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Conditions Influencing the Synthesis of Acid Protease by *Mucor pusillus* Lindt[1]

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Protease synthesis by *Mucor pusillus* Lindt, in a wheat bran medium under submerged conditions, was influenced by substrate concentration, initial *p*H of the medium, and temperature of incubation. A 4% wheat bran (dry weight) concentration was satisfactory for enzyme production. The initial *p*H of the medium had a substantial effect on enzyme synthesis; adjustment of the enzyme production medium to *p*H 5.0 prior to sterilization was desirable. Incubation at 35 C resulted in the best enzyme yields. Under optimal conditions of enzyme production, maximal activity was detected after 5 days of incubation. The enrichment of the medium with glucose increased the yield of mycelia but lowered the amount of enzyme produced.

Recently, certain species of the genus *Mucor* have become of interest because their proteases coagulate milk and may be of value in cheese production (H. Arima and S. Iwasaki, Japanese Patent 12,741, 1963; U.S. Patent 3,212,905, 1965). A process for making cheese with the protease of *M. pusillus* Lindt has been patented (U.S. Patent 3,212,905). The milk-clotting acid protease of this mold has been isolated and its characteristics have been studied (G. A. Somkuti and F. J. Babel, Bacteriol. Proc., p. 18, 1967). This report describes the conditions of cultivation that are most favorable for the synthesis of the acid protease by *M. pusillus* Lindt under submerged conditions.

MATERIALS AND METHODS

Culture and inoculum. The culture, *M. pusillus* Lindt, was obtained from S. Kristof, Department of Biological Sciences, Purdue University. The mold was maintained on potato malt (Difco) agar slants at 37 C, and transferred weekly. The inoculum used in these studies was prepared by gently washing the lawn from 96-hr-old agar slants with sterile distilled water and centrifuging the suspension at 1,100 × *g* for 15 min in a Servall SS-1 centrifuge. The washing and centrifuging of the mycelia were repeated three times. Finally, the pellet was suspended in sterile distilled water, and various amounts of the suspension, as specified for individual experiments, were used to inoculate the medium for enzyme production.

Medium for enzyme synthesis. A wheat bran medium was used throughout this investigation. In all cases, the inoculated medium was agitated on a New Brunswick model G-25 gyrotary shaker, operating at 240-cycles/min.

Determination of protease activity. Protease activity was measured by two different methods. With method I, which was a modification of Anson's method (1), 1 ml of mycelium-free culture broth was mixed with 4 ml of 1% casein in 0.05 M potassium phosphate buffer (*p*H 6.0), and incubated at 30 C for 4 hr. After the precipitation of protein with an equal volume of 5% trichloroacetic acid, the mixture was filtered through two layers of Whatman no. 42 filter paper, and the absorbance of filtrates was determined at 280 mμ in a Beckman DU spectrophotometer. Method II, essentially that of McDonald and Chen (2), was much more sensitive than method I, and permitted the use of smaller amounts of crude enzyme for assay, and reduction of incubation time at 30 C to 1 hr or less. The preparation of samples for the estimation of protease activity was identical with that of method I. In both methods, a standard curve for tyrosine allowed conversion of absorbancy units to microequivalents of tyrosine solubilized per 5 ml.

Determination of milk-clotting activity. Skim milk was adjusted to *p*H 6.0 and preincubated at 35 C for 30 min. Milk clotting was tested by mixing 1 ml of enzyme with 9 ml of skim milk and incubating at 35 C. Clotting time was recorded in minutes.

RESULTS AND DISCUSSION

Effect of wheat bran concentration. Wheat bran in varying concentrations was suspended in distilled water and, without adjustment of *p*H (6.0), dispensed in 30-ml amounts in 125-ml Erlenmeyer flasks. The medium was sterilized at 121 C for 20 min. After cooling, the equivalent of 0.4 mg (dry weight) of mycelia was added to each flask. The medium was agitated at 34 C for 72 hr.

