# Optimization of protease production by *Ps. aeruginosa* PAO1 and Physico-chemical characterization of the enzyme

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**Abstract:** Microbial proteases represent one of the commonly used industrial enzymes. *Ps. aeruginosa* PAO1 was used in the present study for the production of protease. Maximum protease production was observed in 10 g/l tryptone basal medium with 5 g/l glucose at pH 7.0 when inoculated with 4% of 24h broth culture and incubated at 37 ºC for 48h with agitation speed of 150 rpm. Removal of $Na^{+}$ or $K^{+}$ or $Mg^{++}$ or $Ca^{++}$ or $PO_4^{---}$ from fermentation medium has an inhibitory effect on protease production. The enzyme was characterized biochemically; it has maximum activity at pH 8 and 40-50ºC in crude extract after 60 minutes of incubation. The enzyme was stable in the presence of $K^{+}$ or $Ca^{++}$, $Na^{+}$ and $Ba^{++}$ slightly enhanced its proteolytic activity whereas $Mg^{++}$ and EDTA reduced the activity of protease. The activity of protease enzyme increased in B-mercaptoethanol (BME) and Triton X-100 in all concentrations tested. The protease enzymatic activity was measured in the presence of detergents, Cortex®, Arial®, Persil® and Elnabolsi® retained residual activity more than 60% after 60 min incubation while in Feba® 10% of its activity was retained. Also, the enzyme is stable in the presence of 1g/l SDS, which is the common ingredient in modern bleach-based detergent formulations, while its activity decreased with increasing the concentration of SDS. Based on these findings *Ps. aeruginosa* PAO1 protease shows a good potential for application in detergents.
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## 1.Introduction

Proteases are important group of industrial enzymes, as over 60% of the total enzyme market relies on proteases, whose function is to hydrolyze peptide bonds in proteins and break them down into polypeptides or free amino acids [49].

They are produced by a number of eukaryotic and prokaryotic organisms including fungi, animal and bacteria [59]. Proteases produced by microbial sources are preferred over those produced by plants or animal source, since they possess some advantages like its broad biochemical diversity, rapid growth, limited space required for cell cultivation and the ease with which the enzymes can be genetically manipulated to generate new enzymes for various applications [55].

Proteases are classified into three groups, depending on their activity at different pH, i) acidic, ii) neutral, and iii) alkaline protease. Acid proteases showed maximum performance at pH range 2-5 and mostly produced by fungi. Neutral proteases have optimum pH ~7 and mainly of plant origin, while alkaline proteases have optimum activity at pH ≥8 [21]. They have got wide usage commercially in detergents, leather processing, silk industry, food, pharmaceutical industries, preparation of organic fertilizer and waste processing industries [13].

Among bacteria, Pseudomonas species are specific producers of extracellular proteases which act as virulence factor for development of *Ps. aeruginosa* biofilms. *Pseudomonas aeruginosa* PAO1 was reported to produce exotoxin A, alkaline protease and elastase [23], however few studies have been done on proteolytic enzymes from PAO1.

Studies on other strains of Pseudomonas showed that nutritional, chemical and physical factors can influence protease production. Nutritional factors include the sources of carbon, nitrogen and metal ions while physical factors such as inoculum size, temperature, pH and incubation time have significant effect on production [53]. To use an enzyme as detergent additive, it should be stable and active in the presence of detergent ingredients such as surfactants builders, bleaching agents and various other formulation aids [3].

The present study aims to optimization the growth conditions which have been predicted to play a significant role in enhancing the production of proteases. Also, extracellular protease from *Ps. aeruginosa* strain PAO1 was isolated and its enzymatic properties were characterized.

## 2. Material and Methods

**Microorganism**.

Standard strain of *Pseudomonas aeruginosa* PAO1 used was provided by Prof. Dr. Martin Schuster (Oregon State University, Corvaillis, Oregon, US). Culture was maintained on nutrient agar slopes at 4°C

and sub-cultured every 4 weeks and also was kept at -80°C as glycerol stocks.

**Reagents and solutions**

All reagents used were of analytical grade (A.R. from Fluka, Aldrich or Merck). All solutions were prepared using double distilled water and used without further purification.

**Screening for protease production**.

*Pseudomonas aeruginosa* PAO1 was inoculated onto 5% skimmed milk agar plates and incubated at 37°C for 48 h [2].

**Enzyme production medium.**

The medium (BSM) consisted of a basal salt solution containing (g/l): NaCl (0.1); KCl (0.2); $K_2HPO_4$ (0.2); $CaCl_2.2H_2O$ (0.5); $MgSO_4.7H_2O$ (0.5). The pH of the medium was adjusted to 7.2 before autoclaving [66].

