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Thermostable Alkaline Protease Production via Solid State Fermentation in a Tray Bioreactor Using *Bacillus licheniformis* ATCC 21424

Pouryafar, F., Najafpour, G. D.*, Noshadi, N. and Jahanshahi, M.

Faculty of Chemical Engineering, Noshirvani University of Technology, Babol, Iran

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ABSTRACT: In solid state fermentation using tray bioreactor, several agricultural residues such as rice bran, sugarcane bagasse, wheat bran, barely bran, corn meal and corn husk were used as substrates. Among them, wheat bran was found to be the most suitable substrate with high enzyme activity of 798.83 and 776.91 U/gds for top and middle trays, respectively. Process parameters such as incubation time (48 hours), cabin temperature (35 °C) and cabin humidity (90%) were optimized. It was found that the desired carbon and nitrogen supplementary sources were rice bran (1%) and corn meal (2%), respectively. The protease was stable in broad temperature range (30-75 °C) and pH values of 7-13, with maximum activity was defined at 65 °C and pH value of 8.

Key words: Solid state fermentation, Protease, *Bacillus licheniformis*, Wheat bran, Tray bioreactor

INTRODUCTION

Protease (EC 3.4.21-24) is an enzyme that is used for the hydrolysis of protein (Kumar and Takagi 1999). Protease is one of the most important enzymes and represents 60% of total industrial enzyme sale. They are widely used in several industrial applications like detergent, food, pharmaceutical and leather industries. Proteases being found in a wide diversity of sources such as plant, animals and microorganisms (Xu *et al.*, 2013, Gupta *et al.*, 2002).

Microbial proteases are preferred to the other sources, because their production cost is less and renewable resources can be used. Generally, proteases are produced from bacteria, fungi and yeast. However, bacillus species have the ability to produce significant extracellular protease during post-exponential and stationary phases (Potumarthi *et al.*, 2007, Bhunia *et al.*, 2012). At present, large proportion of the most commercial proteases, mainly neutral and alkaline, are produced using bacillus strains (Mani *et al.*, 2012). Different species of *Bacillus* such as *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus firmus* and *Bacillus lentus*, are reported to produce proteases (Prakasham *et al.*, 2006, Bhunia *et al.*, 2010). Bacteria usually produce protease by submerged fermentation, but recent reports showed that solid state fermentation is more efficient than submerged fermentation for the bacterial enzyme production (Imtiaz and Mukhtar 2013, Subramaniyam and Vimala 2012). Solid-state fermentation (SSF) is refers to the growth of microorganisms on moist solid surface in absence of free water (Pandey 2003). Cost of the growth medium, is the major factor for the overall production cost of enzyme. In SSF, by utilization of agro-industrial wastes and by-products, which they are good sources of nutrients for the growth of microorganism, production cost of the enzyme, can be reduced (Vijayaraghavan and Vincent 2012). Although in many cases, treatments are required to optimize the conversion of wastes into value added products such as enzymes (Arvanitoyannis and Tserkezou 2008a, Arvanitoyannis and Tserkezou 2008b). Several Agricultural byproducts such as cow dung (Vijayaraghavan and Vincent 2012, Vijayaraghavan *et al.*, 2014), feather meal (Azeredo *et al.*, 2006), rice and wheat (Chutmanop *et al.*, 2008), red gram husk (Rathakrishnan and Nagarajan 2011), green gram husk (Prakasham *et al.*, 2006) etc were used as the substrate for the production of protease. Moreover, solid state fermentation have several advantages over submerged fermentation, such as reduced production cost, higher yield, low energy consumption and simple downstream processing (Ali and Zulkali 2011). For industrial production of biologically compounds like proteolytic enzymes via solid state fermentation, efficient design of bioreactor systems is very important (Pandey 2003). Many different kinds of bioreactors from laboratory-scale to

