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Research Article

Optimization and production of alkaline protease by solid state fermentation using *Bacillus subtilis* NS isolated from marine water sample

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Abstract

Proteases are enzymes occurring everywhere in nature be it inside or on the surface of living organisms such as plants, animals and microbes, Proteolytic enzymes (proteases) are ubiquitous being found in all living organisms and are essential for cell growth and differentiation. The extracellular proteases are of commercial value and find multiple applications in various sectors. In the present study, fifteen bacteria were isolated from the coastal region of Tamil Nadu and based on alkaline protease production, one isolate *Bacillus subtilis* was selected. To produce the protease enzyme in inexpensive manner, using *Bacillus subtilis* NS, various agro-industrial waste were tested and solid state fermentation was used for production parameters. Wheat bran was proved as the best substrate for protease production. The result revealed that the temperature 50°C, pH 9, carbon source glucose and nitrogen source yeast extract is optimum for protease production using wheat bran as agro industrial waste.

Keywords: Alkaline Protease, *Bacillus subtilis*, Solid state fermentation, Optimization and Marine water.

Introduction

Proteases, one among the three largest groups of industrial enzymes, accounts for about 60% of the total worldwide sale of enzymes from biological sources (Adinarayana *et al*., 2003). Although, proteases are found ubiquitously in plants, animals and microorganisms, the microorganisms are the preferred sources of proteases and dominated the commercial application.

About 30 to 40% of the production cost of industrial enzymes is estimated to account for the cost of the growth medium. Microorganisms utilize various substrates as a source for growth and its metabolic activities. Selection of growth medium is itself has become tough task for scientist as it directly impacts on the production cost. It is proven that by choosing appropriate media production cost can be slashed by 30% - 40% and anything which helps to reduce overall production cost is highly recommended in industrial perspective. Solid State Fermentation (SSF) processes are usually simpler and can use wastes or agro-industrial substrates, such as defatted soybean cake, gram bran, wheat bran, rice bran, banana waste, etc. for enzyme production (Germano *et al.*, 2003; Kashyap *et al.*, 2003). Cultures reported for the production of protease by solid state

fermentation are limited to the genus *Bacillus* (Tunga *et al.*, 2003). In the present study, *Bacillus subtilis* NS isolated from marine water sample was used to optimize the production parameter, for the alkaline protease production by solid state fermentation using different agro industrial waste.

Materials and Methods

Culture used

Fifteen bacteria were isolated from coastal region of Tamil Nadu and based on alkaline protease production one isolate *Bacillus subtilis* was selected. It was maintained on Nutrient agar slants at 4°C and sub-cultured every four weeks.

Optimization of culture conditions for alkaline protease production

Agro-industrial waste materials namely wheat bran, rice bran, green gram bran, black gram bran and coconut cake was selected as substrates for protease production by solid state fermentation in the production media (Glucose - 1.5 g, Urea - 2 g, KH_2PO_4 - 0.2 g, $MgSO_4$. $7H_2O$ - 0.1 g, $CaCl_2$ - 0.1 g, substrates - 7.5% (w/v) and Distilled water – 100 ml). For this, 10 g of substrate was taken in separate flasks and autoclaved at 121°C and 15 lb pressure for 20 min. After cooling, the flasks were incubation, the enzyme was extracted using 0.2 M phosphate buffer (pH 9) and extracted activity was assayed according to Mukhtar and lkram-Ul-Haq (2008). The best substrate which secreted high protease activity was selected for further process. Carbon sources namely glucose, sucrose, lactose, fructose and xylose were amended separately in flasks inoculated with equal quantity of inoculums. Organic and inorganic nitrogen sources namely beef extract, yeast extract, urea, ammonium sulphate, ammonium chloride, sodium nitrate and peptone were amended in the production media separately. The extraction procedure was followed and protease activity was assayed.

To observe the effect of initial pH on enzyme production, 10 g of production media of different pH (7, 8, 9, 10, 11 and 12) was taken in each flask. Similarly, the culture media was incubated to find out the effect of different temperatures (30, 40, 50, 60, 70 and 80°C) on protease production.

