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Metal ion and pH Stable Protease Production Using Agro-industrial Waste

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Keywords

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Abstract

Protease from a newly isolated *Bacillus* strain exhibited stability in presence of many monovalent and divalent ions and a wide pH range. Agro-industrial waste as Wheat Bran, Gram Husk, Rice Bran and Mustard Oil Cake were used for the production of the enzyme in Solid state fermentation. Maximum enzyme production was observed with rice bran at moisture content 1:3 (w/v) and 20% inoculum size (v/w). The optimum temperature and pH of the enzyme activity were found to be 60oC and 8 respectively. It was found to be stable at 30-50°C for 2 h and pH range of 6-9. The enzyme activity enhanced in presence of metal ions as Na^{+}, K^{+}, Ca^{++}, Mg^{++} Cu^{++}, Mn^{++}, Zn^{++} and Hg^{++} the activity.

1. Introduction

Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively. *Bacillus* species are prolific producers of extracellular proteases that have a wide range of applications, particularly in the detergent, food, pharmaceutical, leather, paper and pulp and chemical industries [1-3]. The industrial demand of proteolytic enzymes, with appropriate specificity and stability to pH, temperature, metal ions, surfactants and organic solvents continues to stimulate the isolation of new promising strains that can utilize cheap carbon and nitrogen source for high production of enzyme with novel characteristics at a low cost [4]. The metabolic processes of the microorganisms are influenced to a great extent by the change of pH, temperature, substrate, water content, inoculum concentration, etc. In general, each microbial strain is unique in its molecular, biochemical, and metabolic enzyme production properties. Therefore, it becomes very important to know the environmental conditions of the microorganisms for maximum production and thorough characterization of isolated individual microbial species to evaluate its potential at commercial level [5-6]. Agricultural wastes contain high moisture content and can be converted to useful agricultural and industrial products by microbes with the supplementation of nitrogen sources [7], thus providing an alternative avenue and value-addition to these otherwise under or non-utilized residues. Hence they are considered the best substrates for the enzyme production in solid state fermentation [8]. SSF process is believed to mimic natural environment and encourage the microorganism to work at its best for the production of the product [9]. There are several reports describing the use of agro-industrial residues for the production of protease [6-10].

This paper reports the production of a metal ion and pH stable protease by a newly isolated strain by the utilization of agro-industrial waste as Wheat Bran (WB), Gram Husk (GH), Rice Bran (RB) and Mustard Oil Cake (MOC) which otherwise contribute to tons of waste globally. Optimization of fermentation parameters for protease production and the process parameters of the enzyme activity was performed for possible commercialization of the enzyme.

2. Materials and Methods

Isolation and screening of protease producing bacteria

Twenty five strains were isolated from soil samples collected from various sites at Delhi and NCR, India. Out of 25, 3 strains were selected on the basis of zone of hydrolysis they exhibited on the milk agar plates. The selected strains were inoculated in sterilized nutrient broth and incubated at 37°C to get a standard inoculum (3.2 x 10^5 cfu/ml).

Substrate

Four types of agro-industrial wastes to be used as substrate viz., WB, GH, RB and MOC were procured from local market of Delhi and powdered to size about 2mm before use.

Enzyme production

Experiments were conducted in 100ml Erlenmeyer flasks. 5g of the substrate was taken into the flask and impregnated with 10ml of sterile

liquid nutrient medium [%: KH_2PO_4 - 0.1, NaCl- 0.25, $MgSO_4.7H_2O$- 0.01, $CaCl_2$- 0.01]. The flasks were autoclaved, inoculated with the prepared inoculum, and incubated at 37°C for the desired time period. Samples were aseptically withdrawn and assayed for protease activity.

Assay of proteolytic activity

Protease activity was measured by modified method of Kembahvi et al. [11] using casein as substrate. The enzyme extract suitably diluted, was mixed with 50mM glycine–NaOH buffer to make 1 ml volume.1ml of 1% casein was added and incubated for 10 min at 60°C. The reaction was stopped by addition of 0.5 ml TCA (20% w/v). The mixture was allowed to stand at room temperature for 30 min to 1h and then filtered to remove the precipitate. 1 ml of the filtrate was mixed with 5 ml of 0.5M Na_2CO_3 solution. 0.5ml of Folin &Ciocalteu's (phenol reagent) reagent was added and the mixture was incubated in dark to develop the blue colour. The blue coloured solution was then was estimated spectrophotometrically at 660nm. One unit of protease activity was defined as the amount of enzyme required to liberate1 μg tyrosine per ml in 1 min under the experimental conditions used. The experiments were carried out in triplicates and standard error was calculated.

