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BIOSYNTHESIS OF PROTEASES BY SUBMERGED CULTURE FERMENTATION

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Evaluation of wheat bran, soybeans meal, maize bran, rice bran or rice husk as substrate was carried out for the synthesis of both alkaline and neutral proteases by *Bacillus subtilis* in shake flasks. Of all these subtrates, however, wheat bran was found to be an ideal substrate for enzyme formation. Partial replacement of wheat bran by rice bran or soybeans meal resulted in reducing enzyme production. The initial pH of the fermentation broth showed great influence on the biosynthesis of both neutral and alkaline proteases.

INTRODUCTION

Proteases break down proteins to smaller molecules – peptides, and finally to the binding units of proteins – amino acids. According to their mode of action the proteases fall into three groups: acid, neutral and alkaline [1-4].

Proteases are produced by all organisms. Some proteases formed are excreted in the surrounding medium, and are called extra-cellular proteases; others remain inside the cell and are called intracellular proteases. Food digesting proteases and microbial proteases produced on a large-scale by the fermentation industry are extra-cellular. Both bacteria [5-9] and moulds [10,11] produce extra-cellular proteases. In many cases more than one protease is excreted. Strains of *Bacillus subtilis* produce both a neutral and an alkaline type protease. The proteases may be excreted in the surrounding medium during a certain period in the life of the organism. Some, like the strains of the species of *Bacillus megatrium,* produce an extra-cellular protease during the logarithmic growth period [12]. Others, like the representatives of *Bacillus subtilis* and *Bacillus cereus,* produce a protease after a stationary phase (logarithmic phase) in the period between growth and sporulation [13,14].

Proteases are used in food, pharmaceutical preparations as digestive enzymes, in tanneries for bating hides and as a component of detergents. This study gives an account of the production of neutral and alkaline proteases by *Bacillus subtilis* by submerged culture fermentation using various easily available cereal wastes/cereals such as wheat bran, maize bran, rice bran, rice husk and soybeans as substrates.

MATERIALS AND METHODS

Organism and growth: The strain of *Bacillus subtilis,* used in the present study, was isolated locally from the suspension of soil. The following nutrient medium was used for the maintenance of the bacterial cultures:

(g/l): beef extract, 1.5; glucose, 2.0; yeast extract, 3.0; casein hydrolyzate, 4.0; peptone, 6.0; and agar, 25.0. The cultures were incubated at 37° for 48 hr. and then stored in a refrigerator at 5°. All isolates were gram positive spores forming rods and were identified as Bacillus species using the criteria of Bergey's Manual of Determinative Bacteriology. The culture expressing maximum activity of enzyme production was further identified as *Bacillus subtilis.*

Inoculum preparation: In a 500 ml conical flask 100ml of nutrient broth were sterilized at 121° for 15 min. The medium, after cooling, was inoculated from the agar slant aseptically and allowed to grow at 30° for 24 hr on a rotary shaker (designed and fabricated in the workshop of the PCSIR Laboratories, Lahore rotating at the rate of 125 rpm).

Fermentation procedure: For the production of protease, protein-free Le Mense Medium [15] was prepared by omitting bactopeptone from the medium whose composition is shown below:

(g/l): glucose, 3.30; yeast extract 1.0; $CaCO_3$, 1.0; peptone 3.0; pH 7.0 or pH 9.0 adjusted as desired. (Tap water was replaced by distilled water, as large amount of salts present in the former retards the growth of micro-organisms). The wheat bran, or bran of other cereals or defatted soybeans, along with protein-free 50 ml of Le Mense medium contained in a 300 ml conical flask was sterilized at 121° for 15 min. The sterilized mash was inoculated by transferring 2 ml of 48 hr old inoculum. The culture was incubated at 30° for optimum period (48 hr in the case of wheat bran, maize bran, rice bran or rice husk; and 4 days when defatted soybeans meal was used as substrate) for growth and enzyme synthesis.

Extraction of enzyme: Enzyme extraction was carried out by adding 50 ml of phosphate buffer (1.5 g KH_2PO_4 and 3.5 g K_2HPO_4/100 ml) to each flask and rotated at 125 rpm for 1 hr. The suspension was centrifuged in a refrigerated centrifuge and the enzyme activity determined.

