ml)
200
195
190
185
180
175
170
165
160
155
150
Gelatin
Casein
Bovine serum albumin
Substare


Pseudomonas aeruginosa
Bacillus subtilis
Protease Activity (U/ml)
250
200
150
100
50
0
12 h
18 h
24 h
30 h
36 h
48h
Incubation period


Pseudomonas aeruginosa
Bacillus subtilis
Protease Activity (U/ml)
280
260
240
220
200
180
160
Glucose
Starch
Dextrose
Fructose
Sucrose
Galactose
Maltose
Lactose
Different Carbon sources


Pseudomonas aeruginosa
Bacillus subtilis
Protease Activity (U/ml)
280
260
240
220
200
180
160
140
120
100
Peptone
Potassium Nitrate
Urea
Sodium Nitrate
Ammonium Chloride
Ammonium Nitrate
Ammonium Carbonate
Different Carbon sources

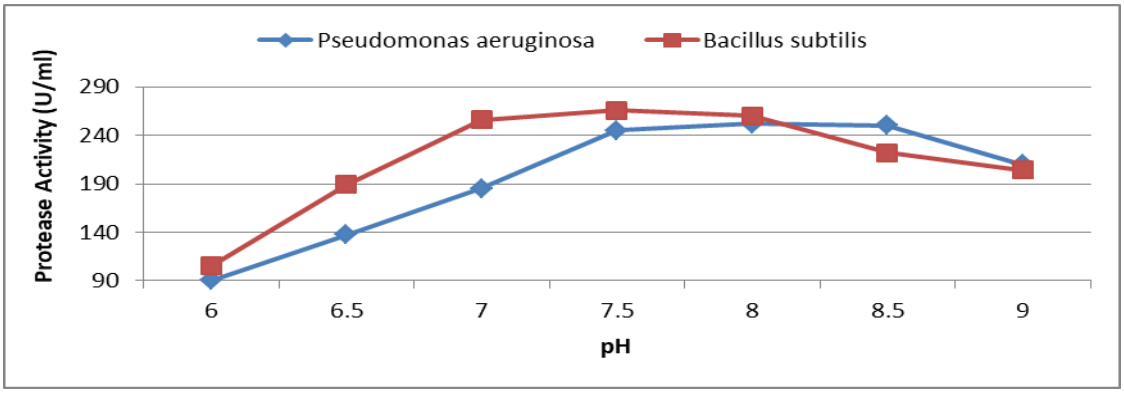


Fig.2 .Different factors affecting on protease production by *P. aeruginosa* and *B. subtilis*

The optimal temperature of alkaline proteases ranges from 50 to 70 ºC**,** as well as, protease produced by *B. anthracis*, S-44 recorded an optimum incubation temperature for purified enzyme activity was 35 ºC **(Abdul-Raouf, 1990; Gupta *et al.*, 2002)**. Moreover, **Secades and Guijarro (1999)** reported that, the protease enzyme was purified from the culture supernatant of *Yersinia ruckeri* was more active in the range of 25 ºC to 42 ºC and the optimal was 37 ºC.

On the other hand, the effects of several metal ions on the activity of protease enzyme were measured (**Fig.3),** the results referred to the activity of enzyme extracted from *P. aeruginosa* was enhanced by Zn^{2+}, Ca^{2+} and Co^{2+}, however, Ba^{2+}, Na^{+}, and Mg^{2+} didn't have any significant effects on the enzyme activity. On the other hand, in the presence of Mn^{2+} and Cd^{2+}, the activity of the enzyme was slightly reduced, whereas Ni^{2+}, Cu^{2+}, and Al^{3+} significantly lowered the enzyme activity. Fe^{3+} inhibited the activity of protease enzyme.

Fe^{3+} metals inhibited the proteolytic activity of alkaline protease enzyme produced by *Bacillus cohnii* APT5 (**Tekin *et al*., 2012**). Otherwise**, Larsen and Auld (1991); Luciano *et al*. (1998),** who mentioned that in some cases Zn^{2+} metals inhibit protease enzyme activities when existing at higher concentrations.

Furthermore, the protease enzyme produced by *B. subtilis* different cations was tested to evaluate the effect on the protease enzyme activity. Co^{2+} is the best metal that enhanced proteolytic activity of protease enzyme, also proteolytic activity was enhanced by Mg^{2+}, Cu^{2+}, and Mn^{2+}, otherwise in case of the presence of Ni^{2+}, Al^{3+}, and Cd^{2+}, the enzyme activity was partially reduced. It was inhibited by $Zn^{2+}$and Fe^{3+}. However, Ca^{2+}, Ba^{2+}, and Na^{+} did not affect the activity. These results are relatively similar to those reported by **Ibrahim (2012)**. In addition to that, the activation of alkaline serine protease enzyme was occurred in the presence of Co^{2+} and Mn^{2+} metals and inhibited by Fe^{3+} (**Pushpam *et al*., 2011**). Whereas, Ca^{2+} and Zn^{2+} metals were supplying strong stability to metalloproteases (**Rao *et al*., 1998**).

Application studies of protease enzyme (wash performance studies).

Protease produced by *P. aeruginosa* and *B. subtilis* was evaluated its potential industrial applications for the formation of detergents. So, the application of the partially purified protease enzyme with the commercial detergents like Ariel was studied in the current study; the results were taken by the visual examination and were indicated that the enzyme was supported vigorously by the detergent in the stain removal process; when the detergent washed the blood-stained cloth piece without enzyme addition the cloth piece was not removed completely and some traces of the stain remained after washing process but by addition of the enzyme to the detergent the blood-stain was removed completely.

