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Production and Optimization of Protease from *Bacillus licheniformis* NRRL-NRS-1264 using cheap source substrates by submerged (SmF) and solid-state fermentation (SSF)

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Abstract: Alkaline protease production was studied in *Bacillus licheniformis* NRRL-NRS-1264 by submerged and solid state fermentation using cheap source substrates such as wheat bran and rice bran. The maximum enzyme activity was obtained for solid-state fermentation containing wheat bran (2.5%) media. Enzyme optimization studies and metal ion effects were also carried out.
Keywords: Protease, *Bacillus licheniformis* NRRL-NRS-1264, Submerged and Solid-state fermentation.

Introduction:

Proteases are enzymes that break the proteins to smaller peptides or amino acids. Proteases are of various classifications namely serine, cysteine, metallo,aspartate, threonine [1]. Proteases are generally found in plants, animals and micro-organisms[2]. For industrial production of the proteases micro-organisms are preferred so that mass production of enzymes can be achieved without the limitation of raw materials.Some of the organisms that produce protease are Bacillus [3], Aspergillus [4], Pseudomonas[5], etc [6].The production of microbial protease from bacteria depends on the type of strain, composition of medium, method of cultivation, cell growth, nutrient requirements,metal ions, pH, temperature, time of incubation and thermo-stability[7]. Almost many of the large-scale enzyme producing facilities use the proven technology of submerged fermentation due to better monitoring and ease of handling whereas solid-state fermentation is receiving much attention in recent years.

Proteases also have wide applications in laundry, detergents, leather, food, pharmaceuticals, baking, brewing, cosmetics, medical diagnosis and meat tenderization [9,10,11]. Proteases are also useful and important components in biopharmaceutical products such as contact-lens enzyme cleaners and enzymic-debriders. They reported the use of an alkaline protease to decompose the gelatinous coating of X-ray films, from which silver was recovered[12]. For an enzyme to be used as an detergent additive it should be stable and active in the presence of typical detergent ingredients, such as surfactants, builders, bleaching agents, bleach activators,

fillers, fabric softeners and various other formulation aids. In textile industry, proteases may also be used to remove the stiff and dull gum layer of sericin from the raw silk fibre to achieve improved lustre and softness [13]. Proteases are also used for meat tenderization, recovery of protein from bones and fish wastes [12].

In this study, production and optimization of protease from *Bacillus licheniformis* NRRL-NRS-1264was carried out using submerged and solid-state fermentations.The pH and temperature optimization studies were carried out and the effect of various metals on the enzyme activity was also studied.

Materials and Methods:

Micro-organisms:

Bacillus licheniformis NRRL-NRS-1264 was obtained from the Agricultural Research Service (ARS), USA .The cultures weregrown and sub-cultured in nutrient agar media at 37°C for 24 h and subsequently stored at 4°C.

Chemicals and reagents:

Beef extract type-1 (HIMEDIA), protease peptone A (HIMEDIA), Trichloroacetic acid (MERCK), casein (HIMEDIA), Tyrosine (HIMEDIA) and other chemicals of analytical grade were used.

Submerged Fermentation (SmF):

Stock cultures of *Bacillus licheniformis* NRRL-NRS-1264 were stored on nutrient agar slants at 4°C. A loopful of inoculum from the nutrient agar slant was used for further level production studies. Production level was carried out in 1L flask containing 300 mL production media with 10% inoculum, incubated at 150 rpm, 37 °C for 24 h.

Enzyme Extraction for SmF:

After incubation for 24h, the culture was filtered with Whatman No. 1 filter paper and the filtrate was further centrifuged at 3000 rpm for 20 min [14]. The insoluble materials and cell debris were separated from protease enzyme (crude enzyme). If required the crude enzyme was again filtered using cellulose nitrate filter paper (pore size 0.45 µm). The supernatant which contains the crude protease enzyme was stored at 4°C and was used for further assay studies.

Solid State Fermentation (SSF):

2.5% cheap source components (Wheat bran, rice bran), sodium di-hydrogen phosphate (0.5%), sodium chloride (1%), dextrose (5%), peptone (1%), ammonium sulphate (1%) and soyabean meal (5%) were mixed with proportionate amount of distilled water in a 500mL Erlenmeyer flask. The flasks were sterilized, inoculated uniformly with 10% inoculum and incubated at 37°C under static condition for 24 h.

Enzyme Extraction (SSF):

The solid state enzyme extract was extracted by adding 25 mL distilled water and mixed well.Then the solid materials and bacterial biomass were separated by centrifugation at 3000 rpm for 20 min. The clarified supernatant used for enzyme characterisation studies.