**Preparation of inoculum**.

The organism was subcultured from a nutrient agar stock culture into nutrient broth media and incubated for 24h at 37°C. The cultures were then pipetted into BSM medium containing 10 g/l of peptone and at inoculum sizes of 2, 4, 6, 8 and 10% (v/v). The samples were then incubated at 37°C at the agitation speed of 150 rpm. The optimum inoculum size determined from this was used to inoculate fresh media in order to test the effect of different factors.

**Quantitative test for protease.**

Total protease activity in culture supernatant was measured according to Keay and Wildi with minor modifications using a casein substrate [28]. Briefly, 1 ml of culture supernatant was mixed with 1 ml buffered casein solution 10 g/l (w/v) at pH 7.5. Freshly mixed 0.1 M sodium phosphate buffer was added to maintain this pH. The mixture was then incubated for 10 min at 37°C and the reaction was terminated by addition of 2 ml 0.4 M trichloroacetic (TCA) acid. The mixture was then vortexed and incubated for 30 min at room temperature followed by centrifugation at 10.000 rpm for 5 min. The resulting supernatant (1ml) was then mixed with 5.0 ml of 0.4 M $Na_2CO_3$, and after 10 min, 1 ml of Folin reagent: distilled water (1:3 v/v) was added to each tube. The tubes were allowed to stand for 30 min at 37°C and then the absorbance were measured at 660 nm. The similar method was used to prepare a control, however, TCA was added at the surface and casein was added only after 10 min of incubation. A standard curve was generated using solutions of 0-60 µg/ml tyrosine. One unit of protease activity was defined as the amount of enzyme required that releases 1 µg/ml tyrosine under the above experimental conditions [60].

**Optimization of culture conditions for protease production**

Different culture conditions, like pH (5-11), temperature (28, 37 and 42°C), agitation (static, 75 and 150 rpm) were optimized for production of protease from *Ps. aeruginosa* PAO1 in the formulated BSM medium containing 10g/l peptone. Samples were collected after 24, 48 and 72 hours and centrifuged at 10000 rpm for 10 minutes at 4 °C. Supernatent was used as crude extract. Proteolytic activity was measured under standard assay conditions [27, 33].

**Effect of nutrients on enzyme production**

The effects of some nutrients such as carbon, nitrogen sources and some metal salts on enzyme production were investigated. Carbon sources chosen for the study were glucose, fructose, xylose, mannose, sucrose, starch, glycerol and ethanol. These carbon sources were added to peptone medium at concentration of 5 g/l. Different organic, inorganic nitrogen sources and amino acids including tryptone, yeast extract, beef extract, corn steep liquor, gelatin, urea, ammonium sulphate, arginine, glycin, aspargine, methionin, alanine and ornithin were tested. These nitrogen sources were used to replace peptone which was the original nitrogen source in growth medium. When inorganic nitrogen sources and amino acids were used as sole nitrogen source, glycerol (0.5%) was added to BSM [60]. The influence of various metal ions on protease production was determined by inoculating the BSM with the absence of different metal ions such as sodium, potassium, calcium, sulphate, phosphate, magnesium using tryptone as nitrogen source and glucose as carbon source [60]. In all experiments, samples were harvested at 24, 48 and 72 h post inoculation and processed as described previously.

A new medium containing the best source of carbon, nitrogen and metal ion was constructed and inoculated with *Pseudomonas aeruginosa* PAO1 for maximum protease production. All the experiments were carried out in triplicate and the results presented are the mean of the three values.

**Physico-chemical characterization of *Pseudomonas aeruginosa* PAO1 protease enzyme.**

*Pseudomonas aeruginosa* PAO1 protease enzyme was produced under the optimized environmental and nutritional conditions. Cell free supernatant was taken and used for the characterization of the enzyme. The effect of pH (4-11), temperature (40, 50, 60 °C), various metal ions (20 mM solutions of NaCl, KCl, $CaCl_2$, $MgSO_4$ and $BaCl_2$), chelating agent EDTA (20 mM), denaturants [β-mercaptoethanol (BME) at concentration (10, 30, 50, 70 and 90 g/l) and Triton X-100 at concentrations (2, 4, 6, 8 and 10 g/l )], SDS (1-5 g/l) and various commercial detergents brands Cortex®, Arial®, Persil®, Feba®, and Elnabolsi® at final concentration 0.7 mg/ml on the enzyme activity were determined. Commercial detergents were heated at 100°C for 10 min before use [66]. The enzyme was incubated for 60 min at room temperature under the

tested conditions and the enzyme activity was measured before and after the treatment under standard assay conditions. Buffers (0.02 M) of different pH values were used to test the pH stability of the enzyme: acetate buffer (pH: 4, 5), phosphate buffer (pH: 6, 7, 8) and glycin-NaOH buffer (pH: 9, 10, 11) [58].

## 3.Results

**Qualitative and quantitative analysis.**

*Pseudomonas aeruginosa* PAO1 produce protease leading to clear zones around the bacterial colonies on 5% skimmed milk agar plates at 37°C after 24 h and reaches to maximum, (44 U/ml) after 48h incubation at 37°C, pH 7 and agitation speed of 150 rpm in BSM supplemented with peptone 10 g/l.

