International Journal of Engineering

Journal Homepage: www.ije.ir

Growth Media Optimization for Production of Alkaline Protease from Industrial Wastewater using *Bacillus subtilis* PTCC 1254

Z. Jafari[a], G. Najafpour Darzi*[a], H. Zare[b]

[a] *Biotechnology Research Laboratory, Faculty of Chemical Engineering, Babol Noshirvani University of Technology, Babol, Iran*
[b] *Department of Chemical Engineering, Buein Zahra Technical University, Buein Zahra, Qazvin, Iran*

PAPER INFO

Paper history:
Received 05 August 2022
Received in revised form 05 October 2022
Accepted 14 October 2022

Keywords:
Alkaline Proteases
Bacillus subtilis
Low-Cost Substrate
Starchy Wastes
Kinetic Model

ABSTRACT

Alkaline proteases are widely used in industrial processes due to their high pH tolerance and thermal stability. In present work, the protease producing ability of *Bacillus* strains (*Bacillus subtilis* PTCC 1254, *B. subtilis* PTCC 1156 and *B. subtilis* PTCC 1715) was studied. *B. subtilis* PTCC 1254 showed the highest proteolytic activity and therefore, the strain was selected as the biological agent in the submerged fermentation. Cell growth kinetic model was investigated using Malthus and Logistic equations, which were relatively well fitted to the experimental data. The maximum specific growth rate for Malthus and Logistic models were 0.187 and 0.377 h^{-1}, respectively. The optimum culture conditions were defined as follows: pH 9, temperature 37°C, fermentation time 72 h, agitation speed 150 rpm and 4% inoculum with medium contained 1 g/l $CaCl_2$, 0.6 g/l K_2HPO_4, 1 g/l KH_2PO_4, 0.2 g/l $MgSO_4.7H_2O$, 2 g/l sugarcane bagasse and 4 g/l corn bran as carbon and nitrogen sources. A 25% v/v industrial wastewater containing starchy waste was used as main substrate. Under optimum conditions, maximum alkaline protease activity of 117.43 U/ml was achieved. Also, the obtained protease was able to remove blood stain from cotton fabric and hydrolyze gelatin of X-ray film. Thus, this protease showed potential applications in detergent and photographic industries.

doi: *10.5829/ije.2023.36.03c.11*

1. INTRODUCTION

Enzymes as biocatalysts are used in biochemical reactions. Enzymes are also commercially used in number of industrial processes [1]. Among various enzymes, about 60% of total enzyme marketplace are devoted to protease which are the most important group of industrial enzymes [2, 3]. Protease catalyze the hydrolysis of peptide bonds linking amino acids in the polypeptide chain of the protein molecule [4]. Enzymes are specifically classified based on enzyme structure and properties of enzyme active site. Proteases are categorized based on their activities with special functional group such as carboxyl, serine, metallo, neutral, acidic and alkaline proteases [5]. The most important group of enzymes so far used are the alkaline serine proteases [6]. Alkaline proteases as additives are extensively used in pharmaceuticals, dehairing of leather, textile, food and detergent industries [7, 8]. The major sources of alkaline proteases consist of plants, animal organs and microorganisms. The microorganisms including bacteria, molds and yeasts can produce alkaline proteases [5, 9, 10]. Presently, *Bacillus* species are commercially used to obtain alkaline proteases due to their high pH and temperature tolerances [11]. Selection of the right microorganism is very important in production of high yield of the desirable enzymes. Furthermore, protease production by microorganisms is affected by the medium composition; the nitrogen and carbon sources and process parameters such as pH, temperature, agitation speed, incubation time and inoculum size [12, 13]. Achieving high enzyme productivity significantly depends on selection of suitable fermentation technique and optimization of

*Corresponding Author Institutional Email: Najafpour@nit.ac.ir
(G. Najafpour Darzi)

Please cite this article as: Z. Jafari, G. D. Najafpour, H. Zare, Growth Media Optimization for Production of Alkaline Protease from Industrial Wastewater using *Bacillus subtilis* PTCC 1254, ***International Journal of Engineering, Transactions C: Aspects,*** Vol. 36, No. 03, (2023) 513-522

media composition [14, 15]. Proteases and other enzymes are often produced by submerged and solid state fermentation techniques [16, 17]. Currently, more than 90% of the commercial enzymes are produced through implication of submerged culture due to its obvious advantages in consistent enzyme production characteristics with defined medium, process conditions and advantages in downstream despite of the cost-intensiveness for medium components [18, 19]. The cost of enzyme production is the most important factor which effects on process economics. The most important factor in the cost of enzyme production in submerged fermentation is devoted to the growth medium [20]. One of the environmental problems in recent years is the treatment and release of industrial wastewater. Certainly, special wastewater, in particular those collected from food factories can be used for production of value-added products. Through, specific process and recyclable materials can be utilized for production of protein, enzyme and etc. [21, 22]. Another low-cost substrate that can be used for enzyme production is agricultural waste. The cost of enzyme production is reduced by utilization of agro-waste like rice bran, wheat bran and corn bran [23, 24]. In this study, specific strain of bacterial strains with high proteolytic activity was selected. In addition, considering the economic aspect of enzyme production, low-cost substrate was used for enzyme production. The purpose of present work was to demonstrate a thermal tolerant species of bacteria for the production of protease by means of media and process parameters optimization in order to maximize enzyme productivities.

2. MATERIALS AND METHODS

2. 1. Organisms and Media *B. subtilis* PTCC 1254, *B. subtilis* PTCC 1156, *B. subtilis* PTCC 1715 were supplied by the Iranian Research Organization for Science and Technology, Iran. These microorganisms were cultivated in a medium containing 4 g/l glucose, 2 g/l peptone, 2 g/l yeast extract, 1 g/l KH_2PO_4 and 0.2 g/l $MgSO_4.7H_2O$, and were incubated in an incubator-shaker (IKA KS 4000 ic Control) at 33°C and 110 rpm for 24 h. The stock cultures of all strains were maintained on culture tubes and stored at -4°C for long term preservation. To prepare solid phase media for culture studies, 2% agar was added to the liquid media. The microorganisms were cultured for purity and stock cultures were regenerated in every month on a fresh plate.

2. 2. Materials Industrial wastewater was collected from the starch manufacturing plant (Mahshad, Yazd, Iran). The wastewater consisted: starch (3.65 g/l), glucose (3.321 g/l), total nitrogen (8.8 mg/l N), NH_4 (11.7 mg/l), NH_3 (10.7 mg/l), total phosphate (52.48 mg/l P), amount of ash (0.2%), total suspended solid (1.06%). The substrates screened were sugarcane bagasse, rice bran, wheat bran as carbon supplements; corn bran and soybean meal as nitrogen sources. All substrates were washed with distilled water and dried at 60°C for 48 h to remove undesired debris. In order to obtain proper powder, dried substrate was milled and kept in a dry place. All substrates were purchased from local market.