Protease assay:

The protease activity was determined by the method proposed by Yang *et al*. 0.5 mL of curde enzyme was added to 0.5 mL of 1% casein and 1 mL of glycine-NaOH buffer (25 Mm, PH 7.5), the mixture was incubated at 40°C for 30 min. The reaction was terminated by the addition of 2 mL 10% TCA solution. The solution was then incubated at 37°C for 30 min, in dark andsubsequently centrifuged. Theabsorbance for the supernatant was measured at 660 nm [15].

One protease unit was defined as the amount of enzyme that released 1 µg of tyrosine per ml per minute under the above assay conditions.

Effect of metal ions:

The effect of metal ions on enzyme activity was determined by addition of 10mM of different metal ions such as calcium, magnesium, copper, sodium and Ethylene diamine tetraacetic acid (EDTA) to 0.5 mL of crude enzyme.

Media Optimization:

Two different media containing one of the following carbon sources: wheat bran and rice bran were selected. The media components of each were: media (1) : wheat bran 2.5%, Di-sodium hydrogen phosphate – 0.5% , Sodium chloride – 0.1%, Ammonium sulphate – 0.1%, dextrose – 0.5%, soya bean meal – 0.5%, (2)Rice bran 2.5%,rice 2.5%, Di-sodium hydrogen phosphate – 0.5% , Sodium chloride – 0.1%, Ammonium sulphate – 0.1%, dextrose – 0.5%, soya bean meal – 0.5%.

Protein Assay:

Soluble protein was estimated by Lowry's method using bovine serum albumin (BSA) as standard [16].

Enzyme Activity Parameters

pH:

Enzyme activity of cell-free crude enzyme extract was measured at various pH values. The buffers used were Glycine-NaOH buffer for pH 8, Tris-Hcl buffer for pH 9, Tris buffer for pH 10, Carbonate-bicarbonate buffer for pH11.

Temperature:

The enzyme activity of protease was measured at various temperatures ranging from 45°Cto 100°C.

Results:

The maximum protease activity obtained in submergedfermentation was 126.57µg/mL/min, for wheat bran media at pH 9, temperature 80°C.

The maximum enzyme activity of 238.3 µg/mL/min was obtained for wheat bran containing media in solid stead fermentation where temperature and pH were 80°C and 9.

Discussion

Submerged Fermentation (SmF):

Among the two media taken into consideration wheat bran containing media had higher activity of protease compare to rice bran. Jaswal *et al* had mentioned that for wheat bran containing media 120.51 µg/mL/min activity had been obtained whereas 126.57 µg/mL/min was obtained for wheat bran containing media (1) [17] (Fig 1).

Similarly for rice bran based production 109.4 µg/mL/min of activity had been obtained (**Fig 2)** whereas Naidu and Devi reported an activity of 42.1 unit/mLfor media containing 1% rice bran [18].

In contrary Vijay Anand *et al* had reported a lower activity of 52 µg/mL/min for wheat bran and 64µg/mL/min for rice bran where rice bran seems to be the better carbon source [19].

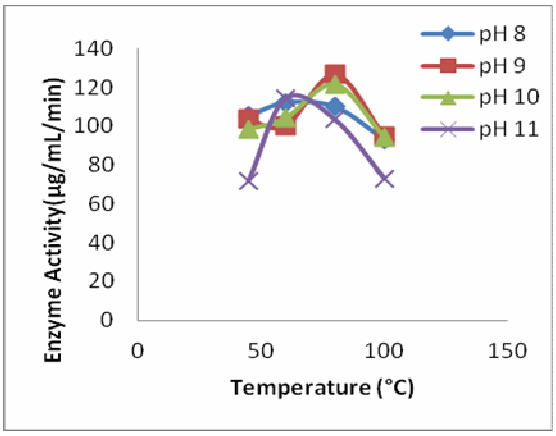

Fig 1: Submerged fermentation in wheat bran containing media

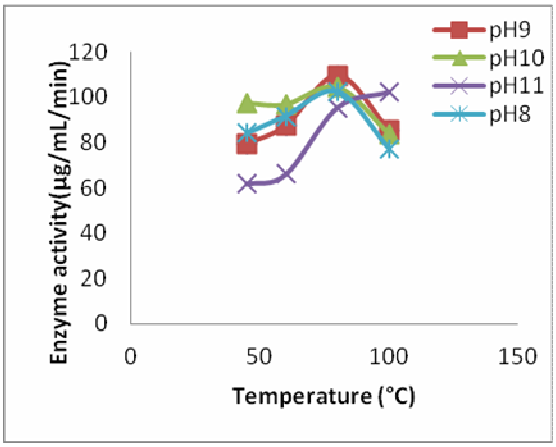

Fig 2: Submerged fermentation in rice bran containing media

Solid State Fermentation (SSF):

For efficient and increased enzyme activity, solid-state fermentation proves to be a better production mode for industrial scale [20]. 238.3 µg/mL/min activity was obtained for wheat bran where as a lesser activity of 190.2µg/mL/min was noted for rice bran (Fig 3, 4). Divakar*et al* had reported protease production in wheat bran and rice bran substrate where 1620 units/g and 86 units/g activity was achieved respectively [21]. Other carbon source such as glucose [22], sucrose [23], maltose [24] etc., [25] had been mentioned.

