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Economic Production of Polyamide Surface Modifying Protease Enzyme under Solid State Fermentation Using Wheat Germ Meal and Sand.

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ABSTRACT

Agro –industrial by-product residues were used as substrates in the presence of sand as a carrier material for production of polyamide surface modifying protease enzyme under solid-state fermentation from *Bacillus* isolate P16. Wheat germ meal supported the highest enzyme production at 6% (699 U/g of fermented culture). Maximum protease production was achieved at 10% initial moisture content and 30 °C incubation temperature. Enrichment of the medium with different carbon sources inhibited the enzyme production to different levels. However, the nitrogen source, ammonium phosphate, enhanced the enzyme production by 6%. Optimum incubation time was 7 days. Calcium chloride at 1% and supplementation the medium with Tween 60 or Tween 80 enhanced the enzyme productivity by 8% and 15%, respectively. This enzyme showed the highest activity at 60 °C and pH 8. It was stable at 30 °C- 60 °C for ten min however it was unstable in pH range 5-9 for one h. Calcium, magnesium and sodium ions are activators for the enzyme under study. Pilot scale production of protease enzyme under the optimum SSF conditions was tested in aluminum trays. The protease activity was 900 U/g fermented culture. This result was comparable with that obtained from bench scale production (in flasks). The fermentation medium cost for production of 900000 U protease enzyme was estimated as US $ 2.8. The results presented in this work show the potential of utilization of wheat germ meal as a substrate and sand as a carrier for the production of industrial enzyme under SSF.

Keywords: Polyamide surface modifying protease, wheat germ meal, sand, *Bacillus*, solid state fermentation.

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INTRODUCTION

Polyamide (PA) based textiles show the great disadvantage to be uncomfortable to wear and difficult to finish due to their hydrophobicity. Therefore, enhancement of the hydrophilicity of PA is a key requirement for many applications and can be achieved by chemical, physical and enzymatic methods. Enzyme treatment can be chosen as a green alternative for synthetic polymer surface modification as they offer many advantages over chemical and physical methods such as they are very specific, act under moderate reaction conditions which lead to less or negligible damage of the strength properties of the synthetic polymers, easier to control, cost effective, eco-friendly and the scale up is possible [1, 2].

Enzymes that can hydrolyze PA are proteases, cutinases and polyamidases [3]. These enzymes were successfully used for surface targeted hydrolysis of PA leading to hydrophilization [4, 5, 6]. Surface hydrolysis of PA leads to increasing of polar groups (free amino and carboxylate end groups) on the surface. The enzymatic hydrolysis of surface moieties of PA has been demonstrated to be a powerful mild strategy for improving hydrophilicity and activating materials for further processing, which is the key requirement for many applications including painting, inking and antifogging [3].

Many proteases such as protex Gentle L, protex 40L, protex multiplus L, and protex 50FP were used to investigate changes in PA fiber. Protease treatment of PA fibers showed significant decrease in thermal degradation temperature whereas reactive and acid dyes showed higher dye bath exhaustion [7].

Enzymatic surface modification of synthetic fibers can improve their use nowadays. A lot of experimental research work has been carried out on the surface modification of PA but their large scale and industrial application will be undertaken and optimized [2].

Proteases are one of the most important commercial enzymes constituting 60-65% of the global enzyme market [8]. At present, the overall cost of enzyme production is very high due to the high cost of the substrate.

Over the past couple of years, solid-state fermentation (SSF) involving growth of microbes on moist solid substrate(s) in the absence of free flowing water, has advantages over submerged fermentation, like lower production cost, saving of water and energy, less waste effluent problem and stability of the product due to less dilution in the medium [9-12].

There are some reports about using agricultural wastes for protease production under SSF [13-20].

The authors have previously screened 19 protease enzymes produced by bacilli isolated from Egypt for specific surface modification of PA fabric. One of these enzymes produced by *Bacillus* isolate P16 (identified as *Bacillus pumilus* showed good and promising results in modifying PA surface [21, 22]. The purpose of this study was to establish the best conditions for economic production of PA surface modifying protease enzyme by *Bacillus* isolate P16 under SSF using agro-industrial by-products as substrates and sand as carrier material. Also, pilot scale production of this enzyme applying the best cultural conditions in trays under SSF was evaluated. In addition, biochemical characterization of the enzyme under study was assessed.

