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OPTIMIZATION OF ALKALINE PROTEASE PRODUCTION FROM *BACILLUS* SP.AGT UNDER SOLID STATE FERMENTATION

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ABSTRACT

Bacillus sp. AGT which produces an alkaline protease enzyme was isolated from the tannery effluent. It is important to produce the protease enzyme in inexpensive and use of cheaper raw material will slash the total production cost drastically. Production of alkaline protease by *Bacillus* sp.AGT using solid state fermentation was optimized. The effect of various substrates like green gram, rice bran and black gram were examined and found that green gram showed highest enzyme production. In addition to identification of suitable substrate, optimization of various process parameters such as inoculum concentration and initial moisture content were performed. Optimum inoculum size and initial moisture content were found to be 25% and 30% respectively.

INTRODUCTION

Proteases are among the commercially most viable enzymes and microbial alkaline proteases dominated the worldwide enzyme market, accounting for two-third share of the detergent industry (Niehaus *et al.*, 1999; Gupta and Roy, 2002). Microbial enzymes especially proteases draws more and more attention as it has a long history of application in the food and detergent industries. Their application in the leather industry for dehairing and bating of hides to substitute currently used toxic chemicals, is a relatively new development and has conferred added biotechnological importance (Rao *et al.*, 1998). *Bacillus* sp. among all other microbes were found to be predominant and a prolific source of alkaline proteases. A large proportion of the commercially available alkaline proteases are derived from *Bacillus* strains (Gupta *et al.*, 2002 and Joo *et al.*, 2002).

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 and Krishna, Chandrasekeran, 1996). Culture reported for the production of protease by solid state fermentation are limited to the genus *Bacillus* and some fungi (Tunga *et al.*, 1998, Tunga *et al.*, 1999 and Tunga *et al.*, 2003). SSF is generally simpler process and requires less pre-processing energy than submerged fermentation and other advantages are superior productivity, low waste water output and improved product recovery(Uyar and Baysal, 2004). In this study, attempts made to enhance the production of alkaline protease through cheaper substrate by means of solid state fermentation for *Bacillus* sp. AGT which were isolated from tannery effluent.

MATERIALS AND METHODS

Microorganisms

Alkaline protease producing *Bacillus* sp. AGT isolated from tannery effluent

Screening of bacteria

Bacillus sp. AGT was isolated from the tannery effluent and it was grown in 100 mL of production media with pH 8.0 containing glucose 5%, peptone 7.5%, calcium chloride 0.5%, magnesium sulphate 0.4%, Potassium di hydrogen phosphate 0.5%, and ferrous sulphate 0.01%. The culture medium was incubated at 37°C and 200 rpm on a shaker incubator for the period of 48hrs. After 48hrs, 15% (v/v) of this culture were inoculated into fresh medium and it was incubated at 37°C and 200 rpm on a shaker incubator for the period of 24hrs to serve as inoculum for solid state fermentation.

Alkaline protease production in SSF

Different substrates like green gram, rice bran and black gram were used as substrate for solid state fermentation.

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About 30g of each substrate in 100 ml of distilled water was sterilized in 250 ml flasks and inoculated with appropriate level of *Bacillus* sp. AGT. The inoculated flasks were incubated at 37°C and 200 rpm on a shaker incubator upto 6 days. The contents of the flasks were harvested and assayed every 24hrs.

Enzyme extraction

About 20ml of 0.1 M phosphate buffer was poured in each flask kept shaking for 10 min at 200 rpm and the resulting suspension was filtered through two layered cheese cloth, centrifuged at 10,000 rpm for 15min and finally filtered through Whatman No.1 filter paper (0.45 µm pour size). This solution was used as the source of enzyme for assays.

Alkaline protease activity

Enzyme assay performed as per Mac Donald & Chand method. The proteolytic activity of the enzyme was determined using 2% casein as a substrate. Crude culture filtrate of about 1.0 ml was boiled in a water bath at 100°C for 20 minutes. Samples were incubated at 37°C for one hour. After incubation, 1.0ml cold TCA (Trichloraceticacid 10%) was added and centrifuged at 8000 rpm for 10 minutes, the supernatant was collected and mixed with 2.5ml of reagent (sodium carbonate and sodium hydroxide) and then 0.7ml of Folin-Phenol reagent was added and incubated at room temperature for 20minutes. The samples were read at 660nm using Spectrophotometer.