2. 3. Screening for Alkaline Protease Activity Casein-agar medium consisted 10 g/l casein, 5 g/l NaCl, 12 g/l agar, 5 g/l beef extract and 5 g/l peptone. The respective strains were inoculated on the plates as a single line and incubated at 37°C for 48 h. The production of alkaline protease was proved by the formation of clear zones surrounding the colonies [25]. The microorganism with maximum zone formation was selected for further analysis.

2. 4. Inoculum Preparation and Production of Enzyme A loop-full of the agar culture of each strain was added into 50 ml of the medium containing 10 g/l glucose, 5 g/l peptone, 5 g/l yeast extract, 0.2 g/l $MgSO_4.7H_2O$ and 1 g/l KH_2PO_4, and were incubated at 37°C for 24 h. This formulation is known as modified Horikoshii medium [26]. The inoculum was prepared by inoculating 5% of the prepared solution into prior medium and incubated for 16 h. Then 5% of the inoculum was added into medium containing (g/l): peptone 5, KH_2PO_4 1, K_2HPO_4 0.6, and 25% (v/v) starchy waste. Submerged fermentation was carried out at 37°C and 130 rpm for 72 h. The fermentation residue was centrifuged at 8000 rpm and kept at 4°C for 10 min. The supernatants were used to measure protease activity.

2. 5. Growth Kinetics The culture containing 10 g/l glucose, 5 g/l peptone, 5 g/l yeast extract, 0.2 g/l $MgSO_4.7H_2O$ and 1 g/l KH_2PO_4 was inoculated with 5% (v/v) inoculum and incubated in a shaking incubator at 37°C for 24 h. Samples were taken at certain time intervals and the sugar concentration and cell density were determined. Cell dry weight was determined using a 0.25 µm filter by drying biomass at 60 °C for 24 h. 3,5-dinitrosalicylic acid (DNS) method was used to determine reduced sugar concentration [27].

Malthus and logistic equations are known as unstructured kinetics models that are used for investigation of kinetic growth of *B. subtilis* PTCC 1254. Malthus rate model is defined as the changes of biomass concentration which is proportional to the cell growth:

$$\frac{dx}{dt} = \mu x \quad (1)$$

where x is cell dry weight (g/l) and µ is specific growth rate (h^{-1}). Apply separation of variables then integration with initial condition, the above equation is simplified as:

$$\ln\left(\frac{x}{x_0}\right) = \mu t \quad (2)$$

where x_0 is the initial cell dry weight (g/l) and t is the incubation time (h). Logistic equation is derived by the following equation:

$$\mu = \mu_m(1 - \frac{x}{x_m}) \quad (3)$$

where μ_m and x_m are the maximum specific growth rate (h^{-1}) and the maximum cell concentration (g/l), respectively. By substitution of the above equation into the Malthus equation and performing integration, cell dry weight can be expressed as the following equation [28]:

$$x = \frac{x_0 \exp(\mu_m t)}{1-(\frac{x_0}{x_m})(1-\exp(\mu_m t))} \quad (4)$$

2. 6. Assay for Proteolytic Activity Alkaline protease activity was assayed by the suggested method of Sigma-Aldrich. 5 ml casein solution (0.65%, w/v casein in 50 mM potassium phosphate buffer, pH 7.4) was placed at the water bath at 37°C until equilibrium was achieved. One ml of the enzyme solution was poured to the mixture and allowed to react for 10 minutes. Then the reaction was stopped with 5 ml TCA solution (0.11 M trichloroacetic acid). After 30 min the sample was centrifuged at 8000 rpm for 10 min. A 2 ml of the supernatant was mixed with 5 ml sodium carbonate solution (0.5 M) and 1 ml Folin-Ciocalteu's phenol solution and the mixture was incubated at water bath at 37°C for 30 min. The amount of tyrosine released was determined using a UV-VIS spectrophotometer (UNICO, 2100 series, USA) at 660 nm against the blank. The concentration of tyrosine released was obtained by a spectrophotometer at a wavelength of 660 nm. One unit of protease activity is equivalent to the protease required to release 1 µg tyrosine under standard conditions in 1 min.

2. 7. Optimized Culture Conditions for the Growth of *B. Subtilis* PTCC 1254 The basal media with 25% (v/v) starchy waste as a carbon source was used for production of alkaline protease by *B. subtilis* PTCC 1254. Initial experiments showed that optimum percent of waste for production of protease was 25% (v/v). Several nitrogen sources such as 5 (g/l) yeast extract, casein, potassium nitrate, ammonium chloride, sodium nitrate, soybean meal, corn bran and corn flour were replaced instead of peptone. The basal medium was supplemented with various carbon sources such as 5 (g/l) glucose, fructose, lactose, arabinose, starch, sugarcane bagasse, rice bran and wheat bran. Effect of different parameters such as temperature (30, 35, 37, 40 and 45°C), pH (5, 6, 7, 8, 9, 10, 11 and 12), $CaCl_2$ concentration (0.5, 1, 1.5 and 2 g/l), inoculum concentration %v/v (1, 2, 3, 4, 5 and 10), agitation speed (110, 130, 150, 180 and 200 rpm) and incubation time (24, 48, 72, and 96 h) were studied. All the experiments were carried out in triplicates.

2. 8. Cell Growth and Protease Production The *B. subtilis* PTCC 1254 culture was inoculated once in the basal medium (pH 7, agitation rate 130 rpm) and once in the optimal medium under optimal conditions. Samples were aseptically taken at different time intervals and cell density along with enzyme activity was determined.

2. 9. Applications of Alkaline Protease from *B. Subtilis* PTCC 1254 For assessing blood stain removing ability, human blood 100 µl was injected on 100% cotton fabric, then was placed at oven for 5 min (95–100 °C). The alkaline protease (10 ml, 117.43 U/ml) was applied to the stain. The removal of blood stain was investigated at room temperature. Ability of gelatin hydrolysis from X-ray films was also investigated. The X-ray films were washed with double distilled water and dried with ethanol; then were placed in an oven at 40°C for 30 min. X-ray films were placed in the enzyme solutions and incubated in a shaking incubator. Finality, the effect of enzyme activity on the X-ray film was investigated after 10, 20, 30 and 40 minutes. Also, the turbidity of reaction mixture was determined at 660 nm.

