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Influence of Physical and Chemical Parameters on Protease Production by *Bacillus megaterium* IBRL MS 8.2 in Submerged Fermentation

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Abstract: Proteases are a group of enzymes that catalyze the hydrolysis of protein and among the various proteases, bacterial proteases are the most significant when compared to animal and fungal proteases. In this study, a local bacterial isolate *Bacillus megaterium* IBRL MS 8.2 was found to produce the highest protease production of 155.38 U mL^{-1} when cultivating it in the improved medium which consisted of initial medium pH of 7.5, inoculum size of 7.5% (3×10^7 cells mL^{-1}), agitation speed of 150 rpm, incubation temperature of 30°C, 0.5% of sucrose and 1.0% of beef extract. The highest protease production was achieved on the 30 h of cultivation. On the other hand, the highest cell growth of 2.33 g L^{-1} was achieved on 18 h of cultivation time. The data obtained suggest that enzyme production by *B. megaterium* IBRL MS 8.2 was not growth dependant.

Key words: *Bacillus megaterium*, submerged fermentation, proteases, enzyme production, Malaysia

INTRODUCTION

Proteases are a group of enzymes whose catalytic function is to hydrolyze peptide bonds of proteins and break them down into polypeptide or free amino acids. They are also called proteolytic enzymes or proteinases and are one of the most important groups of industrial enzymes. Proteases account for nearly 60% of the total industrial enzyme market (Chu, 2007; Oskouie *et al*., 2008). Based on their acid-based behavior, proteases are classified in to three groups that is acid (performed best at pH range of 2-5), neutral (performed best at pH 7) and alkaline (performed best at pH ≥8) proteases (Padmapriya and Williams, 2012). Neutral proteases have wide application in baking, protein modification and in the leather, animal feeds and pharmaceutical industries (Beg and Gupta, 2003) whereas alkaline proteases are widely used in detergent industry besides leather and textile industries (Chen *et al*., 2004; Kasana and Yadav, 2007). Acid proteases are used mainly in food processing industry, such as dairy industry as milk clotting agents for the manufacturing of cheese and to improve food flavor (Shivakumar, 2012).

Proteases are widespread in nature and microorganisms serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications (Kocher and Mishra, 2009). Microbial proteases are produced from high yielding strains including species of *Bacillus*, *Alcaligenes*, *Pseudomonas*, *Brevibacterium* and *Aeromonas* (Boominathan *et al*., 2009; Shabbiri *et al*., 2012). Among these, a large proportion of commercially available proteases are derived from Bacillus strains (Mehrotra *et al*., 1999). Bacillus proteases are predominantly extracellular and can be concentrated in the fermentation medium. Several Bacillus species involved in protease production are *B. licheniformis* (Ferrero *et al*., 1996), *B. cereus* (Nilegaonkar *et al*., 2007), *B. sterothermophilus* (Sookkheo *et al*., 2000), *B. mojavensis*, *B. megaterium* and *B. subtilis* (Agel *et al*., 2012; Boominathan *et al*., 2009; Padmapriya and Williams, 2012; Shumi *et al*., 2004; Soares *et al*., 2005). To get a high yield of protease production by microorganisms, selection of the right organism plays a key role. Besides that the growth of microorganism and the product yields are also influenced by nutritional and physical parameters. Therefore, the objective of the present research was to improve the different environmental factors, such as initial pH of the medium, cultivation temperature, inoculum size, agitation speed and also cultivation period together with the chemical; factors including carbon and nitrogen sources for the maximal production of proteases by *B. megaterium* IBRL MS 8.2. These information are important as prerequisite for scale up production of enzyme by the organism.

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MATERIALS AND METHODS

Microorganism, culture maintenance and inoculum preparation: *B. megaterium* IBRL MS 8.2 culture was obtained from the Industrial Biotechnology Research Laboratory culture collection, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia and was used throughout the study. The bacterial culture was grown on nutrient agar slant at 37°C for 24 h aerobically and stored at 4°C for further use. The subculturing was performed every month to ensure its survival.

