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PRODUCTION OF NEUTRAL PROTEASES BY SERRATIA MARSCENS USING RICE BRAN

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Abstract

For the utilization of rice bran, a cheap and widely available agricultural by-product, to produce extracellular protease enzyme, *Serratia marscens* isolated from soil and skim milk was used.

Studies of nutritional factors for getting the optimal composition of rice bran media showed that 33 % (v/v) rice bran hydrolysate (6% carbon), 0.06% N-source (4.3% potassium nitrate) and 1% NaH_2PO_4 gave the highest yield.

The highest proteolytic activity in the culture supernatant (650 T.U./ml) determined after 72 h incubation at 35 °C and 120 rpm, was obtained when the strain had been grown in natural medium. Optimum temperature for production of protease was found to be 35 °C whereas maximum activity was recorded at 30 °C and pH 7.5.

Based on the EDTA sensitivity and its neutral pH spectrum, the protease of *S. marscens* may be classified as neutral protease.

Keywords: proteases, neutral proteases, Serratia marscens, rice bran.

Introduction

Bacterial proteases are a group of hydrolytic enzymes overshadowing all others in industrial importance and utilization, especially in the field of detergent and food technology (AKIBA and FUKIMBARA, 1967; MORIHARA, 1974 and ABDEL-ALI et al., 1977).

In Egypt, many milling companies dispose rice bran as the main by-product. Due to its chemical composition, it is a good substrate for enzyme production by microorganisms (GAMAL, 1976; NATARAJAN, 1977; GAMAL et al., 1983 and EL-HAWARY and IBRAHIM, 1988).

In Egypt, high quantities of bacterial proteases are imported, thus it is quite reasonable to think of producing these enzymes locally.

Therefore a screening program was set about in the present work for selection of the most active proteolytic bacteria as well as studying factors affecting the production of proteases from rice bran as natural medium.

Materials and Methods

Isolation of Proteolytic Bacteria

Dilutions of 50 aerobic heterotrophic bacteria originally isolated from soil and whey samples were plated on skim milk agar medium. After 24 h incubation at 30 °C, the plates were flooded with 8–10 ml of concentrated HCl (SMITH et al., 1952). The presence of a clear zone around the colonies against an opaque white background was a preliminary indication for proteolytic activity. The diameter of each clear zone was measured as a rough estimate of proteolytic activity of the organism. The organisms which showed large zones of protein solubilization were selected for further study (SRINIVASAN et al., 1964).

Media

a) *Natural medium*: Rice bran kindly offered by Dakahlia Mills Company, Mansoura, A.R.E. Chemical analysis resulted 12.2% crude protein; 41.8% organic carbon; 13.8% fat; 8.9% crude fibers; 12.5% moisture and 13.3% ash.
Rice bran was hydrolysed using 0.5 mol/l H_2SO_4 for 60 min at 121 °C. After hydrolysis, the filtrate was neutralized with 1 mol/l NaOH solution and added to water (5.5% (v/v)), while the carbon content is 1%, the pH was adjusted to 7.5.

b) Synthetic medium (MORIHARA and TSUZUKI, 1977); glucose 70.0 g.; $(NH_4)_2HPO_4$ 10 g; Na_2HPO_4 10 g.; KH_2PO_4 2.0 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; $CaCO_3$ 20.0 g.; yeast extract 2.0 g and H_2O 1000 ml.

Determination of Organic Carbon

Organic carbon was determined according to the method of Walkley and Black given by RICHARDS (1954).

Characterization and Identification of the Selected Isolates

The schemes used for characterizing these heterotrophic proteolytic organisms have been described by BAUER et al. (1974) and KREG and HOLT (1984).

Culture Conditions

The experiments were carried out in 250 ml Erlenmyer flasks, each containing 50 ml of medium adjusted to pH 7.5. The medium was inoculated with 1.0 ml of 24 h old culture and then incubated at 30 °C for 72 h on a rotary shaker at 120 r.p.m. The content of each flask was centrifuged at 4000 r.p.m. for 30 min to obtain the extract containing the enzyme (BROMKE and HAMMEL, 1979).

Determination of Enzyme Activity

a) Screening Method

The activity of the proteolytic enzymes was indicated by the clear zones surrounding the colonies. The diameters of these zones were measured in millimeters (SRINIVASAN et al., 1964).