3. RESULTS AND DISCUSSION

3. 1. Screening for Alkaline Protease Activity Protease production ability of all three bacterial samples cultured on casein-agar medium was investigated. Figure 1 shows colonies and the clear zone created by each one of the bacterial colonies. All strains showed a positive result for protease production on casein-agar medium, but the best protease producer displayed the highest clear zone of inhibition was *B. subtilis* PTCC 1254. Several studies have demonstrated that the formation of clear zone on casein-agar or skim milk agar by a bacterial colony is an indication of alkaline protease production [29, 30]. The results showed that all three strains can produce alkaline proteases.

3. 2. *B. Subtilis* PTCC 1254 Growth Kinetic Figure 2 shows the changes of sugar concentration and cell growth during the fermentation time. The growth curve showed that *B. subtilis* PTCC 1254 had a short lag phase. Exponential growth phase was extended from 2 to 16 h of incubation time; while after 16 h, the substrate concentration was reduced by 70%.

Based on obtained data, Malthus and Logistic kinetic growth rate modes are illustrated in Figures 3a and 3b, respectively. The data were relatively well fitted with a regression of 0.94 and 0.97 for Malthus and Logistic models, respectively. The maximum specific growth rate for Malthus and Logistic models were 0.187 and 0.377 h^{-1}, respectively. Malthus model is based on simple exponential growth while Logistic model can project the

Figure 1. Bacteria cultured on casein-agar medium, (A) *B. subtilis* PTCC 1156, (B) *B. subtilis* PTCC 1715 and (C) *B. subtilis* PTCC 1254

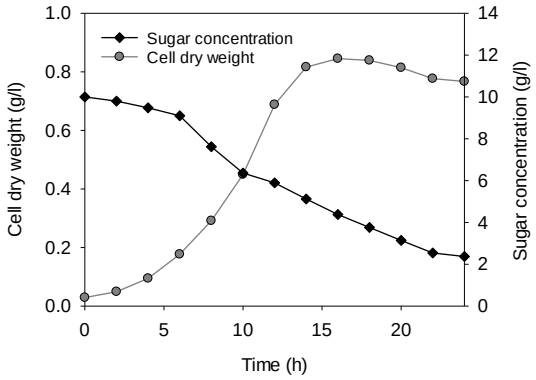

Figure 2. Cell dry weight and sugar concentration profiles

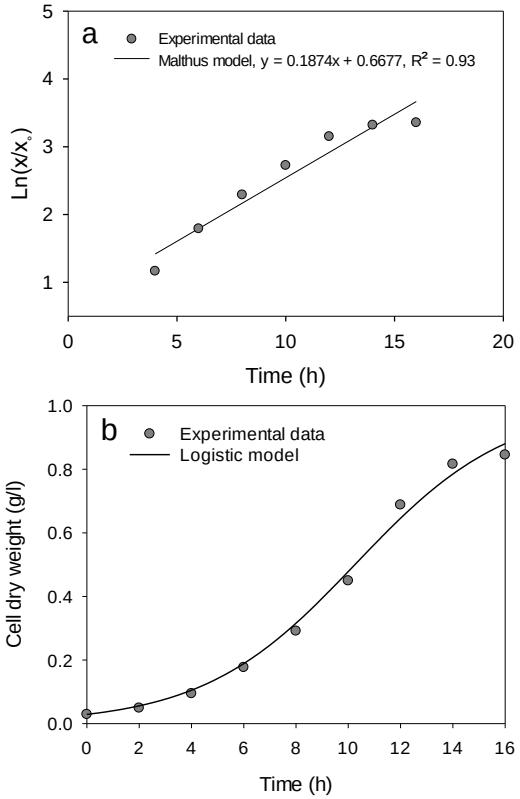

Figure 3. Fitting the experimental data to (a) Malthus kinetic model and (b) Logistic kinetic model

growth curve while considering any growth inhibition term. Based on obtained μ_{max} Logistic rate model was able to describe cell growth and kinetic rate of *B. subtilis* PTCC 1254 in submerged fermentation. Batch kinetics and modeling of alkaline protease production by isolated *Bacillus* sp. was also reported by other researcher using Moser -Boulton kinetic model [31].

3. 3. Production and Enzymatic Activity of Strains

Temperature, agitation and incubation time for the production of alkaline protease by *Bacillus* species were 30-37°C, 100-200 rpm and 24-120 h, respectively [32]. Accordingly, 37°C temperature, 130 rpm and 72 h were selected for incubation of all samples and then bacteria were cultivated in the basal medium. Enzyme activity in each strain was considered with respect to the above-mentioned method. Table 1 summarized the results of enzyme production for each bacterium. The obtained results showed that the unit activity of enzyme production by *B. subtilis* PTCC 1254 was more than other two samples, so the specified strain was selected for further study.

3. 4. Effect of Nitrogen Sources

In primary production of amino acids, nucleic acids, protein and cell wall components are metabolized via utilization of desired nitrogen source. The regulatory effect of these nitrogen sources on the enzyme synthesis was investigated. Presence of both carbon and nitrogen sources in the medium should highly be affected on production of enzyme protease [33]. By evaluating the effect of different nitrogen sources on protease production, it was determined that corn flour and corn bran resulted in the highest enzyme activity (73.20 and

TABLE 1. Enzyme activity of different strains of bacteria

Sample of bacteria	Enzyme activity (U/ml)
B. subtilis PTCC 1254	29.55
B. subtilis PTCC 1715	6.7
B. subtilis PTCC 1156	1.44

72.07 U/ml). Organic nitrogen sources had significant effect on enzyme production, whereas simple inorganic nitrogen sources showed reduced alkaline protease production (see Table 2). Present study is in agreement with previous research as reported that inorganic nitrogen sources significantly reduced protease production in compare to organic nitrogen sources [12, 34, 35]. Use of low-cost feedstock and optimizing the media composition with minimum requirements for maximum enzyme production has a critical role in industrial scale enzyme production. Since corn bran is by-product of the corn industry, it was preferred over corn flour due to its low cost. The advantage of corn bran could be because the organism prefers proteins with slow metabolism or partially hydrolyzed. In many microorganisms, accumulated amino acids that may be produced by highly metabolized nitrogen substrates, repress production of protease [36]. Based on obtained results, it was obtained that the best nitrogen source for enzyme production may be quite different which is depended on selection of suitable microorganism.