**Optimization of protease production.**

**1. Environmental factors**

**1.1. Inoculum concentration**. Results showed that maximum production was achieved at 4% inoculum concentration. The enzyme activity gradually decreases when using higher inoculum concentrations (6%, 8% and 10%) as shown in figure (1). Thus, 4% inoculums concentration was used for the rest of this study.

**1.2. Incubation time.** *Pseudomonas aeruginosa* PAO1 produces protease at maximum level after 48h, figure (2).

**1.3. pH.** Maximum production was found at pH 7 after 48 h incubation (figure 3). Above pH 7 there was a decrease in the enzyme production.

**1.4. IncubationTemperature.** *Pseudomonas aeruginosa* PAO1 produces protease in the range 28-42°C with maximum at 37°C (figure 4).

**1.5. Agitation.** Continuous agitation at 150 rpm was found to be obligatory for high enzyme elaborating by *Pseudomonas aeruginosa* PAO1 since stationary as well agitation at 75 rpm failed to synthesis higher level of the enzyme even after 72h of incubation (Table 1).

**2. Effect of some nutrition.** The medium components effects on protease production by *Pseudomonas aeruginosa* PAO1 were studied**.**

**2.1 Effect of nitrogen sources.** Almost all the organic nitrogen compounds tested support good growth and protease production by *Pseudomonas aeruginosa* PAO1 except urea. Maximum protease production was obtained when tryptone was used as the sole nitrogen and carbon source. However, employing inorganic compounds as sole nitrogen source significantly reduces protease production where ammonium sulphate completely inhibits the production. Also, the use of amino acids as nitrogen source repressed and reduces the production (Figure 5).

**2.2. Effect of carbon sources.** The presence of additional carbon source in peptone medium resulted in increase in protease production. Using glucose, fructose and xylose increase the production level by 36.7%, 63.8% and 14.8%, respectively, relative to control. While addition of sucrose, mannose, starch, glycerol and ethanol resulted in reduction of production (Figure 6).

Thus, combination of high effecting nitrogen and carbon sources into BSM were studied. As shown in figure 7, mixture of yeast extract medium with glucose or fructose as carbon source leads to low protease production (28% and 35% reduction respectively as compared to yeast extract medium). In case of tryptone medium and glucose as carbon source, the protease level increases by 47% with respect to tryptone medium. Using fructose instead of glucose reduces the enzyme level by 19% (Figure 7).

**2.3. Effect of metal ions**. Table (2) shows that metal ions affect on protease production where removal of $Na^{+}$, $K^{+}$, $Mg^{++}$, $Ca^{++}$, or $PO_4^{---}$ reduced the protease yield by 12%, 13.9%, 27.9%, 33.7% and 40.6%, respectively. This means that production of protease is $Ca^{++}$ and $PO_4^{---}$ dependent. While removal of $SO_4^{--}$ led to slight increase in protease production by 12%.

**Characterization of *Pseudomonas aeruginosa* PAO1 protease enzyme.**

**1. Temperature.** The enzyme stability examined at different temperatures, the results showed that *Pseudomonas aeruginosa* PAO1 produced mesophilic protease that remains active in 40-50°C range. The enzyme lost its activity at temperature above 50°C.

**2. pH.** The enzyme activity was tested in the pH ranged from 5.0 to 11.0 and the optimum activity was found at the alkaline pH (pH 8), after which the activity is declined.

**3. Denaturants.** The protease form *Ps. aeruginosa* PAO1 showed a high activity in all concentrations of BME (Fig. 8) indicating the absence of disulphide bonds in the protease. The protease retained more than 100% activity in presence of Triton X-100 with maximum at 0.8% v/v (Fig. 9).

**4. Surfactant and various commercial detergents.** The stability of mesophilic protease toward surfactant and various commercial detergents was measured by incubating the crude enzyme extract for 1 h with SDS (1-5 g/l), Cortex®, Arial®, Persil®, Feba®, and Elnabolsi® at room temperature followed by measuring its activity under standard assay conditions. **Generally,** the protease from *Ps. aeruginosa* PAO1 was stable and compatible in presence of the locally available detergents (Cortex®, Arial®, Persil®, Feba®, and Elnabolsi®) and SDS. Protease enzyme activity is slightly increased in the presence of the detergent brand Cortex® after 60 min incubation, while the presence of brands Elnabolsi®, Arial® and Persil® reduced its activity to be 73-60%. In case of the Feba® brand, the enzyme almost loss its activity (10%) . In the presence of 1 g/l SDS , the enzyme retained full activity for 60

min. However, usage of SDS higher concentrations led to lose its activity (Table 3).