MATERIALS AND METHODS

Materials

Azocasein was purchased from Fluka. Other chemicals used in this study were of analytical grade.

Agro-industrial by-products were supplied by Oil Extraction Unit and Animal Nutrition Department at National Research Center of Egypt. These agro-industrial by-products included sugar beet pulp, coconut meal, jojoba meal, cress meal, linen meal, wheat germ meal, cotton seed meal, pea peels, potato peels, orange peels, banana peels, bean peels and carrot pomace.

Bacteria used and inoculum preparation

Bacillus isolate P16 was isolated from Giza in Egypt and used in this study for production of PA modifying protease enzyme [21]. For inoculum preparation, the tested organism was grown in nutrient broth at 30 °C for 24 h under shaking at 150 rpm.

SSF and optimization of the process parameters

A group of dried ground agro-industrial by-products was employed as the main source of nutrients for growth and protease production by *Bacillus* isolate P16 without any pretreatment in the presence of sand as a carrier material.

Initially 50 g of sand and 3% of each substrate were taken individually in 250 mL Erlenmeyer flasks, moistened with tap water at 10% moisture and autoclaved. Inoculum of 14×10^6 CFU/g was added and incubated at 30 °C for 7 days under static conditions. Each fermentation test was repeated twice in triplicate. Effect of wheat germ meal (WGM) concentration (1.5 – 12%), moisture content (5-70%), incubation temperature (20-40 °C), carbon source at 1% (glucose, galactose, arabinose, fructose, raffinose, maltose, sucrose, lactose, starch, cellulose, pectin, acetate, citrate, glycerol), nitrogen source at 1% (sodium nitrate, ammonium sulphate, ammonium phosphate, urea, peptone, yeast extract), incubation period (3-15 days), inoculum size (2.4×10^6 - 75.8×10^6 CFU/g), metal ions at 1% (Na^+, K^+, Mg^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}) and Tweens at 0.1% (Tween 20, Tween 40, Tween 60, Tween 80) were evaluated.

Also, pilot-scale production of protease enzyme by *Bacillus* isolate P16 in aluminum trays (30×30×10 cm^3) under the selected SSF conditions was evaluated. Each tray contains 850 gram sand, 6% WGM, 1% ammonium phosphate, 0.1% Tween 80, 1% $CaCl_2$ and 10% moisture content was inoculated and incubated for 7 days at 30 °C.

Protease activity

The enzyme from the fermented WGM was extracted with tap water. The slurry was squeezed through a damp cheese cloth. Extracts were pooled and centrifuged at 4 °C for 15 min at 10.000 rpm. The supernatant was used as the enzyme source.

Protease activity was assayed according to [23]. One milliliter of azocasein solution (0.2% w/v in 50 mM of phosphate buffer, pH 8) and enzyme source (0.5 mL) were incubated initially at the tested temperature for 30 min. The reaction stopped by the addition of 1mL of 20% (w/v) tri-chloroacetic acid (at 0 ^oC) and left on ice for 10 min. The mixture was centrifuged and the optical density of the supernatant was measured at 420 nm (OD_{420}). One unit of the enzyme activity was defined as a 0.1 increase in OD_{420} under specific reaction conditions.

Biochemical properties of the enzyme

Temperature and pH profiles were determined at different temperatures (30-70 °C) and pH rang 7-9, respectively. The thermal and pH stabilities of the enzyme were ascertained by measuring the activity of the residual enzyme exposed at various temperatures (30-70 °C) for ten minutes and at pH range 7-9 for one h, respectively. Effect of metal ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, Fe^{2+}, Co^{2+}) at 5mM were tested. All experiments were conducted in triplicates.

Statistical Analysis

All the experiments were carried out independently in triplicates in 250-ml Erlenmeyer flasks. The data represented here are in the form of mean ± SE. All the values were subjected to one way analysis of variance (ANOVA) and significance is presented as Duncan's multiple range test results in the form of probability ($P \leq 0.05$) values which were obtained using [24].