*Corresponding author E-mail: najafpour@nit.ac.ir

industrial-scale have been used in SSF processes; that, they can be divided into groups based on mixing and aeration. The most commonly used SSF bioreactors, are tray type, packed bed and rotating drum bioreactors. Tray bioreactors are the simplest SSF bioreactors that are used (Ali and Zulkali 2011, Durand 2003). The objective of present study was production and characterization of protease using *Bacillus licheniformis*. The organism is grown on agro-industrial residues in a tray bioreactor. In addition, optimum conditions should be defined for the achievements of high activity protease production such as, incubation time, cabin temperature, cabin humidity and supplementation of co-carbon and co-nitrogen sources. *B. licheniformis* is a gram positive bacterium that commonly found in the soil and bird feathers (Chen *et al.*, 2012). Several findings indicated that the bacterium grows well in alkaline conditions and produces thermostable protease can remains stable at high pH level. These enzymatic properties suggest the enzyme is suitable for industrial applications such as an additive in detergent formulation and in the leather industry, so that the main industrial protease was one produced by *B. licheniformis* for use as a cleaning aid in detergents (Sellami-Kamoun *et al.*, 2008, Anuraj *et al.*, 2012, Sarker *et al.*, 2013). No significant work has been reported in terms of thermostable alkaline protease production in tray bioreactors. In this bioreactor, by controlling the temperature and humidity, problems of heat and mass transfer have been overcame (Vaseghi *et al.*, 2013). Consequently a thermostable protease with high activity in broad range of pH was obtained.

MATERIALS & METHODS

All chemicals used in present study were purchased from Merck (Darmstadt, Germany); while tyrosine was supplied from Sigma-Aldrich (St. Louis, MO, USA). Rice bran, sugarcane bagasse, wheat bran, barely bran and corn meal and corn husk were obtained from local market, Iran.

The organism, *Bacillus licheniformis* ATCC 21424 was obtained from IROST (Iranian research organization for science and technology), Tehran, Iran. The strain was cultivated on nutrient agar medium at 37 °C for 24 hours and stock culture stored in nutrient agar slant at 4 °C. Inoculum was prepared in 250 mL conical flask containing 50 mL of sterilized nutrient medium (g/L): (glucose 10, peptone 5, yeast extract 5, KH_2PO_4 1, $MgSO_4.7H_2O$ 0.2, pH 8), and was aseptically inoculated with a loop-full of bacteria from a fresh slant and allowed to grow at 37 °C and 150 rpm for 24 hours in a rotary shaker. After 24 hours of growth, the bacterial culture was used as inoculum.

Various agricultural residues such as rice bran, sugarcane bagasse, wheat bran, barely bran, corn meal and corn husk, were used as solid support/substrate matrices for protease production. All substrate washed with distilled water and dried at 60 °C. Then ground in a mixer grinder and used. Protease activity was evaluated for each of the substrate. The best substrate which secreted high protease activity was selected for further process.

SSF was carried out in a Plexiglas tray bioreactor (45 × 35 ×55 cm), have three aluminum trays (35 × 25 ×5 cm) located horizontally inside the chamber with equal space (18.3 cm). As the experimental set up is shown in Fig. 1, for monitoring and controlling of temperature and humidity inside the bioreactor, temperature and humidity probes were used and placed approximately in the center part of bioreactor chamber, that had perfect control of temperature and humidity in the bioreactor. In addition, four air recirculation fans (PC fan 12 volts, 0.18 Amperes, 1.68 Watts), were subjected to provide uniform temperature distribution inside the cabin. These fans were placed beside trays 1 and 2 (Vaseghi *et al.*, 2012, Mohseni *et al.*, 2012). Due to good heating and air circulation the cabin's temperature and humidity were stable and reliable.

A 5 gram of dried substrate was filled in paper bags were placed on top and middle trays and inoculated with bacterial inoculum under sterile conditions. Bottom tray was filled with supplementary nutrient solution containing (%): yeast extract 0.1, KH_2PO_4 0.1, $MgSO_4.7H_2O$ 0.05, NaCl 0.25 and $FeSO_4.7H_2O$ 0.004. Desired moisture content of the bioreactor was provided by these media. The peristaltic pump (ETATRON, Italy), were used to circulating the

