Production and Characterization of a Novel Protease from *Bacillus* sp. RRM1 Under Solid State Fermentation

Renganathan, Rajkumar*, Jayappriyan Kothilmozhian Ranishree, and Rengasamy Ramasamy

Centre for Advanced Studies in Botany, University of Madras Guindy Campus, Chennai- 600 025, India

Received: January 5, 2011 / Revised: March 19, 2011 / Accepted: March 21, 2011

A commercially important alkaline protease, produced by *Bacillus* sp. RRM1 isolated from the red seaweed *Kappaphycus alvarezii* (Doty) Doty ex Silva, was first recognized and characterized in the present study. Identification of the isolated bacterium was done using both biochemical characterization as well as 16S rRNA gene sequencing. The bacterial strain, *Bacillus* sp. RRM1, produced a high level of protease using easily available, inexpensive agricultural residues solid-state fermentation (SSF). Among them, wheat bran was found to be the best substrate. Influences of process parameters such as moisture content, moisture level, temperature, inoculum concentration, and co-carbon and co-nitrogen sources on the fermentation were also evaluated. Under optimized conditions, maximum protease production (i.e., 2081 U/g) was obtained from wheat bran, which is about 2-fold greater than the initial conditions. The protease enzyme was stable over a temperature range of 30–60°C and pH 6–12, with maximum activity at 50°C and pH 9.0. Whereas the metal ions Na⁺, Ca²⁺, and K⁺ enhanced the activity of the enzyme, others such as Mg²⁺, Cu²⁺, Fe³⁺, Co³⁺, and Zn²⁺ had rendered negative effects. The activity of the enzyme was inhibited by EDTA and enhanced by Ca²⁺ ions, thus indicating the nature of the enzyme as a metalloprotease. The enzyme showed extreme stability and activity even in the presence of detergents, surfactants, and organic solvents. Moreover, the present findings opened new vistas in the utilization of wheat bran, a cheap, abundantly available, and effective waste as a substrate for SSF.

Keywords: Protease, 16S rRNA gene sequence, *Bacillus* sp. RRM1, agro-residues, SSF

Proteases constitute one of the commercially important groups of extracellular microbial enzymes widely used in several industrial sectors such as detergent, food, pharmaceutical, chemical, leather, and silk, apart from waste treatment. These enzymes, owing to their characteristic nature of aided digestion, have the potential to contribute to the development of value-added products [24]. This has created an increasing attention towards the exploitation of exotic microbial strains for the production of alkaline proteases from novel sources.

In recent years, solid-state fermentation (SSF) has shown much promise in the development of bioprocesses and products. SSF has been known for centuries and used effectively for the production of oriental foods. More recently, it has gained importance in the production of microbial enzymes owing to several economic advantages over conventional submerged fermentation. Several reports on SSF have been documented with reference to the production of new chemicals [38], enzymes [1], antibiotics [23], and immunosuppressants [27]. In SSF, the solid substrate not only supplies the nutrient to the culture but also serves as an anchorage for the microbial cells, where cost and availability are important considerations, and therefore, the selection of an appropriate solid substrate plays an important role in the development of efficient SSF processes. SSF is generally a simpler process and requires less preprocessing than submerged fermentation.

The metabolic processes of the microorganisms are influenced to a great extent by the change of pH, temperature, substrate, water content, inoculum concentration, etc. These conditions vary widely from species to species. Therefore, it becomes imperative to know the environmental conditions of the microorganism for the maximum production. Nevertheless, research about SSF has been neglected not only because of the popularity of the submerged culture process but also because of the difficulties associated with the measurement of parameters in SSF, such as microbial biomass, substrate consumption, and concentration of products formed as well as the measurement of the physical properties of the system [17].

Although there are many microbial sources available for producing proteases, only a few are recognized as commercial...
producers. In fact, major industrial units are continuously trying to identify enzymes that have potential industrial applications, either to use them directly or to create popular enzymes with enhanced catalytic activity for well-adapted large-scale industrial processes [14]. However, the new enzymes would have to offer a competitive advantage over the existing products. One such example is the detergent protease produced by Tetradinobacter turnirae, a shipworm bacterium involving a symbiotic relationship with a marine shipworm, Psiloteredo healdi [16]. It has been reported that T. turnirae produces protease of which greater than 80% are extracellular [15].

In this context, solid-state fermentation has gained renewed interest and fresh attention from researchers owing to its importance in biomass energy conservation and solid waste treatment, and its application to produce secondary metabolites. Production of these biocatalysts using agro-residue substrates under SSF conditions provides several advantages in productivity, and cost-effectiveness in labor, time, and medium components, in addition to environmental advantages like less effluent production, waste minimization, etc. [30]. There are several reports describing the use of agro-residues for the production of alkaline protease, for example, mug meal by Bacillus sp. AR 009 [13], pigeon pea by Bacillus sp. JB-99 [19], and wheat bran by Rhizopus oryzae [2]. However, these production characteristics would have to offer a competitive advantage over the existing products. In general, each microbial strain is unique in molecular, biochemical, metabolic, and enzyme production properties. This warrants thorough characterization of isolated individual microbial species to evaluate its potential at the commercial level. The demand for highly active preparations of proteolytic enzymes with appropriate specificity and stability over a wide range of pH and temperature, and retention of activity in the presence of ions and organic solvents continues to stimulate the search for new enzyme sources.