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RESULTS AND DISCUSSION

Effect of agro-industrial by-products as substrates on enzyme productivity

The selection of an ideal agro-industrial residue for enzyme production in a SSF process depends upon several factors, mainly related with the cost and availability of the substrate material, and thus may involve screening of several industrial residues [25].

Among the tested agro-industrial by-products, WGM enhanced the enzyme production by *Bacillus* isolate P16 (592 U/g fermented culture) followed by coconut meal (524 U/g fermented culture) as shown in Table 1. The selected WGM concentration for the maximum protease production was 6% (Table 2).

Table 1: Effect of different agro-industrial by-products as substrates on protease production by tested organism under SSF

Agro-industrial by-products	Protease activity* (U/g fermented culture)
Sugar beet pulp	175.3 ± 3.3 e
Coconut meal	524.3 ± 12.6 b
Jojoba meal	103.3 ± 4.4 f
Cress meal	11.0 ± 0.58 h
Linen meal	210.3 ± 5.8 d
Wheat germ meal	591.7 ± 10.1 a
Cotton seed meal	210.3± 6.1 d
Pea peels	104.0 ± 4.5 f
Potato peels	50.3 ± 3.0 g
Orange peels	419.0 ± 9.5 c
Banana peels	440.0 ± 18.9 c
Bean peels	154.3 ± 4.7 e
Carrot pomace	218.0 ± 10.4 d

* Protease activity is expressed as mean value ± standard error. Values for each treatment per tested organism followed by different letters are significantly different at $P \leq 0.05$.
Fermentation conditions: 3% by-products, 10% moisture content, 30 °C incubation temperature and 7 days incubation period.

Table 2: Effect of wheat germ meal concentration on protease production by tested organism under SSF

WGM concentration (%)	Protease activity* (U/g fermented culture)
1.5	311.3 ± 6.1 d
3.0	536.7 ± 8.8 c
4.5	636.7 ± 18.6 b
6.0	699.3 ± 12.1 a
7.5	651.7 ± 19.2 b
9.0	641.7 ± 11.7 b
12.0	552.5 ± 12.5 c

* Protease activity is expressed as mean value ± standard error. Values for each treatment per tested organism followed by different letters are significantly different at $P \leq 0.05$.
Fermentation conditions: 10% moisture content, 30 °C incubation temperature and 7 days incubation period.

WGM is a product obtained by finely grinding the cake that remains after removal of most of the oil from wheat germ seeds by a mechanical extraction process. It contains 25-30% protein, 47% carbohydrate, 2-10% fat, 9% fiber, 5-10% ash, 0.13% phosphorous, 0.11% potassium and very low concentrations of other numerous elements Therefore, WGM medium contains most of the requirements for growth and protease production by *Bacillus* isolate P16.

In the literature different natural substrates namely rice bran, wheat bran, soya bean meal, ground nut cake, green gram husk, *Jatropha curcas* seed cake, nug meal, banana waste, potato peels and coconut oil cake were used for protease production under SSF [13,14,15,17,18,20,26].

The authors found no published reports regarding use of sand as a carrier material in SSF. The sand has some benefits as a carrier material in SSF as for instance it does not absorb water, allowing more air to penetrate, occupies a small volume and it can be reused several times. WGM at 6% will be used in the subsequent experiment.

Influence of moisture content on enzyme production

SSF process is different from submerged fermentation culturing, since microbial growth and product formation occurs at or near the surface particle having low moisture content [25, 27]. Therefore, moisture content is a crucial factor in SSF system that influences the microbial growth as well as product yield [28]. In fungal and bacterial SSF, lower moisture levels stated to reduce solubility of the nutrients in the solid substrates, a lower degree of substrates swelling and higher water tension. Similarly, higher moisture content were reported to cause decreased porosity, loss of particulate structure, development of stickiness, reduction in gas volume and decreased gas exchange [29].

In this study, the maximum enzyme production was observed at 10% moisture content (699U/g fermented culture) as shown in Table 3. At lower or higher initial moisture levels than 10%, the enzyme synthesis decreased.

