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Cost Effective Method of Protease Production in Solid State Fermentation Using Combined Substrate Corn Cob and Lentil Husk

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Abstract

An alkalophilic Bacillus cereus strain was found to produce a thermal and pH tolerant extracellular alkaline protease which showed very good compatibility and stability with commercial laundry detergents. Considering important industrial application of B. cereus protease, its production needs to be enhanced. Different agro-industrial waste products, viz., wheat bran, lentil husk, green gram husk and corn cob were used as substrates for the production of B. cereus protease by solid state fermentation (SSF). The production of alkaline protease in SSF was compared with that in submerged fermentation (SmF) by using the same substrates. This comparative study revealed that production of B. cereus protease is significantly higher in SSF than in SmF. Among all the substrates used, corn cob followed by lentil husk showed best production in SSF. Combined effect of corn cob and lentil husk was examined when mixed in 1:1 ratio. The production of protease was enhanced almost double in comparison to individual substrate. Other important parameters like inoculum size, substrate concentration, fermentation time, incubation temperature and moisture content were optimized to get maximum protease production in SSF. Maximum protease activity of 580.80 u/mL/min (4640 U/g solid substrate) was obtained in SSF when initial moisture content was maintained at 60%, 3 g of corn cob and lentil husk were used in 1:1 ratio, 2.0–2.5 mL (2 mg/mL) of inoculum was added and culture was incubated at 30 °C for 7 days.

Keywords: *Bacillus cereus, alkaline protease, solid state fermentation, corn cob, lentil husk*

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INTRODUCTION

Proteases (EC 3.4) are very important industrial enzymes and account for about 60% of the total world enzyme market [1]. Alkaline proteases from various *Bacillus* species have been reported to be used in laundry formulations [2, 3]. An alkalophilic bacterial strain, *Bacillus cereus* was grown under submerged condition for the production of protease that showed better compatibility and stability in commercial laundry detergents [4]. In account of industrial application, yield of microbial proteases is very important and number of literatures support high production of it in solid state fermentation (SSF) [5, 6].

SSF has gained much attention from industry because it becomes a better alternative to submerged fermentation with simpler cultivation equipment's, lower capital investment, higher productivity, low energy requirement, less water output, better product recovery, and allows easy control of contamination due to low moisture content [7]. Production of protease in SSF is found be common from fungal sp. due to high growth in low moisture conditions [8–10]. However, number of literatures also showed protease production by bacterial sources using SSF processes [11–13]. SSF appears as an interesting low cost alternative for the production of biomolecules because agro-industrial residues can be applied as culture media which reduce production costs [14]. On the other hand, their extensive use through SSF processes solves the pollution and waste management problems. *Bacillus* protease production has been carried out under SSF using substrates wheat bran, green gram husk, soy cake and potato peel, etc. [11–13, 15].

The present work shows the production of laundry detergent compatible protease from *Bacillus cereus* under SSF using agro wastes like wheat bran, green gram husk, corn cob and lentil husk. Effect of combined substrate corn cob and lentil husk was studied for production of protease that was not reported earlier. Culture conditions were optimized by using different concentrations of agro-industrial wastes used, inoculum concentration, production time, and moisture level to get better production of protease. Laundry detergent compatible *B. cereus* protease production was compared in solid state fermentation and submerged fermentation process.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions

Bacillus cereus MTCC 3105 was maintained at 4 °C on nutrient agar medium containing beef extract 1.0%, NaCl 0.5%, peptone 1.0%, agar 2.0%. The organisms were grown in a medium having composition (%w/v): glucose 3.0%, soybean meal 1.0%, $CaCl_2$ 0.04% and $MgCl_2$ 0.2% [4]. The pH of the medium was adjusted to 7.5 by dissolving the medium constituents in 0.2 M Tris/HCl buffer. The growth medium was inoculated with the organism and incubated for 24 h at 30 °C and 200 rpm in an orbital shaker to develop inoculum.

Protease Production

Alkaline protease from *B. cereus* was produced by two methods: submerged condition and solid state fermentation.

Submerged Fermentation

The liquid medium used for the production of alkaline protease had the following composition (%w/v): glucose 2.0%, soybean meal 2.0%, $CaCl_2$ 0.04% and $MgCl_2$ 0.02% [4]. The pH of the medium was adjusted to 8.5 by dissolving the medium constituents in 0.1 M phosphate buffer. Fermentation was carried out using 50 mL of the production medium in 250 mL Erlenmeyer flask. The production medium was inoculated with 5% inoculum (3×10^6 cfu/mL). The flasks were incubated for 72 h in a temperature-controlled (30 °C) shaking incubator (200 rpm). The contents were then centrifuged (10,000 g, 30 °C, 20 min) and the cell-free supernatant was used for determining extracellular protease activity. Submerged fermentation was also supplemented with agro-wastes; those are used in solid state fermentation.

