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Optimization of protease production process using bran waste using *Bacillus licheniformis*

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Abstract−Protease enzyme production by *Bacillus licheniformis* bacteria was investigated. Various agricultural wastes as substrate such as wheat bran, rice bran, and sugarcane bagasse were considered. The most important effective parameters on enzyme production, like incubation time, various substrates and solid substrate particle size, media pH, different nitrogen sources in a bench-scale designed bioreactor, were optimized. The optimum protease production conditions, for both Erlenmeyer flask and batch bioreactor, at 37 °C, pH of 8, incubation time of 48 h, wheat bran (5 wt%) with the particle size of 1 mm, an equal amount of peptone and yeast extract (1% w/w) and agitation rate of 180 rpm were defined. In addition, maximum protease activity in the Erlenmeyer flask and batch bioreactor was 596 and 683.93 U/mL, respectively. The pH and thermal stability of produced protease were studied; the highest amount of remaining activities at pH 8 and 60 °C were 97 and 63% of initial activities, respectively. Also, shelf-life of the produced protease enzyme retained up to 88% of its initial activity after 30 days of storage at 4 °C. However, the produced enzyme was exposed remarkably compatible with the commercial detergent; the enzyme perfectly washed and removed the stains from the sample cotton textile.

Keywords: Protease, Wheat Bran, *Bacillus licheniformis*, Bioreactor

INTRODUCTION

Enzymes are proteins that act as biological catalysts and are used in various industrial processes. Proteases are known as one of the most important groups of industrial enzymes. The main enzyme production in the world belongs to protease enzyme family (approximately 60%) [1,2]. This enzyme can hydrolyze peptide bonds (R-OC-NH-R') in proteins and peptide groups. Based on the Enzyme Commission (EC), proteases belong to group 3 of the hydrolases and subgroup 4, hydrolysis of peptide bonds [2]. Due to proteases' high activity and stability over a wide range of pH and temperature, they can be applied in various industries such as detergent, food, silk, leather, dairy, beverage, brewing, textile, bioremediation and pharmaceutical production [1,3-5].

The global laundry detergent market size was estimated to be 109.6 billion tons in 2018 and is expected to grow at a CAGR[1] of 6.5% from 2019 to 2026; so developing an efficient laundry detergent is demanded and economically valuable [1]. It is obvious that a good detergent must remove various types of stains from blood, food, and other secretions; whereas adding various enzymes like protease in detergents can be effective [6]. Proteases can be produced from animals, plants, and microorganisms; in particular, its microbial production from bacteria is noticeable because its properties can be easily manipulated [1,2,7]. It can be noted that important prerequisites for the use of protease in detergents are activity and stability of enzyme at alkaline pH and temperature as well as compatibility with other ions and oxidizing agents. Also, due to economic reasons and saving energy consumption is reasonable to use protease enzyme which can be active at low temperature [1,6,8].

Based on the literature, the ingredients of culture medium are strongly affected on produced protease enzyme properties by the specific microorganism [9]. Each kind of microorganism species has a specific condition for achieving maximum enzyme production and cell growth rate; these significant factors, including temperature, pH, aeration, humidity and other substrate properties, must be considered [10]. Whereas, various microorganisms from different fungal and bacteria species such as *Aspergillus niger*, *Bacillus* and *Pseudomonas* species have been commonly used to produce protease [11-13]. It is important to optimize the components of the culture medium to reduce unused materials at the end of the fermentation and facilitate enzyme downstream process and purification [14]. There are several strategies available that are mostly used to improve the efficiency of the enzyme production media, such as one-factor-at-a-time, artificial neural network and genetic algorithm. In one-factor-at-time method, changing a parameter at a time while keeping others at a constant level is used to optimize medium variables to obtain optimum growth of the microorganisms and high efficiency of enzyme production. The important benefit of one-factor-at-a-time method is its uncomplicatedness; in this way, a sequence of experiments can be conducted and their obtained results can be analyzed simply by not using statistical programs [15,16]. Therefore, this paper preferred using one-factor-at-a-time method to optimize the culture medium for protease enzyme production.

Furthermore, on an industrial scale, using an economical culture

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[1]Compound Annual Growth Rate

medium is essential to reduce the enzyme production costs [14]. More than 50% of enzyme production capital cost is related to the substrate; although the use of agricultural waste as nitrogen and carbon sources in the substrate can reduce the final enzyme price [9,17]. As a result, the use of low-cost sources, including agricultural wastes such as wheat bran and rice bran as carbon sources for the growth of microorganisms, followed by the production of the protease enzyme, is desired [2,14]. Note that there is research that investigates protease enzyme production with various nutrients and different methods. In many cases, optimization of the process to reach a high amount of productivity and enzyme activity has been considered. As a case, Potumarthi et al. [18] conducted research on the effects of aeration and agitation on alkaline protease production and yield of submerged fermentation in a batch stirring tank bioreactor using *B. licheniformis*. The maximum produced enzyme activity was 102.04 U/mg DC at agitation of 200 rpm and airflow rate of 3 vvm. Also, Banerjee et al. [19] studied alkaline protease from *Bacillus brevis*. The enzyme from a shake flask culture displayed maximum activity at pH 10.5 and 37 °C. It also has been reported that soybean meal was the best organic nitrogen source and the produced protease enzyme omitted blood stains completely when used along with detergents in the presence of Ca^{2-} and glycine. In addition, Emran et al. [9] produced an enzyme by submerged fermentation using the bacterial strain *Bacillus licheniformis* ALW1 and, with optimizing the process, reached to enzyme activity of 22.903 U/mL. The produced enzyme saved more than 80% of its activity as additive to a commercial detergent during washing performance in the removal of blood stain after 30 min at 50 °C.