*b) Determination of Enzyme Activity by Casein Digestion Method (*LEONARD *and* WIDI, *1970)*

The protease activity was generally determined by a modified Anson's method. Suitably diluted enzyme solution (2.100 units in 1.0 ml, 0.1% calcium acetate solution) was preincubated in a water bath at 37 °C for 5 min. Prewarmed (37 °C) casein solution (1.0 ml) was added to the enzyme and the reaction mixture was incubated at 37 °C for 10 min. The reaction was then terminated by the addition of 2.0 ml of 0.4 mol/l trichloroacetic acid (TCA) solution. The reaction mixture was incubated at 37 °C for additional 20 min, and then filtered off through a Watmann No. 1 filter paper into a clean dry test tube. 1.0 ml of the clear filtrate was transferred to another clean dry test tube, 5.0 ml of 0.4 mol/l sodium carbonate solution were added, followed by 1.0 ml of diluted Folin-phenol reagent (one part of reagent diluted with three parts of distilled water). After mixing thoroughly, the mixture was incubated at 37 °C for another 20 min and the absorbance was measured at 660 nm with a Pye Unicam Spectrophotometer (400 UV).

A unit of protease activity was defined as the quantity of enzyme which produced TCA-soluble fragments giving blue colour equivalent to 0.5 μg tyrosine under the conditions of the assay. A calibration curve was set up with a series of different quantities of a standard solution containing 0.60 μg/ml tyrosine in 0.2 mol/l HCl.

Measurement of Thermal Stability

Portions of culture supernatant were kept in water bath at 20, 30, 40, 50 or 60 °C. After 2 h, the glass tubes containing the samples were removed from the bath, quickly cooled with ice water, and the remaining proteolytic activity was assayed at 30 °C.

Determination of the Optimum pH

The optimum pH for proteolytic activity in culture supernatant was determined using 3 buffer solutions (0.05 M) citrate-NaOH-HCl (pH 5.4–6.2), Tris-HCl (pH 6.6–7.8) and borate-HCl (pH 8.0–9.2).

Portions of culture supernatant previously diluted (1 : 5 v/v) with each of the buffer solutions were mixed with casein solution (2%) dissolved in the same buffer. The proteolytic activity was measured at each pH value.

Results and Discussion

Isolation of Bacteria Producing Extracellular Proteases

Isolation was accomplished in two steps. In the first step, portions of enrichment cultures which had been made as described in the Materials and Methods section, were plated on skim milk agar medium. After incubation at 30 °C for 24 h, colonies which had a distinct halo exceeding the colony diameter fourfold or more were considered as good producers of extracellular proteases. 14 isolates were picked up, purified by several transfers on agar, and designated by code numbers.

In the second step the isolated strains were subjected to further selection in liquid cultures. They were grown in both synthetic media suggested by MORIHARA and TSUZUKI (1977), shaken at 120 rpm for 72 h at 30 °C, and the proteolytic activity of their culture supernatants were determined. Results shown in *Table 1* indicate that the culture supernatant of isolate No. 8–3 exhibited the highest proteolytic activity compared with the tested isolates. Isolate 8–3 was, therefore, chosen for further experiments, and the morphological, cultural and physiological characteristics of the isolate proved to be identical with those of *Serratia marscens*.

Production of Bacterial Proteases on Natural Media

The effect of different additions of rice bran hydrolysate to the medium as a substrate to secure high yield of protease was investigated. The data ob-

Table 1
Production of bacterial protease using submerged technique

Isolate No.	Skim milk agar clear-zones (mm)	* Enzyme productivity T.u/ml
5–1	32	296
5–2	18	146
5–3	24	150
5–4	26	109
5–5	30	190
5–6	28	178
5–7	21	112
5–8	21	131
5–9	23	96
5–10	23	59
8–1	30	81
8–2	18	46
8–3	38	380
8–4	24	121

* Isolates were grown in synthetic medium for 72 h and the proteolytic activity was measured in culture supernatants.

tained are illustrated by *Fig. 1.* It can be clearly seen that there was a stimulation of enzyme production with higher ratios of rice bran hydrolysate. The increase in enzyme production was more pronounced at concentrations exceeding 4.0% carbon. A maximum yield was obtained at 6% carbon concentration (33% v/v rice bran hydrolysate) by *S. marscens.*

On the other hand, it was observed that enrichment of the natural medium with yeast extract to a concentration of 0.2% increased the enzyme production to 520 T.u/ml for *S. marscens* (*Fig. 2*). This may be attributed to the vitamins, minerals and growth factors present in yeast extract (SMITH et al., 1952).