Optimization of protease production parameters

One at a time strategy was used for various physicochemical factors optimization.

Optimization studies were carried out by varying substrate (WB, GH, RB and MOC), Moisture content from 1:2 to 1:5 (w/v) and inoculum size at 1%, 5%, 10% and 20% of bacterial culture with colony count at 3.8 x 10^5 cfu/ml.

Biochemical characterization of the enzyme

The optimum temperature of the enzyme activity was examined by varying the incubation temperature of the assay from 30°C to 70°C (for 10 min at pH 9). The thermal stability of the enzyme was assessed by incubating the enzyme without the substrate fractions at various temperatures between 30 to 70ºC for 2h. Enzyme was taken at every 30min intervals, and was assayed for activity.

Optimum pH for the enzyme activity was assessed by performing the assay with buffers with pH ranging from 6-10. The stability of the enzyme in different pH was assessed by incubating the enzyme for 2h in buffers of different pH. The enzyme activity was investigated in every 30min.

Effect of metal

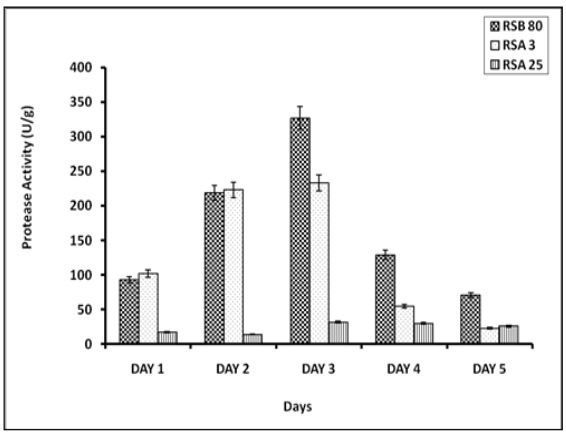
To investigate the effect of metal ions on enzyme activity, the casein hydrolysis was performed in the presence of various metal ions (Na^+, K^+, Ca^{++}, Mg^{++}, Zn^{++}, Mn^{++}, Cu^{++} and Hg^{++}) in chloride and sulphate salts, at a concentration of 5 mM and 10mM.

3. Results

Three best protease producing strains were selected on the basis of the zone of hydrolysis they exhibited on the milk agar plates (Fig.1). Strain RSB-80 showed the maximum activity of 327 U/g, while strain RSA-3 and RSA 25 showed the maximum activity of 233 and 35 U/g on the third day using wheat bran as substrate (Fig 2). Hence strain RSB 80 was selected for further optimization. The selected isolate was a *Bacillus* strain as identified on the basis of microscopic and biochemical analysis.

Figure 1: The selected strain RSB 80 exhibiting zone of hydrolysis on skim milk agar plate.

Figure 2: Protease production by strains RSB 80(▩), RSA 3(⊡) and RSA 25 (▥) by using Wheat Bran as substrate.

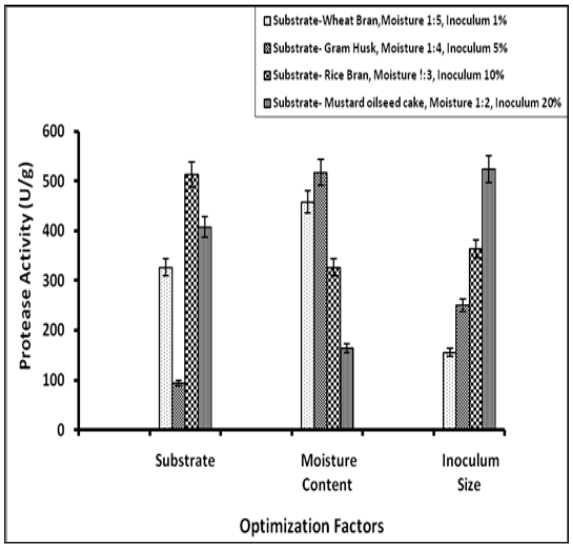


Optimization of protease production parameters

RB was found to be the best utilized substrate resulting in maximum enzyme production (513U/g) as compared to WB (307 U/g), GH (94 U/g) and MOC (408 U/g) (Fig 3).