However, alkaline protease is currently used as a cleaning additive to synthetic materials; adding protease to detergents can increase the washing efficiency up to 35-40% [13]. Commercial alkaline proteases are usually produced from *Bacillus* spp. for a variety of reasons, like high growth rates, short process time, their ability to secrete enzyme in the extracellular environment; generally recognized as safe, and they can grow on inexpensive substrate and waste agriculture material [12,17]. The enzyme production of protease can be carried out via two methods, submerged and solid-state fermentation, which may vary depending on the type of microorganism and its subsequent applications [11]. The main difference between them is the amount of water content [7]. In submerged fermentation, the microorganisms grow in liquid media culture with high free water available, and in solid-state fermentation, the microorganisms grow in solid support materials in the absence or very low water content. The importance of protease enzyme in Iran is very crucial; as annually is spent a huge amount of currency for import because there is no industrial or commercial protease production plant at present. Consequently, investigation on production of protease enzyme from agricultural waste has national interests, as well as achievement of low production cost.

This study aimed to produce a protease enzyme in the lab scale using agricultural waste and then applying in a bench scale batch bioreactor. Protease enzyme production was considered on three agriculture wastes, including wheat bran, rice bran and sugarcane bagasse. In the next step, enzyme production was optimized based on nitrogen source, pH, the solid substrate particle size and incubation time. The thermal stability and enzyme activity of the produced enzyme at different conditions were examined. Finally, the efficiency of produced enzyme as additive on commercial detergent was considered and the obtained data were reported.

MATERIALS AND METHODS

1. Chemicals

All the chemicals and reagents used in this study were analytical grade and provided by Scharlau Chemicals (Barcelona, Spain), Sigma Aldrich and Merck (Darmstadt, Germany). Tyrosine was supplied by Sigma-Aldrich (St. Louis, MO, USA). The wheat bran, rice bran, sugarcane bagasse and various commercial detergents were obtained from a local supermarket (Babol, Iran).

2. Microorganism

Bacillus licheniformis ATCC 21424 was purchased from Persian Type Culture Collection (PTCC). The bacteria were incubated on agar plate consisting (in g/L): (agar 15, peptone 5, and yeast extract 3) at 37 °C for 24 hours. The prepared culture was kept at 4 °C until the next use. For preparing pre-culture, a single colony from fresh agar medium was inoculated in 250 mL flask containing 50 mL of sterilized broth medium (g/L): (glucose 5, peptone 5, yeast extract 5, magnesium sulfate heptahydrate 0.2 and dipotassium phosphate 1) followed by stirring at 37 °C in 180 rpm for 24 h.

3. Protease Production

The pre-culture 5% (v/v) inoculum was prepared in two flasks: liquid culture contains a composition of pre-culture and another flask contains wheat bran 15 g/L; fermentation was conducted at 37 °C, 180 rpm and incubation period of four days. The samples were collected in each 12 h and the growth rate of bacteria and produced protease activity were measured. At the end of the fermentation process, the medium was centrifuged at 4 °C in 10,000 rpm for 15 min. To determine enzyme activity, the supernatant was used in the next stage.

4. Protease Enzyme Activity Assay

One of the important parameters in enzyme production is the activity of the produced enzyme. In this method, casein was used as a standard substrate. While casein is digested by protease enzyme, the amino acid tyrosine is released along with other amino acids and peptide fragments. One-unit activity of protease (U) was described as the amount of enzyme required that released 1 μg tyrosine per min under the assay conditions. After centrifugation of the medium, 1 mL supernatant containing enzyme solution was added into 5 mL substrate (1% v/v casein with 50 mM buffer Tris/HCl, pH: 8) and incubated at 37 °C for 30 min. For ending reaction, 5 mL of trichloroacetic acid (TCA) 5% was added to the solution and kept at room temperature for 30 min and then it was centrifuged again at 4 °C, 13,000 rpm for 15 min. The supernatant was filtered and its absorbance was measured at 280 nm via UV-spectrophotometer [20,21].

5. Protein Assay

Protein concentration of produced protease was evaluated by the Bradford protein assay via bovine serum albumin as a standard solution [22].

6. Effect of Different Parameters on Protease Production

In this study, the parameters were optimized with 'one-factor-at-

a-time. The agriculture waste as a substrate was replaced with expensive synthetic materials in the submerge fermentation process. Because of high amounts of protein and nutrients, these agricultural wastes are valuable materials for growing microorganisms. The effect of wheat bran, rice bran and sugarcane bagasse on growth rate and protease enzyme production at a concentration of 10 g/L was considered. All agricultural residues were washed with distilled water and then dried at 60 °C in an oven for 48 h; after drying, they were milled by a mixer-grinder and then sieved using standard ASTM sieves with a mesh size of 80-18. The collected obtained substrate with different particle sizes was stored in isolated packages at 4 °C for subsequent experiments. Also, the produced enzyme activity and protein concentration were measured through the fermentation process for each of the substrates. The best substrate which secreted most protease enzyme was selected for further experiments. After the suitable substrate was selected, its concentration in the medium in the range of 1-10% (w/w) was evaluated, and then the optimum value was determined.

However, the fermentation time is one of the important factors that should be considered in order to achieve maximum bacteria growth rate and simultaneously to produce protease enzyme at desired experimental condition. Therefore, fermentation process time was considered as the duration of 96 h and samples were withdrawn aseptically every 24 h; then the protease activity and protein concentration were determined to find optimum fermentation time. Based on the literature, alkaline pH is suitable for the *Bacillus* species to grow and produce protease enzyme. So, the influence of different pH in the range of 7-11 was investigated and the optimum value was defined. In the next step, in order to improve the protease enzyme production and growth rate, the effect of various organic and inorganic nitrogen sources such as peptone, yeast extract, ammonium sulfate, urea and ammonium chloride was investigated. All nitrogen sources were examined at different concentrations in the range of 0.5-7% (w/w); the best nitrogen source and its optimum concentration for the next stage of the experiment were evaluated.

