Protease production using *Bacillus licheniformis* by submerged fermentation

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Abstract

Enzymes such as protease, occupies the pivotal position in the world of enzymes with respect to their applications in both the physiological and commercial applications. Bacillus like *Bacillus licheniformis* produce protease and was used throughout the present study. The *Bacillus licheniformis* is a potential microorganism for producing proteins with verities of functions including protease with substantial activity and effective pH and temperature tolerance, at laboratory and industrial scale. Herein effect of several temperature 25-50 ºC, pH 7-10.5, shaker's speed 100-200 and furthermore inoculum concentration 3-12 %V/V on enzyme production investigated. The medium cultivated at about pH (8.5), temperature of 35 ºC and gyratory speed of 180 rpm these parameters influenced protease production. It was found that yeast extract, soybean meal corn and steep liquor (powder), as nitrogen sources enhanced protease production. Cornstarch and glucose of 3 %W/V was found to be effective as carbon source for protease production. The protease stability was almost retained for 35 minutes heating at 60 ºC and pH 8.5. Moreover, as heating continued at the same temperature and pH the enzyme had 40% of its activity. The effect of metal ions types on the stability of the partially purified protease is reported. It was found that the protease activity at 60 ± 0.5 ºC, pH 9.5 and the presence of 15 mM CaCl2 and 10 mM NaCl during an hour of treatment retained approximately 40 percent of its activity. A batch period of 24 protease production showed similar proteases activity in comparison with 48 h operation thus the following fermentation conducted within 24 h. The partially purified protease had sound stability in the alkaline pH of 9-9.5 and temperature range of 60 ±0.5 ºC in the presence of certain ions like calcium and sodium salt.

1. Introduction

Alkaline protease (EC 3.4. 21. 14) is one of the most important groups of industrial enzymes, which is exploited mainly in degradation of large polypeptide substrates, such as exogenous proteins into peptides and amino acids before cellular utilization. Microbial protease is classified into two major groups of, peptidase and proteins, which is based on their nature of hydrolyses *(Kennedy, 1987; Rao, et al.,1988)*. The *Bacillus* genus is also industrially very important for producing valuable proteins differing in applications, and terms of pH and temperature tolerance and in retaining optimal activity. *Bacillus licheniformis* is a facultative aerobic bacterium which is used in fermentation industry for the production of extracellular

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enzymes such as alkaline proteases which are widely used in, laundry detergents *(Outtrup & Boyce, 1990; Fogart & Kelly, 2012)* leather processing (*Malathi & Chakraborty,1991*), food industries *(Jisha, et al., 2013; Contesini,et.al.,2018;Johnvesly & Naik, 2001)* and other industries worldwide *(Crueger& Crueger, 1990)*. Further, alkaline protease may be produced by various microorganisms like yeast, molds, bacteria and other sources including mammalian tissues, plants (like papain, figs and bromelain) *(Uyar & Baysal, 2004)* and certain insects (*Moreia, et. al., 2003*). Alkaline protease accounts for at least 25% of the total enzyme sales, with two-third of the commercially produced being of microbial origin (*Kalisz, 1988*). Detergent chemicals used for formulation of the medium mixture, which have higher affinity for proteinaceous materials are preferred by detergent and leather tanning industries (*Gerhartz,1988; Rao, et. al., 1988; Godfrey & Reichet, 1982; Singh &Bajaj, 2017*). There is always a tendency to look for new detergent compositions either for environmental or energetic considerations to substitute new bleaching systems for washing at lower cost. In general, microorganisms employed for production of bulk chemicals such as protease and amylase utilize complex carbon and nitrogen sources in industrial practice. Production of bulk enzymes take place over a long period and requires maintenance of high cell mass concentration, and within this period the cell growth should be kept minimal.