Inoculum was prepared by transferring a loop-full culture of *B. megaterium* IBRL MS 8.2 from the slant aseptically into 250 mL Erlenmeyer flasks containing 50 mL of nutrient broth. The flasks were kept on a rotary shaker at 100 rpm for 24 h at 37°C. The content of the flasks were centrifuged 6000 rpm for 10 min and the supernatant was decanted. The cell pellet was washed thoroughly with sterile distilled water. Finally, the cell mass was suspended in sterile distilled water and used as inoculums for subsequent experiments.

Medium composition and cultivation conditions: The cultivation was carried out in 250 mL Erlenmeyer flask containing 90.00 mL of cultivation medium consisted of (%; w/v): Sucrose, 1.0; yeast extract, 1.0; $CaCl_2.2H_2O$, 0.01; KH_2PO_4, 0.1, $MgSO_4.7H_2O$, 0.01 and Ca $(CH_3COO)_2$ 0.01. The pH of the medium was adjusted to 7.0 prior of sterilization. After sterilization, the medium was cooling down to room temperature (30±2°C) and the medium was inoculated with 10% (v/v) of the inoculums (3×10^7 cells mL^{-1}) and incubated on an orbital shaker incubator (Certomat-H, B. Braun) at 37°C, agitated at 200 rpm for 72 h of cultivation period for enzyme production. The samples were withdrawn at every 6 h intervals and were assayed for protease activity and cell growth determination. All experiments were conducted in triplicate and results represent the average values with standard deviation.

Improvement of cultural conditions for maximum protease production: The optimization of cultural conditions (physical parameters) for maximum protease production was carried-out to determine the optimal temperature (25, 30, 35, 37 and 40°C), initial medium pH (6.0, 6.5, 7.0, 7.5 and 8.0), agitation speed (100, 150, 200 and 250 rpm) and inoculum sizes (2.5, 5.0, 7.5, 10.0 and 12.5%; v/v of 3×10^7 cells mL^{-1}). The medium compositions (chemical parameters) were also optimized, including the various carbon (citric acid, glucose, lactose, maltose, molasse, starch and sucrose) and nitrogen (beef extract, peptone, yeast extract, KNO_3, $NaNO_3$ and $(NH_4)_3SO_4$) sources at 1.0% (w/v). Samples were collected for analysis with each flask representing a single sample (destructive sampling). Time course profile was carried out for 72 h before and after the improvement of physical and chemical parameter conditions. The samples were withdrawn at every 6 hourly intervals and were assayed for protease activity and cell growth determination. All the experiments were performed in triplicate and the values were reported as standard deviations.

Crude enzyme extraction: The culture broth was filtered through a Buchner funnel containing a filter paper (Whatman No. 4) to separate the cells. The cell-free culture filtrate containing the crude enzyme was centrifuged at 5000 g for 10 min and the supernatant was then assayed for protease activity.

Cell growth determination: The cell growth was determined spectrophotometrically at 540 nm and the actual concentration was compared to standard curve prepared prior determination. The cell growth was expressed as g/L.

Determination of protease activity: Protease activity was determined using the method previously described by Hagihara *et al.* (1958). In this assay, the proteolysis activity was determined by detecting the release of amino acid (tyrosine) upon casein hydrolysis. About 0.5% (w/v) casein which was previously dissolved in 200 mM $Na_2CO_3/NaHCO_3$ buffer, pH 10 (prepared by titrating disodium carbonate with sodium bicarbonate), containing 2 mM $CaCl_2.5H_2O$ and 0.02% sodium azide, NaN_3) was heat up to 42°C in a shaker incubator. Then, 0.5 mL of crude enzyme was transferred to each 0.5 mL pre incubated casein buffered solution in Eppendorf tubes and mixed on a vortex. The enzyme-substrate mixture tubes were further incubated in a shaker incubator at 42°C and 200 rpm for 30 min. To stop the reaction, 1.0 mL of 10% (w/v) Trichloroacetic Acids (TCA) was added, followed by the mixing of tube's reaction contents on a vortex and the centrifugation at 10,000×g for 10 min. The enzyme activities were determined from the absorbance of the supernatant at 275 nm with a UV-VIS spectrophotometer. The blank was prepared under the similar assay conditions to use the same method with an exception in the sequence of transferring (TCA) where 1 mL Trichloroacetic Acid (TCA) was added to the pre incubated assay solution before enzyme. Stock solution of Trichloroacetic Acid (TCA) 100% (w/v) was prepared by adding 45.4 mL H_2O to 100 g TCA. One unit of protease activity was defined as the activity that liberates

1 μ mol of tyrosine per minute. A standard curve was generated using solutions (0-200 μg mL^{-1}) tyrosine.