7. Effect of pH and Temperature on Stability & Activity of Produce Protease

To define the optimum pH of protease production, the activity of enzyme was examined in a wide range of pH 6-12. These experiments were carried out via various buffers and casein 1% (w/v) as substrate using the standard method [19]. A different buffer solu-

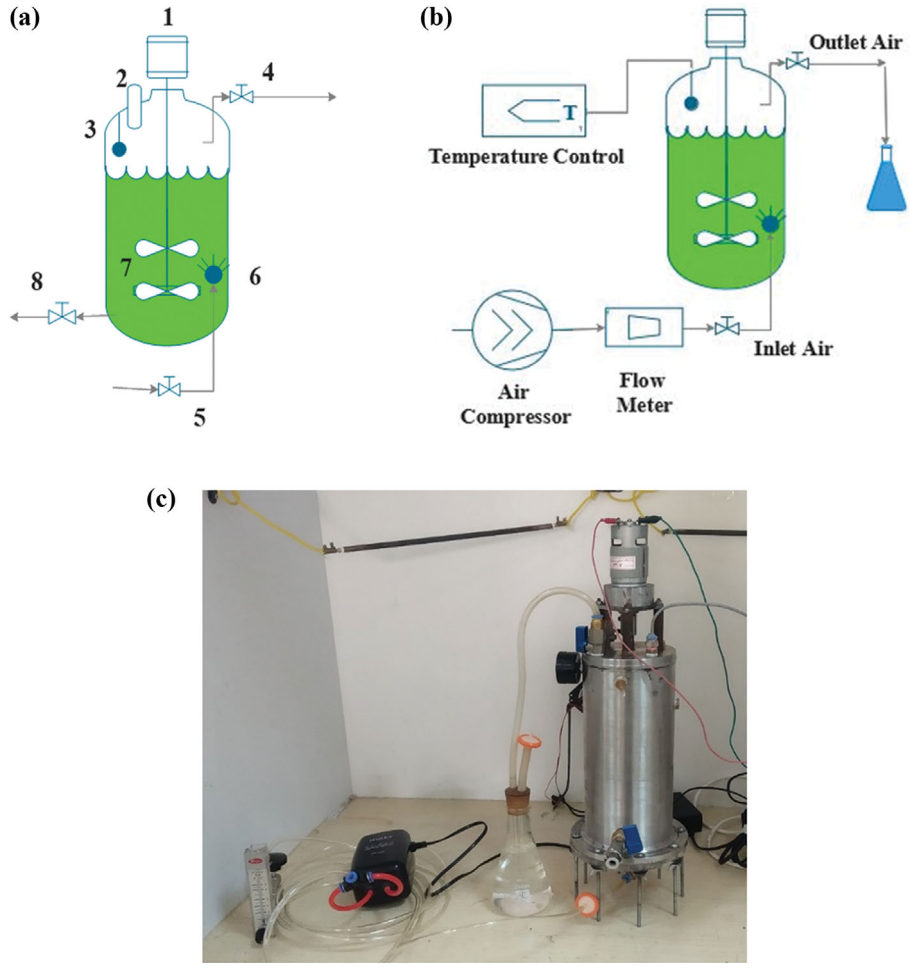

Fig. 1. (a) Schematic of bioreactor. 1) agitator, 2) feed input, 3) temperature sensor, 4) air outlet, 5) air inlet, 6) sparger, 7) blade, 8) sampling valve. (b) Schematic of enzyme production process. (c) The enzyme production process and photo of handmade bioreactor for protease production.

tion, such as citrate solution (pH: 6), potassium phosphate (pH: 7), Tris/HCl (pH: 8), carbonate bicarbonate (pH: 9, 10), bicarbonate/NaOH (pH: 11) and potassium chloride/NaOH (pH: 12), was prepared. To evaluate pH stability, the produced protease enzyme was incubated at 40 °C for 1 h then its activity was measured according to the standard method [19,23].

Additionally, the effect of temperature on produced protease activity was considered in pH: 8 and range of temperature at 30-80 °C under standard condition, then the best temperature was specified. Furthermore, protease thermal stability was assessed at three different temperatures (50, 60, 70 °C) for 120 min and enzyme activity was measured every 20 min; the obtained results were reported [19,24].

8. Bioreactor Design

In the lab scale, an Erlenmeyer flask (250 mL) with 50 mL working volumes was used. In addition, the designed bioreactor was applied for enzyme production on a bench scale up. To fabricate the bioreactor, a stainless steel cylindrical with a working volume of 700 mL was used; a DC stirrer motor (ZGA60FM7) with a maximum speed of 300 rpm was operated to rotate mixing shaft. In designing of the bioreactor, one sample port and a medium inlet with sufficient overhead the bioreactor were planned. The temperature sensor was installed on the upper part of the bioreactor; for controlling and displaying the temperature, the Samwon ENG controller was utilized. To seal the system, at the top and bottom part (rotary inlet section) of the bioreactor, suitable fittings such as a washer and radial shaft seal were used. A schematic diagram and actual image of the fabricated bioreactor is shown in Fig. 1. To control the bioreactor stirrer, a 12 V dimmer was utilized. Also, aeration was carried out by an air compressor (model HAILEA ACO 5501). In addition, for monitoring the inlet air flow rate, a flowmeter (Dwyer) was used. To produce the protease enzyme on a bioreactor, the optimum conditions which were obtained from earlier stage of batch runs were applied.

9. Application of Produced Protease

The application of produced protease as an additive to detergent in the removal of stains, stability and compatibility with commercial detergents was investigated.

9-1. Protease Compatibility with Detergents

The compatibility of the produced protease was studied. Stock solutions with commercial detergents such as Softlan, Golrang, Yekta, Rakht and Derakhshan at a concentration of 7 mg/mL were prepared. Then, 1 mL of the produced enzyme was added to 4 mL of each stock solutions and their relative activity at various temperatures (40, 50, 60 °C) was determined after 1 h. The stability of the above solution with enzyme at temperature 50 °C for 2 h was considered. Then, the activity was measured every 30 min by the standard method [19,23].

9-2. Additive to Detergents

The action of protease as an additive to commercial detergents with pieces (5*5 cm) of white cotton textile with blood stains was examined. To fix the stain on clothes, 100 μL of blood was sprayed on the cloth. After 24 h and fixing the stain on the clothes, the samples were placed in separate flasks and the following steps were performed, respectively.

1. The first flask with 100 mL distilled water with blood stain, as blank.

2. The second flask with 100 mL distilled water and 1 mL detergent solution added at a concentration of 7 mg/mL.

3. The third flask with 100 mL distilled water and 1 mL detergent solution added at a concentration of 7 mg/mL and 1 mL enzyme solution.