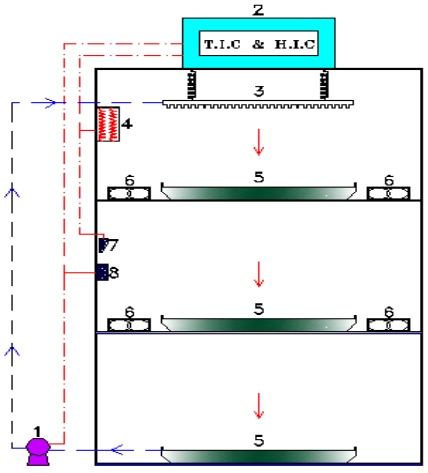

Fig. 1. Schematic diagram of the tray bioreactor set up, (1) Pump, (2) Temperature and Humidity Indicator and Controller, (3) Nozzle, (4) Heater, (5) Tray, (6) Fan, (7) Temperature Sensor, (8) Humidity Sensor.

nutrient solution from the bottom tray to the surface of top and middle trays. Finally, the remaining solution in the middle tray was poured into its original sources in the bottom tray. In fact, by recirculation of the media creates uniform distribution of moisture, bacteria can access to the nutrients that are required for their growth and protease production (Mohseni *et al.*, 2012, Vaseghi *et al.*, 2012).

The effect of process parameters on protease production such as fermentation period (24-120 h), temperature of bioreactor (30, 35, 40, 45 and 50 °C), bioreactor humidity (70-95%), simple and complex carbon sources (glucose, lactose, saccharose, soluble starch, fructose, wheat straw, bagasse**,** barely bran and rice bran) and nitrogen sources (yeast extract, peptone, corn meal, corn husk, soybean meal, casein, urea, NH_4Cl and $NaNO_3$) were investigated. Optimization of various parameters was carried out with "One factor at a time" strategy keeping all other variables constant except one. Results reported in this study are averages of triplicate findings.

The enzyme extracted from fermented medium using 50 mL of Tris-HCl buffer (pH 8, 0.01 M) by shaking on a rotary shaker for 1 hour at 30 °C and 200 rpm. Fermented solid substrate was then filtered with whatman filter paper (No.41: diameter of 125 mm) by means of a vacuum pump. The clear supernatant was used as the crude enzyme.

Protease activity was determined by a slightly modified method using casein as the substrate (McDonald and Chen 1965). A 1 mL of enzyme solution was added to 5 mL of casein solution (0.65% solution in 0.05 M Tris-HCl buffer of pH 8.0) and incubated at 37 °C for 30 min. The reaction was stopped by addition of 5 mL of TCA solution (110 mM). After 30 min the entire mixture was centrifuged at 5000 rpm for 10 min. A 2 mL of supernatant was mixed with 5 mL of sodium carbonate solution and then 1 mL of Folin and Ciocalteau reagent was added to the test tubes; well mixed and incubated at 37 °C for 30 min. The blue colour produced was detected using spectrophotometer at wavelength of 660 nm. One unit of protease activity was defined as the amount of enzyme liberating 1 ìg of tyrosine per min under assay conditions.

The effect of pH on protease activity was determined by incubating the reaction mixture at pH values ranging from 4 to 13.0, and then protease activity was measured, according to the standard assay procedure. The pH was adjusted using the following buffer systems 0.05 M, acetate (pH 4), citrate (pH 5-6), phosphate (pH 7), Tris-HCl (pH 8-9), carbonate (10-11) and KCl-NaOH (pH 13.0). In order to determine the pH stability, the protease was incubated in different buffer solution at 30 °C for 2 hours, after which the activity was determined. The effect of temperature on enzyme activity was studied by conducting the reactions in temperature range of 30 to 75 °C, using the standard assay method. The thermo-stability of enzyme was studied by incubating the enzyme preparation at varying temperatures ranging from 30-75 °C for 1 hour in a constant temperature and the activity was evaluated.

RESULTS & DISCUSSION

Wheat barn, rice bran, sugarcane bagasse, barely bran, corn meal and corn husk were used as the substrate for the production of protease. Fig. 2 shows enzyme activities with respect to different substrates. Maximum enzyme activities obtained from wheat bran were 798.83 and 776.91 U/gds for top and middle trays, respectively. Wheat bran is a potential substrate in solid state fermentation process; that is a good source of protein, carbohydrate and minerals required for the