Solid State Fermentation

The agro-industrial wastes to be used as substrate were procured from local Indian market and grinded to size about 2–3 mm before use. The production media was prepared in Erlenmeyer flask (250 mL) containing 5 g of agro-industrial substrates and 10 mL liquid media (0.5% glucose, 0.5% soybean meal, 0.04% $MgCl_2$, 0.2% $CaCl_2$). The substrates were soaked for 4 h and then autoclaved for 20 min at 121 °C and 15 psi/m^2. Initial moisture level of the media was maintained at 50% by soaking media substrate in appropriate amount of distilled water [16]. After cooling the flasks to room temperature, 2 mL (3×10^6 cfu/mL) of overnight inoculum was added to each flask. The flasks were then kept in an incubator at pH 8.5, 30 °C under static condition for 3 days.

Enzyme Extraction

Protease enzyme from solid state broth was extracted by taking 1 g of fermentation broth from each substrate flask and 8 mL of distilled water was added to it. This was kept in shaker-incubator for 1 h at 4 °C and 150 rpm. Fermentation broths were centrifuged for 15 min at 10,000 rpm, 4 °C and the supernatant was checked for the protease activity [12].

Assay of Protease Activity

Alkaline protease activity was estimated by taking 5.0 mL of 1.2% casein solution in Tris-HCl buffer at appropriate pH, and suitably diluted enzyme (1.0 mL) was incubated at 50 °C for 10 min [4]. The reaction was terminated by adding 5.0 mL of 0.3 M trichloroacetic acid, and after 30 min of incubation (30 °C), the reaction mixture was centrifuged (10,000 g, 30 °C, 20 min). The absorbance of clear supernatant was measured spectrophotometrically at 275 nm. Enzyme activity was expressed as protease unit, in which 1 U of protease activity was defined as the quantity of the enzyme that liberated the digestion product not precipitated by protein precipitating reagent and gave an absorbance at 275 nm equivalent to 1 µg/mL of tyrosine/min under assay conditions and protease activity was determined in 1 g dry

fermented substrate is equivalent to 8 mL broth.

Optimization of Process Parameters for Alkaline Protease Production in SSF

The protocol adopted for the optimization of process parameters was to evaluate the effect of an individual parameter at a time and to incorporate it at the standard level before optimizing the next parameter.

Optimization of Incubation Period

Solid state fermentation was performed with all four substrates by incubating the culture (pH 8.5, 30 °C) for different time periods (1, 3, 5, 7 and 9 days). The protease production in each was estimated by standard assay method.

Optimization of Amount of Substrate

Amount of agro-wastes (wheat bran, gram husk, lentil husk and corn cob) was optimized by varying the amount in different flasks (1, 3, 5, 7 g). The flasks were incubated for 7 days under the same culture conditions. Substrates, corn cob and lentil husk were also tried as combined substrates in 1:1 ratio and followed for protease production in SSF as before.

Effect of Inoculum Size on Protease Production

Protease production was performed by varying the size of the inoculum as 1, 1.5, 2, 2.5 and 3.0 ml of the same 12 h inoculum. Other optimized conditions were followed and protease activity was determined.

Optimization of Incubation Temperature

The inoculated substrates (corn cob and lentil husk) were incubated at different temperatures to determine the optimum fermentation temperature for alkaline protease production (20, 30, 40, 50, 60 °C).

Optimization of Moisture Level

The initial moisture content of the fermentation substrate (mixture of lentil husk and corn cob) was optimized by changing the percentage of water levels (40, 50, 60, 70 and 80, 90%). The percent moisture content was estimated by drying 3 g of combined substrate to constant weight at 100 °C and the dry weight was recorded. To fix the initial moisture content of the solid medium, the substrate was soaked with the appropriate quantity of distilled water. The sample was then dried to calculate moisture content (%).

Initial moisture content of solid medium (%) = [(wt. of substrate – dry wt.)/dry wt] × 100

Chemical and Statistical Analysis

All media components and chemicals used were of highest purity grade available commercially. Agro-byproducts (wheat bran, green gram husk, lentil husk and corn cob) were obtained locally. All the experiments were performed independently in triplicates and the results given here are the mean of three values.