Effect of Nitrogen Source on Protease Production in Natural Medium

Experiments were designed to determine the most favourable nitrogen source for high protease production by the test isolate. Sixteen different nitrogen sources were tested, six of them inorganic and ten organic. The different nitrogen sources were added to the natural medium in amounts calculated to give a final nitrogen concentration of 0.5%. The results are illustrated in *Fig. 3.* They show that inorganic nitrogen sources were more

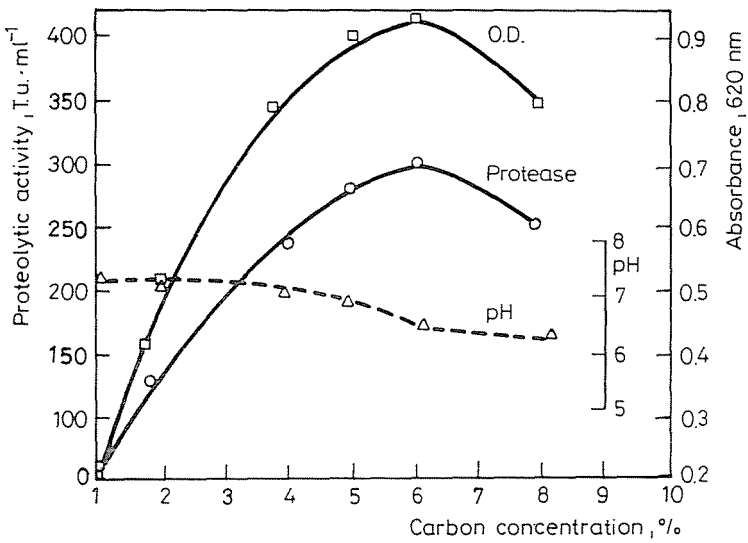

Fig. 1. Growth of *S. marscens* and production of proteolytic enzymes. Cells were grown in natural medium (50 ml in 250 ml flasks) at 30 °C and 120 rpm. Samples were taken at intervals and the absorbance, pH and proteolytic activity in the supernatant were measured.

favourable than organic ones for protease production. Potassium nitrate gave comparatively the highest yield of protease by *S. marscens.*

Effect of Nitrogen Concentration on Protease Production on Natural Media

As KNO_3 was the favourable inorganic N-source with *S. marscens* isolate, an experiment was conducted to investigate the effect of different concentrations on protease production. Concentrations were adjusted to give N-level of 0.1 to 0.8%.

The results obtained were recorded as enzyme activity per ml in culture filtrate as illustrated in *Fig. 4.* They reveal that a nitrogen level of 0.6% was most favourable to *S. marscens* isolate grown in natural medium the enzyme production being 570 T.u/ml.

Nitrogen supplementation plays an important role in C/N balancing ratio in the natural medium (GAMAL et al., 1983).

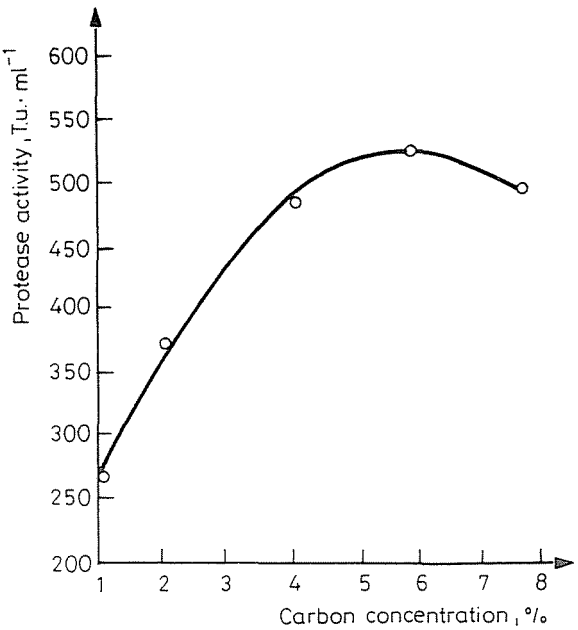

Fig. 2. Effect of carbon concentration on protease production by *S. marscens* grown in natural medium supplemented with 0.2% yeast extract

Effect of Phosphorus Concentration

Different concentrations of NaH_2PO_4 were added to the medium containing 6% carbon (*Fig. 5*). The results show that the maximum enzyme activity was reached at a concentration of 1% of NaH_2PO_4. Our results are in agreement with those reported by DWORSCHAK et al. (1953).