**5. Metal ions.** The enzyme retained its activity in presence of $K^{+}$ or $Ca^{++}$. Both of NaCl and $BaCl_2$ increase the enzyme activity up to 115% and 122.6%, respectively. However, $Mg^{++}$ and EDTA reduce the activity by 45% and 80%, respectively.

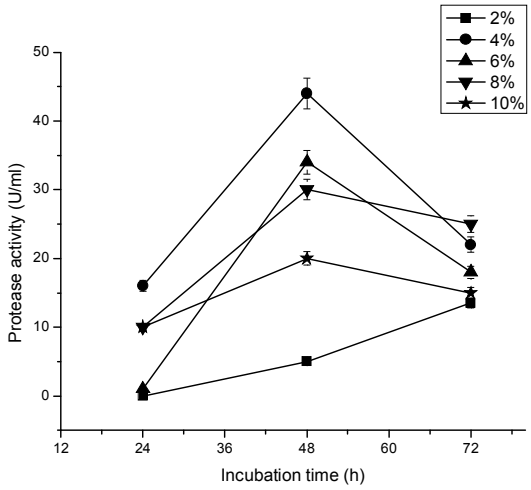


**Figure 1 Effect of inoculum concentration on protease production by *Ps. aeruginosa* PAO1. Symbols: 2% inoculums size (■),4% inoculums size (●),6% inoculums size(▲),8% inoculums size (▼),10% inoculums size (◄)**

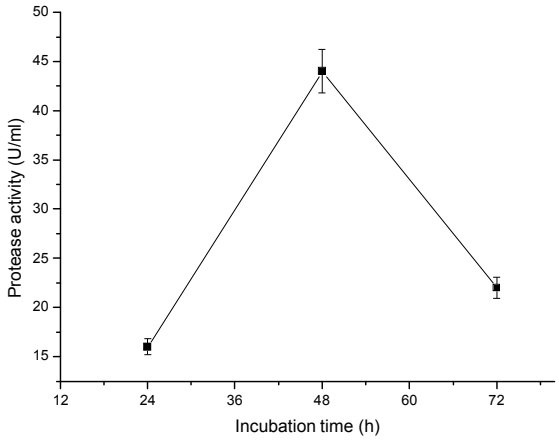


**Figure 2 Effect of incubation time on protease production by *Ps. aeruginosa* PAO1.**

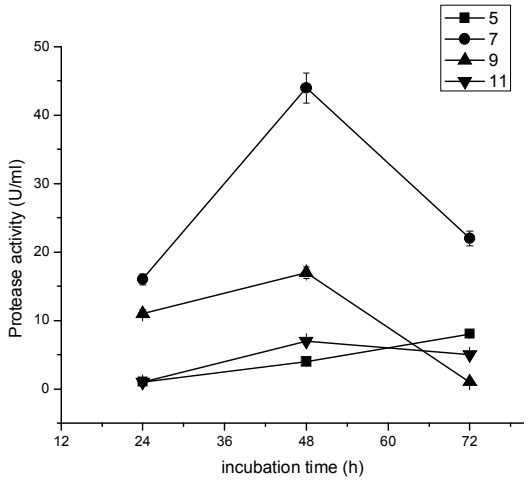


**Figure 3 Effect of pH on protease production by *Ps. aeruginosa* PAO1. Symbols:pH 5 (■), pH 7 (●), pH 9 (▲) and pH 11(▼).**

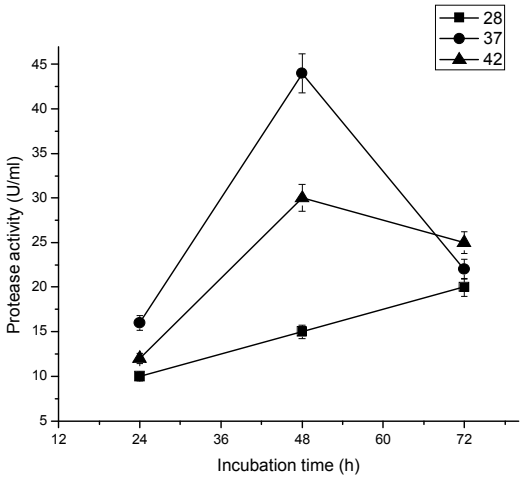


**Figure 4 Effect of incubation temperature on protease production by *Ps. aeruginosa* PAO1. Symbols: 280 C (■), 370 C (●) and 420 C (▲).**

**Table 1. Effect of agitation on production of protease enzyme from *Ps. aeruginosa* PAO1**

| Agitation | Enzyme activity (U/ml) at different incubation periods | | |
|---|---|---|---|
| | 24 h | 48 h | 72 h |
| Static | ‹ 1±8.75119 | 18.5±8.75119 | 10±8.75119 |
| Agitation at 75 rpm | 15±2.51661 | 20±2.51661 | 17±2.51661 |
| Agitation at 150 rpm | 16±14.74223 | 44±14.74223 | 22±14.74223 |