Assay method: The proteolytic value or activity was determined by the method of Oshima and Church [16].

The extracted enzyme suspension was filtered till it was clear. One ml of the filtrate was diluted with water to make 5 ml. portions of this diluted enzyme solution (such as 0.10 ml, 0.2 ml, 0.3 ml, 0.4 ml, 0.5 ml, 0.6 ml, 0.7 ml, 0.8 ml, 0.9 ml and 1. ml) were allowed to digest 5 ml of 0.5% casein solution in separate test tubes for 1 hr at 37-40°. The appearance of a precipitate or cloudiness after adding 0.5 ml of a mixture of saturated $MgSO_4$ solution and conc. HNO_3 (4:1) to the digestion tubes indicated an incomplete digestion of casein by the enzyme. The density of cloudiness or precipitate depends upon the amount of undigested casein. The clear tube next to one showing opacity was taken as the tube containing the minimum amount of the enzyme which digested completely the casein present in the 5 ml of 0.5% solution.

To express proteolytic activity the following formula devised by Oshima and Church [16] was adopted:

When 0.025 g (or ml) of the original enzyme substances (solution) digests completely 5 ml of 0.5% casein solution (i.e., 0.25 g casein) in one hour at 37-40°, the proteolytic value (PV) of this substance is 100.

RESULTS AND DISCUSSION

Effect of concentration: The concentration of substrates, such as wheat bran or defatted soybeans meal, in the culture broth play an important role in aerobic fermentation. The amount of both substrates was varied from 5-30 g/100 ml (Fig. 1). The optimum levels of both substrates was found to be 10 g/100 ml of the fermentation medium and further increase in their concentration reduced agitation, hence protease formation.

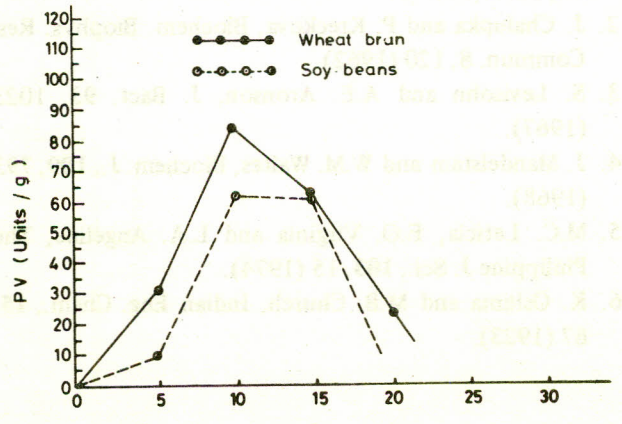

Fig. 1. Concentration (g/100 ml)

Rate of enzyme synthesis: Fig. 2 shows the comparison of the rates of synthesis of neutral protease by *Bacillus subtilis* using wheat bran or defatted soybeans meal in shake flasks. The amount of protease produced in wheat bran was maximum two days after inoculation and further incubation greatly reduced enzyme formation. In defatted soybeans, however, the rate of enzyme formation was slow and its concentration reached maximum (62.5 units/g) 4 days after inoculation and further increase in incubation period resulted in reducing enzyme formation.

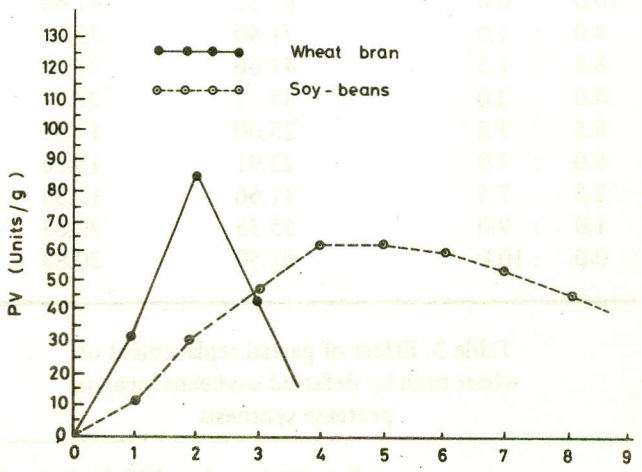

Fig. 2. Incubation period (Days)