We used *Bacillus licheniformis* PTCC 1525 for possible production of protease. It was desirable to study the effect of several parameters such as pH, temperature, nitrogen, carbon source and concentration and shaker's speed on the protease production. Further effect of temperature, pH and metal ions on the stability and activity of partially purified protease studied. Further it was found that glucose of (3 %W/V), nitrogen (yeast extract, (2.5 W/V%), temperature (35 ºC), pH (8.5), shaker's speed (180 rpm) had effective influence on the protease production. Furthermore, the effect of metal ions on the stability of partially purified protease at temperature 60 ºC, pH 8.5 retains found to resists its activity for 35 minutes. Moreover, the stability of partially purified protease at the presence of 15 mM CaCl2 and 10 mM NaCl and heating at 60 ºC which maintained its 40 % of stability for an hour.

2. Materials and Methods

2.1 Microorganism and batch cultivation

Bacillus licheniformis PTCC 1525 was gifted by Tehran MIRCEN, Iran, which was originally isolated from soil of waste stream of a detergent manufacturing company, and it was identified according to Bergey's Manual of Systematic Bacteriology (Garrity, et. al., 2011). Stock culture of Bacillus licheniformis was maintained at 4 oC on brain heart infusion agar (BHIA) medium and subcultured every 2-3 weeks. The same medium (BHI A) used for preparation of slant. A loop full (2.5 mm diameter loop) of inoculants applied to cultivate 100 mL seed liquid culture at 35 oC (± 1 oC). About five milliliters of the seed culture (V/V%) used to incubate 100 mL of production culture. The production medium initially consisted of soy meal (SM), glucose, peptone, di-potassium hydrogen phosphate, sodium carbonate, respectively. The final media consisted of: in W/V%, glucose (3), yeast extract (2.5), peptone (2), di-potassium hydrogen phosphate (0.1), sodium carbonate (1), CaCl2 (0.01), NaCl (0.1), MgSO4, FeSO4, MnCl2, ZnCl2 of 0.005.Sodium carbonate (1) and di-potassium hydrogen phosphate (0.1) W/V % sterilized separately and added to the medium. In addition glucose sterilized separately for 10 min at 100 ºC and added aseptically. The medium initial pH was adjusted to about 8.5. Cultures after inoculation were maintained at 35 ^{O}C ± 1 ºC and agitated at 180 rpm using a gyratory shaker (Clime-O-Shack, Adolph-Kuhner Co. Germany).

2.2 Chemicals

Bacteriological peptone, skim milk agar, and yeast extract was procured from Oxoid. Bovine serum albumin (BSA), and soy meal were of

analytical grade and purchased from Sigma Alderich Chemical Co. Ltd., St., Louis, MO; USA. Corn steep liquor (CSL), was gifted by Glucosan Company Ltd., Qazwin-Iran, which spray dried to form powder. Brain heart infusion (BHI) broth, mineral salts, trichloroacetic acid, tyrosine, casein, glucose, starch, Hammersten casein, sodium sesquicarbonate, di-potassium hydrogen phosphate, sodium carbonate, and maltose were Merck made.

2.3 Analytical methods

2.3.1 Proteolytic activity determination

The initial proteolytic activity of partially purified enzyme determined by applying skim milk agar and the medium pH was maintained at about 8.5 using 0.1 M sodium sesquicarbonate buffer. The pettry dish maintained at 30 ºC (± 1 ºC) for 48 h. A distinct clear zone on agar containing casein was observed. Proteolytic activity determined using modified method of Kunitz (*1947*). The enzyme solution of 0.1 mL mixed with 2 mL of 0.5% Hammersten casein solution at 40 ºC for 10 min., and 4 mL of trichloroacetic acid (TCA) solution was added to the reaction mixture. The mixture was further incubated at 40 ºC for 20 min and filtered through filter paper (Whatman No.1). The optical density at 280 λ nm measured in a silica cuvette with 1-cm path length using Unicom 8620 UV/VIS spectrophotometer. One unit of the protease activity defined as the amount of the enzyme that produced trichloroacetic acid, soluble materials equivalent to 1 μmole of tyrosine from casein per minute under the defined assay conditions *(Suaifan, et. al., 2017).*

2.3.2 Protein determination

Protein concentration of the enzyme sample was estimated by Lowry's method (*Lowry et. al., 1951*) using bovine serum albumin (BSA) as standard. A calibration chart was constructed and used.