± Standard deviation

**Table 2 Effect of metal ions on protease production by *Ps. aeruginosa* PAO1**

| Metal ion removal | Enzyme activity (U/ml) |
|---|---|
| **Control** | 246±12.3 |
| **Sodium** | 216±10.8 |
| **Potassium** | 212±10.6 |
| **Calcium** | 163±8.15 |
| **Sulphate** | 258±12.9 |
| **Phosphate** | 146±7.30 |
| **Magnesium** | 177±8.85 |

± Standard deviation

**Table 3 Stability of protease in presence of commercial detergents and SDS**

| Detergents (0.7 mg/ml) | Residual activity (%) after 60 min |
|---|---|
| **Cortex®** | 104±5.2 |
| **Arial®** | 65±3.25 |
| **Persil®** | 62±3.1 |
| **Feba®** | 10.36±0.518 |
| **Elnabolsi®** | 73.3±3.665 |
| **SDS 0.1%** | 100±5 |
| **SDS 0.2%** | 60±3 |
| **SDS 0.3%** | 55±2.75 |
| **SDS 0.4%** | 47±2.35 |
| **SDS 0.5%** | 39.5±1.975 |

± Standard deviation

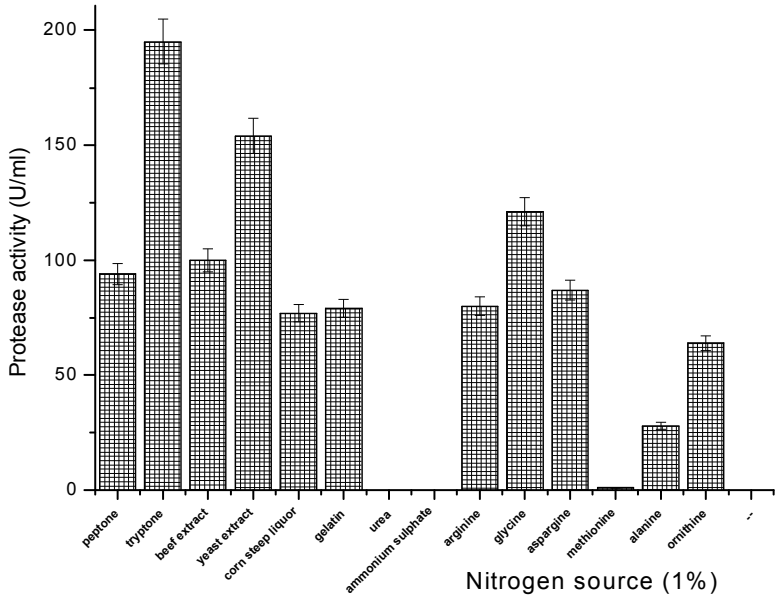


**Figure 5 Effect of nitrogen sources on protease production by *Ps. aeruginosa* PAO1.**

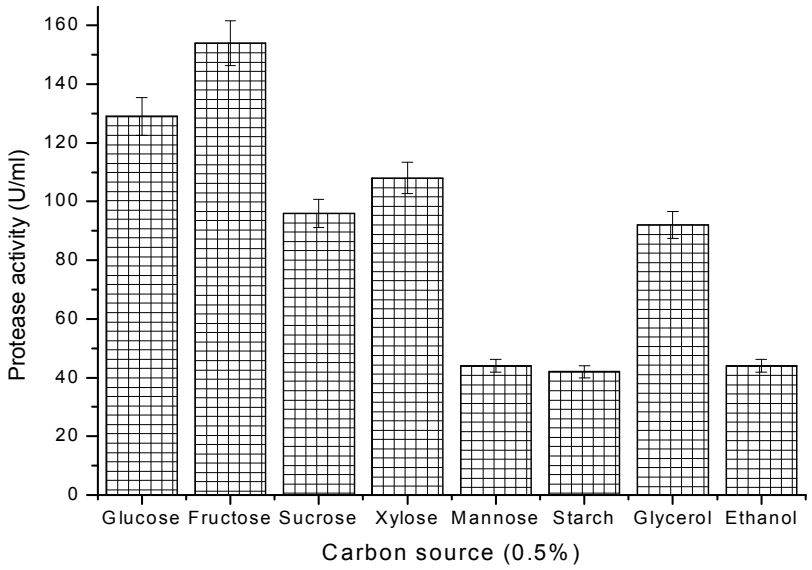


**Figure 6 Effect of carbon sources on protease production by *Ps. aeruginosa* PAO1.**

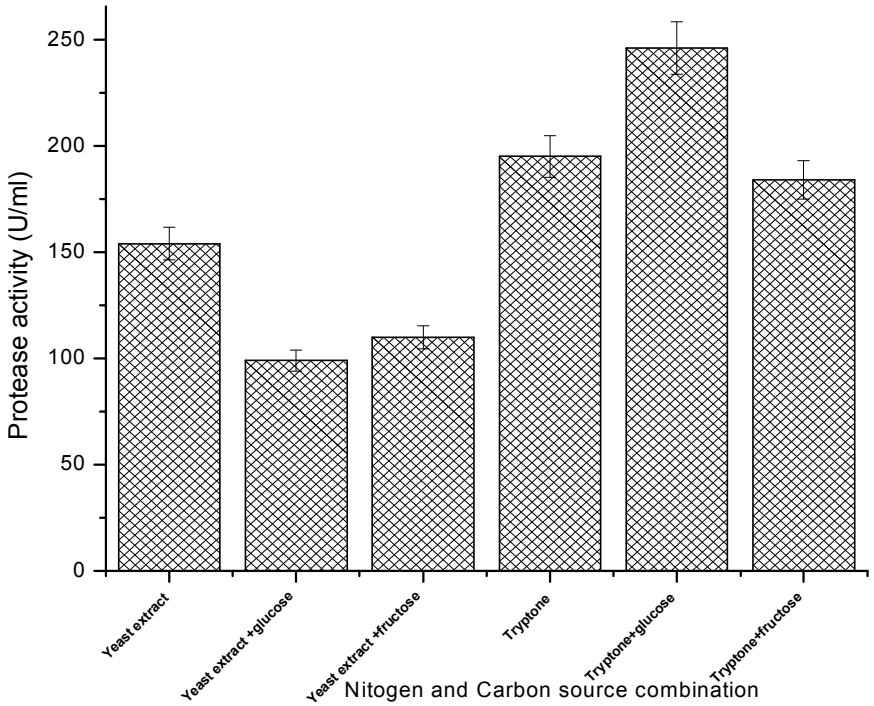