Optimization of process parameter for SSF

Various process parameters which are influencing enzyme production were optimized inorder to find best optimal conditions to have more enzyme activity. Inoculum concentration(v/w) (10%, 15%, 20%, 25% and 35%) and initial moisture content (20%, 30%, 40%, 50% and 60%) were optimized for maximum enzyme production.

RESULTS

Alkaline protease activity

The contents of the flasks containing different substrate such as green gram, rice bran and black gram for SSF were harvested and assayed every 24hrs for enzyme activity. Among all the substrates selected green gram (420U/g)-Fig.1 was a better substrate for alkaline protease production than other substrates such as rice bran (300U/g)-Fig.2 and black gram(220U/g)-Fig.3.

Optimization of process parameter for SSF

Among various substrates since green gram had shown higher enzyme activity various process parameters which tend to influence enzyme production were optimized for green gram inorder to find best optimal conditions to exploit more enzyme activity. Inoculum concentration(v/w) of 10%, 15%, 20%, 25% and 35% for the period of 24hrs and initial moisture content of 20%, 30%, 40%, 50% and 60% with 25% inoculum concentration for the period of 24hrs were optimized for maximum enzyme production.

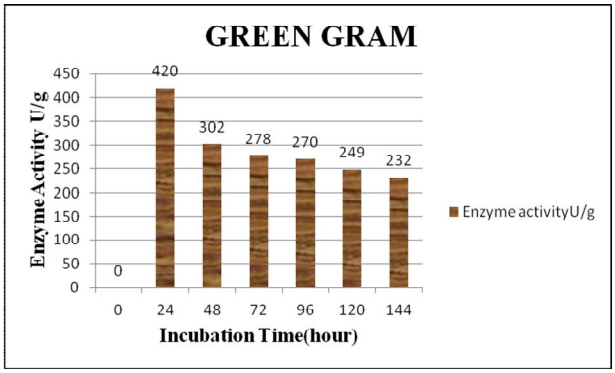

Fig. 1 Effect of incubation period on alkaline protease production in a SSF system for Green Gram.

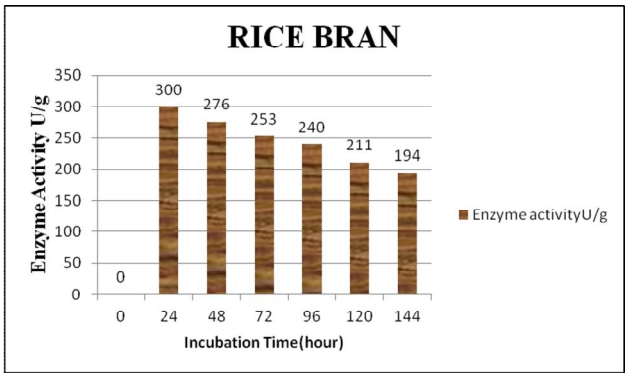

Fig. 2 Effect of incubation period on alkaline protease production in a SSF system for Rice Bran

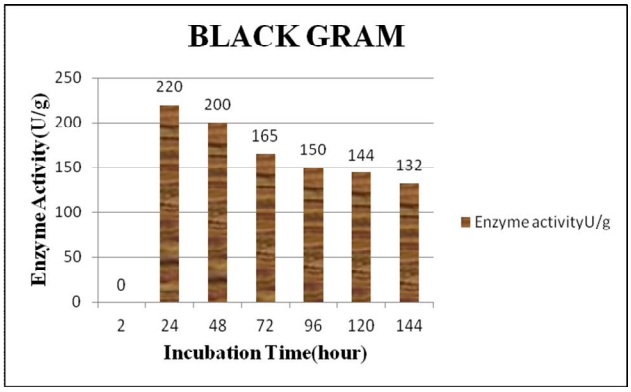

Fig.3 Effect of incubation period on alkaline protease production in a SSF system for Black Gram