The present investigation is aimed to exploit the locally available, inexpensive agro-residues for protease production using Bacillus sp. RRM1 under solid-state fermentation.

**Materials and Methods**

**Microorganism and Taxonomic Study**

The strain Bacillus sp. RRM1 was isolated from the live specimens of red seaweed, Kappaphycus alvarezi (Doty) Doty ex Silva, collected in the month of September 2008 along the Mandapam coast, Tamil Nadu, India. The isolate was identified according to the methods described in Bergey's Manual of Systematic Bacteriology [36] and confirmed based on its 16S rRNA sequence [26]. The sequence was initially analyzed on the NCBI server (http://www.ncbi.nlm.nih.gov/) using the BLAST (blastn) tool, and corresponding sequences were retrieved. A similarity matrix was prepared using Dnadist program in PHYLIP analysis package [18] using Jukes-Cantor corrections. The phylogenetic tree was constructed by the neighbor-joining method using the MEGA software package. The bacterium was deposited in the Culture Collection maintained at the Centre for Advanced Studies in Botany, University of Madras, Chennai, India. The stock culture of the isolate was maintained in glycerol stocks [50% (v/v)] and stored at -20°C in a deep freezer. The culture was revived from the stock in nutrient broth followed by streaking on nutrient agar plates. The isolate, Bacillus sp. RRM1, produced a significant amount of alkaline protease in the culture medium.

**Preparation of Substrates**

Different agro-residues such as wheat bran, rice bran, black gram hull, pigeon pea, and green gram hull were obtained from a local market, whereas orange peels were collected from the University of Madras canteen. These waste materials were washed first with tap water followed by distilled water to remove the adhered surface dust particles. Then blending operation was carried out by immersing them in hot water (75–80°C) for 20 min followed by oven drying at 45°C. The dried material was ground in a mixer grinder and then sterilized at 121°C, 15 lb pressure for 15 min and stored at 4°C before further use.

**Solid-State Fermentation and Optimization of Process Parameters**

To screen the various substrates/supports for protease production, initially 10.0 g of coarse substrate was taken in a 250 ml Erlenmeyer flask and to this a predetermined quantity of distilled water was added, mixed thoroughly and autoclaved at 121°C, 15 lbs pressure for 15 min. After cooling, the flasks were inoculated with 10% inoculum (v/w) of 24 h grown culture (0.8 OD at 600 nm) broth and incubated at 37°C for various time periods (12, 24, 36, 48, 60, 72, and 84 h).

To investigate the influence of moistening agents (MA), the following were prepared and used. MA I (g/l): MgSO₄·7H₂O (5.0), KH₂PO₄ (5.0), FeSO₄·7H₂O (0.1), pH 9.0; MA II (g/l): KH₂PO₄ (1.0), MgSO₄·7H₂O (0.2), Na₂CO₃ (10.0), pH 9.0; MA III (g/l): MgSO₄·7H₂O (0.5), K₂HPO₄ (2.0), KCl (0.5), NH₄NO₃ (10.0), pH 9.0; MA IV (g/l): MgSO₄·7H₂O (0.5); K₂HPO₄ (1.5), pH 9.0. All the MAs were prepared in distilled water. Besides these salt solutions, seawater (MA V), tap water (MA VI), and distilled water (MA VII) were employed as the moistening agents. The above MA were prepared separately. To an Erlenmeyer flask, 10 g of wheat bran and a predetermined quantity of the above moistening agents were added and mixed thoroughly. The influence of other culture parameters on protease production, effects of initial moisture content of the substrate (20%, 30%, 40%, 50%, 60%, 70%, and 80%), temperatures (20, 30, 37, 45, 50, 55, and 60°C), inoculum size [5, 10, 15, 20, and 25% (v/w)], co-carbon sources [glucose, fructose, sucrose, galactose, xylose, maltose, mannose, and lactose at 1.0% (w/v) and co-nitrogen sources [NH₄Cl, (NH₄)₂SO₄, NH₄NO₃, NaN₃, urea, yeast extract, beef extract, and peptone at 1.0% (w/v)] were studied. For initial moisture content, solid substrate was mixed with a predetermined amount of water and 100% moisturization was achieved by adding 5.0 ml of water to 5.0 g of substrate and vice versa [31]. Maximum production of protease at various concentrations (0.5%, 1.0%, 1.5%, and 2%) of maltose as carbon source and yeast extract as nitrogen source was also investigated. Un-inoculated flasks served as negative controls, whereas the inoculated flasks without any co-carbon or co-nitrogen source served as positive controls. Alkaline protease production was expressed as the mean and standard deviations based on the results obtained from triplicate flasks.