**Figure 7 Effect of combined nitrogen and carbon sources on protease production by *Ps. aeruginosa* PAO1.**

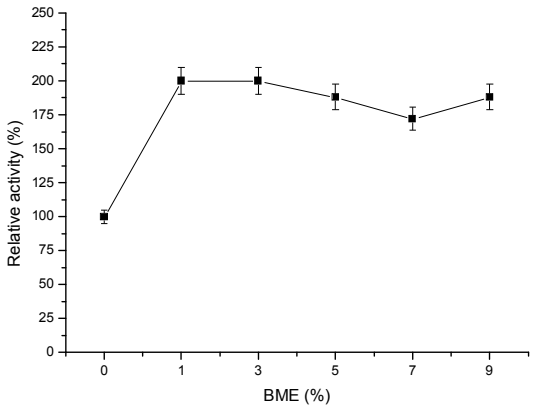


**Figure 8 Stability of protease in β-mercaptoethanol (0-9 % v/v)**

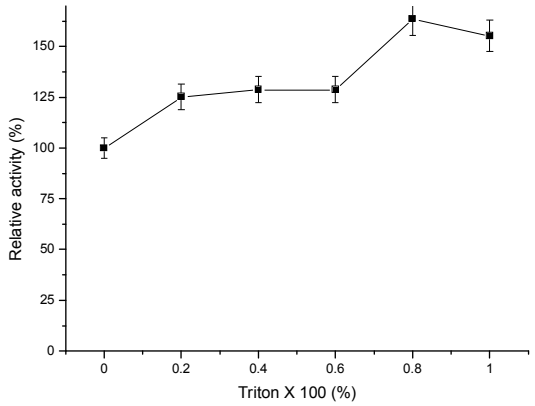


**Figure 9 Stability of protease in Triton X-100 (0.2-1% v/v)**

## 4. Discussion

It has been reported that the production of extracellular proteases by different microorganisms can be strongly influenced by the culture conditions [53]. The results of the present study showed that protease production by *Pseudomonas aeruginosa* PAO1 was affected by some environmental conditions. According to the obtained results, the optimum inoculum size for protease production in *Ps. aeruginosa* PAO1 was 4.0% in accordance with Norazizah *et al*. [44]. The increase in protease production using small inoculum size was suggested to be due to higher surface area to volume ratio which improved distribution of dissolved oxygen and more effective uptake of nutrient [53]. Moreover, at 6.0% and 10.0%, the protease activity was found to be decreased due to that higher inoculum sizes resulted in reducing dissolved oxygen and increasing competition towards nutrient [53].