RESULTS AND DISCUSSION

Bacillus cereus protease has an important application in detergent industry and so it is purified and characterized in previous work [17]. Due to its potential application, methods for enhanced and cheaper production of protease are needed. One alternative low cost production method is solid state fermentation and the process is highly affected by the nature and cost of solid substrate used in the process [18].

Agro-industrial wastes wheat bran, corn cob, green gram husk and lentil husk are used in the present study as low cost substrates. Bacterial fermentation was carried out for three days in both SmF and SSF by using 5 g agro-byproducts (Figure 1). Among all the substrates used corn cob followed by lentil husk gave the best production of alkaline protease in both the fermentation systems, while wheat bran was reported commonly in earlier studies [15, 19]. *Bacillus cereus* produced 22.34 u/mL/min alkaline protease in SmF and 45.86 u/mL/min in SSF by using corn cob as substrate. The result shows almost double production of protease in SSF as compared to SmF, which was also reported by previous workers [19, 20]. Enzyme synthesis in SSF is resistant to catabolite repression, mainly due to existence of microscopic gradients within the mass of cell aggregates, or the changes in cell permeability to sugars. Catabolite repression is not observed in SSF even at sugar concentration as high as 100 g/L while showing repression whenever sugar concentration is higher than 10 g/L [20].

Different production parameters were optimized for enhanced production of *Bacillus* protease in SSF. The effect of fermentation time on protease production by using different agro-byproducts is shown in Figure 2. *Bacillus cereus* showed maximum protease activity up to 7 days with all the four substrates used. Maximum protease production was found with corn cob (179.52 u/mL/min or1440 U/g) at pH 8.5, 30 °C and 7 days. The production is significantly higher in comparison to three days production (45.86 u/mL/min) that was commonly opted in previous works [8, 12]. Yang and Wang reported maximum protease production from *Streptomyces* sp. after 10 days in SSF [20].

Effect of substrate concentration on enzyme production is shown in Figure 3. It was observed that 3 g substrate in the fermentation medium yielded maximum enzyme production (237 u/mL/min or 1896 U/g solid substrate). A further increase in substrate did not increase the enzyme yield significantly because 2 mL inoculum was added to each flask and increase in substrate level only could not affect the growth of organism [21]. Due to maximum yield of protease with corn cob followed by lentil husk, both the substrates were mixed in 1:1 ratio in further experiments.

Effect of inoculum concentration on protease production was observed as shown in Figure 4. The highest protease productions (297.3 u/mL/min) were obtained for inoculum concentrations in the range of 2.0–2.5 mL. This result indicates that 2.0 mL inoculum is best amount to balance between substrate availability and initial cell concentration under SSF.

Figure 5 shows effect of incubation temperature on protease production by *B. cereus* in SSF using corn cob and lentil husk as substrates. It can be observed that the highest enzyme level was obtained at 30 °C in SSF which was also reported by *B. cereus* in submerged fermentation [4]. An increase in temperature to 60 °C resulted in significant decrease in protease production, probably due to heat denaturation of the enzyme.

Initial moisture content is a crucial factor affecting the formation of products through solid state fermentation. A moisture level of 60% was found to be optimum for alkaline protease production (Table 1). The results pointed to a marked improvement (580.80 u/mL/min or 4160 U/g dry SS) was achieved by optimizing moisture content. Different *Bacillus* sp. showed maximum protease production in initial moisture content of 40–70% [15, 22]. Lower moisture content causes a reduction in solubility of nutrients provided to organism by solid substrate (SS), a lower degree of swelling and higher water tension [23].

On the other side, reduction in enzyme production at high moisture content may be due to the reduction in substrate porosity, changes in the structure of substrate particles, reduction of gas volume and decreasing in bacterial growth [21]. *B. cereus* showed very high production of protease under optimized SSF, even more in comparison to high protease productive fungal strains like *Aspergillus* sp. [24].

CONCLUSIONS

Protease produced from the bacterial sp., *Bacillus cereus* proved to be very important enzyme for laundry detergent industry. The protease production was improved approximately double by solid state fermentation in comparison to submerged fermentation. Agro-industrial wastes as substrate for SSF is being used since long time, but use of lentil husk and corn cob as combined substrate is reported first in the present study.