Table 3: Effect of moisture content on protease production by tested organism under SSF

Moisture content (%)	Protease activity* (U/g fermented culture)
5	535.0 ± 12.2 d
10	698.7 ± 5.8 a
20	663.3 ± 7.8 b
30	602.0 ± 4.7 c
40	294.3 ± 8.7 e
50	220.0 ± 11.9 f
60	175.7 ± 6.5 g
70	126.0 ± 8.7 h

* Protease activity is expressed as mean value ± standard error. Values for each treatment per tested organism followed by different letters are significantly different at $P \leq 0.05$.
Fermentation conditions: 6% WGM, 30 °C incubation temperature and 7 days incubation period.

In the literature, the recommended moisture level for protease production by bacilli has been reported to be 30% -140% [13, 17, 28, 30, 31] Low moisture level used in this study is related to the negative water absorbance of the carrier material (sand). In the subsequent experiment, 10% moisture will be applied.

Effect of incubation temperature

It is known that the temperature is one of the most critical parameter that has to be controlled in any bioprocess [32].

Bacillus isolate P16 exhibited the highest enzyme production at 30°C (698U/g fermented culture) as shown in Table 4. Increasing or decreasing the cultivation temperature resulted in reduction of enzyme production. In the available literature, the optimum incubation temperature for protease production by bacilli was 30- 50°C as reported by [18, 19, 26, 30, 33]. Incubation at 30°C will be applied in the following experiment.

Table 4: Effect of incubation temperature on protease production by tested organism under SSF

Temperature (°C)	Protease activity* (U/g fermented culture)
20	365.7 ± 13.3 d
30	698.3 ± 7.3 a
40	588.3 ± 9.3 b
50	538.7 ± 9.8 c

* Protease activity is expressed as mean value ± standard error. Values for each treatment per tested organism followed by different letters are significantly different at P ≤ 0.05.
Fermentation conditions: 6% WGM, 10% moisture content and 7 days incubation period.

Effects of carbon and nitrogen sources on enzyme production

Addition of various carbon sources separately to fermentation medium resulted in a decrease in proteolytic activity (data not shown). This may be attributed to the repressive effect of readily metabolizable sugars on the mechanism of protease production [34].

Culture environment has a dramatic influence on enzyme production especially nitrogen sources which play a crucial role in enzyme induction in bacteria [35]. Among the various tested nitrogen sources, ammonium phosphate at 1% was found to be the best nitrogen source for the enzyme production where it leads to 6% increase in protease production (743.3 U/g fermented culture) as shown in Table 5. Other tested nitrogen sources decreased protease enzyme production to different levels.

Table 5: Effect of nitrogen sources (1%) on protease production by tested organism under SSF

Nitrogen sources	Protease activity* (U/g fermented culture)
None (control)	701.0 ± 4.6 b
Sodium nitrite	491.7 ± 10.1 c
Sodium nitrate	305.7 ± 3.5 e
Ammonium sulphate	312.7 ± 5.2 e
Ammonium phosphate	743.0 ± 12 a
Urea	504.7 ± 10.4 c
Peptone	447.0 ± 9.6 d
Yeast extract	463.7 ± 3.8 d

* Protease activity is expressed as mean value ± standard error. Values for each treatment per tested organism followed by different letters are significantly different at P ≤ 0.05.

It has been reported that beef extract, yeast extract and sodium nitrate supported different bacilli for maximum protease production [14, 17, 33, 19, 31]. On the other hand, [36] found that protease production by *B. subtilis* Y-108 was repressed by most of the nitrogen sources employed by SSF. Ammonium phosphate at 1% will be applied in the subsequent experiment.

Effect of incubation period on enzyme productivity

It is very essential to detect the optimum incubation time at which an organism exhibit the highest enzyme activity since organisms show considerable variation at different incubation periods.

The time course study for enzyme production under SSF condition showed an increase in protease production with increasing the incubation period up to 7 days (751.7 U/g fermented culture) as shown in Figure 1. Incubation for 15 days dramatically decreased the enzyme activity to 276.7 U/g fermented culture. The decline in enzyme activity might be due to denaturation and/or decomposition of protease enzyme as a result of interactions with other compounds in the fermented medium [13].

It was reported that the best incubation periods for the maximum production of protease enzyme by different bacilli species under SSF were 24h, 48h, 60h, 72h, 96h and 120h [13, 19, 26, 31, 33].