Statistical analysis: In order to evaluate and determine the significant of the findings and also to compare the differences among the findings, the statistical analysis was used. One way Analysis of Variance (ANOVA) and Duncan Multiple Range Test (DMRT) with PASW Statistics 18 version were used to analyze the significant different of the mean of experimental data. A 5% confidence level or $\alpha = 0.05$ were used to test all experimental data. All enzyme activities and fungal growth were made in triplicates. The error bars showed the standard deviation of triplicates.

RESULTS AND DISCUSSION

Incubation time for highest protease production: To study the optimal incubation time for maximum protease production, the fermentation samples were withdrawn periodically at every 6 h up to 72 h and assayed. The results are shown in Fig. 1. Protease activity was detected after 6 h of cultivation and increased gradually until achieved the highest production of 15.47 U mL^{-1} at 48 h of cultivation. However, the production dropped after achieving the maximum production and maintained after 60 h of cultivation until the end of the experiment. The growth of the cells increased until after 30 h of cultivation with 2.53 g L^{-1} as it reached the stationary phase with a declining level of growth rate. The results also revealed that the maximum protease production was obtained at the stationary phase of growth. Hence, the protease production by *B. megaterium* IBRL MS 8.2 showed partial growth dependence. The pH of the cultivation medium was slightly dropped at the end of the experiment. Therefore, 48 h was used as a cultivation time for the subsequent experiments.

Effect of initial medium pH on protease production: The bacteria was allowed to grow in media of different pH ranging from 6.0, 6.5, 7.0, 7.5 and 8.0 (Fig. 2). The highest protease production was observed in the medium of pH 7.5 with 22.67 U mL^{-1}. Lower or higher pH values than the optimum level produced lesser enzyme production. The maximum cell growth of 2.73 g L^{-1} was also obtained at pH 7.5. Therefore, the protease produced by *B. megaterium* IBRL MS 8.2 might be the neutral protease. Protease production by microbial strains strongly depends on the extracellular pH because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes which in turn support the cell growth and product production. The findings are in agreements with those findings by Anuraj *et al.* (2012) who isolated neutral protease producer, *Paenibacillus polymyxa* EMBS 024. The results were contradictory to Kuberan *et al.* (2010) who reported that the maximum protease production was at pH 8. It is likely that changes in pH cause denaturation of enzyme resulting in the loss of catalytic activity. Therefore, each enzyme has specific pH optima for its activity. Since microbes can produce acid, neutral or alkaline proteases, the results obtained in this study revealed that *B. megaterium* IBRL MS 8.2 produced neutral pH.

Effect of inoculums size on protease production: Initial microbial load or inoculums size to a medium does affect the growth and in-turn metabolite production. To study the effect of inoculum size, the experiments were conducted using 2.5, 5.0, 7.5, 10.0 and 12.5% of 3×10^7 cells mL^{-1} inoculum size (Fig. 3). The results indicated that protease production was increased with increase in size of inoculum up to 7.5% level with 74.04 U mL^{-1} protease production and 2.73 g L^{-1} of cell growth. Further increase in inoculums level did not increase the protease production, even though increased in inoculums size definitely increased the cell growth. This condition could be due to a competition for the nutrients among the bacterial cells. Similar observations are

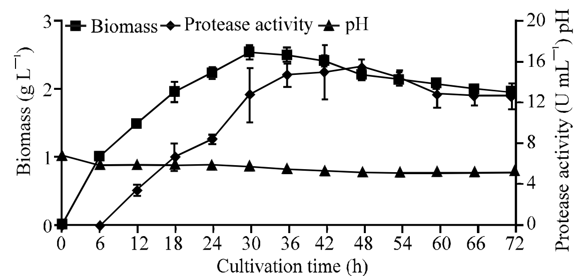

Fig. 1: Time course profiles of protease production and cell growth by *Bacillus megaterium* IBRL MS 8.2 before improvement