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Mycelia and wheat bran flakes were eliminated by centrifugation, and protease activity was estimated by method I.

The concentration of wheat bran in the medium influenced protease synthesis by *M. pusillus* Lindt. Results in Fig. 1 show that, at 72 hr, the greatest gain in activity occurred when wheat bran concentration was increased from 0.5 to 1.0%. Concentrations of wheat bran greater than 2% gave further increases in protease activity, but the increases were small. Since wheat bran absorbs considerable moisture, it is difficult to work with a concentration greater than 4%.

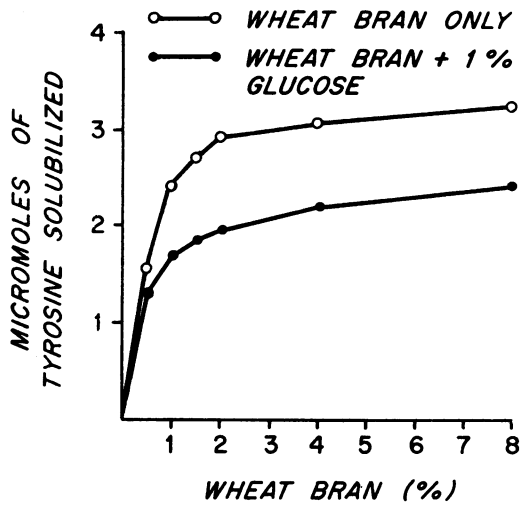

FIG. 1. *Effect of wheat bran concentration on protease production in the absence and presence of glucose.*

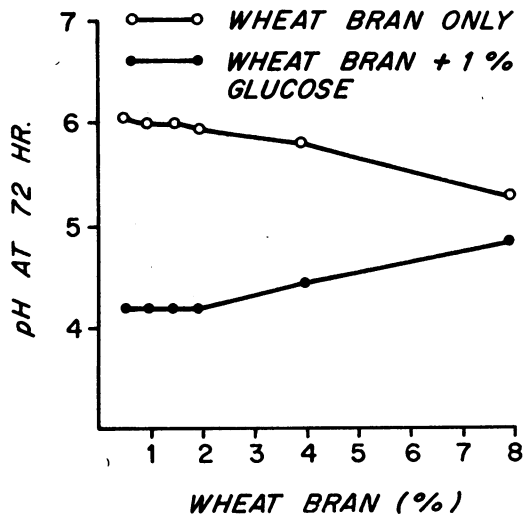

FIG. 2. *Effect of wheat bran concentration on the pH of 72-hr-old cultures grown in the absence and presence of glucose.*

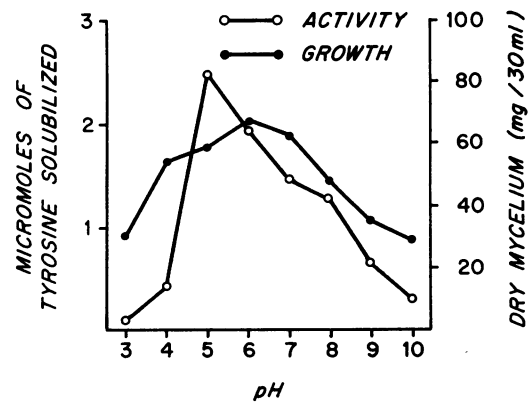

FIG. 3. *Effect of initial pH of wheat bran medium on protease yields after 72-hr incubation, 34 C.*

