Appendix
List of author publications

1. Optimization of thermostable alkaline protease production from species of *Bacillus* using rice bran. Krishna Suresh Babu Naidu, Kodidhela Lakshmidevi Devi, *African Journal of Biotechnology* Vol. 4 (7), pp. 724-726, July 2005 (ISSN 1684-5315 © 2005 Academic Journals).
Short Communication

Optimization of thermostable alkaline protease production from species of Bacillus using rice bran

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A protease producing microorganism was isolated from soil collected from a detergent industry and identified as Bacillus species. Isolate K-30 produced thermostable alkaline protease utilizing rice bran. The optimum conditions for protease activity was 55°C at pH 9 with 4% inoculum in the medium containing 1% rice bran after 96 h of incubation. Beef extract, tryptone and yeast extract were good nitrogen sources while lactose, starch, and sucrose were suitable for enzyme production. The extracellular production of the enzyme, its thermostable nature and compatibility with most commercial detergents are features which suggest its application in the detergent industry.

Keywords: Alkaline protease, rice bran, thermostability, detergent.

INTRODUCTION

Proteases are essential constituents of all forms of life on earth including prokaryotes, fungi, plants and animals. Proteases are highly exploited enzymes in food, leather, detergent, pharmaceutical, diagnostics, waste management, and silver recovery. The protease enzyme constitutes two thirds of total enzymes used in various industries and this dominance in the industrial market is expected to increase by the year 2005 (Gupta et al., 2002). Of all proteases, alkaline proteases produced by Bacillus species are of great importance in detergent industry due to their high thermostability and pH stability. For production of enzyme for industrial use, isolation and characterization of new promising strains using cheap carbon and nitrogen source is a continuous process (Parekh et al., 2002). Rice bran, a by product of the milling of rice is a good source of proteins at present underutilized as a food material. The potential of producing rice bran at the global level is 27.3 million ton. The present study was undertaken to examine the effectiveness of rice bran as potential alternative protein source in production medium and optimization of enzyme production conditions using rice bran.

MATERIALS AND METHODS

Screening of microorganism

For isolation of protease producing organism, soil samples were collected from the vicinity of Nellore detergent industry in Andhra Pradesh. Briefly, 1 g of the sample was suspended in 100 ml sterile distilled water, agitated for 45 min on a shaker at 30°C and 0.2 ml was spread on casein agar plates (nutrient agar with 1% casein) and incubated at 30°C for 7 days. Enriched sample was plated over nutrient agar containing 0.4% gelatin (Harrigan et al., 1966). After incubation for 24 h, plates were flooded with 1% tannic acid. Colonies showing clear zone were picked and purified. A total of 75 isolates were screened for protease production by using casein digestion method. Two isolates K-21 and K-30 which showed maximum activity were selected and maintained on nutrient agar at 4°C. The cultures were examined for various morphological and biochemical characteristics as per Bergey's Manual of determinative Bacteriology (Holt et al., 1994).

Protease assay

The proteolytic activity was assayed by casein digestion method (Manachini et al., 1989) at 55°C, pH 8.0 in 50 mM Tris-HCl buffer. One unit of protease activity is defined as the amount of which liberates 1 μg of tyrosine min⁻¹ under experimental conditions.
Figure 1. Production of protease by *Bacillus* sp. K-30 at different pH using rice bran.

Figure 2. Production of protease by *Bacillus* sp. K-30 at different temperature using rice bran.

Figure 3. Protease Production pattern of *Bacillus* sp. K-30 at optimum pH and temperature.

**RESULTS AND DISCUSSION**

Using morphological and biochemical characteristics (Holt et al., 1994) these isolates were identified as *Bacillus*. The optimum pH and temperature for production of protease by K-30 using rice bran was 9 and 50°C, respectively (Figures 1 and 2). The pH of the culture strongly affects many enzymatic processes and transport of compounds across the cell membrane. Majority of the thermophilic bacilli are found to grow at pH and temperature range of 5.8-8.0 and 50-65°C, respectively (Zeikus, 1979). Alkaline protease production is found to be maximum at pH 9-13 (Borris, 1987). The mechanism of temperature control in enzyme production is not well understood (Chaloupka, 1985). A link exists between enzyme synthesis and energy metabolism in *Bacillus*, which is controlled by temperature and oxygen uptake (Frankena et al., 1986).

By growing these cultures at optimum pH and temperature, the activity of protease was estimated at different intervals of growth. The maximum protease production was recorded after 96 h of incubation at 50°C (Figure 3). *Bacillus* usually produces extra cellular protease during late exponential phase (Ward, 1985).

The use of cheap sources of carbon and nitrogen like wheat bran, rice bran, casein, soy meals are important as these can significantly reduce the cost of production of protease. Therefore, the effects of various carbohydrates...
Table 1. Effect of different carbon and nitrogen sources on protease production by Bacillus sp. K 30.

| Carbon source (1%, w/v) | Protease activity (U ml⁻¹) | Nitrogen source (1%, w/v) | Protease activity (U ml⁻¹) |
|-------------------------|-----------------------------|---------------------------|-----------------------------|
| Wheat bran              | 81.1                        | Beef extract              | 75                          |
| Rice bran               | 42.1                        | Yeast extract             | 65                          |
| Casein                  | 40                          | Tryptone                  | 62                          |
| Maltose                 | 35                          | Potassium nitrate         | 28                          |
| Skin milk               | 35.3                        | Glycine                   | 23                          |
| Lactose                 | 32                          | Peptone                   | 65                          |
| Starch                  | 62                          | Gelatin                   | 55                          |
| Sucrose                 | 60                          | Soybean meal              | 45                          |

and organic nitrogen sources were evaluated at optimum pH, temperature and incubation time with respect to enzyme yield. Wheat bran supported the maximum production of protease enzyme however rice bran also had comparable results along with casein and soy meal. The best nitrogen source for protease production was beef extract, while yeast extract and tryptone were comparable (Table 1). Addition of inorganic nitrogen sources in the production medium resulted in low enzyme yield as also reported by Fujiwara et al. (1987). Among the ten carbon sources studied, starch, sucrose, and lactose proved appreciably good for the protease production. Lactose, starch, soy meal and sucrose are considered good for industrial protease production (Sonnleitner, 1983).

The appreciable high enzyme activity and stability at high temperatures and pH, suggest that K-30 can be a potential producer of alkaline protease by using cheap substances like wheat bran, rice bran, and soy meal. Bacillus species have been successfully used in degradation of pertinacious waste into useful biomass by many investigators (Atalo et al., 1993; Yeng et al., 2000). In this study, we further demonstrate that Bacillus sp. are useful in deproteinisation of rice bran and can be used as production medium for thermostable alkaline protease which can find application in detergent industry.

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