Further optimization studies in terms of moisture content and inoculum size showed that moisture content 1:3 (w/v) and 20% inoculum size (v/w) gave the maximum enzyme production of 525U/g (Fig 3).

Figure 3: Effect of variation in Substrate, Moisture Content and Inoculum Size in enzyme.



Biochemical characteristics of the enzyme

The temperature at which the enzyme exhibited its best activity was 60°C (Fig 4). The enzyme was found to be stable for 2h at 30°C. At 40 and 50°C the enzyme lost only 16- 22% of its activity after 2h. However at 60 and 70°C the enzyme retained only 25 and 20% respectively after 30 min (Table 1). The protease was active and stable at a broad range of pH 6.0–9.0 losing only 28- 30% activity after 2h (Table 1) with maximum activity at pH 8 (Fig 4). At pH 10 it retained 60% of its activity after 2h.

Figure 4: Enzyme activity at different temperatures (30°C to 70°C) and pH (6-10)

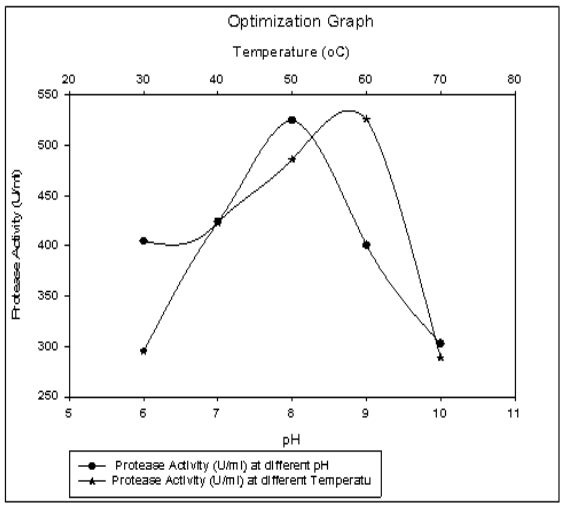


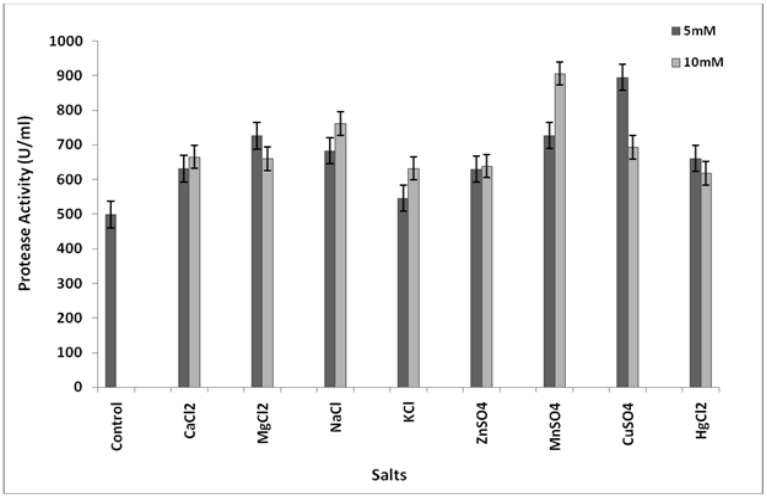
Table 1: Residual Enzyme activity at different temperatures (30°C- 70°C) and pH (6-9)