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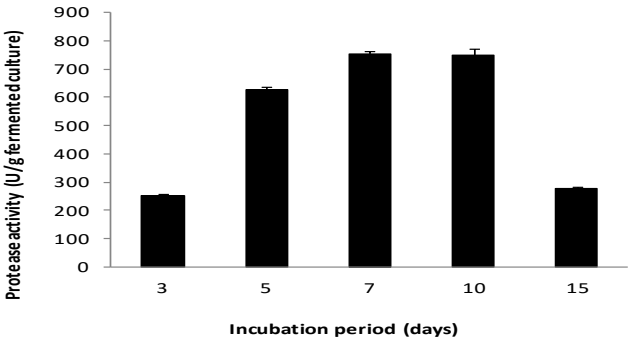

Figure 1: Effect of incubation period on protease production by tested organism under SSF. Fermentation conditions: 6% WGM, 1% ammonium phosphate, 10% moisture content and at 30 °C incubation temperature.

Influence of inoculum size

Size of inoculum is an important biological factor, which determines biomass production in the fermentation process. The results showed no significant effect on protease production with inoculum size range from 2.4×10^6 - 75.8×10^6 CFU/g (data not shown).

Effect of metal ions

Among the tested metal salts, calcium chloride enhanced the protease production about 8% as shown in Figure 2. On the other hand, protease production was completely inhibited by manganese, ferrous and cobalt ions.

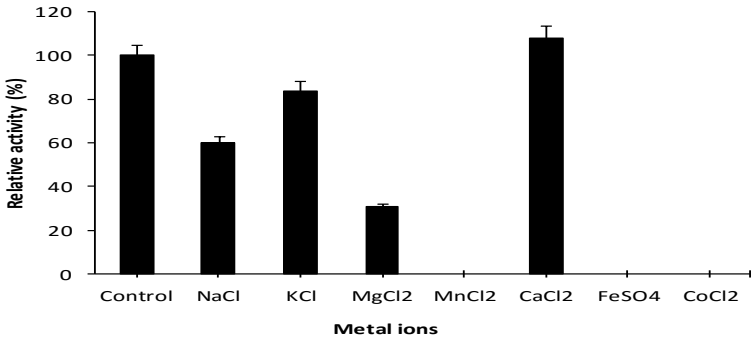

Figure 2: Effect of metal ions on protease production under SSF. Fermentation conditions: 6% WGM, 1% ammonium phosphate, 10% moisture content, 30 °C incubation temperature and 7 days incubation period.

Positive effects of metal cations on protease production have been reported by [37, 38, 39]. However, supplementations of metal ions like Co, Mn, Mo, Zn, and Ca, Mg, and Fe ions showed repressive effect on protease production as reported by [40]. In the following experiment $CaCl_2$ will be added.

Effect of Tweens on enzyme production

As shown in Figure 3, Tween 60 and Tween 80 enhanced the protease production about 15 % however; Tween 20 and Tween 40 had inhibitory effect.

It was suggested that the surfactants altered the cell permeability which resulted in a higher release of the enzyme [41]. Tween 80 has been reported to increase the yield of extracellular enzymes and other microbial metabolites [42].

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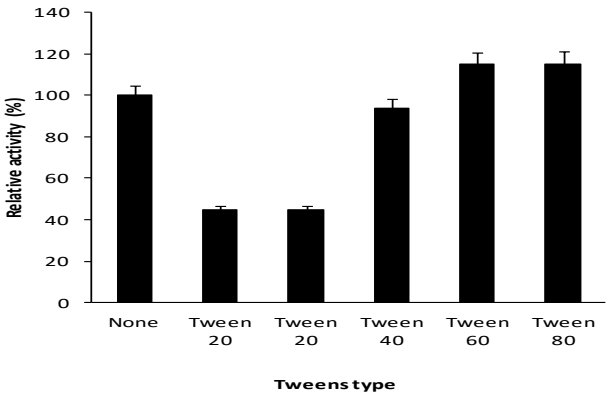

Figure 3: Effect of Tweens on protease production under SSF. Fermentation conditions: 6% WGM, 1% ammonium phosphate, 1% $CaCl_2$, 10% moisture content, 30 °C incubation temperature and 7 days incubation period.