Results and Discussion

Different agro-industrial waste *viz*., green gram bran, black gram bran, wheat bran, rice bran and coconut cake there used for the production of protease by SSF. Among these substrates, wheat bran produced highest protease activity of (490.12 U/ml) followed by black gram (325.09 U/ml), green gram (112.88 U/ml) and rice bran (12.34 U/ml) (Table - 1). The lowest activity was recorded in coconut cake (8.64 U/ml). The medium with wheat bran contains the protein components and mineral nutrients required for the growth of the bacterium, thereby enhanced protease production as compared to other solid substrates (Joo and Chang, 2005). Based on protease enzyme wheat bran was selected and optimized for various parameters like carbon, nitrogen, pH and temperature.

Influence of pH and temperature on alkaline protease production of wheat bran

The enzyme production gradually increased with increase in the pH of the medium, and the maximum production (497.34 U/ml) was recorded at pH 9. The enzyme production was 230.54 U/ml, 356.65 U/ml, 344.87 U/ml, 221.78 U/ml and 121.67 U/ml of agro industrial wastes at pH 7, 8, 10, 11 and 12 respectively. The protease production was decreased at above and below optimal pH. Saurab and Praveen Kumar also reported maximum protease production by *Bacillus* sp. at pH 9.0. Optimum protease production (488. 13 U/ml) was observed at 50°C. The protease production was decreased at above and below optimal temperature. Temperature affects microbial cellular growth and microbial physiology, thus affecting product formation in turn (Table - 2).

Effect of carbon source and nitrogen on protease production wheat bran by SSF

Although, wheat bran supports the growth of *Bacillus subtilis* NS and protease production, it may not provide enough carbon sources needed by the

Table 1. Alkaline protease production by *Bacillus subtilis* NS by SSF using different Agro-industrial wastes

Substrates	Enzymatic activity (U/ml)
Green gram bran	112.88
Black gram bran	325.09
Wheat bran	490.12
Rice bran	12.34
Coconut cake	8.64

Table 2. Effect of pH and temperature on protease production by solid state fermentation

pH	Enzymatic activity (U/ml)	Temperature (°C)	Enzymatic activity (U/ml)
7	230.54	30°C	230.67
8	356.65	40°C	275.54
9	497.34	50°C	488.13
10	344.87	60°C	244.12
11	221.78	70°C	121.44
12	121.67	80°C	92.12

Table 3. Effect of carbon and nitrogen source on protease production of wheat bran

Carbon source	Enzymatic activity (U/ml)	Nitrogen source	Enzymatic activity (U/ml)
Lactose	120.67	Peptone	330.67
Fructose	321.54	Yeast extract	461.54
Glucose	491.13	Ammonium chloride	201.13
Sucrose	144.12	Ammonium sulphate	144.12
Xylose	221.44	Sodium nitrate	121.44

organism for maximum protease production. Hence, the exogenous addition of various carbon sources to the medium has enhanced the protease production. Among all carbon sources, maximum protease production was observed with glucose (491.13 U/ml) followed by fructose (321.54 U/ml) (Table - 3). Srinivas *et al*. (2010) also reported that *Bacillus subtilis* KHS-1 (MTCCNO - 10110) exhibited maximum protease production in glucose in medium. The protein content in wheat bran is very low and hence, the exogenous addition of various nitrogen levels to the solid medium was studied. As shown in Table - 3, among different nitrogen sources, maximum protease production were observed with yeast extract (461.54 U/ml), followed by peptone (330.67 U/ml). Optimized production medium and production conditions enhanced the alkaline protease production by *Bacillus subtilis* NS by 461.54 U/ml. Ataloand Gashe (1993) showed that yeast extract and peptone can induce the alkaline protease production in glucose medium.

Conclusion

The results of the present study revealed that the agro industrial waste wheat bran can be used as a cheaper source for the production of protease enzyme by solid state fermentation. Regarding the optimum parameters for the production of protease enzyme using *Bacillus subtilis* NS in wheat bran the pH 9.0, temperature 50°C, carbon source (glucose) and nitrogen source (yeast extract) proved the best.

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