Each flask was incubated at 50 °C for 1 h in an incubator-shaker to simulate the washing machine. Then pieces of clothes were washed with water and dried. Visual inspection of remaining stains on pieces of cloth represented the effect of enzyme on the removal of stains [19].

RESULTS AND DISCUSSION

1. Protease Production

Agricultural waste can be used as a substitution substrate for the expensive synthetic media due to its high amounts of protein and nutrients. In addition, it can be used as the valuable additive substance into the medium. Based on the literature, while agriculture waste is used as a substrate at industrial scale, protease enzyme production would be increased; consequently, the cost of produced enzyme noticeably would be reduced. Bacteria growth rate and produced enzyme activity were evaluated in two different cultures for the incubation period of 96 h (according to section 2.3). Based on obtained results, which are illustrated in Fig. 2, wheat bran is a suitable source for protease enzyme production. The protease enzyme used wheat bran and glucose as a substrate possessing enzyme activities of 210.8 and 90.3 U/mL, respectively. Using wheat bran as a substrate is appropriate for both, cell growth rate and enzyme activity. In fact, the bacteria life in exponential rate was extended till 48 h, which is a positive signal for continuous processes or industrial scale and commercial plants.

2. Effect of Various Parameters on Protease Activity

Agriculture waste such as wheat bran, rice bran, and sugarcane bagasse was used as a substrate for protease enzyme production and the obtained results were compared. These experiments were carried out at 180 rpm and 37 °C for four days. The amount of bacteria growth rate, activity of the produced enzyme and synthesized protein were assessed based on explained methods in previ-

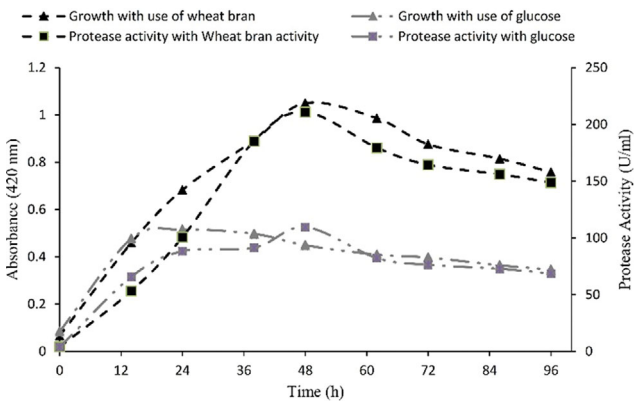

Fig. 2. Bacteria growth rate and produce enzyme activity in submerge fermentation.

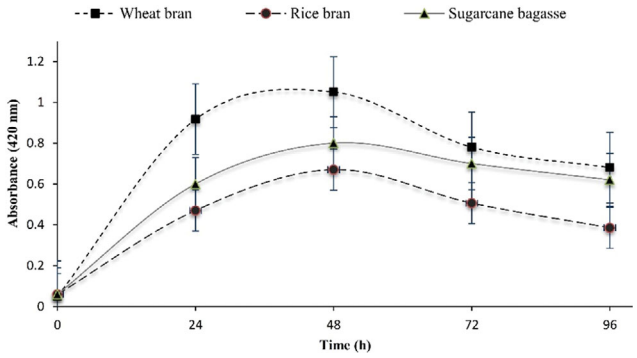

Fig. 3. Effect of different substrates on growth of *B. lichniformis*, which is measured by culture density (OD) in 420 nm via spectrophotometer.

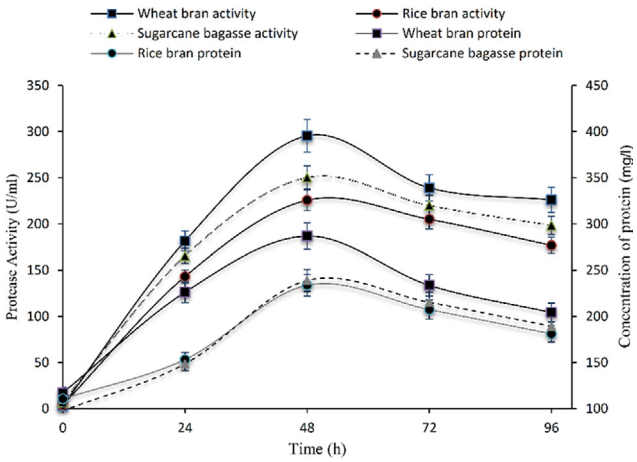

Fig. 4. Effect of different substrates on protease production and protein concentration.

ous sections. As illustrated in Figs. 3 and 4, the highest growth rate of bacteria and maximum protease enzyme activity, by means of wheat bran, were 48 h and 295.53 U/mL, respectively. It can be noted that the reduction of nutrients and the alteration of the enzyme structure were the main reason for the decrease in enzyme production after 48 h. Similar results were reported by Sarker et al. [25]; they produced protease by *Bacillus licheniformis P003* and the maximum enzyme activity achieved was 48.10 U/mL after 48 h. In addition, Olajuyigbe et al. [26] reported maximum protease activity by *B. licheniformis LBBL-11* was 18.4 U/mL after 48 h. The amount of synthetic protein is also shown in Fig. 4. The investigated parameter has been confirmed that the obtained results of produced protease activity were obviously related to the high protein concentration, which was achieved 286.8 mg/L. It can be clearly concluded that wheat bran had produced protease enzyme more than other substrates. Also, similar results were reported in literatures [27,28]. They suggested that wheat bran is an appropriate substrate for protease production in the fermentation process. On the other hand, results indicated that simple carbon sources like sucrose, soluble starch and fructose did not increase protease activity due to the absence of supplementary nutrients and vitamins. However, wheat bran is a suitable source of carbohydrates, proteins and other essential nutrients which are required for the growth of microorganism and the protease enzyme production.

Wheat bran was sieved by ASTM standard with a different mesh size of 18-80. It was observed that approximately 70% of wheat bran was passed from the sieve with mesh 18; 20% mesh 30 and 10% remained. The inoculum was prepared in three flasks with a working volume of 50 mL consisting of fresh medium and different particle sizes of wheat bran. The obtained results for protease activities and protein concentrations are summarized in Table 1. The obtained data indicated that the smaller particle size had the higher amount of enzyme produced. It can be mentioned that the bacteria are interested in adhering to the substrate surface, and the smaller particle size of substrate possesses the greater surface area for the growing bacteria. When enzyme production was done by two meshes, 18 and 30, the obtained results were similar and had no significant differences (see Table 1). Since most of wheat bran was sieved by mesh 18, it may used quite similar for industrial conditions without any pre-treatment. Thus, it was selected to be used for subsequent experiments.

