ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 3, Issue, 7, pp. 598 - 600, July, 2012

International Journal of Recent Scientific Research

RESEARCH ARTICLE

PURIFICATION AND CHARACTERIZATION OF A PROTEASE FROM *BACILLUS* SP.AGT UNDER SOLID STATE FERMENTATION

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ARTICLE INFO

Article History:

Received 11th June, 2012
Received in revised form 20th, June, 2012
Accepted 10th July, 2012
Published online 30th July, 2012

Key words:

Solid State Fermentation, *Bacillus* and Alkaline protease, SDS-PAGE, Ion Exchange Chromatography and Precipitation

ABSTRACT

Alkaline protease was produced by *Bacillus* SP.AGT by solid state fermentation. The protease was purified to the homogeneity. Purified protease was obtained from acetone precipitation and ion exchange chromatography. The molecular weight of the protease was 29 kDa as estimated by SDS-PAGE. The enzyme was stable and active with pH 9.0 and with temperature of 40° C. However the enzyme was quite unstable at temperature beyond 60° C and pH below 8.0 and above 10.0. Parameters such as pH and temperature with respect to enzyme activity demonstrated clearly the impact of these process parameters on the yield of enzymes as well as their independent nature in influencing the ability of bacteria to synthesize the enzyme

INTRODUCTION

Proteases one of the most important groups of industrial enzymes and account for at least a quarter of the total global enzyme production (Layman 1986). Most of these enzymes find applications in the food industry, in the meat tenderization process, peptide synthesis, for infant formula preparations, baking and brewing. (Genckar *et al.*, 2005). It is widely accepted that its use has increased significantly in various other industries such as detergent, feed additives, and dehairing. Although a number of microorganisms produced proteases, *Bacillus* strains are recognized as important sources of commercial alkaline proteases because of their ability to secrete large amounts of enzymes with high activity (Jacobs 1998; Kobayshi *et al.*, 1998, Yang *et al.*, 2000, Christiansen *et al.*, 2002, Joo *et al.*, 2002 Beg *et al.*, 2003, Joo *et al.*, 2004 and Joo *et al.*, 2003). It is well known fact that key contributor for protease enzyme production is *Bacillus* sp. It is well documented that media components greatly influence the extracellular production of proteases in microorganisms and their interaction plays an important role in the synthesis of enzyme(Puri *et al.*, 2002). Although many enzymes have their way into biotechnological application still there is drought as far as industrial demand is concerned and to overcome this industrial demand there is a need to emulate a focus towards optimization of protease production from microorganisms. Screening new organism for protease production and optimization of its production with different growth parameter has been done in our previous study (Ashok *et al.*, 2012). Though various growth parameters plays key role in enzyme production, purification and characterization of protease enzyme plays a key role towards commercialization of these enzymes. In this paper, we report the purification and characterization of *Bacillus* sp AGT.

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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%. All assays were performed by cell-free supernatant of the fermentation broth. The proteolytic activity were performed by using pour plate and spread plate technique in skin milk agar medium. Culture were regenerated every Month on a fresh plate from the frozen stock culture

Production of protease enzyme

The enzyme protease was produced by *Bacillus* sp. AGT by Solid State Fermentation (SSF). About 30g of green gram 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 for further analysis.

Enzyme extraction

About 20ml of 0.1 M phosphate buffer was poured in 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. This solution was used as the source of enzyme for assays.

Enzyme and protein activity

Enzyme assay performed as per Mac Donald & Chand method. Enzyme activity was determined using 2% casein as a substrate. Filtrate of about 1.0 ml was boiled in a water bath at 100°C for 20 minutes. After incubation at 37°C for one hour, 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 sodium carbonate and sodium hydroxide reagent. To this mixture 0.7ml of Folin-Phenol reagent was added and incubated at room temperature for 20minutes. The samples were read at 660nm using Spectrophotometer. Protein was measured by Brad ford method 1976 with bovine serum albumin (BSA) as standard.

Enzyme purification

Purification was carried out at 4°C. Cell free culture filtrate of *Bacillus* sp. AGT was collected and its protein was precipitated by using 90 % of acetone. The precipitated protein was resuspended in tris-HCl buffer and dialyzed against the same buffer for further analysis. Chromatography was performed on a column of DEAE cellulose powder. Column had been equilibrated with tris buffer. A known amount of protein (approx 20 mg) was loaded on the top of the column. Elution was started with tris buffer pH 9.0 followed by the same buffer with linear gradient of NaCl from 0.0 to 0.5M. The protein content of each fraction was read at 280 nm. Protease positive fractions were pooled together, dialyzed, concentrated for further analysis.