2.3.3 Partial purification of protease enzyme

A protease is generally concentrated by precipitating with salt or solvents, partially purified protease obtained using different solvent like ethanol, acetone, iso-proponal at various concentrations (*Horkoshi, K., 1971*; *Bhosle, et. al., 1995; Gray, 1993*). Since alcohol precipitation is an exothermic reaction *(Rose, A. H., 1980).* The 50 mL of the cell free supernatant of temperature 4 ± 0.2 ºC added to organic solvents like, acetone, ethanol and isopropanol (-10 ºC alcohols) at various concentrations in a cold room appropriately. After alcohol fractionation, the precipitated was separated by centrifugation (10000 g for 10 min at 4 ± 0.2 ºC). The precipitate immediately recovered in aqueous medium and dissolved in 0.1 mM phosphate buffer of 4 ± 0.2 ºC and used. It was found treatment with acetone (1: 4% v/v) resulted in enzyme of higher activity, however, during fractional purification (1:1% v/v) cell free supernatant and acetone was used to get more partially purified protease.

2.4 Reproducibility:

The experiments were carried out in triplicate and the average values were reported.

3. Results and discussion

The *Bacillus licheniformis* PTCC 1525 originally isolated from soil of waste stream of a detergent producing factory. The organism is aerobic and cultivated in the pH range of 7-8.5 and was found to grow optimally about 8.5. The enzyme was found to be stable at incubated temperature of 35 ºC in the pH range of 7-8.5 for 6 h.

3.1 Effect of carbon and nitrogen sources and inoculum concentrations on protease production

3.1.1 Carbon source

Effect of cornstarch and glucose concentration from 1-5 % W/V as carbon sources on protease production investigated within 48 h. It was

found 3 %W/V glucose resulted (Fig 1) in higher enzyme activity. Figure 1 showed that glucose has more influence on the protease production than cornstarch that may be easier metabolized than corn starch. The result of the present study is on similar trend with the sighted result of Fujiwara (*Payne, J, W. 1980*). However, *Frankena & Yamamoto* (*1987*) used 1.8 g L^{-1} glucose in the cultivating medium of *Bacillus licheniformis* producing protease. Certain published papers (*Gerhartz, 1988; Frankena, et.al., 1985; Glenn, A.R.(1976); Ingram, et. al. 1983; Razak, et. al., 1994*) depicted that the protease production was repressed at the presence of glucose, such results contradict the finding of the present work. In the industrial scale of protease production glucose is preferred as the carbon source, and has a strong effect on the carbon catabolite control (*Fujita, 2009*). In the presence of glucose, repressive genes encoding occurs for enzymes that are responsible for carbon recruitment like proteases (*Wiegand et al., 2013*). Therefore, large scale production of protease is a combination of batch and as the glucose is depleted the operation is switched to defined glucose concentration flow rate in fed batch mode using Stirred Tank Bioreactor.

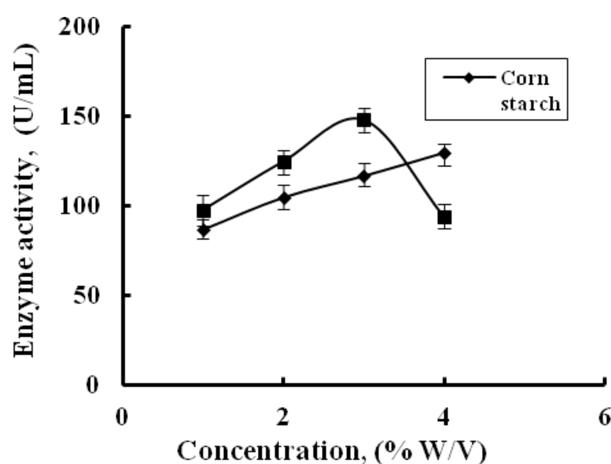

Figure 1. Effect of glucose and corn starch on protease production.