The effect of pH on protease production was evaluated in the range of 7-11. To monitor enzyme production, enzyme activity and produced protein were measured. The obtained results are summarized in Table 2. The enzyme was produced in a broad pH range of 7-11. According to the results, the optimum pH for the production of protease enzyme by *B. licheniformis* was pH 8 with an activity of 543 U/mL. However, the enzyme production significantly decreased under relatively acidic pH values (below pH 7); the data are not reported. Therefore, the produced protease enzyme was found to be a typical alkaline protease [3,19,29].

The substrate concentration is one of the important parameters

Table 1. Effect of wheat bran size on protease activity and protein concentration

Mesh No.	Mesh 18	Mesh 30	No mesh
Protease activity (U/mL)	462.1±3.3	471.6±15.7	431.8±13.8
Concentration of protein (mg/L)	273.58±6.2	307±8.5	266.35±10.6

Table 2. Effect of pH on protease activity and protein concentration

pH	7	8	9	10	11
Protease activity (U/mL)	525.36±12.5	542.92±10.3	466.58±9.92	429.37±1.7	407.61±26.1
Concentration of protein (mg/L)	328.38±9.8	340.67±13.6	275.48±11.2	263.51±10.9	258.61±7.7

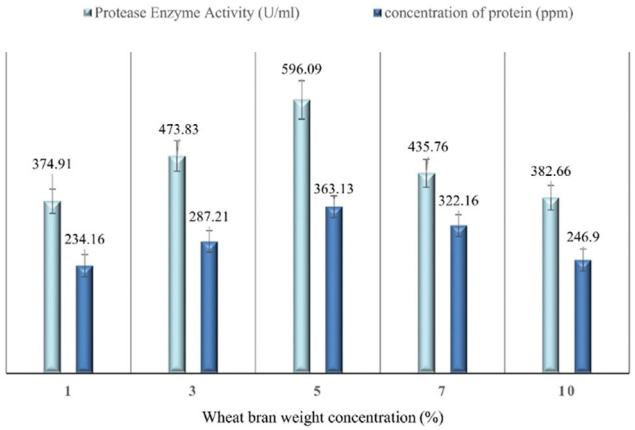

Fig. 5. Effect of wheat bran concentration on produce protease activity.

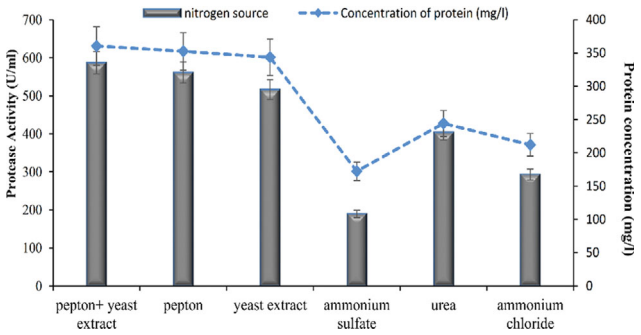

Fig. 6. Effect of nitrogen sources on produce protease activity.

for enzyme production because the high concentration of nutrients can affect the growth rate of microorganism. Based on research, a high concentration of wheat bran in medium can be inhibiting the growth rate of *Bacillus* bacteria and thus it can be reducing the protease enzyme production. Therefore, different percentages of wheat bran concentration in the range of 1 to 10% (w/w) were evaluated based on enzyme activity to finding optimum substrate concentration. The obtained data are presented in Fig. 5. As can be seen, the highest amount of enzyme activity was 596 U/mL, which was achieved at 5% (w/w) of wheat bran concentration. According to the literature, high concentration of nutrient and supplementary material can inhibit enzyme production and reduce its efficiency. It can be concluded from Fig. 5 that protease production was reduced at high concentration of wheat bran due to high amount of nutrient and rare elements, which cause microorganism growth inhibition. In conclusion, 5% concentration of wheat bran was selected as optimum value for future experiments.

In this study, the effect of various nitrogen sources such as peptone, yeast extract, as organic nitrogen sources ammonium sulfate, urea and ammonium chloride as inorganic nitrogen sources for protease production, were investigated. Generally, in microbial cells, nitrogen is used in both organic and inorganic forms to produce amino acids, nucleic acids and proteins. According to researches, bacteria usually consume several nitrogen sources for growing and producing enzyme, while bacteria producing protease enzyme use most nitrogen sources for enzyme production as secondary metabolite. Mukhtar et al. [30] and Sumantha et al. [31], in different investigations, reported that use of organic sources had reduced protease enzyme production, which can be attributed to the high rate of metabolism of these compounds. Therefore, in this work, the effect of nitrogen source was examined and the results are shown in Fig. 6. As can be seen, combining nitrogen sources, the equal mixture of peptone and yeast extract had the highest amount of protease production (589 U/mL), which is consistent with other findings. In several reports yeast extract and peptone had the highest protease production value [3,19]. Subsequently, various amounts of equal concentration of peptone and yeast extract were considered for finding the optimum value for protease enzyme production in wheat bran substrate. Obtained results are depicted in Table 3, and 1% (w/w) of this nitrogen source achieved maximum enzyme activity (587.2 U/mL) and was chosen as the optimum value for further experiments. Concurrent with this observation, Pansare et al. [32] noticed that ammonium nitrate can cause inhibition of protease production by *Aeromonas hydrophilia*. They concluded that increasing fast metabolizable ammonium ion concentrations in the medium can make the catabolite repression of enzyme synthesis. In another research, Snowden et al. [32] illustrated that ammonium ion might interfere with the utilization and metabolism of peptides through catabolite repression. Also, the presence of ammonium ion, along with carbon source and oxygen in medium, might inhibit sporulation of *Bacillus licheniformis*, which indirectly decreases the alkaline protease enzyme production. Therefore, based on our achievement and other observations, inorganic ammonium source can inhibit protease enzyme production.