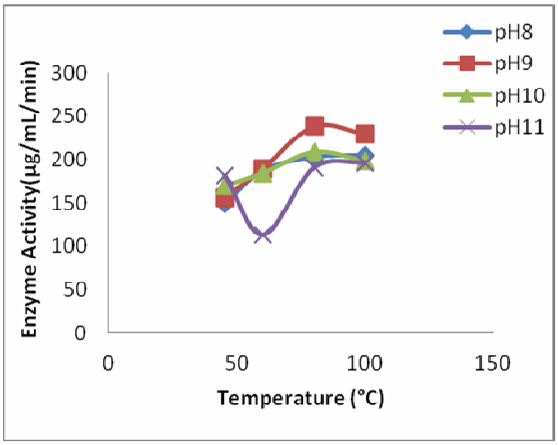

Fig 3: Solid state fermentation in wheat bran containing media

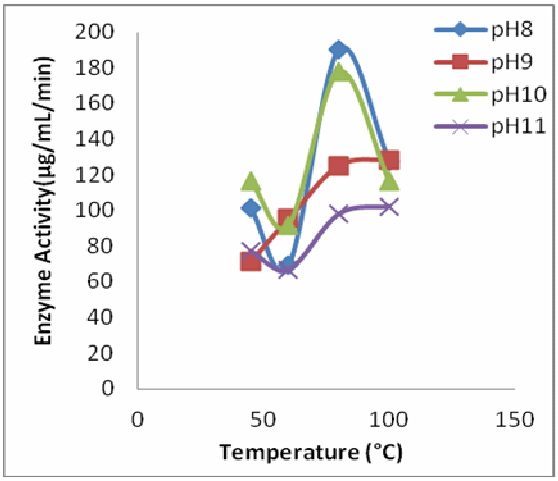

Fig 4: Solid state fermentation in rice bran containing media

Effect of metal ions:

Of various metal ions used at a concentration of 10 mM, all metals including calcium and magnesium had lower enzyme activity compared to that of controls (Fig 5). Nilegaonkar *et al.* had described the effect of various metal ions on the alteration of the enzyme activity [26]. Nilegaonkar had reported a higher enzyme activity for calcium ion (3%) whereas other metals ions had inhibitory effects only. EDTA (10 mM) was also an effective inhibitory agent for protease activity but the inhibition was at par with other metal ions and was not drastically inhibited.

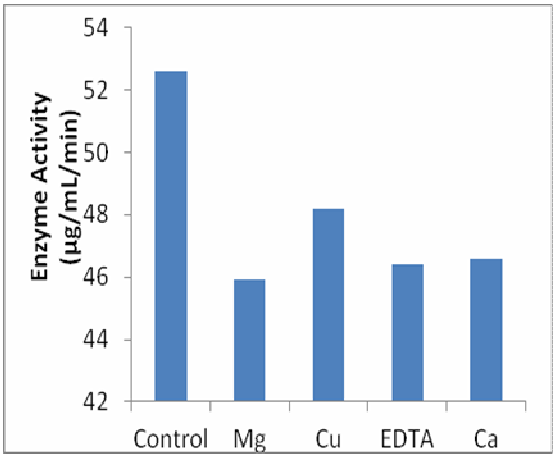

Fig 5: Effect of enzyme activity on various metal addition

Effect of pH and temperature:

For both Submerged Fermentation and Solid State Fermentation, more protease activity was obtained for pH8, temperature 80°C in wheat bran carbon source. In case of rice bran both Submerged Fermentation and Solid State Fermentation the enzyme activities were low compared to wheat bran. The pH and temperature dependent enzyme activities were shown from the result obtained by Oliveira *et al* where the maximum activity was at pH 9 and 10, temperature 55°C. The lowest enzyme activity was at pH 5 and temperature 80°C [27]. Nadeem *et al* had also mentioned pH and temperature influence for *Bacillus licheniformis* N-2 where the optimised pH and temperature were 10 and 37°C [28].

Conclusion:

Thus *Bacillus licheniformis* NRRL-NRS-1264 was found to be an excellent and efficient producer of alkaline protease which can be used, on further purification strategies, for wide range of applications. Further studies on enzyme kinetics and fermenter scale data will enable to translate the production of protease enzyme for commercial avenues with much ease.

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