3. 5. Effect of Carbon Supplementation In general, it is clearly projected that the desired medium composition for different microorganisms may significantly influence on production of alkaline proteases. Availability of both the carbon and nitrogen sources within the medium had significant effect on protease production and both of these parameters applied regulatory affecting on enzyme production. In this study, complex and simple carbon sources were investigated. Protease production was decreased when simple carbon sources were used in the culture medium, that is most probably caused by the catabolite repression of the enzyme. Unlike simple carbon sources, complex carbon sources were desired substrates for protease production by *B. subtilis* PTCC 1254. The obtained result is in agreement with previous reported data regarding protease production [37-39]. The amount of proteolytic enzyme reached to the maximum in the presence of sugarcane bagasse. Enzyme activity was enhanced by addition of 2 (g/l) sugarcane bagasse (82.09 U/ml) when compared to a basal medium without sugarcane bagasse (see Table 3). A decrease in enzyme production was observed at low and high concentrations of sugarcane. The results suggested that proper concentration level of sugarcane bagasse played a significant role in enhancing the production of alkaline protease. Catabolic repression or substrate inhibition resulted in repressed enzyme production at high substrate concentrations.

3. 6. Effect of Incubation Time A 50 ml of optimized medium was incubated at 37°C (130 rpm) for duration of 24, 48, 72 and 96 hours. Figure 4 presents the effect of incubation time on protease activity. The optimum enzyme production of 81.44 U/ml was observed after 48 h; However, numerically, the highest production of protease was observed at 72 hours (82.09 U/ml). It should be noted that whether increase in fermentation time and hence increase in operational cost can be compensated by extra enzyme production. According to the findings in our experiments, a short fermentation time would be more cost effective compare to the extra enzyme production. A decrease in enzyme units was

TABLE 2. Effect of nitrogen sources on the protease production in basal medium

Nitrogen Sources	Concentration (g/l)	Enzyme activity (U/ml)
Blank	0	29.55
Soybean meal	5	63.71
Corn flour	5	73.20
Corn bran	2	72.07
	4	78
	5	72.03
	6	67.27
	8	64.96
	10	56.48
Yeast extract	5	50.49
Casein	5	61.34
Potassium nitrate	5	11.58
Sodium nitrate	5	23.74
Ammonium chloride	5	25.58

TABLE 3. Effect of carbon sources on protease production in basal medium containing 0.4% corn bran and supplemented with carbon source

Carbon Sources	Concentration (g/l)	Enzyme activity (U/ml)
Blank	0	78
Starch	5	66.68
Wheat bran	5	70.53
Rice bran	5	73.20
Sugarcane bagasse	1	72.01
	2	82.09
	3	79.78
	4	79.13
	5	78.66
Glucose	5	48.41
Fructose	5	51.79
Lactose	5	46.75
Arabinose	5	42.54

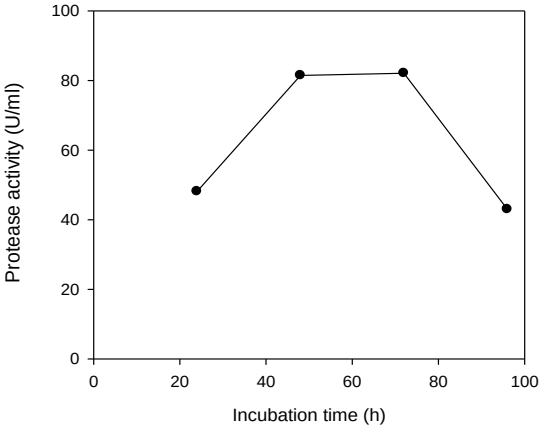

Figure 4. Effect of incubation time on protease activity

observed with increasing incubation time, which could be due to hydrolysis of the protease.

3. 7. Effect of Medium Initial pH In general, the morphological and physiological characteristics of an organism are affected by pH of culture medium. In addition, pH affects many enzymatic processes and the Transport of compounds through the cell membrane [40]. The effect of initial pH for *B. subtilis* PTCC 1254 was obtained by adjusting the culture medium at different initial pH. A 5% (v/v) inoculum was added to the culture medium and incubated at 37°C and 130 rpm for 48 hours.

The protease activity profile at different pH values are shown in Figure 5. This strain produced alkaline protease enzyme in pH wide range from 5 to 9; maximum protease production was found at pH 9 (88.14 U/ml).

3. 8. Effect of Temperature Temperature is the major rolling parameter in growth of microorganism, and it regulates the synthesis and excretion of the enzyme through changes of the physical properties of the cell membrane for extracellular enzymes. One temperature can inhibit the growth of microorganisms while another temperature can activate it. Therefore, in order to obtain an maximum protease production, incubation temperature should be controlled as a critical parameter [41]. The effect of incubation temperature on proteolytic activity was determined by inoculation of 5% inoculum. The effect of temperature on the enzyme activity was obtained by inoculating 5% inoculum into 50 ml of medium at 30, 33, 37, 40 and 45°C for 48 h (Figure 6). Maximum protease activity of 88.14 U/ml was found at 37°C. The reduced enzyme activity at any temperature higher than 37°C might be due to enzyme configuration changes or degradation at high temperature [40, 42].

3. 9. Effect of Agitation Speed Agitation is an influential parameter for mass transfer for the oxygen requirement of microorganisms. Particularly, to make energy for cellular activities, O_2 plays the role of a terminal electron acceptor for oxidative reactions. It has been found that the variation in agitation speed affects the intensity of mixing in shake flasks and nutrient availability [43]. Protease activity was investigated at the agitation speed of 110, 130, 150, 180 and 200 rpm. The effect of the agitation speed on the enzyme production is depicted in Figure 7.

Agitation speed below 150 rpm caused low protease activity caused by limitation not maintaining enough dissolved oxygen for cell growth. Enzyme activity increased with increasing agitation speed, and maximum protease activity was found at 150 rpm. However, keeping the agitation speed at 200 rpm, the enzyme activity (54.82 U/ml) was decreased due to disturbance created at high shear forces. Based on cultivation of the organism, the desired agitation rate was defined at 150 rpm.