Pilot-scale production of protease enzyme under SSF conditions in trays and fermentation medium cost

Production of PA surface modifying protease enzyme in trays (30 × 30 × 10 cm^3) under the best SSF conditions showed protease activity of 900 U/g fermented culture. It was concluded that pilot-scale production of protease enzyme by *Bacillus* isolate P16 using WGM as a medium and sand as a carrier in trays was as powerful as lab-scale production (in flasks).

The medium cost for production of one kilogram of fermented culture containing 900000 U of protease enzyme was estimated at $ 2.8 as shown in Table 6. It was observed that ammonium phosphate and calcium chloride increased the protease production about 14% while they increased the cost of the fermentation medium about 90%. Therefore, these two components could be excluded to reduce the medium cost to $ 0.27 for production of one kilogram of fermented culture containing 774000 U protease.

Table 6: Cost of fermentation medium contents for production of 1 kg of fermented culture.

Contents	Cost ($)
Sand (50 g)	0.00034
WGM (3 g)	0.003
Ammonium phosphate (0.53g)	0.07
Calcium chloride (0.53)	0.08
Tween80 (0.05)	0.0049
Tap water (5.4 ml)	0.0000036
Seed culture	0.0063
Total quantity	59.51g
Total price	$ 0.165
One kg product price	$ 2.765
Units/gram	900

Fermentation conditions: 6% WGM, 10% moisture content, 30 °C incubation temperature and 7 days incubation period.

Enzyme characterization

The optimum temperature for the highest protease activity was around 60°C (Figure 4) and it was stable between 30°C and 60°C (Figure 5) for 10 min. However at 70°C, protease activity dramatically decreased (about 85%). The maximum activity of the enzyme under study (incubated at 60°C) was at pH 8, at 60°C as shown in Figure 6, suggesting that it is an alkaline protease. It was also noted that the activity was unstable at all tested pH values (5-9) for 1 h (Figure 7). These results are in accordance with observations stated by [12]. They found that protease produced by *Bacillus* strain has maximum activity at 60°C and pH 8. [31], reported that the maximum protease activity produced by *Bacillus cereus* was 50°C and pH 8 and was stable over temperature range 40-50 °C and pH 6-9.

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It was observed that the enzyme activity was enhanced by nearly 3% -5% in the presence of calcium chloride, magnesium chloride and sodium chloride as shown in Figure 8. On the other hand, ferrous sulphate, and copper sulphate dramatically inactivated the enzyme. In agreement with these results, [31, 33] reported that Ca^{2+}, Na^{+}, K^{+} and Mg^{2+} were activators while Cu^{2+}, Fe^{2+}, Co^{2+} and Zn^{2+} were inhibitors for protease enzyme produced by bacilli under SSF.

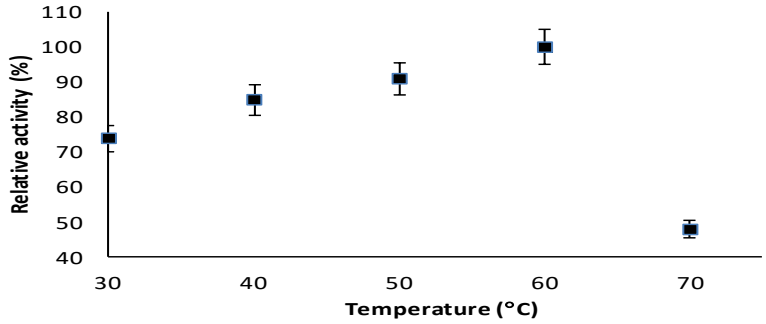

Figure 4: Optimum temperature of protease enzyme produced under SSF. Reaction conditions: pH 8 for 30 min.

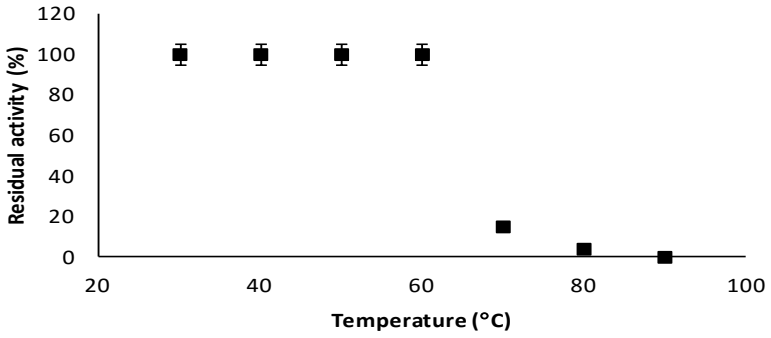

Figure 5: Thermal stability of protease enzyme produced under SSF. Reaction conditions: pH 8 at 60°C for 30 min.

