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STUDIES ON PROTEOLYTIC ACTIVITY OF BACILLUS SUBTILIS

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During bacteriological examination of commercially preserved pickles, some species of *Bacillus* were found to be highly proteolytic. This was indicated during microbial total count determination on nutritive caseinate agar, the colonies of the bacteria hydrolyzed casein, indicating marked proteolytic activity. Proteolytic enzymes of plant origin, bacteria and fungi, have been used extensively in industry during recent years to tenderize meats and clarify beverages.

The present work has been undertaken because of the potential uses of bacterial proteolytic enzymes as meat tenderizers and clarifiers of beverages and the scarcity of information on this enzyme. The objectives of this study were to identify the culture, to determine the proteolytic activity and to develop a simple method for its preparation on a commercial scale.

Experimental

Isolation of Pure Culture.—Various types of locally preserved pickles available in the market were procured, diluted (10^{-1}–10^{-5}) and then added asceptically to the nutritive caseinate agar (Oxoid). The plate cultures were incubated at 37°C for 24 hr. Colonies which hydrolyzed casein, were picked up and pure culture of proteolytic bacteria was isolated by pour plate method (serial dilutions) as reported.[1]

Identification.—A 24-hr plate culture was used for identification purposes. Various physiological and morphological tests, as reported in the literature, were carried out.[6]

Preparation of Cell-free Extract.—Pure culture was cultivated on nutrient agar (Oxoid) slants. The tubes of pure culture after incubation for 48 hr, were washed with sterile nutrient broth (Oxoid), and this inoculum was added to 3 l of nutrient broth (Oxoid), contained in 10–l flask, provided with controlled aeration through the culture broth under sterile conditions. It was kept at a temperature of 32–34°C for one week, to ascertain maximum enzyme formation. The proteolytic activity of the culture broth was determined after every hour. The rate of hydrolysis of gelatin was measured by Formol titration method.

Determination of Proteolytic Activity.—Five ml of the culture broth were added to 25 ml of 6% gelatin solution and incubated at 37°C for 1 hr. The hydrolyzate was titrated against 0.02N NaOH in the presence of 7.5% formalin solution to the end point of pH 8.8.[8]

Preparation of Crude Enzyme Powder.—The culture broth having optimum enzyme activity was centrifuged at 5,000 rev/min and the cell-free extract thus obtained was used for precipitation of the enzyme. The enzyme extract was precipitated with (1) saturated ammonium sulphate (2) ethanol, and (3) methanol separately, with a view to find a suitable precipitating agent, which gives maximum enzyme activity and yield. Two volumes of saturated solution of ammonium sulphate, ethanol and methanol were added separately to each one volume of cell-free enzyme extract, giving a final two-third saturation of the precipitating agents.[7]

Effect of pH on Activity.—The effect of variation of pH on the rate of hydrolysis of gelatin by the crude enzyme was studied using 25 ml of 6.0% gelatin plus 5 ml of 0.1% enzyme buffered with 0.1M citrate buffer pH 5.0, 0.2M phosphate buffer pH 7.0, and 0.2M borate buffer pH 9.2. The rate of hydrolysis of gelatin was determined by Formol titration method at different intervals of time after incubation at 37°C. Comparative studies were carried out for papain on gelatin, under the same conditions except that papain was activated with cystein hydrochloride to give a cystein concentration of 0.025M in 0.1% papain solution, prior to incubation (Table 1).

Results and Discussion

Identification of the organism was carried out by usual techniques given in literature. The strain isolated has been identified as aerobic, spore forming, rod-shaped bacterium. It was found to

TABLE 1.—RATE OF HYDROLYSIS OF GELATIN.

Time (hr)	μ-moles amino N liberated for the reaction mixture pH 5.0		pH 7.0		pH 9.2	
	Papain	Crude enzyme ppt	Papain	Crude enzyme ppt	Papain	Crude enzyme ppt.
½	170.94	110.88	60.06	138.60	49.24	120.12
1	249.48	120.12	166.32	175.56	138.60	147.84
2	267.96	124.74	170.94	194.04	157.08	170.94
12	351.12	147.84	279.20	304.92	240.24	231.00
22	452.76	166.32	300.30	438.90	258.72	277.20
24	498.96	170.94	383.46	512.82	323.40	332.64
26	508.20	175.56	401.94	531.30	341.88	346.50
48	628.32	184.80	415.80	545.16	351.12	351.12
72	600.60	180.18	480.48	623.70	378.84	383.46

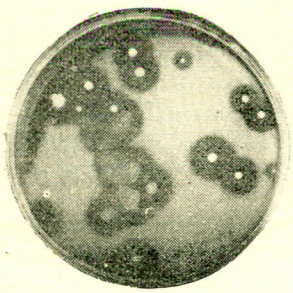

Fig. 1

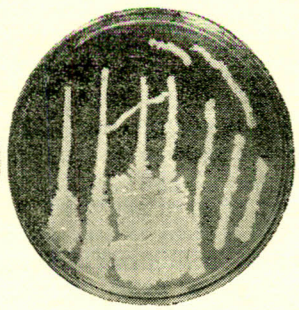

Fig. 2

Fig. 3

hydrolyze casein and liquefy gelatin at 37°C in 3 days. It also hydrolyzed starch, whereas citrate was utilized as a sole source of carbon. All other biochemical characteristics of the organism are in fair agreement with the standard description of *Bacillus subtilis*.[3,4,5] (Figs. 1, 2 and 3). The desired organism when cultivated on nutrient broth and supplied with sufficient amount of air through the culture media, grows best and liberates exo-enzyme. Enzyme formation is at maximum in 72–96 hr producing 78.5–83.2 μ-moles amino nitrogen from gelatin as determined by Formol titration method.[9]

Maximum enzyme yield was obtained with ammonium sulphate solution and least with methyl alcohol. However, the proteolytic activity of the precipitates as determined in μ-moles amino N liberated for the given reaction mixture are shown in Table 2. This clearly indicates that the precipitates obtained by precipitation with ammonium sulphate possessed marked proteolytic activity as compared to the values obtained for the enzymes precipitated with ethanol and methanol. The bacterial residue left after centrifugation of the culture broth possessed meagre activity, thus confirming that the organism liberates exo-enzyme.

The rate of liberation of free carboxylic groups by crude enzyme is maximum at pH 7.0, than at pH 5.0 or at pH 9.2, while in case of papain,

TABLE 2.—PRECIPITATION WITH DIFFERENT ENZYME PRECIPITANTS.

Precipitants	% ppt for the culture broth mg/100 ml	Activity in μ-moles amino N
Ammonium sulphate	200	50.82
Ethanol	107.5	27.72
Methanol	80	18.48

Activity in the residue left after centrifugation=9.24 μ-moles amino N

the rate of hydrolysis is maximum at pH 5.0 than at pH 7.0 or pH 9.2. Though papain and crude enzyme precipitate differ in respect of their pH optima, the amount of amino N liberated for the same reaction mixture at their optimum pH is very nearly equal, thereby indicating that even crude enzyme power possesses marked proteolytic activity almost equal to papain.

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