3. 10. Effect of Inoculum Size Determining inoculum size with regard to microbial fermentation processes is important since lag phase is eliminated. Increasing in the inoculum size up to 4 v/v% (113.11 U/ml) at which enzyme activity achieved maximum

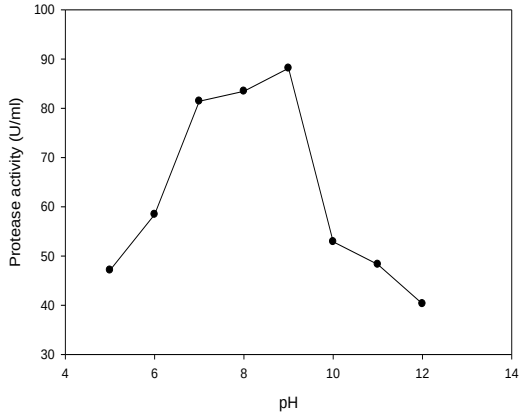

Figure 5. Effect of media pH on production of protease

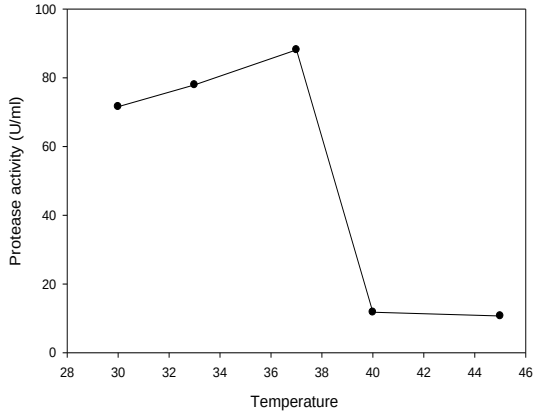

Figure 6. Effect of temperature on production of protease

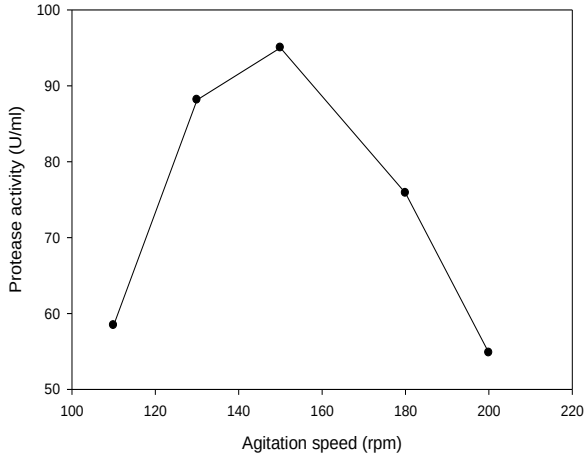

Figure 7. Effect of Agitation Speed on Production of Protease

levels, protease production increased steadily. Thereafter (4%), enzyme activity did not change significantly. Suitable inoculum size can balance between the biomass and available, which improve optimal protease production. [44].

3. 11. Effect of $CaCl_2$ Concentration $CaCl_2$ was used in the composition of the medium to help stabilize the protease secreted by *B. subtilis* PTCC 1254. Addition of $CaCl_2$ also enhanced and maintained the enzyme activity. Regarding protease activities, $CaCl_2$ showed an enhanced effect at a concentration of 1 g/l. Concentrations higher than 1 g/L of $CaCl_2$ had an inhibitory effect which caused decreased in protease activity to 33.05 U/ml.

3. 12. Cell Growth and Protease Production By studying the cell growth of *B. subtilis* PTTC 1254, it was found that in both of medium, the microorganism showed a lag phase of about 4 h. The growth phase was exponential until about 18 h, then it entered the stationary phase. Protease production almost corresponded with cell growth and the maximum value was obtained in the stationary phase (Figure 8). The relationship between growth and enzyme production with different organisms has been reported. Maximum protease production by *Pseudoalteromonas* was achieved at the end of the exponential phase. Whereas, maximal alkaline protease production with *Bacillus* sp VE1 occurred during the early stationary phase [45]. Comparing the production of alkaline protease in basal and optimal medium showed that growth trends were quite similar in both media; however, the amount of enzyme production extensively varied. Maximum alkaline protease was 29.55 U/ml in the basal medium (72 h), while the maximum production of enzyme was achieved after 48 h of fermentation (117.43 U/ml) which is much shorter incubation time in compare to basal medium.

3. 13. Applications of Alkaline Protease from *B. Subtilis* PTCC 1254 The ability of gelatin hydrolysis of X-ray film by the protease obtained from *B. subtilis* PTCC 1254 was investigated. As shown in Figure 9, the enzyme treatment time increased, the gelatin layer was more hydrolyzed and the X-ray films became brighter. The turbidity of reaction mixture was gradually increased with respect to time. In this way, the turbidity was 0.327, 0.926, 1.539 and 1.924 after 10, 20, 30 and 40 min, respectively. Also, alkaline protease from *B. subtilis* PTCC 1254 was able to remove blood stain from cotton fabric (Figure 10). Therefore, the produced protease is suitable for the use in detergent and photographic industries.

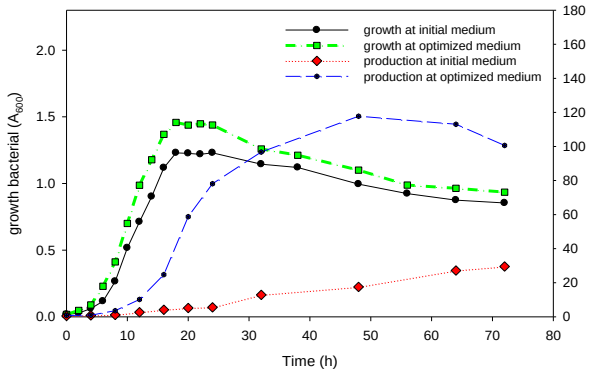

Figure 8. Cell growth and protease production in the basal medium and optimal medium

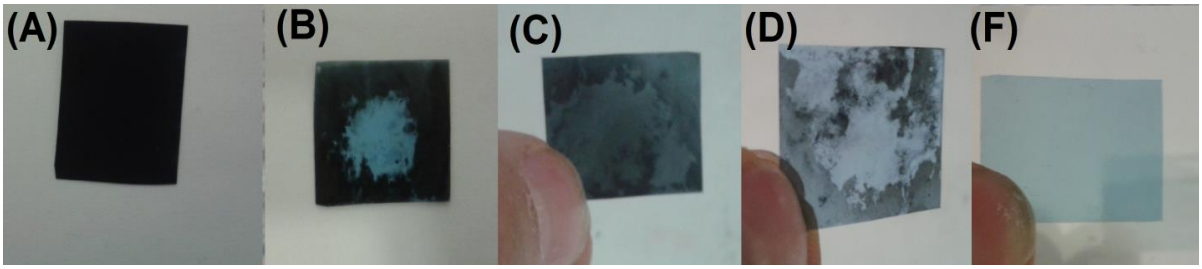

Figure 9. Application of *B. subtilis* PTCC 1254 protease for gelatin hydrolysis of X-ray film, (X-ray film before enzyme treatment (A), X-ray film after 10 min (B), 20 min (C), 30 min (D) and 40 min (F) of enzyme treatment)