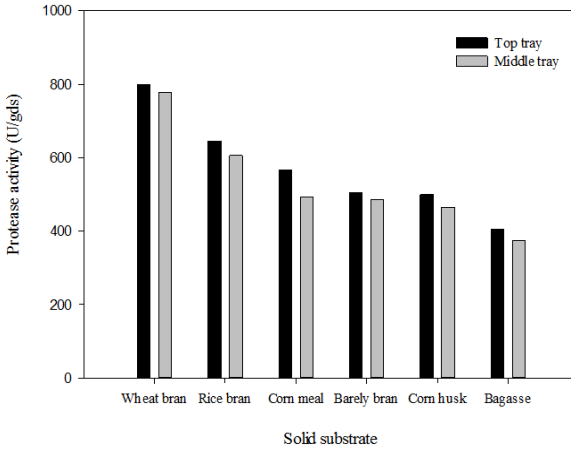

Fig. 2. Effect of different substrates on protease production by *B.licheniformis*

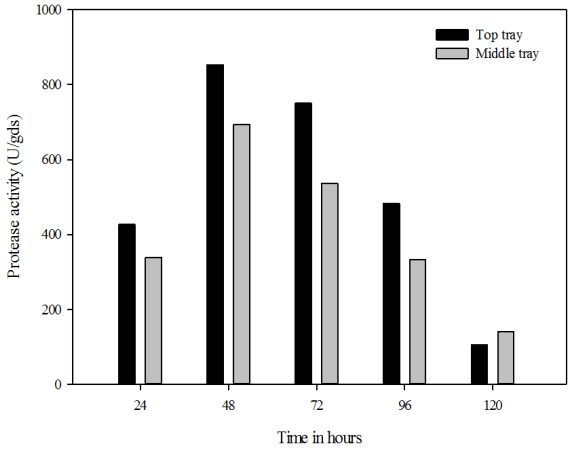

Fig. 3. Influence of incubation time on enzyme production

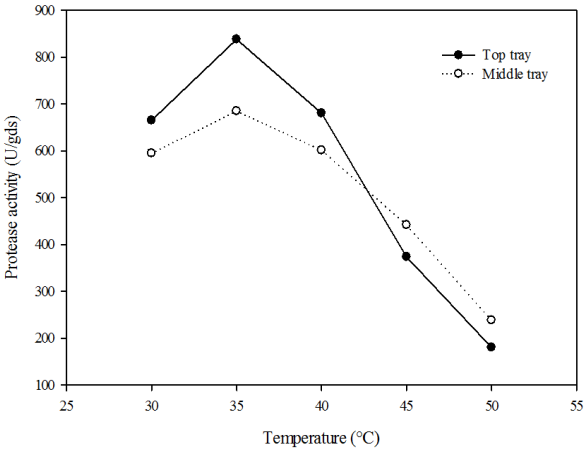

Fig. 4. Influence of incubation temperature on enzyme production

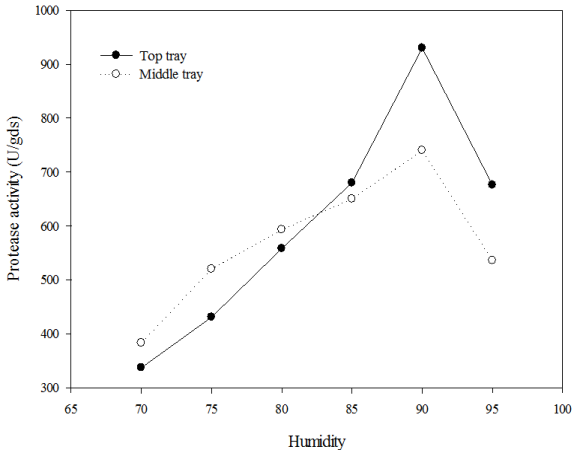

Fig. 5. Effect of Bioreactor Humidity

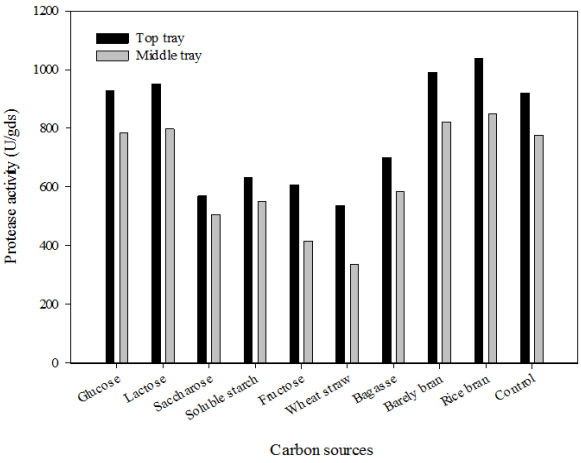

Fig. 6. Effect of different carbon sources

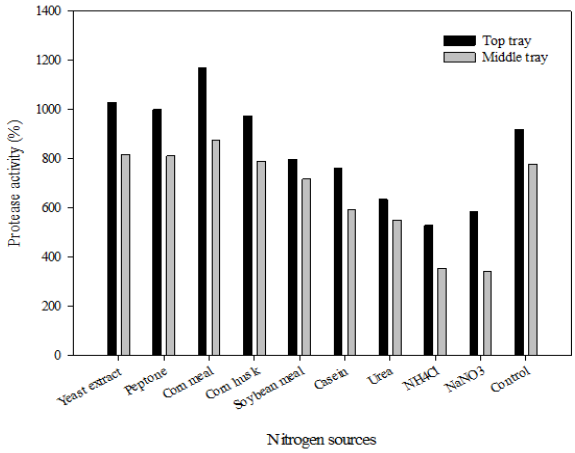

Fig. 7. Role of different nitrogen sources

growth of microorganisms and protease production. Also morphological characteristics of bed of wheat bran caused desired air circulation and oxygen transfer and consequently prefect heat and mass transfer. Further optimization studies were carried out using wheat bran as the substrate. Results showed that circulation of nutrient solution from bottom tray in addition to humidity controlling, can induce significant effect on protease production. Moreover, it was found that protease activity of the top tray was higher than the middle tray except in a few cases. That was most probably due to nutrients directly received in top tray; whereas, the middle tray received the nutritious solution leached out from the top tray.

Protease production was conducted for a period of 120 hours of fermentation in tray bioreactor. Sampling was done for every 24 hours and enzyme activity was determined. Fig. 3 shows that maximum enzyme activity obtained after 48 hours of fermentation from top and middle trays. The lack of nutrients and denaturation of enzyme by other components present in the medium; that may decrease protease production other than the optimum level. These results are in accordance with data reported by Imtiaz et al. (Imtiaz and Mukhtar 2013) and Rathakrishnan and Nagarajan (Rathakrishnan and Nagarajan 2011). Prakasham et al. (Prakasham *et al.*, 2006) reported that the optimal time for *Bacillus* sp. protease production was 60 hours.