3. Comparison Protease Production in Bioreactor and Flask

At first, enzyme production was optimized in flask and then the enzyme produced in the bioreactor with this optimum condition. Subsequently, comparison experiments were executed in three shaking rates (150, 180 and 200 rpm) for Erlenmeyer flask (in the incubator shaker) and designed bioreactor during 72 h. Also, the bioreactor aeration rate was adjusted at 3 vvm. The results in Figs. 7 and 8 show that protease enzyme activity was the highest at 180 rpm and 48 h for both Erlenmeyer flask (592.17 U/mL) and bioreactor vessel (683.93 U/mL). At the same time, protein concentration was measured using Bradford assay, and the results are summarized in Table 4. As can be seen, the highest amount of enzyme protein for Erlenmeyer flask and bioreactor was obtained in 180 rpm and 48 h, 365.3 and 388.2 mg/L, respectively. Based on literatures, increasing agitation rates can enhance mass transfer coeffi-

Table 3. Effect of peptone and yeast extract concentration as nitrogen sources on produce protease activity and protein concentration

Weight percent (%)	0.5	1	3	5	7
Protease activity (U/mL)	535.5±14.8	587.2±7.9	465.4±6.3	259.1±2.1	249.5±0.3
Concentration of protein (mg/L)	358±7.1	360.45±10.8	285.7±8.2	198.06±3.9	193.03±4.8

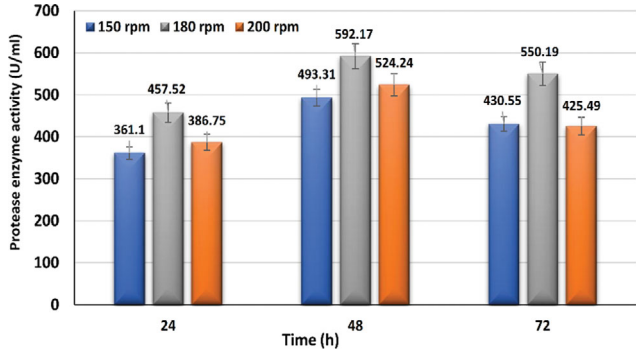

Fig. 7. Effect of stirring rate with constant aeration rate (3 vvm) on protease production in Erlenmeyer flask (150, 180, 200 rpm).

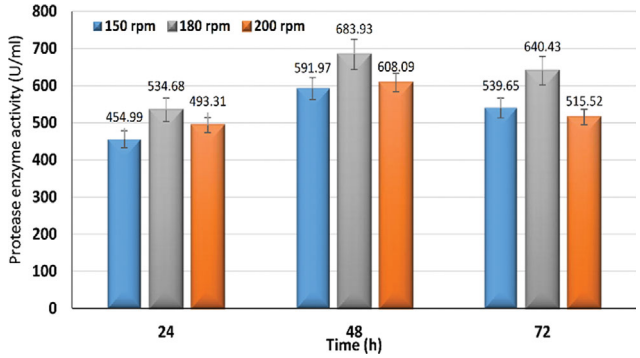

Fig. 8. Effect of stirring rate with constant aeration rate (3 vvm) on protease production in bioreactor (150, 180, 200 rpm).

cients, as the enzyme concentration increases with agitation rate up to 180 rpm. As the agitation increases to 200 rpm, mass transfer limitation may occur while there is limited soluble oxygen available to the living cell. In addition, a very high shear rate did not improve enzyme production, and it may destroy living cell wall of bacteria [33]. Finally, designed bioreactor efficiency is greater than enzyme production in Erlenmeyer flask and it is capable of producing protease enzyme on a large scale, so it can be remarkably valued in industrial scale production.

4. Effect of pH and Temperature on Stability & Activity of Produced Protease

The stability and activity of protease enzyme via various buffers were considered in the form of standard conditions as previously

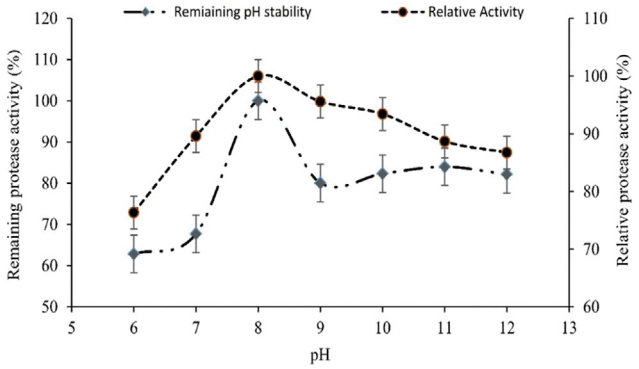

Fig. 9. Effect of pH on produced protease activity and stability.

described in section 2.7. The obtained data are depicted in Fig. 9 (red marker); it can be concluded that the produced enzyme was active in a wide range of pH and its maximum amount of activity was obtained at pH: 8. The protease enzyme relative activity at pH: 9, 10 and 11 was measured 95, 93 and 88%, respectively. Note that the produced protease relative activity at pH 12 retained 86%; while, commercial microbial protease can act in the range of pH 8 to 10, according to reported data in literature [24,26].

The stability of enzyme at different pH values is illustrated in Fig. 9 (blue marker), which indicates good stability in the wide range of pH 6 to 12 and the best activity was at pH: 8. The stability of produced protease at alkaline pH was noticeable, especially at pH 12, while retaining its relative activity 82%. In some studies, the optimum pH for protease enzyme stability was reported between 8 to 10 [24,34], while the working pH of commercial detergents is in the range of 8 to 12. Consequently, our produced protease enzyme can be used as an additive in commercial detergent without any pretreatment.

To determine the optimum temperature, enzyme activity was measured at various temperatures according to the procedure described in section 2.7. The results of experiments in Fig. 10 depicted that the produced enzyme activity increased with rising temperature up to 60 °C and then decreased.