3.1.2 Nitrogen source

The nitrogen is metabolized to produce nucleic acids, primarily amino acids, proteins and cellular components. Alkaline protease is comprised of 8-15.6 % nitrogen (Gassesse & Gashe, 1997; Bhunia & Basak, 2012). Production of protease depends on the presence of nitrogen and carbon sources and concentration in the medium (Anustrup & Andersen, 1974). Deficiency or excess of nitrogen may cause repression of synthesis of protease by prokaryotes and synthesis of protease is affected at low nitrogen strength. The effect of complex organic compounds including: yeast extract, corn steep liquor (powder), and soya meal on enzyme production investigated. Effect of, YE, SM and CSL concentration 1-2.5 % w/v on the enzyme production within 48 h of fermentation was examined individually. It was found that 2.5 % w/v dry weight of CSL has sound effect on enzyme production. (Fig. 2) revealed that 2.5 % w/v yeast extract resulted in higher enzyme production. However, the enhancement of protease production using yeast extract may be due to presence of vitamin B group (as promoting growth), amino acids, carbohydrates, minerals, vitamins and other essential nutrients for cell growth to synthesis enzymes such as protease and amylase. In addition, substantial production of protease using complex nitrogen sources such as, CSL and SM, may be due to release of cells of many biosynthetic materials, resulting in efficient growth and enzyme synthesis (Bhosle, et. al., 1995; Kole,et.al.,1988; Ferrari, et., al., 1993; Dunn,G.M.,1985). Further, the two complex organic nitrogen sources used like soya meal and corn steep liquor (in powder form) influence is due to nitrogenous material like vitamins, minerals, which effect enzyme production.

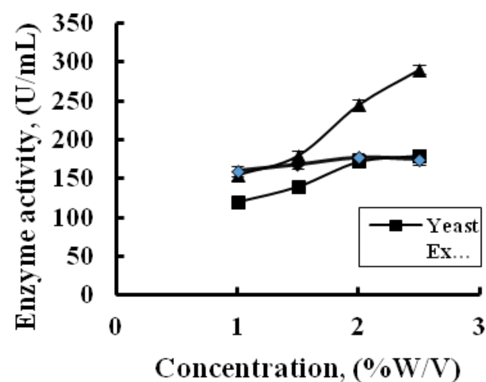

Figure 2. Effect of corn steep liquor powder, yeast extract and soya extract concentration on protease production.

3.1.3 Effect of inoculum

To achieve a high concentration of enzyme in a limited defined volume the inoculum size should be properly determined. Herein, the culture was inoculated with preculture media in

the range of 3 – 12 % V/V having cell density of 10^7CFU mL^{-1}. Stock cultures of the present strain maintained at about 4°C using brain heart infusion agar and pH being maintained at 9.5 with addition of 0.1 M sodium sesquicarbonate buffer. The same medium used to prepare preculture and the result (Fig 3) was presented. It was observed as the inoculums size increased from 3 to 5 % V/V, protease production increased. Furthermore by increasing inoculums concentration more than 5 percent, gradually to 10 % V/V the protease production decreased. It might be due to higher consumption of nutrients by large population of Bacillus lichneiformis in initial phase of log phase resulting in lack of nutrients to be available during the rest of the logarithmic phase. Higher concentration of inoculum usually does not contribute to higher production of protease, which address proper optimization based on the bacterial condition is a work horse of a new species. The finding of (Mabrouk, et.al., 1999) also support the present results.

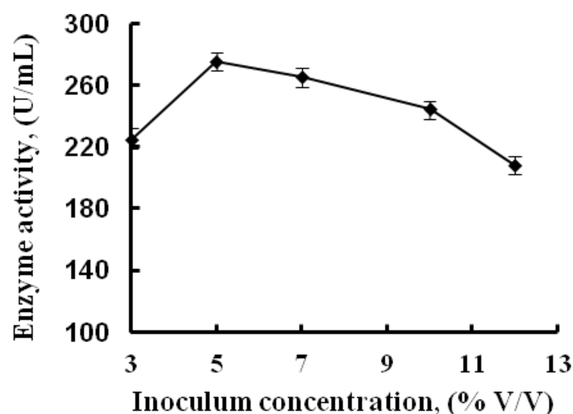

Figure 3. Effect of inoculum concentration on protease production.