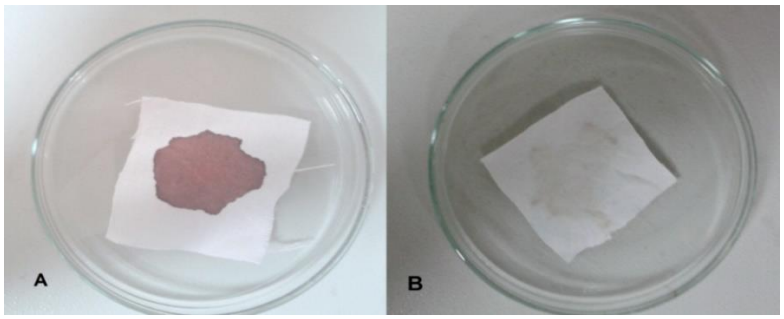

Figure 10. Application of *B. subtilis* PTCC 1254 protease in removing blood from cotton fabric, ((A) bloody cotton fabric before enzyme treatment and (B) cotton fabric after 10 min of enzyme treatment)

4. CONCLUSION

The most important factor affecting on wide application of alkaline proteases for desired use is enzyme cost. In submerged fermentation, a significant part of the total production cost of enzymes is related to the cost of substrate for the cell growth. Under submerged fermentation, the use of organic-based industrial wastewater and agricultural waste such as corn bran and sugarcane bagasse can reduce the cost of producing enzymes. Achievement of high enzyme productivity significantly depends on selection of suitable fermentation technique and optimization of media composition. Malthus and Logistic equations, known as unstructured kinetic models, were used to investigate the growth kinetics of *B. subtilis* PTCC 1254. The data were relatively well fitted with a regression of 0.94 and 0.97 for Malthus and Logistic models, respectively. The maximum specific growth rate for Malthus and Logistic models were 0.187 and 0.377 h^{-1}, respectively. The highest level of protease activity was achieved with the optimized media composition and culture cultivation conditions: industrial starchy wastewater 25% (v/v), sugarcane bagasse concentration 2 g/l, corn bran concentration 4 g/l, $CaCl_2$ 1 g/l, $MgSO_4.7H_2O$ 0.2 g/l, KH_2PO_4 1 g/l, K_2HPO_4 0.6 g/l, pH 9, temperature 37°C, inoculum concentration 4% v/v, agitation speed 150 rpm and fermentation time 48 h. At the optimum condition, alkaline protease production by *B. subtilis* PTCC 1254 increased from 29.55 to 117.43 U/ml. The selected strain showed significant enhancement in protease production under optimized conditions. In addition, the protease produced by *B. subtilis* PTCC 1254 successfully removed the blood stain from cotton fabric and hydrolyzed the gelatin of X-ray film. Thus, the obtained protease found having high potential for applications in detergent and photographic industries.

5. ACKNOWLEDGMENT

The authors would like to thank Biotechnology Research Center, Noshirvani University of Technology, Babol, Iran, for the facilities provided to carry out present research work.

6. REFERENCES

1. Meghwanshi, G.K., Kaur, N., Verma, S., Dabi, N.K., Vashishtha, A., Charan, P., Purohit, P., Bhandari, H., Bhojak, N. and Kumar, R., "Enzymes for pharmaceutical and therapeutic applications", ***Biotechnology and Applied Biochemistry***, Vol. 67, No. 4, (2020), 586-601. doi: 10.1016/j.desal.2011.06.034
2. Naveed, M., Nadeem, F., Mehmood, T., Bilal, M., Anwar, Z. and Amjad, F., "Protease-a versatile and ecofriendly biocatalyst with multi-industrial applications: An updated review", ***Catalysis Letters***, Vol. 151, No. 2, (2021), 307-323. doi: 10.1007/s10562-020-03316-7
3. Pouryafar, F., Najafpour, G., Noshadi, N. and Jahanshahi, M., "Thermostable alkaline protease production via solid state fermentation in a tray bioreactor using *bacillus licheniformis* ATCC 21424", ***International Journal of Environmental Research***, Vol. 9, No. 4, (2015), 1127-1134. doi: 10.22059/IJER.2015.1001
4. Quanten, T., Savić, N.D. and Parac-Vogt, T.N., "Hydrolysis of peptide bonds in protein micelles promoted by a zirconium (iv)-substituted polyoxometalate as an artificial protease", ***Chemistry–A European Journal***, Vol. 26, No. 49, (2020), 11170-11179. doi: 10.1002/chem.202001920
5. Vinoth, J., Murugan, S. and Stalin, C., "Optimization of alkaline protease production and its fibrinolytic activity from the bacterium *Pseudomonas fluorescens* isolated from fish waste discharged soil", ***African Journal of Biotechnology***, Vol. 13, No. 30, (2014), 3052-3060. doi: 10.5897/AJB2014.13863
6. Matkawala, F., Nighojkar, S., Kumar, A. and Nighojkar, A., "Microbial alkaline serine proteases: Production, properties and applications", ***World Journal of Microbiology and Biotechnology***, Vol. 37, No. 4, (2021), 1-12. doi: 10.1007/s11274-021-03036-z
7. Gemechu, G., Masi, C., Tafesse, M. and Kebede, G., "A review on the bacterial alkaline proteases", ***Journal of Xidian University***, Vol. 14, No. 11, (2020), 632-634. doi: 10.37896/jxu14.11/022
8. Hashmi, S., Iqbal, S., Ahmed, I. and Janjua, H.A., "Production, optimization, and partial purification of alkali-thermotolerant proteases from newly isolated *Bacillus subtilis* S1 and *Bacillus amyloliquefaciens* KSM12", ***Processes***, Vol. 10, No. 6, (2022), 1050. doi: 10.3390/pr10061050
9. Arya, P., Jani, S.A., Rajput, K.N. and Raval, V., "Thermostable alkaline proteases from bacteria: A review", ***National Conference on Innovations in Biological Sciences***, 2020. doi: 10.2139/ssrn.3562387