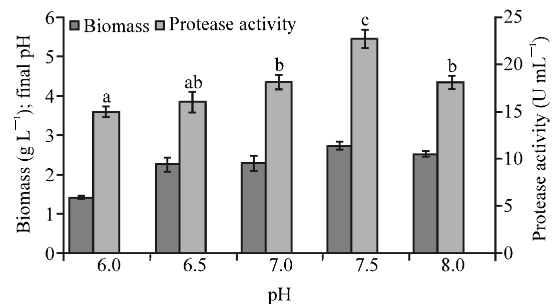

Fig. 2: Effect of initial medium pH on the growth and protease production by *B. megaterium* IBRL MS 8.2

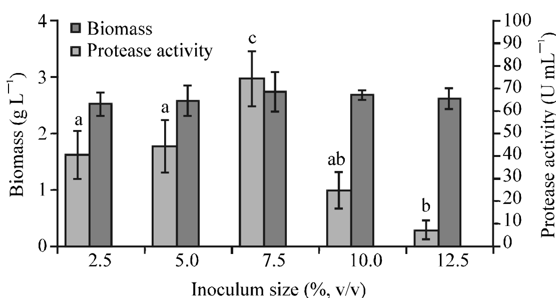

Fig. 3: Effect of inoculum sizes on the growth and protease production by *B. megaterium* IBRL MS 8.2

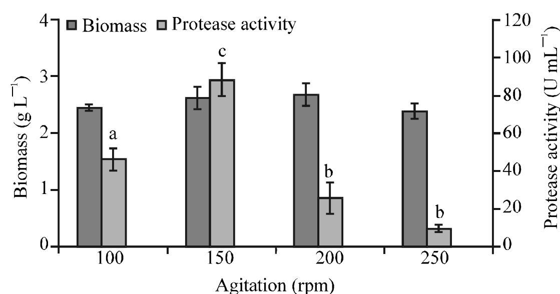

Fig. 4: Effect of agitation rate on the growth and protease production by *B. megaterium* IBRL MS 8.2

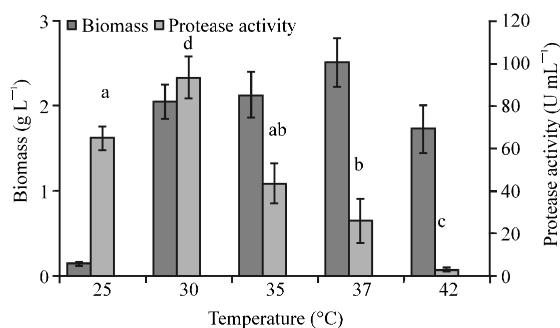

Fig. 5: Effect of temperature on the growth and protease production by *B. megaterium* IBRL MS 8.2

explicitly reported by Ahlawat *et al.* (2009) in the case of pectinase production by *Bacillus subtilis* SS. The enzyme production by inoculums size of 1% (v/v) was much higher compared to 2% (v/v). Adequate nutrient supply could be the reason of the higher enzyme production with lower inoculums size. Moreover, higher inoculums size might not necessarily give higher protease production or cell growth because higher inoculums sizes could result in the lack of oxygen and nutrient depletion in the culture medium.

Effect of agitation speed on protease production: Figure 4 shows the effect of agitation speed on the enzyme production. The enzyme production increased as the agitation speed increased. Maximum protease production of 88.00 U mL^{-1} was achieved at agitation speed of 150 rpm. However, the highest cell growth was obtained at the agitation speed of 200 rpm with 2.67 g L^{-1}. It was observed that increased in agitation speed help the microbial synthesis of enzyme (Potumarthi *et al.*, 2007) which consequently increased the assimilation of sugars (Fontana *et al.*, 2009). Besides that the increased in agitation speed also increased the dissolve oxygen supply in the cultivation medium. Nutrient uptake by bacteria also increased resulting in increased protease production. However at higher agitation speeds (beyond 150 rpm), the enzyme production dropped. This could be due to the higher shear stress when the cells were agitated at higher speeds (Darah and Ibrahim, 1996). Darah *et al.* (2011) found that high agitation speed caused a drop in enzyme production due to excessive breakdown of the enzyme or cell lysis or excessive cell permeability related to abrasion by shear forces. Lower agitation speed than 150 rpm resulted in lower enzyme production and this could be due to the minimum level of dissolved oxygen in the cultivation medium (Saurabh *et al.*, 2007). According to Kao *et al.* (2007), incomplete mixing and/or oxygen transfer might be the reason of low production at lower agitation speeds. Thus, mixing is crucial for better oxygen and nutrient transfer rate in microbial enzyme production.