Molecular weight determination by SDS-page

SDS-PAGE was performed according to the method of Laemmeli 1970**.** The molecular weight was determined by interpolating with molecular standard weight marker

Effect of different ph and temperature on enzyme activity

Optimum pH of the partially purified protease was determined using Tris buffer at pH 7.0, 8.0, 9.0, 10.0, and 11.0. Partially purified protein was incubated for 1 hour in these different pH conditions and assayed for enzyme activity

Partially purified protease was mixed with casein solution prepared by using tris buffer and casein powder and incubated at different temperature 30°C, 40°C, 50°C, 60°C, 70°C, 80°C and 90°C for a period of 1 hour and assayed for enzyme activity

RESULTS AND DISCUSSION

Enzyme purification

By acetone precipitation method, crude protein got precipitated and this partially purified protein was resuspended in tris buffer. After diafiltration with same tris buffer protease was applied to a column of DEAE cellulose. Protease was eluted through linear gradient of NaCl with same tris buffer. Fig-1 shows the protein profile of purified protease by SDS-PAGE method. It is understood from the figure that purified protease from *Bacillus* sp. AGT migrated as a single band of 29 kDa in SDS-PAGE (Fig -1) suggesting that purified protein was homogeneous

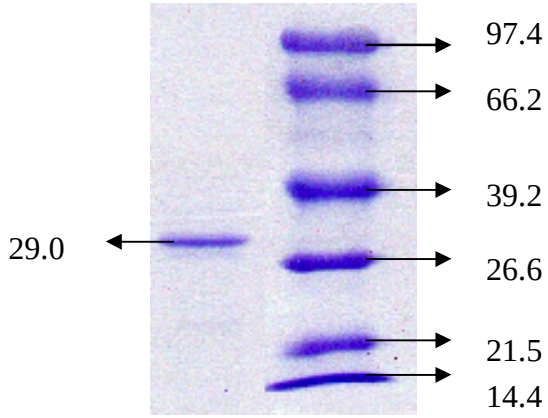

Fig.1 SDS page profile

Effect of different ph and temperature on enzyme activity

The optimum pH of the enzyme was determined to be 9.0 (Fig -2). Results clearly demonstrated that pH below 8.0 and above 10.0 inactivated the enzyme activity. Earlier reports are comparable to our report as greater protease production was reported by *Bacillus* sp. in the alkaline range of 7.0-12.0. The optimum temperature of the enzyme was determined to be 40°C (Fig-3) and as temperature increases, enzyme lost its activity. An optimum temperature of 45°C which is very close to our report has been reported (Sen *et al.*, 1993). Results obtained with these parameters namely pH and temperature with respect to enzyme activity demonstrated clearly the impact of this process parameters on the yield of enzymes as well as their independent nature in influencing the ability of bacteria to synthesize the enzyme. In this study, purification and characterization of protease enzyme from *Bacillus* sp. AGT has been demonstrated along with pH and thermal stability. Further study on its characterization shall show the way of feasibility to commercialize this protease enzyme.

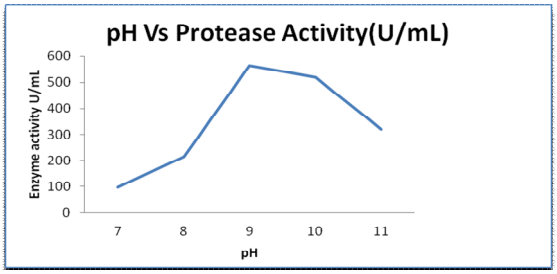

Fig. 2 pH Vs Protease Activity

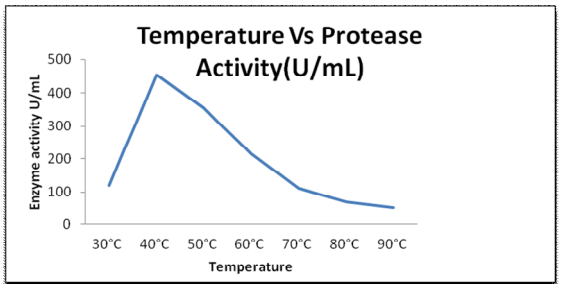

Fig. 3 Temperature Vs Protease Activity

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