B. cereus showed maximum yield of protease, about 580.80 u/mL/min or 4160 U/g dry SS, when 1.5 g corn cob and 1.5 g lentil husk were used. Other production parameters were optimized and the maximum yield was obtained at incubation temperature 30 °C, pH 8.5, 7 days, 2 mL inoculum (colony count 3×10^6 cfu/mL), and 60% initial moisture content.

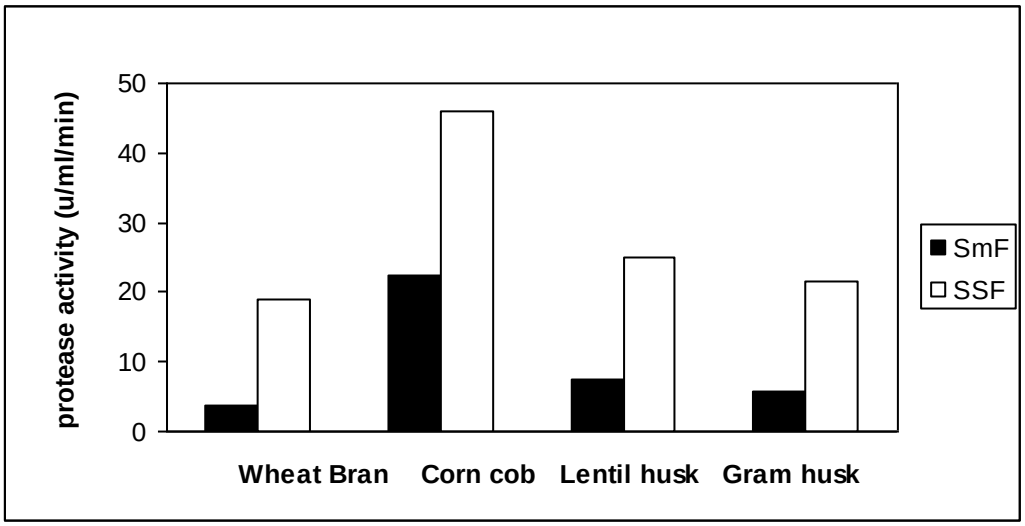

Fig. 1: *Comparison of Protease Production in SmF and SSF.*

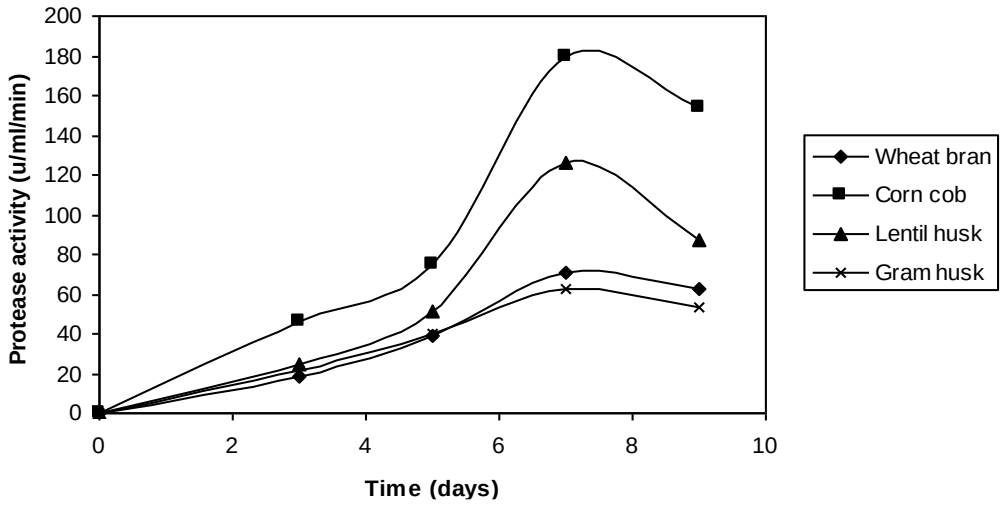

Fig. 2: *Effect of Fermentation Time on Protease Production in SSF by Using Different Substrates.*