Incubation Time	Residual Activity (%) at different temperatures					Incubation Time	Residual Activity (%) at different pH				
	30°C	40°C	50°C	60°C	70°C		6	7	8	9	10
0:00	100	100	100	100	100	0:00	100	100	100	100	100
0:30	100	99.2	97.3	94.3	86.5	0:30	93.2	95.9	96.6	89.2	79.5
1:00	100	96.2	93.1	81.0	67.8	1:00	87.5	89.7	86.5	80.7	69.9
1:30	100	92.2	85.0	74.1	58.1	1:30	81.2	85.8	80.8	71.5	62.3
2:00	100	86.3	81.5	66.3	37.7	2:00	76.8	79.2	75.0	56.4	53.8

Effect of metal ions on enzyme activity

The enzyme activity increased in presence of all the metal ions (Na^+, K^+, Ca^{++}, Mg^{++}, Zn^{++}, Mn^{++}, Cu^{++} and Hg^{++}). Maximum enhancement was seen in the presence of Mn^{++} and Cu^{++} at 10mM and 5mM concentration respectively (Fig 5).

Figure 5: Effect of various metal ions in 5mM and 10mM concentration on the enzyme activity



4. Discussion

The selected strain demonstrated a large zone of hydrolysis around the colony on milk agar medium (Fig.1). This indicates the ability of the strain in hydrolyzing milk by production of extracellular proteolytic enzyme. All the three strains showed maximum production at 72 h, thereafter, the activity decreased substantially till 120h. Similar results have been reported by Kumar et al [12] in production of protease enzyme from *Bacillus* sp. in utilizing agroindustrial waste.

The highest protease activity on rice bran was 525U/g. Rice bran protein is higher in lysine content than any other cereal bran proteins and have digestibility greater than 90% [13]. This explains the better enzyme production by utilization of rice bran.

In SSF, the quantity of water present in the media is function of the substrate water retention capacity, this quantity should be sufficient for the growth of microorganisms without destructing the solid structure or reduce the porosity of substrate or support [14- 15]. Moreover, water has an impact on physico-chemical properties of the substrate, which in turn affect enzyme production [16]. Too much water, however, adversely affects oxygen diffusion in the substrate [17]. In our study the optimal moisture content for the enzyme production was 33%.

Lower number of cells grow in the production medium at lower inoculum size, due to which the incubation time required to form the desired product increases [18]. The inoculum size for the best enzyme production in our study was found to be 20%.

The enzyme showed maximum activity at 60°C, and pH 8, while it exhibited stability in wide pH range (6- 9). Thermal stability and pH stability in wide range has been reported by many researches [19-20].

The enzyme activity was enhanced in presence of all the metal ions used. This phenomenon indicates that the enzyme requires metal ions as cofactors, which also protect enzyme against denaturation, thus maintaining the active conformation of the enzyme at higher temperature [21]. These results correlate with the observations of Kunamneni et al [22] and Beg et al [23].

5. Conclusion

The results of the stability of the enzyme in presence of metal ions and wide pH range indicate that this bacterial strain has a high biotechnological potential for protease production in solid-state fermentation by utilizing various types of agro-industrial wastes, thus providing a low cost production of the most important industrial enzyme throughout the global market.

References

1. Gupta, R., Y.K. Beg and P. Lorenz, 2002. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl. Microbiol. Biotechnol., 59: 15–32.
2. Pastor, M.D., G.S. Lorda, and A. Balatti, 2001. Protease obtention using *Bacillus subtilis* 3411 and amaranth seed meal medium at different aeration rates. Braz. J. Microbiol., 32: 6-9.
3. Giongo, J.L., S.L. Francoise, F. Casarin, P. Heeb and A. Brandelli, 2007. Keratinolytic proteases of *Bacillus sp.* isolated from the Amazon basin showing remarkable dehairing