Among the different incubating temperatures of the chamber in the range of 30 to 50 °C; maximum production of protease by *B. licheniformis*, was obtained at 35 °C (see Fig. 4). Operation at out range of optimum temperature caused decease in enzyme production. That may be caused by thermal denaturation of enzyme at high temperature. For most of the bacteria, the optimum temperature for the growth and protease production is within the range of 30 to 40 °C (Joo *et al.*, 2002). Imtiaz *et al.*(Imtiaz and Mukhtar 2013) has also reported that maximum protease production was obtained for *Bacillus subtilis* at 37

°C. Meena *et al*. (Meena *et al.*, 2013) have shown that using *Pseudomonas aeruginosa*; maximum production of protease was achieved at incubation temperature of 45 °C.

The humidity of the bioreactor has provided by circulation of the nutrients from the bottom tray. The influence of this parameter was investigated by adjusting the humidity of the cabin between 70-95%. Humidity of 90% was the best for maximum protease production for top and middle trays. At optimum level of humidity, protease activities were 930 and 740 U/gds for top and middle trays, respectively (see Fig. 5). Above this level, enzyme activity was decreased in the bioreactor because of the high moisture content. In high humidity values, greater flow of nutrients from the bottom trays pumped in to the bioreactor and consequently created a semisolid medium which has an adverse effect on microbial growth and enzyme production. In addition, high moisture may cause blockage and prevention of air penetration and diffusion problem into the texture of the tray bed.