10. Noshadi, N., Mohammadi, M., Najafpour, G. and Pouryafar, F., "Thermostable α-amylase from lignocellulosic residues using *bacillus amyloliquefaciens*", ***International Journal of Engineering, Transactions B: Applications,*** Vol. 30, No. 8, (2017), 1110-1117. doi: 10.5829/ije.2017.30.08b.01
11. Varia, A.D., Shukla, V.Y. and Tipre, D.R., "Alkaline protease-a versatile enzyme", ***International Journal of Research and Analytical Reviews***, Vol. 6, No. 2, (2019), 208-219. doi: http://doi.one/10.1729/Journal.20854
12. Haddar, A., Fakhfakh-Zouari, N., Hmidet, N., Frikha, F., Nasri, M. and Kamoun, A.S., "Low-cost fermentation medium for alkaline protease production by *Bacillus mojavensis* A21 using hulled grain of wheat and sardinella peptone", ***Journal of Bioscience and Bioengineering***, Vol. 110, No. 3, (2010), 288-294. doi: 10.1016/j.jbiosc.2010.03.015
13. Elgammal, E.W., El-Khonezy, M.I., Ahmed, E.F. and Abd-Elaziz, A.M., "Enhanced production, partial purification, and characterization of alkaline thermophilic protease from the endophytic fungus *Aspergillus ochraceus* BT21", ***Egyptian Pharmaceutical Journal***, Vol. 19, No. 4, (2020), 338-349. doi: 10.4103/epj.epj_31_20
14. dos Santos Aguilar, J.G. and Sato, H.H., "Microbial proteases: Production and application in obtaining protein hydrolysates", ***Food Research International***, Vol. 103, No., (2018), 253-262. doi: 10.1016/j.foodres.2017.10.044
15. Gaddad, S.M., "Enhanced production of extracellular alkaline protease by *Bacillus cereus* GVK21 by optimized formulations", ***International Journal of Pharmacy and Biological Sciences, Transactions B: Applications***, Vol. 9, No. 1, (2019), 1103-1113. doi: 10.21276/ijpbs.2019.9.1.141
16. Vaseghi, Z. and Najafpour, G., "A comparison on lipase production from soybean meal and sugarcane bagasse in solid state fermentation using *rhizopus oryzae*", ***International Journal of Engineering, Transactions B: Applications,*** Vol. 27, No. 2, (2014), 171-176. doi: 10.5829/idosi.ije.2014.27.02b.01
17. Hosseinpour, M., Najafpour, G., Younesi, H., Khorrami, M. and Vaseghi, Z., "Lipase production in solid state fermentation using *aspergillus niger*: Response surface methodology", ***International Journal of Engineering, Transactions B: Applications,*** Vol. 25, No. 3, (2012), 151-159. doi: 10.5829/idosi.ije.2012.25.03b.01.
18. Krishnaveni, K., DJ M, B.M., Ramesh, S. and Kalaichelvan, P., "Production and optimization of extracellular alkaline protease from *Bacillus subtilis* isolated from dairy effluent", ***Der Pharmacia Lettre***, Vol. 4, No. 1, (2012), 98-109.
19. López-Gómez, J.P. and Venus, J., "Potential role of sequential solid-state and submerged-liquid fermentations in a circular bioeconomy", ***Fermentation***, Vol. 7, No. 2, (2021), 76. doi: 10.3390/fermentation7020076
20. Alarid-García, C., Hernández-Calderón, O.M., Rios-Iribe, E.Y., González-Llanes, M.D. and Escamilla-Silva, E.M., "Production of β-glucosidase by *Aspergillus niger* CDBB-H-175 on submerged fermentation", ***The Canadian Journal of Chemical Engineering***, Vol. 100, No. 7, (2022), 1489-1501. doi: 10.1002/cjce.24236
21. Zhu, X., Qi, J., Cheng, L., Zhen, G., Lu, X. and Zhang, X., "Depolymerization and conversion of waste-activated sludge to value-added bioproducts by fungi", ***Fuel***, Vol. 320, No., (2022), 123890. doi: 10.1016/j.fuel.2022.123890
22. Tropea, A., "Food waste valorization", ***Fermentation***, Vol. 8, No. 4, (2022), 168. doi: 10.3390/fermentation8040168
23. Singh, R.S., Chauhan, K. and Jindal, A., "Response surface optimization of solid state fermentation for inulinase production from *Penicillium oxalicum* using corn bran", ***Journal of Food Science and Technology***, Vol. 55, No. 7, (2018), 2533-2540. doi: 10.1007/s13197-018-3173-3
24. Espoui, A.H., Larimi, S.G. and Najafpour, G.D., "Optimization of protease production process using bran waste using *Bacillus licheniformis*", ***Korean Journal of Chemical Engineering***, Vol. 39, No. 3, (2022), 674-683. doi: 10.1007/s11814-021-0965-3
25. Li, S., He, B., Bai, Z. and Ouyang, P., "A novel organic solvent-stable alkaline protease from organic solvent–tolerant bacillus licheniformis yp1a", ***Journal of Molecular Catalysis B: Enzymatic***, Vol. 56, No. 2-3, (2009), 85-88. doi: 10.1016/j.molcatb.2008.08.001
26. Horikoshi, K., "Alkaliphiles: Some applications of their products for biotechnology", ***Microbiology and Molecular Biology Reviews***, Vol. 63, No. 4, (1999), 735-750. doi: 10.1128/MMBR.63.4.735-750.1999
27. Najafpour, G., Younesi, H. and Ismail, K.S.K., "Ethanol fermentation in an immobilized cell reactor using saccharomyces cerevisiae", ***Bioresource Technology***, Vol. 92, No. 3, (2004), 251-260. doi: 10.1016/j.biortech.2003.09.009
28. Najafpour, G., "Biochemical Engineering and Biotechnology, Elsevier, (2015). doi: 10.1016/C2013-0-09819-2
29. Abou-Elela, G.M., Ibrahim, H.A., Hassan, S.W., Abd-Elnaby, H. and El-Toukhy, N.M., "Alkaline protease production by alkaliphilic marine bacteria isolated from marsa-matrouh (egypt) with special emphasis on *Bacillus cereus* purified protease", ***African Journal of Biotechnology***, Vol. 10, No. 22, (2013), 4631-4642. doi: 10.5897/AJB10.2111
30. Bajaj, B.K., Sharma, N. and Singh, S., "Enhanced production of fibrinolytic protease from *Bacillus cereus* NS-2 using cotton seed cake as nitrogen source", ***Biocatalysis and Agricultural Biotechnology***, Vol. 2, No. 3, (2013), 204-209. doi: 10.1016/j.bcab.2013.04.003
31. Zamani, M. and Khayati, G., "Batch kinetics and modeling of alkaline protease production by isolated *bacillus* sp.(research note)", ***International Journal of Engineering; Transactions C: Aspects***, Vol. 28, No. 6, (2015), 841-847. doi: 10.5829/idosi.ije.2015.28.06c.03
32. Aehle, W., "Enzymes in Industry: Products and Applications, John Wiley & Sons, (2006).
33. Blieva, R., Mustafin, K., Akhmetsadykov, N., Suleimenova, Z., Saduyeva, Z., Zhakipbekova, A., Tapenbayeva, I., Kalieva, A. and Narmuratova, Z., "Optimization of culture medium for enhanced protease biosynthesis in *Streptomyces globisporus*", ***Rasayan Journal of Chemistry***, Vol. 14, No. 1, (2021), 270-275. doi: 10.31788/ RJC.2021.1416123
34. Gnanadoss, J.J. and Devi, S.K., "Optimization of nutritional and culture conditions for improved protease production by *Aspergillus nidulans* and *Aspergillus flavus*", ***Journal of Microbiology, Biotechnology and Food Sciences***, Vol. 4, No. 6, (2015), 518-523. doi: 10.15414/jmbfs.2015.4.6.518-523
35. Murthy, P.S. and Naidu, M.M., "Protease production by *Aspergillus oryzae* in solid-state fermentation utilizing coffee by-products", ***World Applied Sciences Journal***, Vol. 8, No. 2, (2010), 199-205.
36. Degering, C., Eggert, T., Puls, M., Bongaerts, J., Evers, S., Maurer, K.-H. and Jaeger, K.-E., "Optimization of protease secretion in *Bacillus subtilis* and *Bacillus licheniformis* by screening of homologous and heterologous signal peptides", ***Applied and Environmental Microbiology***, Vol. 76, No. 19, (2010), 6370-6376. doi: 10.1128/AEM.01146-10
37. Al-Harbi, N.A., "Production and physical characterization of thermo-stable and organic solvents-tolerant protease from mesophilic *Bacillus sp.* NASK P6", ***Biosciences Biotechnology Research Asia***, Vol. 9, No. 1, (2016), 157-164.
38. Reddy, L., Wee, Y.-J., Yun, J.-S. and Ryu, H.-W., "Optimization of alkaline protease production by batch culture of *Bacillus sp.* RKY3 through plackett–burman and response surface

