

## COMPARATIVE STUDIES OF ALKALINE PROTEASE PRODUCTION IN SOLID STATE FERMENTATION: TRAY BIOREACTOR AND FLASK

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### ABSTRACT

In this study, production of protease in solid state fermentation using *Bacillus licheniformis* was investigated. Fermentation was carried out in batch and semi-batch systems including flask and tray bioreactor. Wheat bran, rice bran and mixtures of both agro-wastes were used as substrate. Maximum enzyme production was obtained using wheat bran as sole substrate with high activity of 798.83, 776.91 and 562.11U/gds for top, middle trays and flask, respectively. Results showed that optimum incubation time of 48h was defined. Enzyme production was evaluated with different incubating temperatures and initial moisture content of solid substrates. Maximum protease activity was observed at 35 and 40°C for the tray bioreactor and flask, respectively. The obtained results showed that maximum protease production was achieved with substrate initial moisture of 150, 200 and 150% for the flask, top and middle trays, respectively. In addition, the enzyme activity was improved by supplementary substrate such as corn meal as an inducer, which gave protease activities of 678, 830 and 940U/gds, for flask, middle and top trays, respectively.

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

### INTRODUCTION

In solid state fermentation (SSF), micro-organisms grow on a moist solid surface, without presence of free liquid phase (Raghavarao et al., 2003). In this technique, the substrates are insoluble solid substances that supply nutrients for growth of micro-organism and production of value added products such as enzymes (Pandey, 2003). At present, overall cost of enzyme production is very high due to the cost of growth medium. From commercial view point, in SSF by utilization of agroindustrial substrates, production cost and environmental pollution can be reduced (Prakasham et al., 2006; Vijayaraghavan and Vincent, 2012). Solid state fermentation has many applications in various economic sectors such as industrial fermentation, food industry and environmental friendly process (Mienda et al., 2011). Several parameters including temperature, humidity of substrate bed, depth of bed and particle size play major role on fermentation performance (Mitchell et al., 2006). Moreover, in large scale solid state processes, the efficiency and economy of SSF are affected by issues of heat and mass transfer (Jou and Lo, 2011; Khanahmadi et al., 2004). In SSF, various type of bioreactor including tray, packed bed, rotary drums and gas-solid fluidized bed, have been

used. Tray bioreactors are the simplest type and can be easily scale up for commercial application (Bhargav et al., 2008). In the present study, production of protease using *Bacillus licheniformis* in solid state fermentation process with tray bioreactor and flask was investigated. Proteases are one of the most important groups of industrial enzymes and find widespread applications in detergents, leather, food and pharmaceutical industry (Gupta et al., 2002; Shaheen et al., 2008). Bacteria especially *Bacillus* strain produce significant amount of extracellular protease with respect to industrial demands (Mani et al., 2012; Mukhtar and Haq, 2012). It was believed that bacteria are not suitable for SSF due to the low moisture content of the process; however, recent studies showed that bacterial enzymes could be obtained in SSF with high activity in cost effective medium (Imtiaz and Mukhtar, 2013; Rathakrishnan and Nagarajan, 2011; Subramaniyam and Vimala, 2012; Vijayaraghavan et al., 2014).

Most studies involving protease production in solid state fermentation, utilized flasks, in which few grams of substrates are used. However, no significant work has been reported in terms of protease production in tray bioreactors, the objective

of present study was to evaluate protease production in the flask and tray bioreactor. Wheat bran, rice bran and mixture of both solid substrates were used for protease production. Effect of process parameters such as incubation period, incubation temperature and moisture content were investigated. Meanwhile, several types of protein compounds were employed as inducers for the enhancement of protease activity.

## MATERIALS AND METHODS

**Materials:** 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 and wheat were obtained from local market (Babol, Iran).