Effect of carbon sources for protease production were determined using different simple (glucose, lactose, saccharose, soluble starch and fructose) and complex (wheat straw, bagasse**,** barely bran and rice bran) carbon sources. Among the various carbon sources used, rice bran provided maximum production of protease with enzyme activities of 1040 and 850 U/gds for top and bottom trays, respectively (see Fig. 6). To optimize the rice bran concentration as carbon source, different amount of rice bran ranging from (0.5-2%) were added to the culture medium, and maximum protease production was observed with 1% rice bran. Results showed that simple carbon sources such as saccharose, soluble starch and fructose didn't improved protease activity. That was due to absence of supplementary nutrients and vitamins, while by glucose and lactose as substrates had insignificant increase in protease production**.** The simple carbon sources did not contain supplementary nutrients in compare to control state. Using agricultural wastes such as rice bran as a carbon supplement, in addition to carbohydrate, containing nutrients such as protein, fatty acids, vitamins and other minerals had suitable impact on growth and enzyme production.

Effect of different organic and inorganic nitrogen sources (simple and complex N) such as yeast extract, peptone, corn meal, corn husk, soybean meal, casein, urea, NH_4Cl and $NaNO_3$, on production of protease was investigated. Maximum enzyme production was obtained when corn meal was used as an organic nitrogen source (see Fig. 7). When different concentrations of corn meal were tested, corn meal at 2% provided maximum production of protease. None of these inorganic nitrogen sources have significant improvement in protease activity; that was due to metabolite associated with utilization organic and inorganic nitrogen sources and liberation of ammonia. The availability and quantity of nitrogen sources in the culture medium is a crucial factor for the secretion of protease by microorganisms.

The activity of protease towards casein as substrate at different pH values was determined. The obtained results illustrated in Fig. 8 showed for both trays 1 and 2, the enzyme was active in broad pH range of 7-13. The maximum activity was found at pH 8. The relative activity at pH values of 9, 10 and 11 were found to be about 98, 95 and 88% of the maximum activity at desired pH of 8, respectively. Moreover, the protease retained 80% of the maximum activity at pH value of 13. However, the activity significantly decreased under acidic pH values (below pH 7). Therefore, the enzyme found to be a typical alkaline protease, which is shown its maximum activity in the alkaline pH range of 7-13.

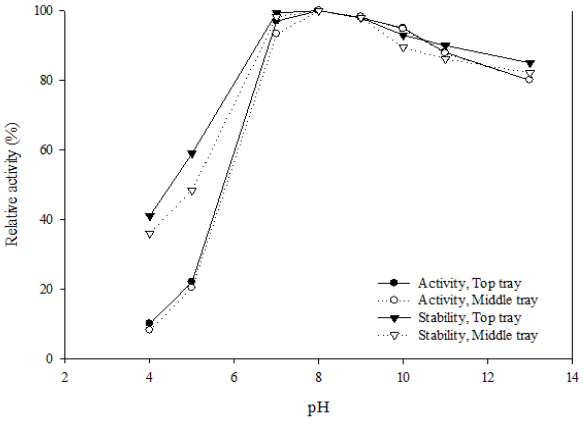

Fig. 8. Effect of pH on protease activity and stability

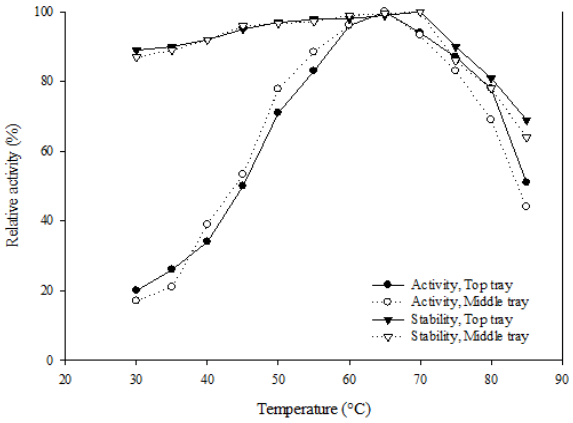

Fig. 9. Effect of temperature on protease activity and stability

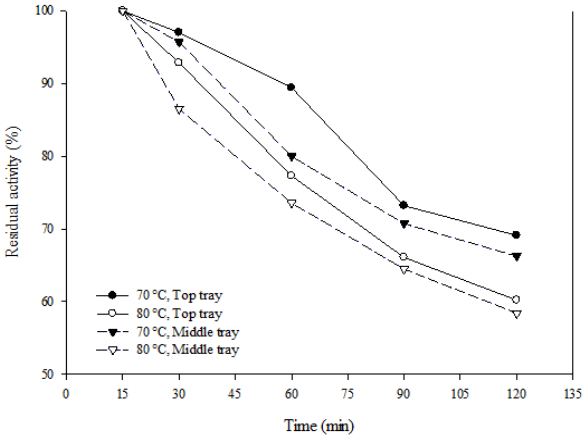

Fig. 10. Heat stability of the protease. The enzyme was incubated at 60 and 70 ÚC for 15-/120 min.