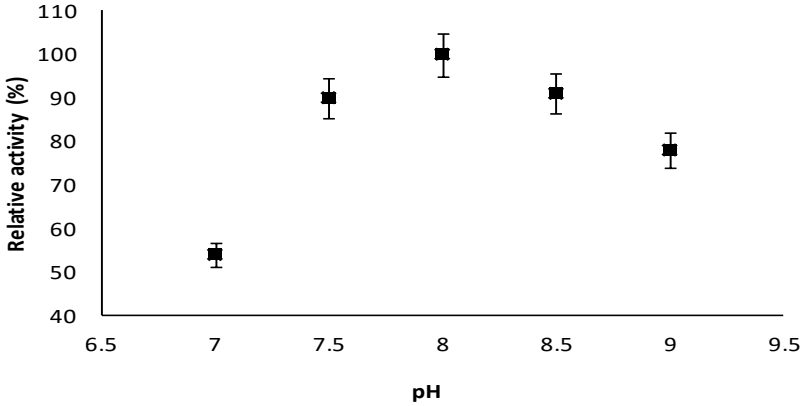

Figure 6: Optimum pH of protease enzyme produced under SSF. Reaction conditions: 60 °C for 30 min.

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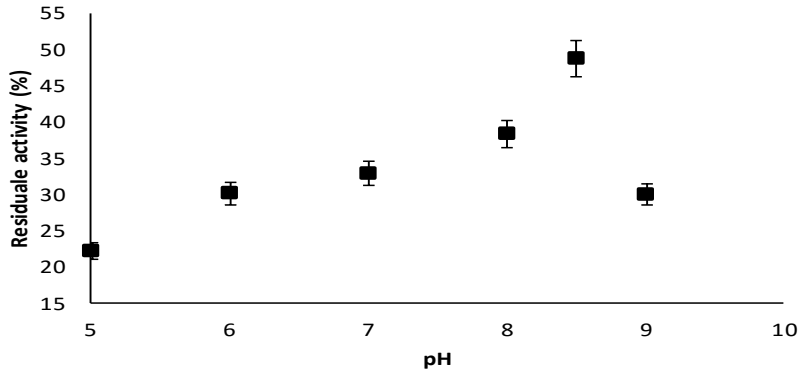

Figure 7: pH stability of protease enzyme produced under SSF. Reaction conditions: pH 8 at 60 °C for 30 min.

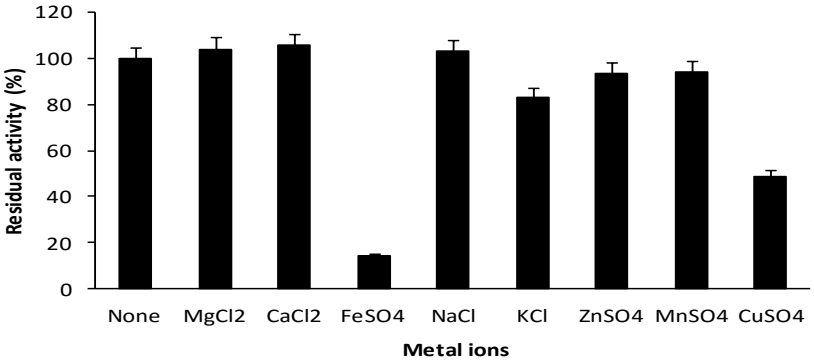

Figure 8: Effect of metal ions on protease activity. Reaction conditions: pH 8 at 60°C for 30 min.

Finally, it is of interest to note that the present work has shown the feasibility of SSF for the first time for production of PA surface modifying protease enzyme produced by *Bacillus* isolate P16 on WGM using sand as a carrier. This approach is expected to be highly feasible and cost-effective for production of protease enzyme and other industrial enzymes.

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