3.1.4 Effect of temperature

In the present study, effect of various temperature on protease production from 25 to 50 ºC during batch cultivation examined. The result (Fig 4) exhibited that the enzyme production increased as the temperature increased from 25 to about 35 º C (± 0.5 ºC). Further, it was observed that by increasing the medium temperature above 37 ºC the enzyme production relatively ceased and Ferero, et.al. (1996) found similar results.

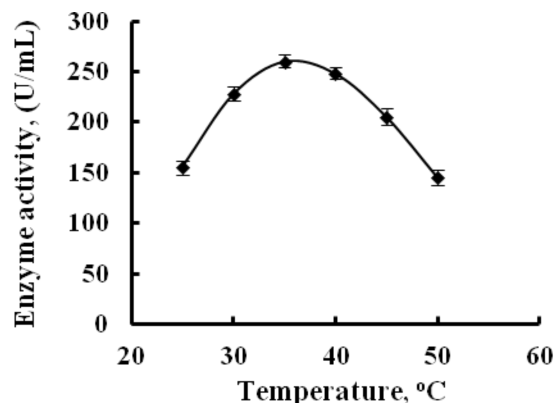

Figure 4. Effect of temperature on protease production.

3.1.5 Effect of pH

The effect of pH on protease that substantially affect many enzymatic processes and transportation of several components across the cell membrane, herein investigated in the range of 7-10.5. The medium pH adjusted with addition of 0.1 M sodium sesquicarbonate and as required by NaOH and sodium carbonate in solution. It was indicated (Fig 5) that as pH increased from 7 to 8.5 the protease production gradually increased, however, further increasing in pH of the medium had adverse effect on protease production. A similar result was reported by (*Haki, & Rakshit, 2003*).

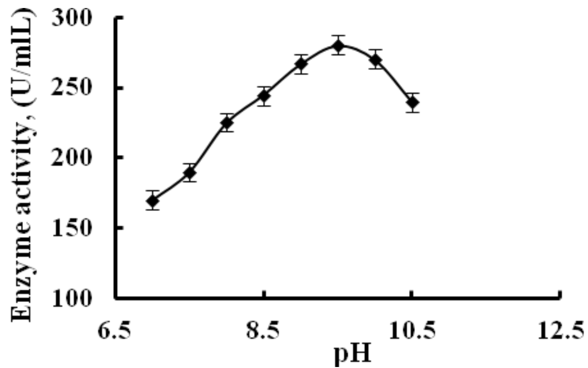

Figure 5. Effect of initial pH on protease production

3.1.6 Effect of shaker's speed

The effect of shaker's speed on protease production studied in the range of 100-200 rpm and the result (Fig 6) was presented. It was observed that as the shaker's speed increased from 120 to 180 rpm the enzyme production also increased. Further increase in shaker's speed had meager effect on protease production. Moreover, higher shaker speed contributes in severe mixing and aeration, resulting in lower protease production, which might be due to inhibiting effect of oxygen concentration and rapid consumption of substrates in early stage of logarithmic phase resulting in shortage of nutrients in the remaining cycle that contribute to surface to volume ratio and consequently in the productivity drop. The effect of agitation on protease described by *Nascimento & Janssen* (2004) found a similar trend. It appears higher agitation reflect on more aeration and resulting in relatively high dissolved oxygen in the fermentative batch medium that may not bring about increase in protease production, and hampers the structure of enzyme. However, at low agitation- aeration rate could result in reduction of protease yields. Therefore, optimized agitation- aeration is required for aerobic spices to reach proper growth and protease production.