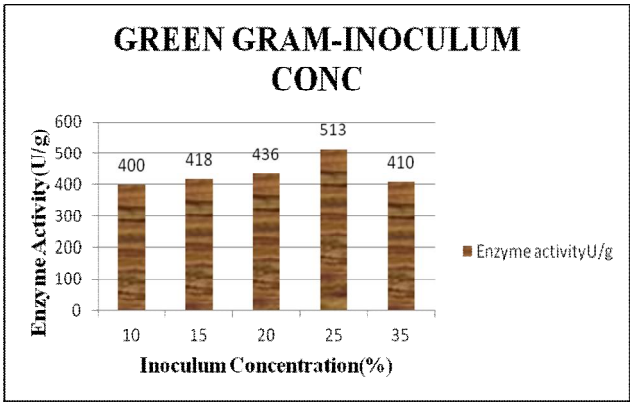

Fig.4 Effect of inoculum concentration(%) on alkaline protease production in a SSF system for Green Gram

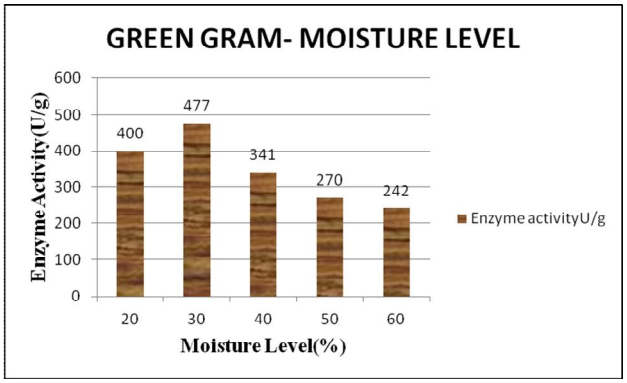

Fig.5 Effect of initial moisture level(%) on alkaline protease production in a SSF system for Green Gram.

25% inoculum concentration(513U/g)-Fig.4 and 30% initial moisture(477U/g) Fig.5 were found to be optimal parameters for maximum enzyme activity for green gram.

DISCUSSION

A number of solid substrates have been used for the production of bacterial enzymes and SSF processes are significantly influenced by the nature of solid substrates (Uyar and Baysal, 2004). Ability to enhance alkaline protease production with the use of green gram, rice bran and black gram as a substrate is well proved by the results shown (Refer Fig.1, Fig.2 and Fig.3). It is understood that green gram supported for higher enzyme production when compared to other substrates like rice bran and black gram. The incubation time is governed by characteristics of the culture and is based on growth rate and enzyme production(Uyar and Baysal, 2004). In some studies, the time employed was 48hrs or 8-9 days for bacteria of fungus(Aikat and Bhattacharyya, 2000, Puri *et al.,* 2002). Higher enzyme activity observed at 24hrs of incubation irrespective of the substrate and our reports aligned with the study of Uyar *et al.,* 2004. Since green gram had shown higher enzyme activity various process parameters like inoculum volume and initial moisture content which tend to influence enzyme production were optimized for green gram to find best optimal conditions to exploit more enzyme activity. The inoculum concentration which may play major role for the production of alkaline protease was also taken into account during optimization. Of various inoculum concentration(v/w) 25% was found to yield more enzyme production. However Sen 1995, reported a 10% inoculum level for the production of alkaline protease by *Bacillus licheniformis* S40. The moisture content of the fermentation medium is one of the main factors which determines the success of SSF process and optimum moisture level is different for strains of the same species of bacteria and moulds(Ramesh and Lonsane 1990). In this study the maximum enzyme yield obtained was at 30% initial moisture level which was comparable to the study reported by Uyar *et al.,* 2004. In conclusion, *Bacillus* sp.AGT isolated from tannery effluent was optimized for protease production with the solid substrate made of cheaper raw material green gram. With the inclusion of various process parameters we have demonstrated increased yield of alkaline protease enzyme and it appears that this cheaper solid substrate may be used for solid state fermentation in industrial enzyme production.

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