Effect of cultivation temperature on protease production: Enzyme activity recorded at different temperatures revealed that the *B. megaterium* IBRL MS 8.2 yielded maximum protease production at 30°C with 93.42 U mL^{-1} (Fig. 5). Higher or lower temperature than the optimum ones produced lower enzyme production. The highest cell growth was at the incubation temperature of 37°C with 2.51 g L^{-1}. The results of the study showed that there was no correlation between cell growth and enzyme production and enzyme production was not growth dependant. Temperature is one of the most important factors affecting the enzyme production. Majority of *Bacillus* sp., can grow and produced proteases in the temperature ranged of 30-40°C. Since, Bacillus strains were mesophilic type and the results were in line with the findings by other researches (Genckal and Tari, 2006; Uyar *et al.*, 2011). The incubation temperature was found to influence extracellular enzyme secretion. The enzyme activity decreased gradually as the temperature increased. This condition could be due to the effects of high temperature on the growth rate of *B. megaterium* IBRL MS 8.2 since, this bacteria is initially was a

mesophilic bacteria. Another possible reason could be the breaking down of enzyme at higher temperatures as enzyme denature above the optimum temperature which possibly caused by changing the physical properties of the cell membrane (Rahman *et al*., 2005).

Effect of different carbon sources: Various source of carbon, such as citric acid, glucose, lactose, maltose, molasse, starch and sucrose was used to replace sucrose which was the original carbon source in the cultivation medium. Results obtained showed that sucrose (disaccharide) gave the highest protease production of 94.31 U mL^{-1} compared to other carbon sources (Fig. 6). Among the monosaccharide tested, fructose showed higher protease production of 70.04 U mL^{-1} compared to glucose which only produced 19.91 U mL^{-1}. Cell growth was enhanced when lactose and starch were added in to the cultivation medium but enzyme productions were inhibited in the medium. The results revealed that maltose and citric acid did not affect protease production by *B. megaterium* IBRL MS 8.2. Although, the highest growth of 2.81 g L^{-1} was obtained in the medium containing molasses, the enzyme production was low of only 5.78 U mL^{-1}. This result suggested that the carbon source used was able to affect the biosynthetic pathway of the microorganism which subsequently affected enzyme activity. It was found that *B. megaterium* IBRL MS 8.2 grew well in the absence of carbon source, however the protease activity was low (5.60 U mL^{-1}). It can be deduced that the yeast extract powder may act as the carbon source which support the growth of *B. megaterium* IBRL MS 8.2. Rahman *et al*. (2005) reported that growth and protease production by *Pseudomonas aeroginosa* strain K were detected using basal medium which contained only peptone and other trace elements. The same finding was obtained by Dube *et al*. (2001) where growth and protease activity by proteolytic anaerobic bacteria (SPA-1-6) were obtained using M-5 broth medium which without any carbon source supplemented.

Effect of concentration of sucrose: The concentration of sucrose was optimized to determine the optimum concentration for protease production. The results are shown in Fig. 7 revealed that higher sucrose concentration reduced the enzyme production and growth by the isolate. The maximum protease production of 99.82 U mL^{-1} was obtained when 0.50% (w/v) of sucrose was added into the cultivation medium. The results showed that enzyme production dropped drastically at the concentration >1.0%. At lower concentration of sucrose of 0.25%, the low amount of

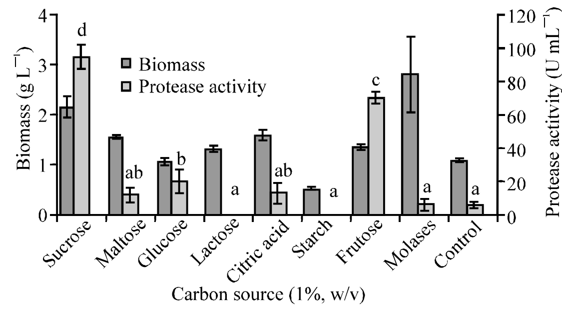

Fig. 6: Effect of different carbon sources on growth and protease production by *B. megaterium* IBRL MS 8.2