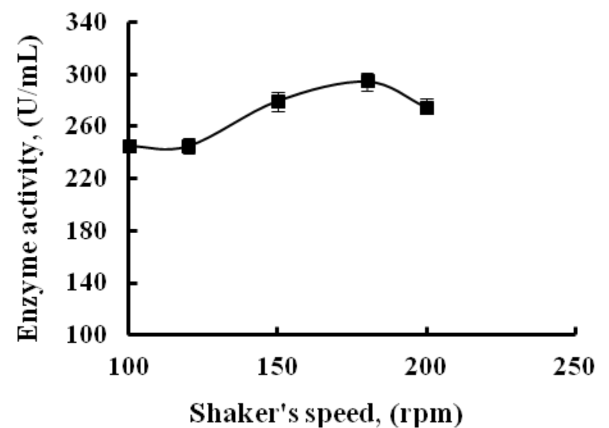

Figure 6. Effect shaker's rotation on protease production

3.2 Effect of temperature on the partially purified protease stability

The result (Fig 7) revealed that the optimum temperature range for the partially purified enzyme stability in medium of pH: 8.5 incubated at about 60 ºC for 30, 45 min and an hour was about 100, 65 and 40%, respectively. The result of the present study corresponded with the reported results in (*Manachi & Fortina, 1998*). The partially purified protease was incubated for 1 h at about 60 ºC and added certain salts ions and at pH: 9.5 that retained about 40% of its original activity. Moreover, the protease mixture retained about 4% and not detectable of its original activity after 90 and 120 minutes of incubation, respectively.

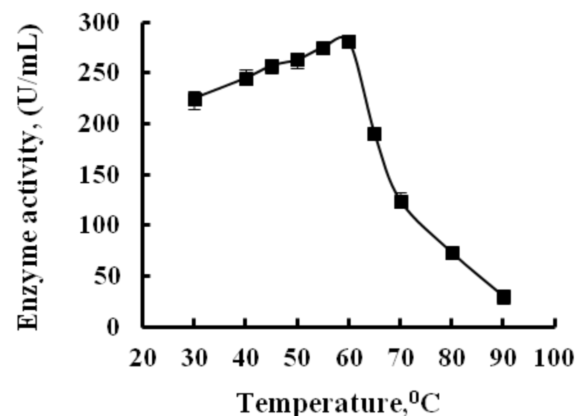

Figure 7. Effect temperature on the stability of the partially purified protease.

3.3 Effect of pH on the partially purified protease stability

The effect of pH on activity of partially purified protease investigated in the range of 7-12 for 1 h at 60 ºC. (Fig 8) illustrated that the optimum pH for the enzyme activity was about 8.5. The protease activity was almost retained for 35 minutes heating at 60 ºC and pH 8.5. Moreover, as heating continued in span of 1 h the protease activity decreased to 40% at about pH 8.5. The present findings were in accordance with several earlier reports (*Moreia, et.al., 2003*; *Hameed, et., al.,1996; Tang, et., al.; Gupta, et., al.,1999*). Furthermore, as the enzyme mixture incubated at various intervals in the pH range of 11-12 at 60 ºC, lost its stability in few minutes.

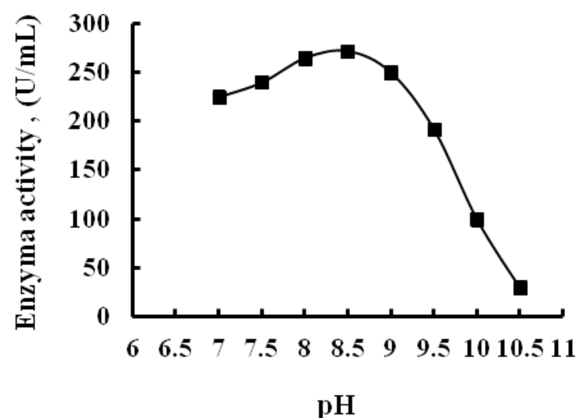

Figure 8. Effect of pH on the stability of the partially purified protease.

