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Green gram husk—an inexpensive substrate for alkaline protease production by *Bacillus* sp. in solid-state fermentation ☆

R.S. Prakasham *, Ch. Subba Rao, P.N. Sarma

Biochemical and Environmental Engineering Center, Indian Institute of Chemical Technology, Hyderabad 500 007, India

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Abstract

Alkaline protease production under solid-state fermentation was investigated using isolated alkalophilic *Bacillus* sp. Among all agro-industrial waste material evaluated, green gram husk supported maximum protease production. Solid material particle size regulated the enzyme production and yield was improved with the supplementation of carbon and nitrogen sources to the solid medium. Optimum enzyme production was achieved with 1.5% maltose and 2.0% yeast extract with 371% increase than control. Glucose did not repressed enzyme production but inorganic nitrogen sources showed little negative impact. The physiological fermentation factors such as pH of the medium (pH 9.0), moisture content (140%), incubation time (60 h) and inoculum level played a vital role in alkaline protease production. The enzyme production was found to be associated with the growth of the bacterial culture.

Keywords: Alkaline protease; *Bacillus* sp.; Enzyme; Green gram husk; Solid-state fermentation

1. Introduction

Proteases constitute one of the commercially important groups of extra-cellular microbial enzymes and are widely used in several industrial sectors, particularly in the detergent, food, pharmaceutical, chemical, leather and silk, apart from waste treatment (Scheuer, 1990). These enzymes also have potential to contribute in the development of high value added products due to their characteristic nature of aided digestion (Kumar and Takagi, 1999). Among all proteases, alkaline proteases are robust in nature and are primarily used as detergent additives (Gupta et al., 2002). They account for 40% of the total worldwide enzyme sales and this trend is expected to increase in near future (Ellaiah et al., 2002). This has created increasing attention in exploitation of exotic microbial strains for production of alkaline proteases.

Several microbial strains including fungi (*Aspergillus flavus*, *Aspergillus melleu*, *Aspergillus niger*, *Chrysosporium keratinophilum*, *Fusarium graminarum*, *Penicillium griseofulvin*, *Scedosporium apiosermum*) and bacterial (*Bacillus licheniformis*, *Bacillus firmus*, *Bacillus alcalophilus*, *Bacillus amyloliquefaciens*, *Bacillus proteolyticus*, *Bacillus subtilis*, *Bacillus thuringiensis*) (Ellaiah et al., 2002) are reported to produce proteases. Among these, *Bacillus* genus has gained importance at industrial scale. Proteases are generally produced using submerged fermentation due to its apparent advantages in consistent enzyme production characteristics with defined medium and process conditions and advantages in downstream in spite of the cost-intensiveness for medium components. In this context, solid-state fermentation has gained renewed interest and fresh attention from researchers owing to its importance in recent developments in biomass energy conservation, in solid waste treatment and in its application to produce secondary

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* Corresponding author. Tel.: +91 40 27193159; fax: +91 40 27193626.
E-mail address: prakasam@iict.res.in (R.S. Prakasham).

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metabolites. Production of these biocatalysts using agro-biotech substrates under solid-state fermentation conditions provide several advantages in productivity, cost-effectiveness in labour, time and medium components in addition to environmental advantages like less effluent production, waste minimization, etc. (Pandey et al., 2000). There are several reports describing use of agro-industrial residues for the production of alkaline protease, e.g. nug meal and *Bacillus* sp. AR009 (Gessesse, 1997), pigeon pea and *Bacillus* sp. JB-99 (Johnvesly et al., 2002), wheat bran and *Rhizopus oryzae* (Aikat and Bhattacharyya, 2000). However, these production characteristics would have to offer a competitive advantage over existing products. In general, each microbial strain is unique in their molecular, biochemical, metabolic and enzyme production properties. This warrants thorough characterization of isolated individual microbial species to evaluate its potential at commercial level.

The present investigation aimed to exploit the locally available, inexpensive agro-substrate, green gram husk, for alkaline protease production using *Bacillus* sp. under solid-state fermentation.

2. Methods

2.1. Microorganism and culture conditions

Alkaline protease producing alkalophilic *Bacillus* sp. was used in this investigation and was maintained on a medium containing (%) yeast extract 0.75, peptone 0.75, glucose 1.0, agar 2.0%, pH 9.0, stored at 4 °C and sub-cultured at monthly intervals.

2.2. Substrates

Different agro-industrial waste materials (green gram, chick pea, red gram, black gram husks and wheat bran) were collected from the local market and processed using USA standard sieve set of Nos. 7, 10, 14, 18 and 50 to obtain mean particle size of 2.8–2.0; 2.0–1.4; 1.4–1.0 and 1.0–0.3 mm and stored till further use.

2.3. Solid-state fermentation

Ten grams of substrate was taken in 250 ml Erlenmeyer flasks and to this a predetermined quantity of water was added, mixed thoroughly and autoclaved at 121 °C for 15 min at 1 kgf/cm^2. After cooling the flasks to room temperature, the flasks were inoculated with 2 ml of 24-h grown (0.8 OD at 600 nm) culture broth under sterile conditions. The contents of the flasks were well mixed and incubated at 33 ± 1 °C for predetermined time period. For investigation of inoculum level, the inoculum concentration was increased accordingly. Whenever required, the different carbon and nitrogen sources were added individually at 1% level to the solid medium before autoclaving unless otherwise stated. In case of incubation time, one-flask contents at every 12 h were used for extraction and estimation of protease activity. For studying the particle size effect, different particle size substrate used while for moisture content, the solid material was provided with calculated amount of water with respect to solid material (for example: for 100% moisturization, 5 ml of water was added to 5 g of solid material and vice versa) and mixed well before autoclaving. pH adjustment of solid medium was achieved by adjusting the pH of moisturizing medium before addition to the solid material. Moisture content of the solid medium was maintained by increasing the quantity of moisturizing medium and through mixing. Results reported in this study are averages of triplicate findings.

2.4. Estimation of bacterial growth

The bacterial growth was estimated by measuring the bacterial DNA content according to Reddy et al. (2000). Fermented matter was powdered finely using liquid nitrogen and used for extraction of bacterial DNA. The resultant DNA content was estimated by measured the absorbance at 260 and 280 nm using Perkin–Elmer (Lambda-25) UV–Visible Spectrometer.

2.5. Enzyme extraction

The enzyme was extracted according to the method described by Nagamine et al. (2003). Fermented medium was mixed thoroughly with 50 mM glycine–NaOH buffer, pH 11 for 30 min and the extract was separated by squeezing through a cloth. This process was repeated three times and extracts were pooled together and then centrifuged. The supernatant was used as enzyme source for protease assay.

2.6. Measurement of protease activity

Protease activity was determined using modified Auson–Hagihara method (Hagihara et al., 1958). In this 1 ml of the enzyme solution was added to 1 ml casein solution (1%, w/v casein solution prepared in 50 mM glycine–NaOH buffer, pH 11) and incubated at 70 °C for 20 min. The reaction was terminated by adding 4 ml of 10% trichloroacetic acid and the contents were filtered through a Whatman No. 1 filter paper. The filtrate absorbance was read at 280 nm using UV–Visible spectrophotometer and the protease activity was calculated using tyrosine standard curve. One unit of alkaline protease activity was defined as 1 μg of tyrosine liberated ml^{-1} under the assay conditions.