Also, the obtained results in Fig. 11 demonstrate that the produced enzyme had excellent stability in a wide range of temperatures and the highest stability was obtained at 60 °C. It is well-known that the optimum temperature and thermo-stability of produced protease is different based on the bacterial species which produced

Table 4. Effect of stirring rate with constant aeration rate on protein concentration of protease production in bioreactor and Erlenmeyer flask at 150, 180, and 200 rpm

Fermentation vessel	Time (h) / Agitation (rpm)	24	48	72
Erlenmeyer flask	150	241.45±4.8	349.9±6.3	322.19±5.7
	180	274.74±5.4	365.38±7.3	362.8±6.1
	200	247.58±6.6	354.41±9.2	332.93±11.6
Bioreactor	150	276.35±2.7	360.54±7.5	351.22±8.3
	180	329.25±5.9	388.22±10.1	380.54±6.4
	200	273.16±8.1	370.22±3.9	354.70±2.5

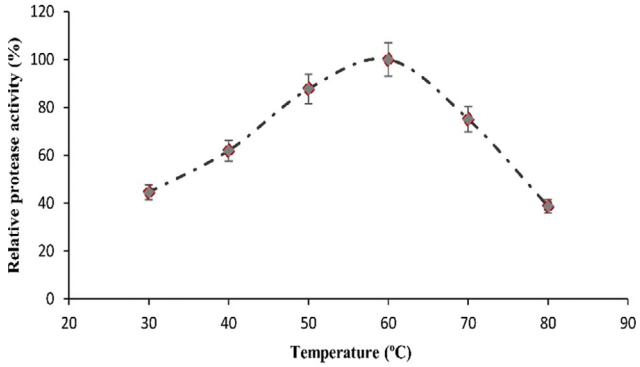

Fig. 10. Effect of temperature on produced protease activity in a wide range of 30 to 80 °C.

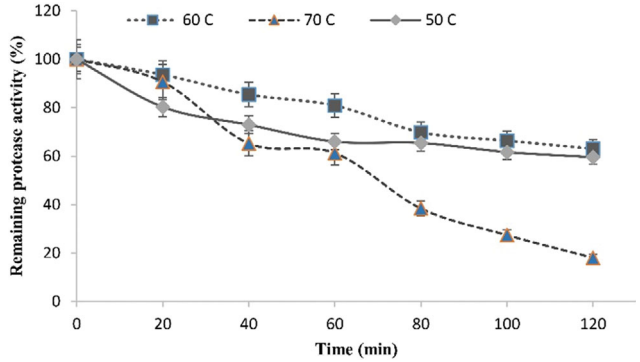

Fig. 11. Effect of temperature on produced protease stability in various temperatures of 50, 60 and 70 °C.

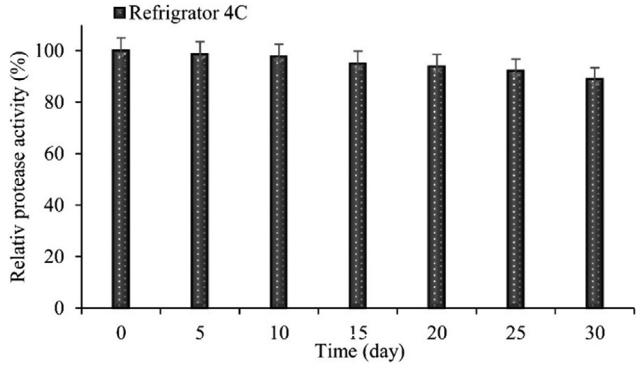

Fig. 12. The effect of shelf life on protease enzyme activity.

the enzyme; in addition, high temperatures can make protein 3D chemical structure change, so it can be reduced in the enzyme activity. However, similar results were reported by Sellami et al. [23], and Hakim et al. [3]; the produced protease was stable at 55 °C and presented good thermal stability in a wide range of temperature (40 to 60 °C).

5. Effect of Storage Time on Protease Activity

The effect of shelf life (4 °C) on protease enzyme activity for a duration of 30 days was investigated. Fig. 12 displayed that the produced enzyme is capable of maintaining 90% of its activity after 30 days which is a good achievement.

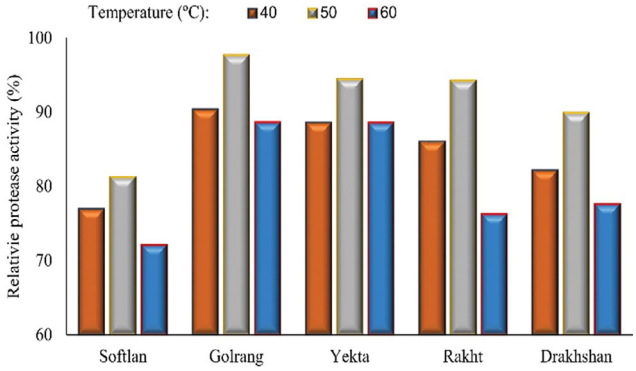

Fig. 13. The effect of commercial detergents on protease enzyme activity in various temperatures 40, 50 and 60 °C for 1 h.

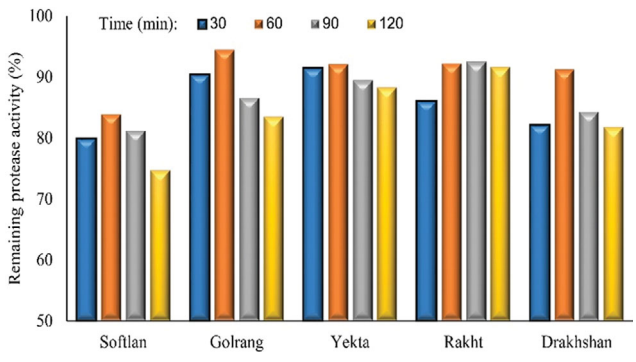

Fig. 14. The effect of local commercial detergents on protease enzyme stability at 50 °C and different times 30, 60, 90 and 120 min.

6. Application of Produced Protease

6-1. Compatibility with Detergent

The activity and stability of produced protease enzyme with commercial detergent in various temperatures were carried out based on the method described in section 2.9.1. According to the results, all commercial detergents had higher relative activity at 50 °C in comparison to other temperatures (see Fig. 13); in addition, the produced protease enzyme as an additive with Golrang detergent retained about 97% of its initial activity after 1 h at 50 °C.