**Microorganism and growth media:** The organism *Bacillus licheniformis* ATCC 21424 employed in this research was supplied from Iranian Research Organization for Science and Technology (IROST, Tehran, Iran). The microorganism was cultivated on nutrient agar medium at 37°C for 24 hours and stored at 4°C. Inoculum was prepared in 250ml Erlenmeyer flasks containing 50 ml of sterilized nutrient medium consisting of (g/l): glucose 10, peptone 5, yeast extract 5,  $\text{KH}_2\text{PO}_4$  1,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.2, pH 8, and was aseptically inoculated with a loopful of bacteria from a fresh slant and allowed to grow at 37°C and 150 rpm for 24 hours in an incubator shaker (IKA, Korea).

**Solid state fermentation:** For solid state fermentation, Rice bran, wheat bran and mixtures (1:1 W/W) of these substrates were used as nutrients for production of protease. These agricultural residues were washed with distilled water three times and dried overnight at 60°C. Then, substrates were sieved to uniform particle size in the range of 0.18-2mm before use. Solid state fermentation was conducted in the following systems under various experimental conditions. The effects of process parameters such as fermentation time (24, 48, 72, 96 and 120h), incubation temperature (30, 35, 40, 45 and 50 °C) and moisture content (50,100, 150, 200, 250 and 300%) for protease activity were evaluated. Also different inducers such as corn meal, soybean meal, yeast extract, peptone, casein and bovine serum albumin were used and protease activity was determined.

**Fermentation in flask:** A 5.0 gram of substrate was taken in 250 ml Erlenmeyer and moisture content of 100 % (wt/wt). These flasks were cotton plugged and autoclaved at 121°C for 20min. After cooling, the medium was inoculated with 1ml of bacterial inoculum as prepared earlier and incubated at 37°C for 120 hours. The fermentation batches were run in triplicates and the average of three values were reported in the obtained results.

**Fermentation in tray bioreactor:** Simultaneously, SSF was carried out in a novel and effective semi batch bioreactor designed and fabricated in previous work. The Plexiglas bioreactor (45 x 35 x55 cm), have three aluminum trays (35 x 25 x5 cm) (Vaseghi et al., 2013). In this bioreactor, the temperature and humidity of cabin were well controlled; consequently maximum protease production under desired condition was obtained. For production of protease in tray bioreactor, 5 gram of dried substrate filled in paper bags were placed on top and middle trays and inoculated with bacterial inoculum. Bottom tray was filled with nutrient solutions containing (%): yeast extract 0.1,  $\text{KH}_2\text{PO}_4$  0.1,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.05, NaCl 0.25 and  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.004. The peristaltic pump (ETATRON, Italy) was used to provide the appropriate moisture content in the cabin by circulating the nutrients from bottom tray along the reactor. Fermentation was done for duration of 5 days and protease activities and total protein contents were determined. Reported results are average values of triplicate recorded data.

**Sampling and protease Extraction from the Fermented Solid:** Samples of fermented solids were taken every 24 hrs and enzyme activity was determined. Protease was extracted from fermented substrates by transferring the solid samples into Erlenmeyer flask containing 50 ml of Tris-HCl buffer (pH 8, 0.01M). The solution was kept in rotary shaker for 1h at 30°C and 200rpm. Then, the whole contents were filtered under vacuum and the clear supernatant was used as the enzyme source and assayed for protease activity and protein content.

**Determination of protease activity:** Protease activity was determined by modified method using casein as the substrate (McDonald and Chen, 1965). A 1.0 ml of crude enzyme was added to 5.0ml of casein

solution and incubated at 37 °C for 10min. The reaction was stopped by addition of 5ml of TCA solution and the content was centrifuged at 5000rpm for 10 min. A 2ml of filtrate was added to 5.0ml of Na<sub>2</sub>CO<sub>3</sub> and 1.0ml of Folin and Ciocalteau reagent. After 30minutes incubation at 37°C, the optical density of the mixture was read at 660nm.

One unit of protease activity was defined as the amount of enzyme required to release one µg of tyrosine per minute under the experimental conditions.

**Estimation of total soluble protein:** The crude enzyme suspension was centrifuged (Hermle, Type: Z293 M-2, Germany) at 6000g for 10 min; and the supernatant was used for protein assay. Protein content of the enzyme solution was measured by Bradford method using bovine serum albumin (BSA) as the standard (Bradford, 1976). The concentration of protein was calculated from the standard curve.