Effect of Incubation Period

To determine the most suitable time at which the production should be harvested, the rate of enzyme secretion was studied during a growth period of 144 h. The data obtained are illustrated in *Fig. 6*. It is clearly shown that the protease activity increased gradually, reaching a peak after 72 h by *S. marscens* in natural medium, being 650 T.u/ml.

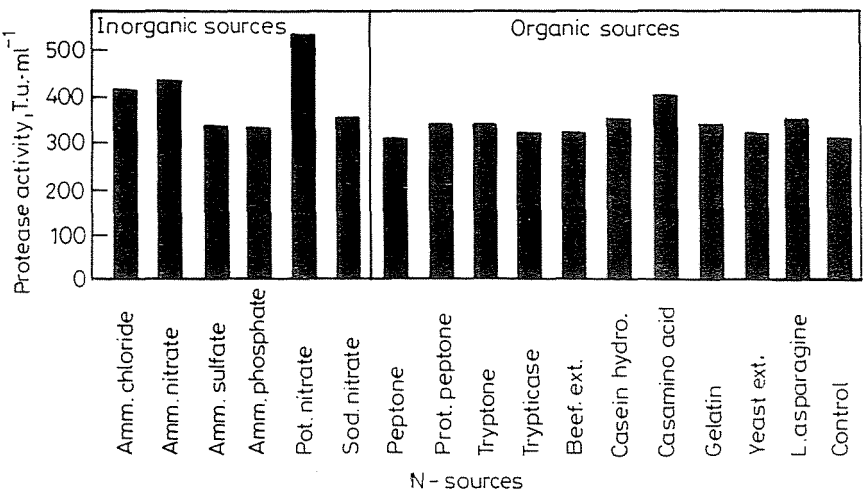

Fig. 3. Effect of N-source (0.5%) on protease production in natural medium by *S. marscens.* Flasks containing 50 ml natural medium, (6% carbon) and different nitrogen sources, (pH 7.5), and incubated at 30 °C for 72 h at 120 rpm. The control medium had no N-source addition.

Effect of Incubation Temperature

The rate of enzyme production was studied at different temperatures during the incubation period. All factors kept constant, only the temperature was varied. The experiments were carried out in natural media. The protease activity was determined after the termination of the incubation period. The results are presented in *Fig. 7.* The data revealed that protease activity increased with rising temperature. The maximum temperature effect was recorded at 35 °C.

On the other hand, the optimum temperature for the proteolytic activity was found to be 35 °C (*Fig. 8*) by *S. marscens* grown in natural medium.

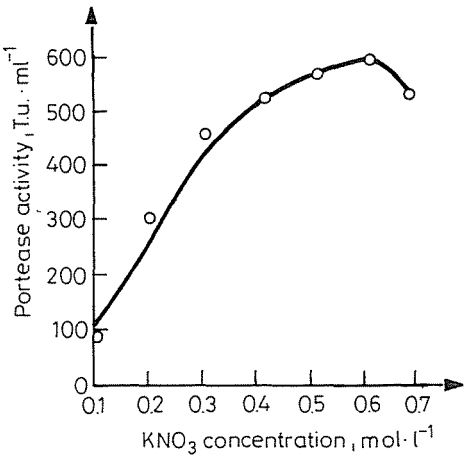

Fig. 4. Effect of different concentrations of KNO_3 on the protease production by *S. marscens* grown in natural medium

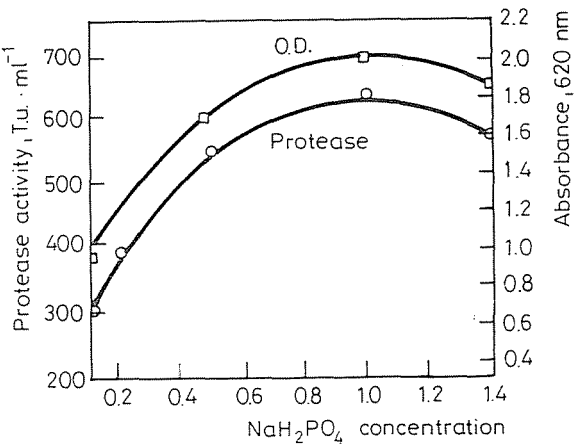

Fig. 5. Production of protease by *S. marscens* grown in rice bran medium supplemented with KNO_3 and different concentrations of NaH_2PO_4