methodological approaches", ***Bioresource Technology***, Vol. 99, No. 7, (2008), 2242-2249. doi: 10.1016/j.biortech.2007.05.006

39. Zare, H., Meiguni, F. and D Najafpour, G., "Production of alkaline protease using industrial waste effluent as low-cost fermentation substrate", ***Iranian Journal of Energy and Environment***, Vol. 12, No. 3, (2021), 264-272. doi: 10.5829/ijee.2021.12.03.11
40. George-Okafor, U. and Mike-Anosike, E., "Screening and optimal protease production by *Bacillus sp*. SW-2 using low cost substrate medium", ***Research Journal of Microbiology***, Vol. 7, No. 7, (2012), 327-336. doi: 10.17311/jm.2012.327.336
41. Balaji, N., Rajasekaran, K., Kanipandian, N., Vignesh, V. and Thirumurugan, R., "Isolation and screening of proteolytic bacteria from freshwater fish cyprinus carpio", ***International Multidisciplinary Research Journal***, Vol. 2, No. 6, (2012), 56-59.
42. Genckal, H. and Tari, C., "Alkaline protease production from alkalophilic *Bacillus sp*. Isolated from natural habitats", ***Enzyme and Microbial Technology***, Vol. 39, No. 4, (2006), 703-710. doi: 10.1016/j.enzmictec.2005.12.004
43. Nascimento, W.C.A.d. and Martins, M.L.L., "Production and properties of an extracellular protease from thermophilic *Bacillus sp*", ***Brazilian Journal of Microbiology***, Vol. 35, No. 1-2, (2004), 91-96. doi: 10.1590/S1517-83822004000100015
44. Sandhya, C., Sumantha, A., Szakacs, G. and Pandey, A., "Comparative evaluation of neutral protease production by *Aspergillus oryzae* in submerged and solid-state fermentation", ***Process Biochemistry***, Vol. 40, No. 8, (2005), 2689-2694. doi: 10.1016/j.procbio.2004.12.001
45. Sanchez-Porro, C., Mellado, E., Bertoldo, C., Antranikian, G. and Ventosa, A., "Screening and characterization of the protease cp1 produced by the moderately halophilic bacterium *Pseudoalteromonas sp*. Strain CP76", ***Extremophiles***, Vol. 7, No. 3, (2003), 221-228. doi: 10.1007/s00792-003-0316-9

Persian Abstract

چکیده

پروتئازهای قلیایی به دلیل تحمل pH بالا و پایداری حرارتی به طور گسترده در فرآیندهای صنعتی استفاده می‌شوند. در کار حاضر، توانایی تولید پروتئاز سویه‌های *باسیلوس* (PTCC 1254، PTCC 1156 و PTCC 1715) بررسی شد. *باسیلوس سوبتیلیس* PTCC 1254 بالاترین فعالیت پروتئولیتیک را نشان داد و بنابراین به عنوان عامل بیولوژیکی در تخمیر غوطه‌وری انتخاب شد. سینتیک رشد سلولی با استفاده از مدل‌های مالتوس و لجستیک مورد بررسی قرار گرفت، که به خوبی با داده‌های تجربی برازش شدند. حداکثر نرخ رشد ویژه برای مدل های مالتوس و لجستیک به ترتیب ۰/۱۸۷ و ۰/۳۷۷ بر ساعت بود. شرایط کشت بهینه به این صورت تعریف شد: pH ۹ ، دمای ۳۷ درجه سانتی‌گراد، زمان تخمیر ۷۲ ساعت، سرعت هم زدن ۱۵۰ دور در دقیقه و ٤ درصد مایه تلقیح با ۱ گرم در لیتر $CaCl_2$، ۰/٦ گرم در لیتر K_2HPO_4، ۱ گرم در لیتر KH_2PO_4، ۰/۲ گرم در لیتر $MgSO_4.7H_2O$، ۲ گرم در لیتر باگاس نیشکر و ٤ گرم در لیتر سبوس ذرت به عنوان منابع کربن و نیتروژن. پساب صنعتی ۲۵ درصد وزنی حاوی پسماند نشاسته‌ای به عنوان سوبسترای اصلی استفاده شد. در شرایط بهینه، حداکثر فعالیت پروتئاز قلیایی ۱۱۷/٤۳ U/ml به دست آمد. همچنین، پروتئاز به‌دست‌آمده قادر به حذف لکه خون از پارچه کتان و هیدرولیز ژلاتین فیلم اشعه ایکس بود. بنابراین، این پروتئاز کاربردهای بالقوه‌ای در صنایع شوینده و عکاسی نشان داد.