The results of present study were in accordance to literature using *Bacillus licheniformis* NRRL-NRS-1264 (Sathyavrathan and Krithika 2014), *Bacillus* sp. (Nascimento and Martins 2004), *Bacillus licheniformis* Lbbl-11 (Olajuyigbe and Ajele 2008) and *Halomonas* sp. PV1 (Vijayaraghavan and Vincent 2012). As illustrated in Fig. 8, the enzyme was very stable within pH range of 7-13, and optimum pH for high stability was observed at pH of 8. Protease from top tray retained 90 and 85% of its activity at pH values of 11 and 13, respectively. Generally, the commercial microbial proteases have optimum pH in the alkaline range of 8 to 10. The important detergent enzyme, subtilisin Carlsberg produced by *B. licheniformis* showed maximum activity at pH val ues of 8-10 (Hadj-Ali *et al.*, 2007). These findings are in accordance to the reported values in literature using *Bacillus licheniformis* P003 (Sarker *et al.*, 2013) and *Bacillus pumilus* (Sarker *et al.*, 2013). In another study, Sellami-Kamoun et al. (Sellami-Kamoun *et al.*, 2008) reported a protease produced by *Bacillus licheniformis* RP1 was very stable over a wide pH range, maintaining 100% of its original activity between 8 and 10. Since the pH of laundry detergents is generally in the range 9.0-12.0, alkaline protease produced in this work, is a potential enzyme for use as a cleaning aid in detergents.

To investigate the effect of temperature on enzyme reaction activity for top and middle trays, enzyme reaction was carried out at different temperatures from 30 to 85 °C. The maximum enzyme activity was obtained at 65 °C. Based on obtained results illustrated in Fig. 9, as temperature increased from 30 to 65 °C, the activity increased to the optimal value, after that a reduction in enzyme activity was observed, though the protease from top tray still retained 87% of its optimal activity at 75 °C. Several investigations have reported the optimum temperature of alkaline proteases defined in range of 60-70 °C (Hadj-Ali *et al.*, 2007, Sellami-Kamoun *et al.*, 2008, Joo *et al.*, 2003). The thermo-stability of enzyme was studied by incubating the enzyme preparation at various temperature ranging from 30-85 °C for 30 min. Fig. 9 shows the protease is very stable and maximum stability at 70 °C was obtained. The enzyme from top tray retained 90, 81 and 69 of its maximum activity at 75, 80 and 85 °C, respectively.

In additional experiment, deactivation of enzyme respect to time courses was investigated by incubating the enzyme solution at 70 and 80 °C, for 15-120 min. The non-heated enzyme was considered as control and also the residual activity was determined. Finally, after 2 hours of incubation at 70 and 80 °C, the protease retained approximately 70 and 60% of its initial activity (see Fig. 10).

Based on obtained results, alkaline protease from *B. licheniformis*, found to be highly thermostable when compared to findings reported in the literature (Vijayaraghavan *et al.*, 2014, Olajuyigbe and Ajele 2008, Xiuqin *et al.*, 2013); that might be a potential enzyme for use as a cleaning aid in detergents and a bating agent in tanneries (Sarker *et al.*, 2013, Hadj-Ali *et al.*, 2007).

CONCLUSIONS

No serious attempt has been reported for production of thermostable alkaline protease in tray bioreactor. In this work, production, optimization and characterization of protease was investigated; while growing *B. licheniformis* on several agricultural residues as substrate. Results showed that maximum production of thermostable alkaline protease was achieved using wheat bran as suitable substrate, after 48hours at temperature of 35 °C and cabin humidity of 90%. Also, rice bran (1%) and corn meal (2%) increased protease production as carbon and nitrogen supplements. Based on the present research findings, the enzyme is very stable in broad range of temperature and pH, and showed maximum activity at pH 8 and 65 °C. These characteristics indicated that alkaline protease produced can be a potential enzyme for use as a cleaning aid in detergents.

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