Optimum pH for Proteolytic Activity

The proteolytic activity in the culture supernatant of *S. marscens* grown in natural medium was assayed at various pH levels ranging between 5.4 and 9.2. The results illustrated in *Fig. 9* show that the optimum pH for

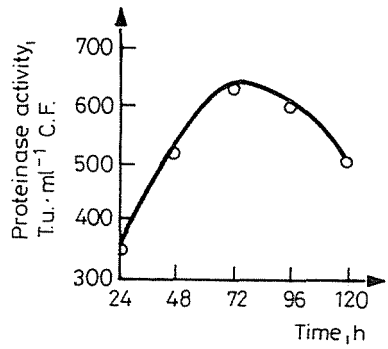

Fig. 6. Effect of incubation period on enzyme production by *S. marscens* grown in natural medium

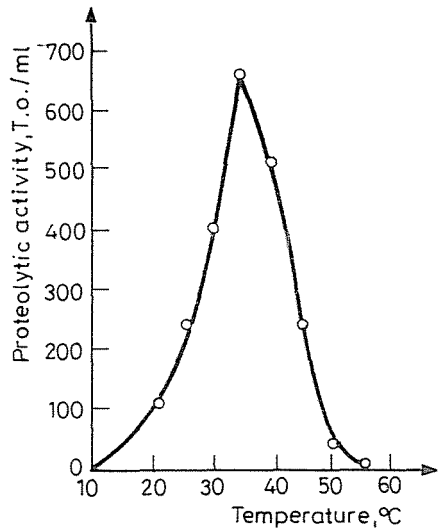

Fig. 7. Effect of incubation temperature on the production of proteolytic enzymes by *S. marscens*. Cells were grown in natural medium and incubated at the designed temperature in a rotary shaker for 72 h.

the enzyme activity was 7.5. Based on the EDTA sensitivity of the enzyme and its neutral pH optimum, the protease of *S. marscens* may be classified

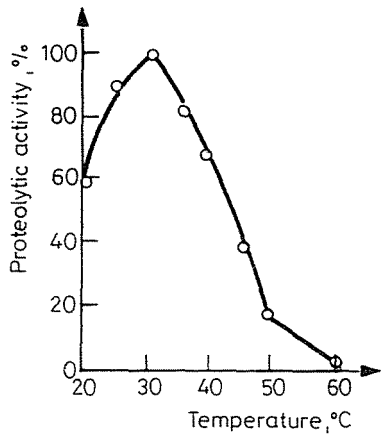

Fig. 8. Effect of temperature on proteolytic activity of culture supernatant of *S. marscens*. Activity at 30 °C was taken as 100%.

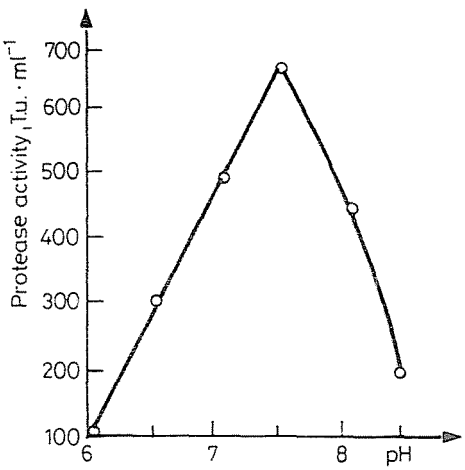

Fig. 9. Effect of pH on the enzyme activity in the culture supernatant of *S. marscens* grown in natural medium. Buffers used were: Citrate-NaOH (pH 5.4–6.2), Tris-HCl (pH 6.6–7.8) and Borate-HCl (pH 7.8–9.2).

as a neutral protease. Neutral proteases were isolated form several bacilli (KEAY, 1969). These enzymes were reported to contain a single zinc atom,

being stabilized by calcium and other divalent ions, and generally lacking cysteine in their structures (FEDDER et al., 1975).

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