3.4 Effect of metal ions on protease stability

The effect of metal ions on the protease stability was shown in (Table 1). It was observed at the presence of 15 mM Ca^{2+} and approximately 10 mM Na the protease treated at about pH 9.5, temperature 60 ºC during 1 h retained 40 % of its activity, which was higher than the control. Further, we understood that the protease of the present study was stabilized by calcium ions and similar result are available in literature (*Takami, et.al., 1989, Stoner, et.al., 2004*). Stabilization of enzymes by metal ions at high temperatures is through metal ion compellation, which is a process with a favorable entropy factor. This is because water previously bounded to the hydrated metal ion in the solution is liberated when the metal ion becomes bound to the protein. Thus, the process is favored at higher temperatures approximately 60 ºC. A number of enzymes require metal ions, like calcium ions, to maintain their stable and active structures. These ions bound strongly to specific binding sites on the surface of the molecules**.**

Table 1. Effect of metal ions on the stability of partially purified protease activity

Metal ions	Concentration (mM)	Enzyme activity, UmL^{-1}
Reference	**------**	**225**
Ca^{2+}	1	276
	5	280.5
	10	287.5
	15	297
Mg^{2+}	1	274
	5	276
	10	270
	50	260
K^{+}	1	274
	5	270
	10	259
Zn^{2+}	1	276
	5	274
	10	258
Mn^{2+}	1	273
	5	262.5
	10	252
Na^{+}	1	275
	5	280
	7.5	284
	10	285

The binding sites are usually constructed from negatively charged carboxylate side-chain groups of aspartyl and glutamyle residues, brought together by folding of the polypeptide chain. Dissociation constants for the binding are low (of the order of 10^{-3} to 10^{-6} M) in the case of E+ Ca^2 $\leftarrow$ $\rightarrow$ E - $Ca^{2,}$ representing very strong binding that take place at low calcium ion concentrations.

This phenomenon supports our results. (Ghorbel-Frikha, et., al.,2005). As can be seen from the above equation, the situation is similar to that of enzyme-substrate complexes. The metal ions bridge cross-link the polypeptide chain and the enzyme-calcium ion complex should, therefore, be more rigid and hence more stable. The bridging by metal ions in this way is compared to that brought about by disulfide formation. In the absence of calcium ions, the binding site would represent a high local concentration of negative charges. The tendency of these groups to move apart to reduce the repulsive electrostatic interaction would contribute to the relative instability of the folded protein. (Voordouw et. al., 2017, Eijsink et. al., 2011). The stability of such protease under defined concentration like 1 mM $CaCl_2.2H_2O$ when added as a metal ion could preserve its stability as reported by (Dong, et. al.,1999). The present partially purified protease stability was examined at the presence of $CaCl_2.2H_2O$ and NaCl and was found effective.

4. Conclusions

It was thought desirable to find the possibilities of production of protease by a Bacillus licheniformis PTCC 1525 that was earlier isolate from a wastewater stream of detergent producing factory located I Qazwin-Iran. The optimum condition range for protease production using the same Bacillus licheniformis was found at pH: 8.5 (± 0.2), 35 ± 0.5 ºC and shaker's speed of 150 rpm. Higher protease production was achieved using 2.5 %w/v yeast extract; however, two more sources were examined. Moreover, protease production was improved using 3% w/v starch.

A batch period of 24 and in continuation to 48 h performed and was observed that 24 h fermentation could be used for the protease production. The partially purified protease had sound stability in the alkaline pH of 9-9.5 and temperature range of 60 ±0.5 ^{O}C in the presence of certain ions like calcium and salt. Low concentration of calcium ions and salt has considerable influence on the stability partially purified protease. It would be more viable to use such a crude culture for tanning than proceeding further to produce pure protease. For scale-up purposes batch and fed batch mode of operation are necessary to be performed to understand the effect of range of variables and acquire data to select appropriate fermenter.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgment

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Ethical approval

This manuscript does not contain any studies with human participants or animals performed by any of the authors.

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