Selection of substrate: The data presented in Table 1 show the evaluation of various substrates for the production of proteolytic enzymes by the bacterium. Of all substrates, however, wheat bran was found to be an ideal sub-

Table 1. Selection of substrate for protease synthesis

Substrate	Proteolytic value (PV) (units/g of substrate)	
	Neutral protease	Alkaline protease
Wheat bran	83.32	41.66
Soybeans (defatted)	62.50	22.91
Rice bran (defatted)	62.50	20.83
Maize bran	55.55	38.68
Rice husk	50.00	8.83

Table 2. Effect of partial replacement of wheat bran by defatted rice bran on protease synthesis

Wheat bran : Rice bran	Proteolytic value (PV) (units/g of substrate) Neutral protease	Alkaline protease
10.0 : 0.0	83.32	41.66
9.0 : 1.0	71.40	35.71
8.5 : 1.5	41.66	31.25
8.0 : 2.0	35.71	27.77
6.5 : 3.5	25.00	17.85
5.0 : 5.0	22.91	16.66
2.5 : 7.5	41.66	17.85
1.0 : 9.0	55.55	20.83
0.0 : 10.0	62.50	20.83

Table 3. Effect of partial replacement of wheat bran by defatted soybeans meal on protease synthesis

Wheat bran (g) : Soy-beans (g)	Proteolytic value (PV) (units/g of substrate) Neutral protease	Alkaline protease
10.0 : 0.0	83.32	41.66
9.0 : 1.0	71.40	31.25
7.5 : 2.5	45.78	17.85
5.0 : 5.0	20.83	10.00
1.0 : 9.0	55.55	17.85
0.0 : 10.0	62.50	22.91

strate providing all nutrients for the synthesis of both neutral and alkaline proteases, i.e. 83.32 and 41.66 units/g wheat bran respectively. The production of both enzymes in the presence of defatted rice bran or defatted soybeans meal was almost the same. The synthesis of alkaline proteases in the presence of maize bran was better than that obtained when culture was grown in defatted soybeans, rice bran or rice husk.

The effect of partial replacement of wheat bran by defatted rice bran or soybeans meal on the synthesis of proteolytic enzymes was also investigated (Table 2 and 3). The production of enzymes by the bactrium, however, was maximum in wheat bran and it was reduced with the increase in the concentration of defatted rice bran or soybeans meal. It shows that wheat bran is a good source of all nutrients needed for the growth of the bactrium and enzyme synthesis.

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REFERENCES

1. B. Hagihara, In *"The Enzymes"*, edited by Boyer, P.D., Lardy, H., and Myrback, M., Academic Press, New York, (1960) Vol. 4, p. 193.
2. D. Tsura, H. Kira, T. Yamamota and J. Fukumoto, Agr. Biol. Chem., **31**, 718 (1967).
3. K. Morihara, H. Tsuzuki and T. Oka, Arch. Biochem. Biophys., **123**, 572 (1968).
4. L. Keay, P.M. Moser and B.S. Wildi, Biotechnology and Bioengineering, **12**, 213 (1970).
5. J. Fedar and C. Lewis, Biochem. Biophys. Res. Commun., **28**, 318 (1967).
6. D. Tsura, *et al.* Agr. Biol. Chem. (Tokyo), **31**, 330 (1967).
7. K. Morihara, Appl. Microbiology, **13**, 793 (1965).
8. T.Y. Liu, *et al.*, J. Biol. Chem. **238**, 251 (1963).
9. A. Hiramatsu, J. Biol. Chem., **61**, 168 (1967).
10. K. Hayashi, *et al.*, Agr. Biol. Chem. (Tokyo), **31**, 642 (1967).
11. G. Danno and S. Yosbimura, Agr. Biol. Chem. (Tokyo) **31**, 1151 (1967).
12. J. Chalupka and P. Kreckova, Biochem. Biophys. Res. Commun. **8**, 120 (1962).
13. S. Levisohn and A.E. Aronson, J. Bact. **93**, 1023 (1967).
14. J. Mandelstam and W.M. Waites, Biochem. J., **109**, 793 (1968).
15. M.C. Leticia, E.O. Virginia and L.A. Angeline, The Philippine J. Sci., **103**, 15 (1974).
16. K. Oshima and M.B. Church, Indian Eng. Chem., **15**, 67 (1923).