The produced protease enzyme stability was also evaluated at 50 °C and the remaining activity was measured at 30, 60, 90 and 120 min; the results are seen in Fig. 14. The produced protease enzyme along with Golrang detergent retained about 83% of its initial activity after 2 h, and so it was used at optimum conditions for further experiments. Finally, the activity and stability of produced protease in washing conditions indicated that it is capable of being used with commercial detergents.

6-2. Additive to Detergents

The produced protease enzyme revealed good compatibility with commercial detergents. Based on the explained method in section 2.9.2, cotton textiles with fixed stains were washed. As can be seen from Figs. 15(a) to 15(c), the cotton textile was washed with hot water, detergent, and detergent with the produced protease, respectively. The results show that detergents along with produced enzyme had good washing ability and stains had been completely vanished.

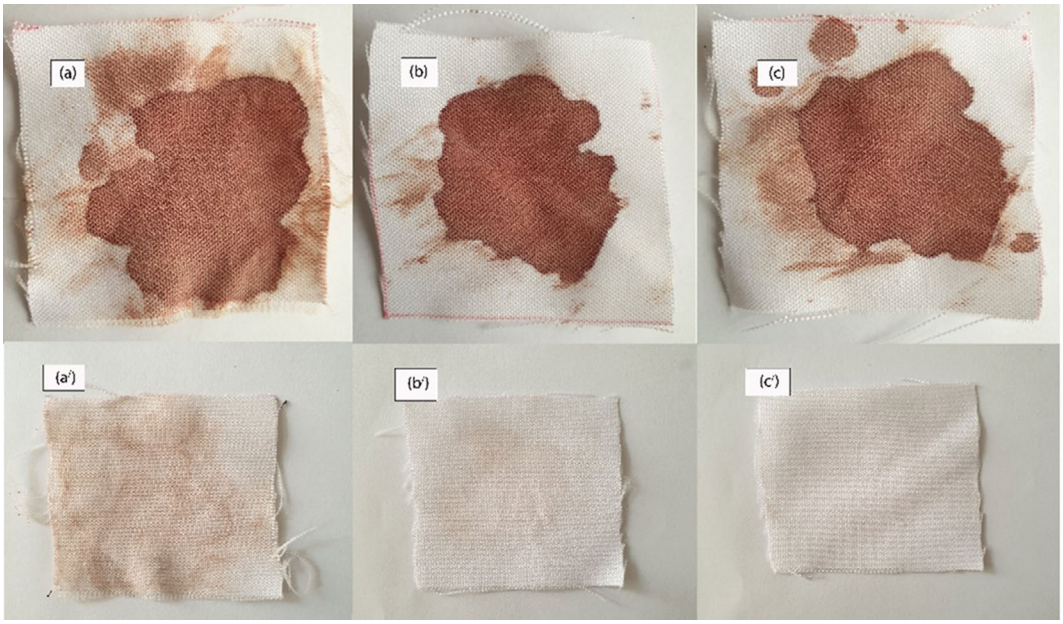

Fig. 15. The effect of blood stain washing with (a) hot water, (b) detergent, and (c) detergent along with the produced protease enzyme.

The obtained results of enzyme compatibility and stability experiments were consistent with other researcher's results. As a case, Haddar et al. [35] produced two types of alkaline serine-proteases from *Bacillus mojavensis A21* and purified them. Both purified proteases had exposed good stability in contact with non-ionic surfactants, oxidizing agents and compatibility with commercial detergents. Similarly, Jellouli et al. [34] conducted research on the production and purification of alkaline serine-protease from *Bacillus licheniformis MP1*. The optimum condition of enzyme activity was reported at pH 10 and temperature 70 °C. It can be noted that the enzyme remained active and stable in the range of pH 8 to 12. Also, it showed high stability against non-ionic surfactants, oxidizing agents and compatibility at 40 °C with liquid and solid detergents available in the market. Also, addition of produced enzyme (40 U/mL) to wash solution enhanced bloodstain elimination. In another instance, Sellami-Kamoun et al. [23] studied the stability of crude extracellular protease produced by *Bacillus licheniformis RP1*. The produced protease enzyme presented satisfactory stability and compatibility toward commercial solid detergents in the range of temperatures 40 to 50 °C using a concentration of 7 mg/mL of detergents. In addition, Abidi et al. [36] investigated the fungus *B. cinerea* which produced extracellular proteolytic enzymes. The optimization of medium composition and cultural conditions was investigated. They reported that the produced enzyme was compatible with laundry detergents and approximately removed the blood, the egg yolk and chocolate stains from cotton cloth.

CONCLUSION

Due to a large amount of enzyme imported to Iran annually, the production of protease enzyme and its usage in various industries is a critical matter. In the present study, to reduce the capital cost of protease enzyme production, agricultural wastes such as wheat bran, rice bran, and sugarcane bagasse by using *Bacillus licheniformis* bacteria under submerged fermentation were considered. To optimize enzyme production, the one-at-a-time method was used and the important factors such as process fermentation time, substrate type and particle size, pH, various kinds of nitrogen sources were investigated. The optimum conditions were extended to the designed bioreactor at a large working volume. The optimum conditions for protease production in the Erlenmeyer flask and bioreactor were gained 48 h, 37 °C, pH 8, wheat bran with a particle size of 1 mm, and 180 rpm agitation rate. The highest protease enzyme activity was obtained by wheat bran (5% w/w) and an equal mixture of peptone and yeast extract (1% w/w). The maximum protease activity was obtained using wheat bran, 596 U/mL for the Erlenmeyer flask, and 683.93 U/mL for the bioreactor. The highest amount of protease activity was obtained at pH value of 8 and its stability retained about 80% of its relative activity at pH 12. Also, the highest amount of protease activity was obtained at 60 °C and retained its 80% initial activity at 70 °C during one hour. The thermal stability of produced protease retained 80% of its initial activity at 50-60 °C during 2 h. The produced protease enzyme had good compatibility with the commercial detergent and washing the cotton textile. According to the results, adding our produced enzyme to detergent was capable of removing fixed stains completely. Shelf-life of produced protease enzyme in the refrigerator showed that even after 30 days of storage time, the enzyme retained up to 88% of its relative activity. Therefore, the produced enzyme was quite stable for at least one month.

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