activity. World J. Microbiol. Biotechnol., 23: 375-382.
4. Kamoun, A.S., A. Haddar, N.E.H. Ali, B.G. Frikha, S. Kanoun and M. Nasri, 2008. Stability of thermostable alkaline protease from *Bacillus licheniformis* RP1 in commercial solid laundry detergent formulations. Microbiol. Res., 163: 299—306.
5. Elibol, M. and A. R. Moreira, 2005. Optimizing some factors affecting alkaline protease production by a marine bacterium *Teredinobacter turnirae* under solid substrate fermentation. Process. Biochem., 40: 1951-1956.
6. Prakasham, R. S., C. H. Subba Rao, and P. N. Sarma 2006. Green gram husk-an inexpensive substrate for alkaline protease production by *Bacillus* sp. in solid state fermentation. Bioresour. Technol., 97: 1449-1454.
7. Yang, S.S., 2002. Application of Solid State Fermentation in Agriculture and Industry. Scient. Agric., 50: 156-167.
8. Ellaiah, P., K. Adinarayana, Y. Bhavani, P. Padmaja, and B. Srinivasula, 2002. Optimization of process parameters for gluco-amylase production under solid state fermentation by a newly isolated *Aspergillus* species. Process Biochem., 38: 615–620.
9. Pandey A., 1994. Solid state fermentation: an overview. In: Pandey A, editor. Solid State Fermentation. New Delhi: Wiley Eastern; 3–10.
10. Sathish, T., G.S. Lakshmi, C.H. Subba Rao, P. Brahmaiah, and R.S. Prakasham 2008. Mixture design as first step for improved glutaminase production in solid state fermentation by isolated *Bacillus sp.* RSP–GLU. Lett. Appl. Microbiol., 47: 256–62.
11. Kembhavi, A.A., A. Kulkarni, and A.A. Pant, 1993. Salt tolerant and thermostable alkaline protease from *Bacillus subtilis* NCIMNo 64. Appl. Biochem. Biotechnol., 38: 83-92.
12. Kumar, P., V. Mathivanan, M. Karunakaran, S. Renganathan, and R.S. Sreenivasan, 2008. Studies on the effects of pH and incubation period on protease production by *Bacillus sp.* using groundnut cake and wheat bran. Indian J.Sci.Technol. 1: 1-4.
13. Wang, M., N.S. Hettiarachchy, M. Qi, W. Burks, and T. Siebenmorgen, 1999. Preparation and Functional Properties of Rice Bran Protein Isolate. J. Agric. Food Chem., 47: 411-416.
14. Gervais, P., M. Bensoussan, and W. Grajek, 1988. Water activity and water content: comparative effects on the growth of *Penicillium roqueforti* on solid substrate. Appl. Microbiol. Biotechnol., 27: 389-392.
15. Gervais, P., and P. Molin, 2003. The role of water in solid state fermentation. Biochem. Eng. J., 13: 85–101.
16. Pandey, A., P. Selvakumar, C.R. Soccol and P. Nigam, 1999. Solid state fermentation for the production of industrial enzymes. Curr. Sci., 77: 149–162.
17. Kashyap, P., A. Sabu, A. Pandey, G. Szakas, and C.R. Soccol, Extracellular L-glutaminase production by *Zygosaccharomyces rouxii* under solid state fermentation, Process Biochem., 38 (2002) 307–312.
18. Chisti, Y., 1999. Solid substrate fermentations, enzyme production, food enrichment. In: Flickinger M.C.; Drew S.W.; editors. Encyclopedia of bioprocess technology: fermentation, biocatalysis and bioseparation, vol.5. New York: Wiley, pp. 2446-2462.
19. Manni, L., K. Jellouli, O.G. Bellaaj, R. Agrebi, A. Haddar, A.S. Kamoun, and M. Nasri, 2010. An Oxidant and solvent-Stable Protease produced by *Bacillus cereus* SV1: Application in the Deproteinization of Shrimp wastes and as a Laundry Detergent Additive. Appl. Biochem. Biotechnol., 160: 2308–2321.
20. Tang, X.Y., B. Wu, H.J. Ying, and B.F. He, 2010. Biochemical Properties and Potential Applications of a Solvent-Stable Protease from the High-Yield Protease Producer *Pseudomonas aeruginosa* PT121. Appl. Biochem. Biotechnol., 160: 1017–1031.
21. Donaghy, J.A., and A.M. McKay, 1993. Production and properties of an alkaline protease by *Aureobasidium pullulans*. J. Appl. Bacteriol., 74: 662 –666.
22. Kunamneni, A., E. Poluri, and S.P. Davuluri, 2003. Purification and partial characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11. AAPS PharmSci. Tech., 4:56.
23. Beg, Q.K., V. Sahai, and R. Gupta, 2003. Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in a bioreactor. Process. Biochem., 38: 1–7.

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