Enzyme secretion is greatly influenced by the change in initial pH of the environment [57]. Enzyme possesses many ionizable groups so pH changes may alter the enzyme conformation [11]. *Pseudomonas* PAO1 elaborated maximum extracellular protease when the medium initial pH was adjusted to 7.0, the enzyme level gradually declined at higher pH values. Similar results were obtained for *Ps. fluorescens* and *Ps. aeruginosa* in peptone yeast extract broth at pH 7.0 [25]. Malik *et al.* [33] had also obtained maximum protease production in the case of *Pseudomonas* SP. B-25 at pH 7.0. However the most of alkaline proteases production have been reported to be at pH 8-9 [4, 27]. Mao and Freedman found that maximum protease production could be achieved by controlling pH and temperature [34]. Enzyme activity recorded at different incubation temperatures revealed that *Ps. aeruginosa* PAO1 yielded maximum protease production at 37ºC as these reported for *Ps. Fluorescens* [27]. Related studies also reported that protease production by *Ps. aeruginosa* was maximal at 30ºC [25]. The temperature influence on extracellular enzyme secretion may attribute to changing the physical properties of the cell membrane [53]. *Pseudomonas* PAO1 also exhibited maximum protease production when incubated at 37 ºC for 48 h on a shaker which is partially agreed with Malik *et al.* [33] who reported that *Pseudomonas SP.B25* showed a peak for protease production at 22 ºC for 48h on a gyratory shaker.

Agitation rates affect protease production in various strains of bacteria [31, 37]. Since *Pseudomonas* are obligate aerobes, continuous aeration of the culture media is generally required to sufficient supply of dissolved oxygen in the media [29] and nutrient uptake by bacteria will be increased [8] resulting in increase of protease production. *Pseudomonas aeruginosa* PAO1 also exhibited maximum protease production when incubated at 150 rpm agitation speed after 48h of incubation. At 75 rpm, insufficient aeration and nutrient uptake caused the inability of bacteria to grow efficiently so decrease in protease activity was observed. The above results indicate that enzyme production decreased by prolonging incubation time after 48 hrs, which may be due to the depletion of nitrogen and other sources present in medium, inactivation of enzyme by acidification of the medium or proteolytic attack by other proteases [12, 14].

Enzyme formulation is largely dependent on the growth condition of the culture and composition of nutrient medium; the medium components play a significant role in enhancing the production of protease [21]. In some organisms organic nitrogren sources were found to be better sources for both growth and also protease production [6, 50]. The results showed that organic nitrogen sources caused increase protease production. Tryptone displayed the highest protease activity among other sources of organic nitrogen, at all the incubation periods examined; this result is in agreement with earlier reported data [33, 43]. In

contrast, Norazizah *et al*. [44] reported that beef extract resulted in miximum protease production by *Bacillus cereus* strain 146.

Organic nitrogen sources were better than inorganic sources to *Pseudomonas* and *bacillus sp.* in growth and enzyme production processes [52, 56]. Addition of ammonium sulphate in the production medium resulted in reduction of enzyme yield, which attributed to the inability of bacteria to utilize ammonia in the media; this finding was agreement with finding of Fujiwara and Yamamoto [19] and Frankena *et al*. [18]. On the other hand, El-Safey and Abdul-Raouf [15] reported that $(NH_4)_2SO_4$ was the best nitrogen source for protease production. Amino acids were found also to decrease the protease production it may be due to growth was very low.

Previous reports suggested that carbon sources in either monosaccharide or polysaccharides form affect on the production of enzymes by bacteria [26, 61]. Among the tested carbon sources, fructose was found to produce the highest protease production level and this in agreement with previously reported data [31, 56]. Also, glucose and xylose showed high protease expression, as observed by Bhatiya [9] and Boominadhan [10]. In contrast, sugars exerted inhibitory effect on protease production by *Pseudomonas spB-25* [33]. Sucrose, glycerol, starch and ethanol caused low protease production due to its use as carbon and energy source for good growth. Which is in agreement with previous studies suggested that larger amount of enzyme was synthesized when carbon sources used were poorly utilized for growth purposes [51, 65]. On contrary, starch caused high level of enzyme expression in bacillus species [5, 32]. Moreover, Mazdak *et al*. reported that sucrose is good substrate for protease production [35].

On the other hand, from commercial point view, sugars, like fructose, lactose, mannitol and sucrose, will be prohibitive due to their cost [62]. Hence, in this study, glucose was found to be the best carbon sources when added to tryptone medium for protease production (246 U/ml).

The metal ions in media are an important factor that affect enzyme production as they act as inducers. The effect of removal some metal ions from the growth medium were investigated. It was found that removal of $Ca^{++}$ and $Mg^{++}$ and $PO_4^{---}$ resulted in low production of protease compared to control. This observation is corroborated by previous studies which suggested that $Ca^{2+}$ and $Mg^{2+}$ ions were affected on protease production [17, 27]. Also Malik *et al*. observed that addition of $K_2HPO_4$ and $KH_2PO_4$ (1:1) to tryptone yeast extract medium led to stimulation of *Pseudomonas sp.B-25* extracellular protease production by 16 to 20% [33]. Removal of $Na^+$ or $K^+$ from the growth medium led to slight inhibition in protease production while removal of $SO_4^{--}$ did not affect on protease production. These observations strongly suggested the requirement of some metal ions for protease production by this organism. The previous results are in agreement with the earlier findings [1, 63]. It was suggested that these metal ions increased stability of proteases [46, 54].