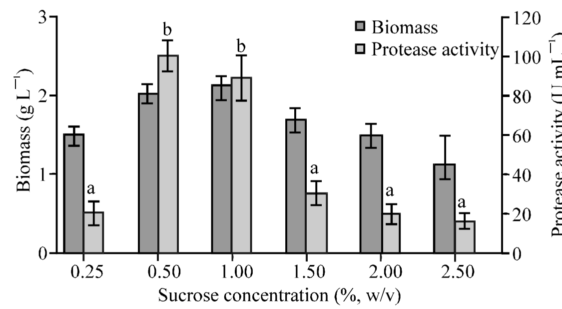

Fig. 7: Effect of sucrose concentration on growth and protease production by *B. megaterium* IBRL MS 8.2

protease production observed and this could be due to the lower growth. The growth of the isolate was maximum at the sucrose of 1.0% with 2.12 g L^{-1} biomass and decreased slowly thereafter. Due to protease production of 0.50 and 1.00% (w/v), sucrose were not significantly different and to reduce the production cost, 0.50% (w/v) of sucrose was selected for used in subsequent experiments.

Effect of different nitrogen sources: Nitrogen is needed for the synthesis of amino acids, purines, pyramidenes, enzyme cofactors and other substances. Figure 8 shows the effect of nitrogen sources on the growth and protease production by *B. megaterium* IBRL MS 8.2. Among the various nitrogen sources tested, it was clearly shown that *B. megaterium* IBRL MS 8.2 preferred organic nitrogen sources than inorganic nitrogen sources. The results showed that by adding organic nitrogen sources, such as yeast extract, beef extract and peptone significantly enhanced the growth and protease production. The enzyme secretion was inhibited when inorganic nitrogen sources, such as $NaNO_3$, KNO_3 and $(NH_4)_3SO_4$ were added into the cultivation medium. All the inorganic nitrogen sources tested did not significantly enhance the growth

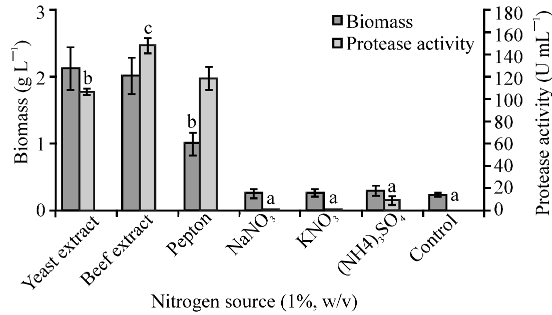

Fig. 8: Effect of different nitrogen sources on growth and protease production by *B. megaterium* IBRL MS 8.2

of the isolate. However, the highest protease production of 147.38 U mL^{-1} was obtained when beef extract was used as the nitrogen source. The protease production in the presence of yeast extract and peptone were found to be 105.33 and 118.40 U mL^{-1}, respectively. As shown in Fig. 8, bacterial growth was detected in the control medium which is without the addition of any nitrogen source. This false result may due to the nitrogen source in nutrient broth which was transferred together with the cells pellet during inoculation into the control medium. Naidu and Devi (2005) reported that medium supplemented with 1% beef extract produced the highest protease activity of 75 U mL^{-1} by *Bacillus* sp.

Effect of concentration of beef extract: The concentrations of beef extract added in the fermentation medium were 0.5, 1.0, 1.5, 2.0 and 2.5 (%, w/v) (Fig. 9). The results obtained revealed that the concentration of beef extract at 1.0% (w/v) gave the maximum protease production of 151.11 U mL^{-1} with the growth of 2.03 g L^{-1}. Increased in the beef extract concentration above 1.0% (w/v) resulted in a drop in protease production. At 0.5% (w/v) of beef extract, the enzyme production was low or of 116.98 U mL^{-1} and the biomass collected was only 1.0 g L^{-1}. On the other hand, the results showed that 1.5% (w/v) of beef extract gave the maximum growth of *B. megaterium* IBRL MS 8.2 of 3.10 g L^{-1}. However, the growth was maintained thereafter even at higher beef extract concentration. Therefore, 1.0% (w/v) of beef extract was selected for further experiments.