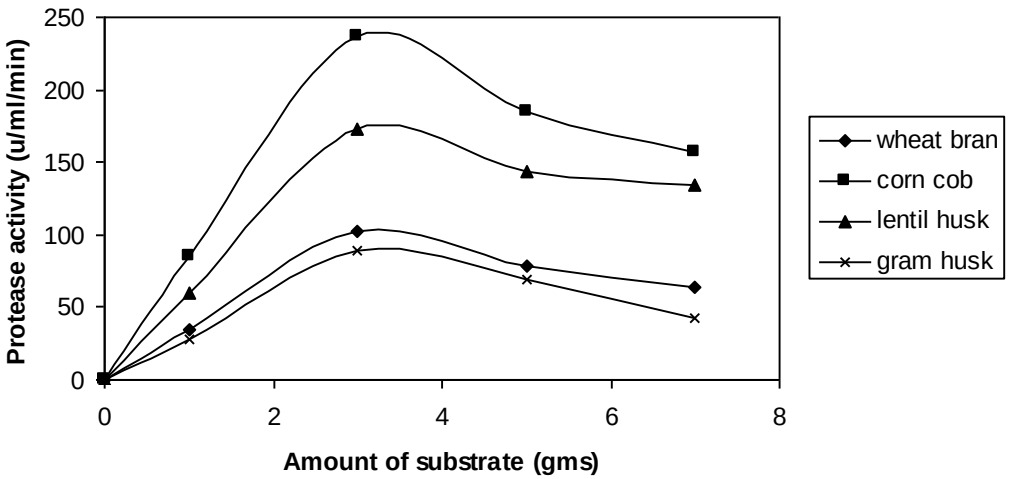

Fig. 3: *Effect of Substrate Concentration on Protease Production in SSF.*

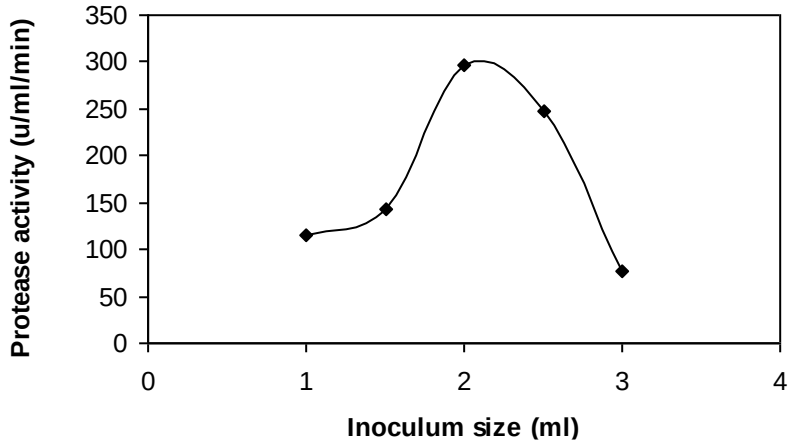

Fig. 4: *Effect of Inoculum Size on Protease Production in SSF (Inoculum Age: 12 h).*

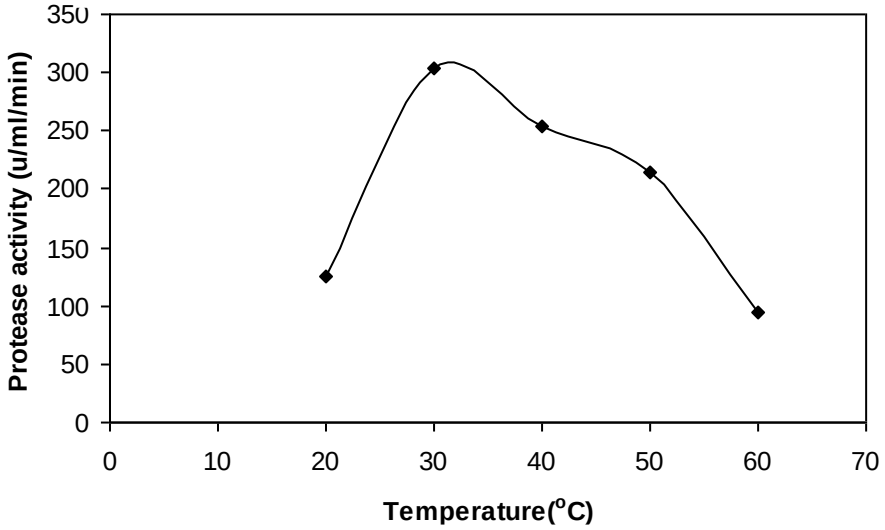

Fig. 5: *Effect of Temperature on Protease Production in SSF by Using Combined Substrate of Corn Cob and Lentil Husk.*

Table 1: *Effect of Moisture Content on Protease Production in SSF.*

Moisture content	**1.5 g Corn Cob + 1.5 g Lentil Husk**
40%	215.4
50%	322.8
60%	580.8
70%	305.52
80%	158.4
90%	155.19

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