Enzymatic properties such as the effect of pH, temperature and metal ions on the activity of protease were studied. High pH value 8 was required for its optimal activity, which is a very suitable for industrial acceptability; most of proteases were reported to have optimal pH 8-9 [4, 47]. The enzyme stability was analyzed at various temperatures showed that the enzyme is a mesophilic protease that remained active at temperatures 40-50°C. The decrease in enzyme activity at higher temperatures may attribute to the destruction of the enzyme. In addition, Lee *et al*. reported that the optimum temperature of purified protease was ranged from 40 to 50 ºC [30]. On the other hand, the maximum activity of purified extracellular protease produced from the *Bacillus subtilis* BS1 was shown at 90 ºC and 50 ºC, 100% and 97% respectively [39]. Furthermore, 45°C was the optimum temperature of the extracellular proteinase (PSCP) produced by *Pseudomonas cepacia* [36].

The stability of enzyme remains a critical aspect in pharmaceutical and industrial biotechnological applications. The stability of mesophilic protease towards denaturants, surfactants, detergents and soap was determined. After 60 min incubation, the BME and Triton X-100 enhance the activity of the enzyme indicating the absence of disulphide bonds in the protease of PAO1 which in agreement with Gupta *et al*. [22] who reported that protease from *Pseudomonas aeruginosa* PseA was stable in urea (1-5 mM), Triton X (0.1-0.5%) and BME (1-5 mM).

The influence of surfactants on protease activity is extremely variable in the previous literatures; Olivera *et al*. [45] observed SDS (1%) and Triton X-100 (1%) enhanced the protease activity of the Bradyrhizobium strain by 12 and 16%, respectively. While Najafi and Deobagkar observed strong inhibitory affects of 1% SDS and Tween-80 on protease activity from *Pseudomonas aeruginosa* PD100 [40], also SDS (0.1%) reduced the activity of the protease enzyme produced by *Ps. aeruginosa* PseA by 39% [22]. While in this study protease enzyme from *Ps. aeruginosa* PAO1 retains its full activity at concentration 0.1% SDS after which the activity decreased. The addition of proteases to detergents considerably increases its cleaning effect by removing protein stains and consumption of surface active substances [16]. Phadatare *et al*. [48] and Gupta *et al*. [20] reported that protease from *Conidiobolus coronatus* and *Virgibacillus pantothenticus* (MTCC 6729), respectively, were stable and compatable in presence of

commercial detergents. In this study, the protease retained 104%, 65%, 62%, 73.3%, and 10.36% of its activity when incubated with Cortex®, Arial®, Persil®, Elnabolsi® and Feba®, respectively.

As the protease produced by PAO1 was stable over a wide range of pH values, temperatures and also showed compatibility with various commercial detergents, so it can be used as an additive component for the detergent.

Metal ions are known to play a role as cofactors for enzyme activities and often act as salt or ion bridges between two adjacent amino acid residues [39]. NaCl and $BaCl_2$ increase the enzyme activity; similar result was obtained by Jaswal and Kocher [24]. On the other hand, inhibition by $Ba^{2+}$ was reported by Bhatiya and Jadeja [9]. However, $Mg^{2+}$ and EDTA reduced the activity of the enzyme by 45% and 80%, respectively, due to chelation of EDTA with calcium ions which is necessary for enzyme activation or participate in the enzyme molecule [64]. On the other hand, Shaheen *et al*. [58] reported that EDTA played important role in stimulation of protease activity from *Bacillus subtilis* SB1 and increased the activity up to 101%. Also, the stability of enzyme in the presence of EDTA was reported by Muderrizade *et al*. [38], while inhibition by EDTA was reported by Sun *et al.* [62] and Olivera *et al.* [45] who have suggested the presence of metalloproteases in the crude enzyme extract. It was reported that the protease activity is stimulated by $Ca^{2+}$ [24, 41]. However other studies showed that the activity was reduced in the presence of $Ca^{2+}$ [7, 42] but in our case, the enzyme retains its activity in the presence of $Ca^{2+}$ and therefore, 100-1000 ppm of $Ca^{2+}$ is normally added to liquid detergents that contain protease [66].

In conclusion, we have determined the optimum parameters for maximum production of alkaline protease by *Ps. aeruginosa* PAO1. This information has enabled the ideal formulation of media composition for maximum protease production by this organism. We also investigated the stability of protease in salts as well as denaturants and detergents. The protease has high optimal pH and thermal stability, which are characteristics of the protease used in detergent formulations. This study highlights the possibility of mixing protease from *Ps. aeruginosa* PAO1 with detergents; this strain also might be suitable for many industrial applications.

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**Conflict of Interest Statement**

All authors declare that there is no financial/commercial conflicts of interest.

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