## RESULTS AND DISCUSSION

Effect of different substrates on protease production: Wheat bran and rice bran are potential substrates for solid state fermenta-

tion process for enzyme production. These substrates are readily available in many regions and employed as the substrates for the production of protease. In order to improve the properties of the sole substrates, solid substrate mixture of wheat and rice bran (1:1 w/w) were evaluated for protease activity. It was observed that, protease activity from the rice and wheat bran as single substrate was higher than the mixtures of bran and maximum enzyme activity was achieved from wheat bran (Fig.-1). Several reported data showed that higher levels of protease activities were observed when wheat bran was as substrate (Agrawal et al., 2005; Meena et al., 2013; Uyar and Baysal, 2004). Since, Wheat and rice bran are good sources of all of nutrients required for the biosynthesis of enzymes by microorganisms, it can be related to the good morphological characteristics of bed of wheat bran. Some physico-chemical properties of wheat and rice bran are summarized in Table 1.

Table -1: Analysis of wheat and rice bran

| Substrate  | Protein (%) | Moisture (%) | Ash (%) | Reducing sugar (%) | Fat (%) |
|------------|-------------|--------------|---------|--------------------|---------|
| Wheat bran | 16.6        | 11           | 7.25    | 9.24               | 5.4     |
| Rice bran  | 12.2        | 8.7          | 10.4    | 7.49               | 12.3    |

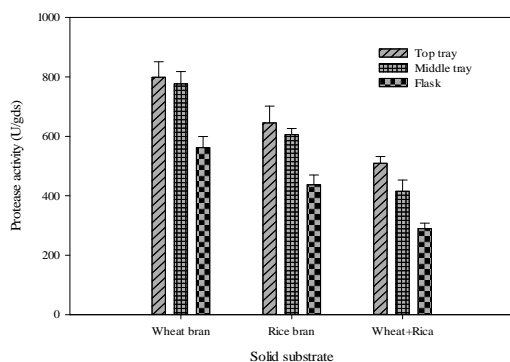


Fig.-1: Effect of different substrates on protease production by *B. licheniformis*

**Influence of incubation time on enzyme production:** To determine the optimum incubation period, fermentation in flask and tray bioreactor was conducted for different time intervals (24, 48, 72, 96 and 120 hrs). As illustrated in Figure 2, maximum protease

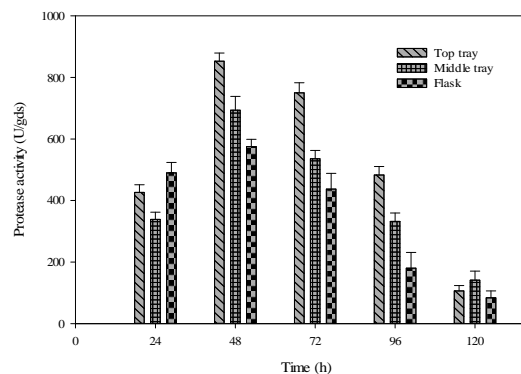


Fig.-2: Effect of time on protease production by *B. licheniformis*

activity was obtained after 48h of fermentation in both systems. After optimum level, with increasing period of incubation, enzyme production was decreased considerably due to the lack of nutrients in the medium, although rate of reduction in tray bioreactor was less than flask. This could be due to the effective design of bioreactor and recircu-

lation of nutrient solution along the set up. These results are in accordance with the observations made with *Bacillus subtilis* by Imtiaz et al., (2013) and *Bacillus cereus* by Rathakrishnan and Nagarajan (2011).

**Influence of incubation temperature on enzyme production:** The effect of temperature on protease activity was investigated by incubating the flasks in incubator at different temperatures (30, 35, 40, 45 and 50°C). While in tray bioreactor, inoculated substrates were incubated at various temperatures of chamber from 30 to 50°C with an increment of 5°C. Results depict optimum temperature for batch and semi batch systems are 40 and 35°C, respectively

(Fig. 3a). Also total protein content of the enzyme solution at different temperatures was measured (Fig. 3b). The obtained results indicated that enzyme solution with high activities has more protein than any enzyme solution with low activities. Protease activity and total protein of tray bioreactor was higher than that of flask except in few cases, which might be due to the fact that the reactor has appropriate design. Based on the results illustrated in Fig. 3 protease activity of top tray was higher than middle tray due to supplementation of nutrients and sufficient gas exchange through the bed.