The growth and protease production profiles after the improvement of physical (cultural conditions) and chemical (medium compositions) parameters were studied for 72 h (3 days). The improved medium which consisted of initial medium pH of 7.5, inoculum size of 7.5% (v/v; 3×10^7 cells mL^{-1}), agitation speed of 150 rpm, incubation temperature of 30°C, 0.5% of sucrose and 1.0% of beef

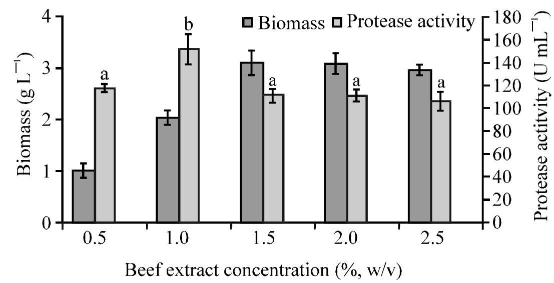

Fig. 9: Effect of beef extract concentration on the growth and protease production by *B. megaterium* IBRL MS 8.2

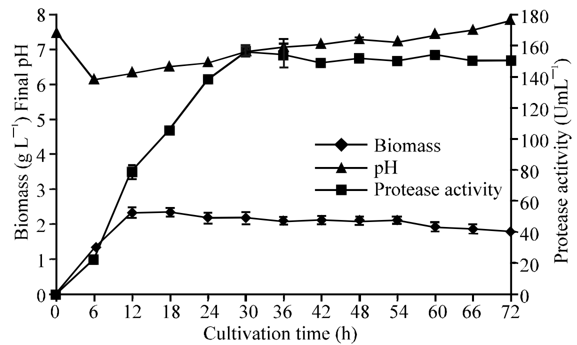

Fig. 10: Profile of growth and protease production by *B. megaterium* IBRL MS 8.2 after cultivation conditions and medium compositions optimization

extract. As shown in Fig. 10, the protease production increased gradually from 6 h and reached maximum production at 30 h of cultivation with an activity of 155.38 U mL^{-1} which remained almost constant thereafter. The growth of the isolate increased drastically and achieved the maximum growth at 18 h of cultivation with 2.33 g L^{-1} cell growth. After the optimal cultivation time, the growth of *B. megaterium* IBRL MS 8.2 declined gradually and reached 1.76 g L^{-1} of cells produced at the end of cultivation. The pH values were decreased and it started to increase after 6 h of cultivation until the end of the experiment (72 h) where in was in the alkaline values. According to Kaur *et al*. (1998), this condition could be due to the production of secondary metabolites that were released in to the cultivation medium.

Production of protease by *B. megaterium* IBRL MS 8.2 before and after the improvement of physical (cultural conditions) and chemical (medium compositions) parameters were examined. Table 1 summarizes the parameters involve in the enhancement of the protease production and cell growth. It was found that there was a

Table 1: A summary on the comparison of non-optimized and optimized physical conditions and medium composition of protease production by *B. megaterium* IBRL MS 8.2

Parameters	Before improvement	After improvement
Physical conditions		
pH	7.0	7.5
Inoculum size (v/v)	10%	7.5%
Temperature (°C)	37	30
Agitation rate (rpm)	200	150
Medium composition (w/v)		
Sucrose	10%	0.5%
Extract	10% (yeast)	1.0% (beef)
KH_2PO_4	0.1%	0.1%
$CaCl_2$	0.01%	0.01%
$MgSO_4.7H_2O$	0.01%	0.01%
Ca $(CH_3COO)_2$	0.01%	0.01%
Protease production	15.47 U mL^{-1} (at 48 h)	155.38 U mL^{-1} (at 30 h)

tremendous increment in protease production after improvement of parameters and also shorter time of cultivation in achieving maximum protease production.

CONCLUSION

The results obtained from this study revealed that culture conditions contributing to the maximal production of proteases. The results showed an increment about 904.4% of protease activity after the optimization (155.38 U mL^{-1}) compared to before optimization (15.47 U mL^{-1}). New isolate *B. megaterium* IBRL MS 8.2 produced neutral proteases which have many applications especially in food, cosmetic and pharmaceutical industries.

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