Sellami *et al.* (2008) studied the protease enzyme extracted from *B. licheniformis* and reported that the enzyme was retained more than 80% of the activity with whole detergents were be tested even after incubation for 1 hr. at 40ºC.

Singh *et al*. (2001) mentioned that a serine alkaline protease produced from *Bacillus* sp. shows the activities of approximately more than 70 % at 40ºC, but at more than 40ºC the stability of the enzyme in the detergents has required some addition like calcium chloride. **Bhosale *et al*. (1995); Banerjee *et al*. (1999)** mentioned that the protease enzyme kept the highest activities within the commercial detergents in the case of the addition of calcium chloride and glycine.

For a long period of time, most varieties of proteases enzyme have been incorporated as bio-builders into the detergents to hydrolyze and remove the proteinaceous materials from clothes **(Ito *et al*., 1998).** proteases enzyme that has been extracted from *A. ramosus* and *B. alcalophilus* was efficient in removing the blood stains from the cloth pieces and cotton fabrics (**Kanekar *et al*., 2002)**.

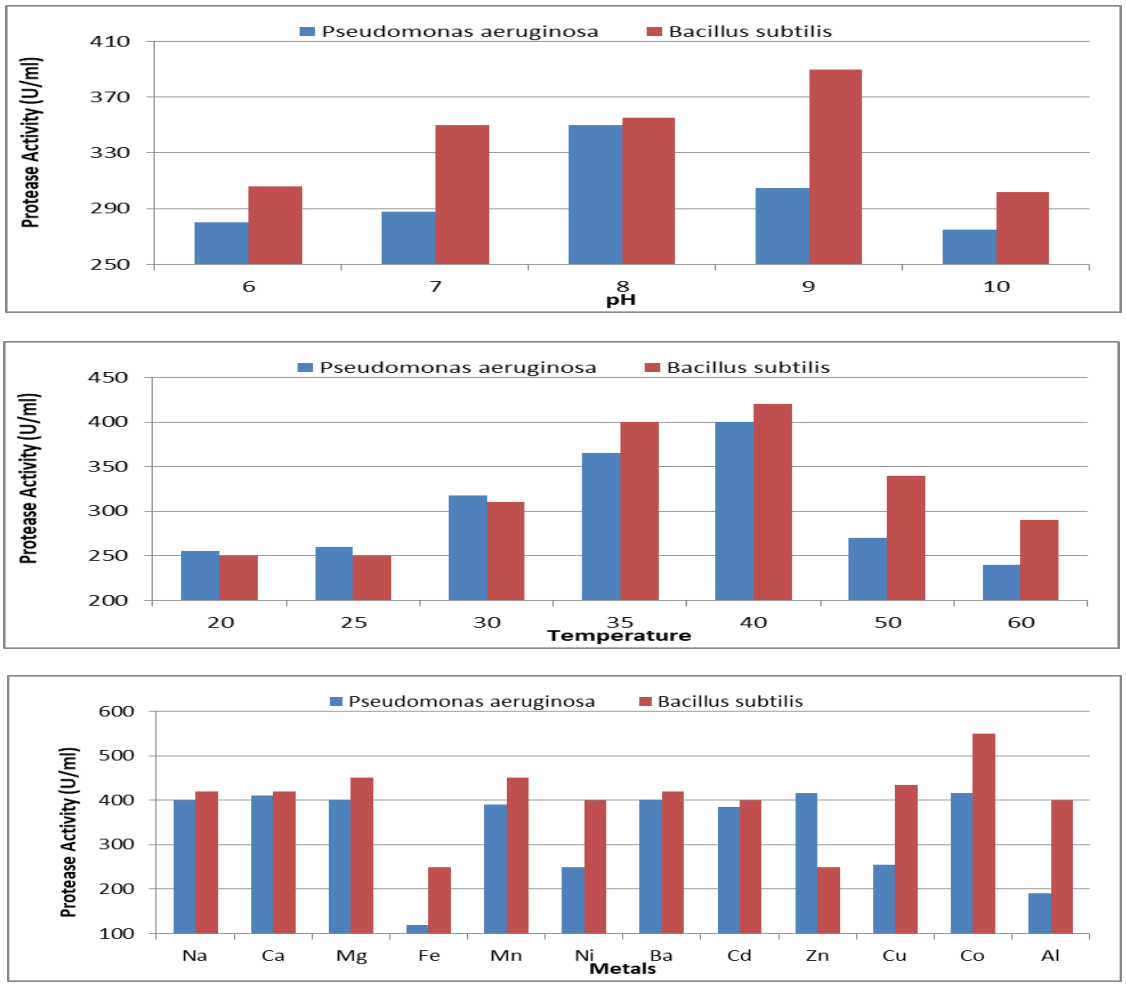


Fig.3.Different factors affecting on protease activity

Finally**,** partialy purified protease enzyme was immobilized by sodium Alginate and chitosan. The immobilized enzyme can use several times with stability of enzyme for different purposes. This immobilization process of protease enzymes was closely similar to those done by **Ohmiya *et al.* (1979)** who used the immobilized protease in cheese making

CONCLUSION

The protease enzyme extracted by *B. subtilis* and *P. aeruginosa* and the optimization of the production process was applied, and the results revealed how maximizing the production of enzyme for each microorganism, as well as, the characterization of protease enzyme was studied, and the maximal activity was achieved. In the present study, the enzyme application on the laboratory scale was applied. and the enzyme showed excellent stability in the existence of the laundry detergent tested like Ariel.

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