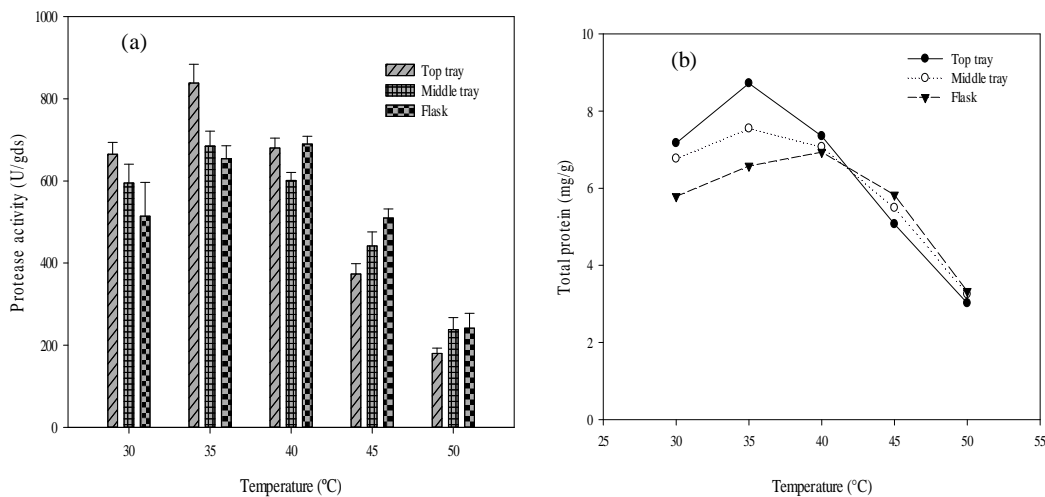


Fig.-3: Effect of different incubating temperature on protease produced by *B. licheniformis*, (a) protease activity, (b) total protein

**Effect of moisture content:** Moisture content of solid substrate is one of the most critical factors which determine the success of the SSF process and effects on microbial growth and protease production. Presence of water in the substrate ensures that the nutrients are accessible for microorganisms (Lazim et al., 2009; Naik, 2012; Sandhya et al., 2005). Effect of moisture content was investigated by adjusting the initial moisture content of substrate between 50-300% with increment of 50%. Results in Fig. 4 showed that a maximum protease activity was obtained with 200% moisture content for top tray. Also the highest enzyme activities were

observed with initial moisture content of 150% for middle tray and flask. After optimum level, protease activity was decreased due to the tendency of particles to stick together, thereby reducing porosity and creating anaerobic conditions. Also, cabin humidity plays an important role for protease production in tray bioreactor. Effect of this parameter was investigated by adjusting the humidity of the cabin between 70-95% and maximum protease activity was obtained at 90% of humidity. At optimum level of humidity, enzyme activities were 930 and 740 U/gds for top and middle trays (Table 2).

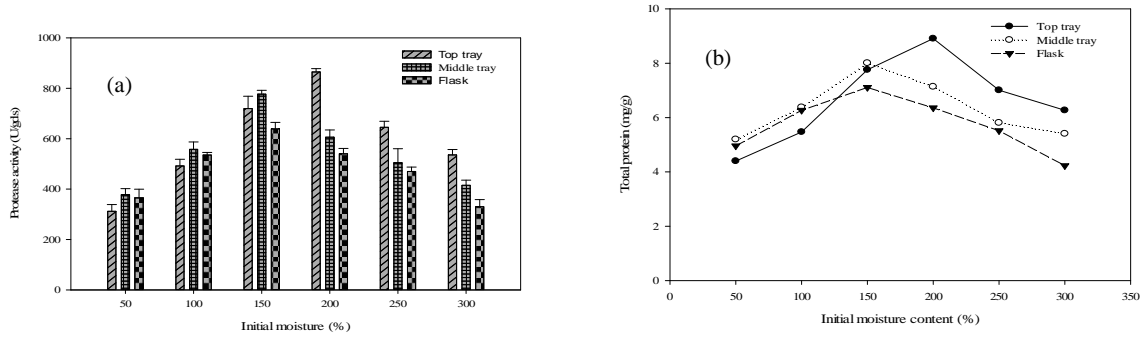


Fig.-4: Effect of initial moisture content on protease produced by *B. licheniformis*, (a) protease activity, (b) total protein

Table- 2: Effect of cabin humidity on protease activity produced by *B. licheniformis*

| Humidity (%) | Protease activity (U/gds) |             | Normalized protease production (Y P/S) |             | Total protein content (mg/g) |             |
|--------------|---------------------------|-------------|----------------------------------------|-------------|------------------------------|-------------|
|              | Top tray                  | Middle tray | Top tray                               | Middle tray | Top tray                     | Middle tray |
| 70           | 337                       | 383         | 0.36                                   | 0.52        | 4.30                         | 5.22        |
| 75           | 430                       | 520         | 0.46                                   | 0.71        | 5.27                         | 6.00        |
| 80           | 558                       | 593         | 0.60                                   | 0.80        | 6.41                         | 6.72        |
| 85           | 680                       | 650         | 0.73                                   | 0.88        | 7.30                         | 7.36        |
| 90           | 930                       | 740         | 1.00                                   | 1.00        | 8.74                         | 7.97        |
| 95           | 676                       | 536         | 0.73                                   | 0.72        | 7.51                         | 6.37        |

**Effect of Different Inducers on protease Production:** Enzymes are inducible products that synthesized by microorganisms in response to the presence of a particular substance. It was found that production of proteases is related to the properties of the protein compound in the fermentation medium (Simkovic et al., 2008; Srinubabu et al., 2007). In this study, effect of various inducers such as corn meal, soybean meal, yeast extract, peptone, casein and bovine

serum albumin (BSA), were screened for protease production (Fig.-5). Maximum enzyme activity was obtained when corn meal was used. Protease activity was 678, 830 and 940U/gds, for flask, middle and top trays, respectively (Fig. 5a). Total protein content of enzyme solution with different inducers, were also shown in Fig. 5b. Results showed that, supplementation of wheat bran with casein and BSA as an inducer did not improve protease production.

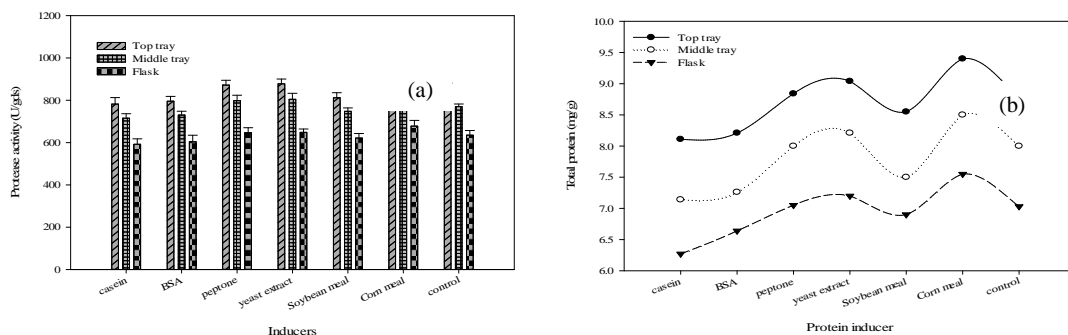


Fig.-5: Effect of Different Inducers on protease Production by *B. licheniformis*, (a) protease activity, (b) total protein

**CONCLUSION**

In this work, protease production in two different bioreactors for solid state fermentation

process including flask and tray bioreactor, were evaluated. Results showed that protease

with high activity level were obtained in tray bioreactor. In order to optimize enzyme production, effects of process parameters were also investigated. Under optimum conditions protease activity was 678, 830 and 940 U/gds for flask, middle and top trays, respectively.

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