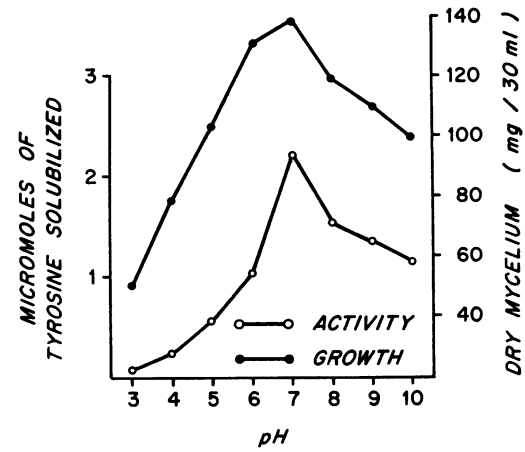

FIG. 4. *Effect of initial pH of glucose-supplemented wheat bran medium on protease yields after 72-hr incubation, 34 C.*

Enrichment of wheat bran medium with glucose. Experimental details were identical with those described in the preceding section except that the medium contained 1% glucose.

The supplementation of the medium with 1% glucose resulted in a substantial loss of protease activity at concentrations of wheat bran greater than 1% (Fig. 1). At 72 hr, the *p*H values of the media supplemented with glucose were much lower than that of the control (Fig. 2). It is very likely that the mold dissimilated the readily available glucose either through what may be called the classical pathways of carbohydrate metabolism or by direct oxidation, and the organic acids produced as end products unfavorably altered the *p*H to a value not conducive to maximal protease synthesis.

Effect of initial pH on protease synthesis and

mycelial growth. A 4% (dry weight) wheat bran suspension was steamed for 1 hr. After cooling, the slurry was centrifuged at 1,500 × *g* for 15 min, and the supernatant fluid was divided into several lots. Each lot was adjusted to the desired *p*H with HCl or NaOH, and 30-ml amounts were dispensed in 125-ml Erlenmeyer flasks. After sterilization and cooling, each flask was inoculated with the equivalent of 0.4 mg (dry weight) of mycelia. Flasks were agitated at 34 C for 72 hr. Protease activity of the mycelium-free broth was estimated according to method I. Mycelial weight was determined by centrifuging the flask contents at 2,000 × *g* for 20 min. The packed mycelia were washed with distilled water and recentrifuged; this step was repeated three times. The mycelia were finally placed in preweighed aluminum dishes, dried at 90 C in a vacuum oven for 48 hr, and weighed. The average weight of four replicates is reported.

The initial *p*H of the medium had a profound effect on protease synthesis by *M. pusillus* Lindt

TABLE 1. *Milk-clotting activity of 72-hr-old cultures of Mucor pusillus Lindt grown in unsupplemented wheat bran medium*

Initial *p*H of medium	Clotting time (min)
3.0	None in 180
4.0	30
5.0	>1
6.0	1
7.0	7
8.0	60
9.0	None in 180
10.0	None in 180

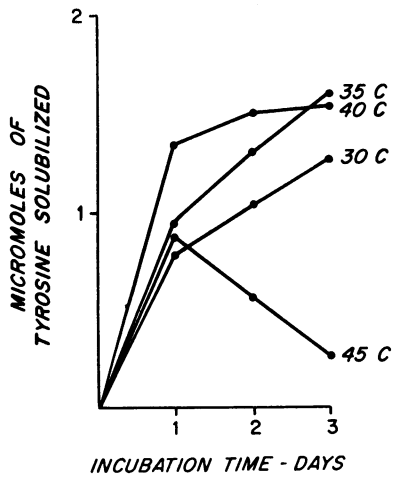

FIG. 5. *Effect of incubation temperature on protease synthesis.*

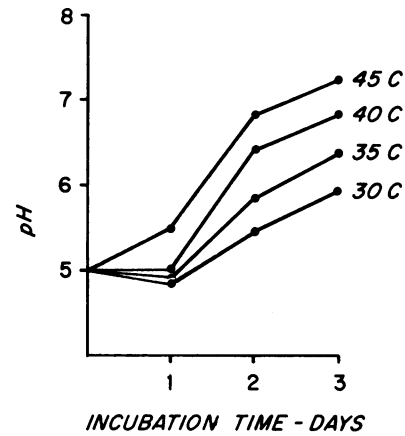

FIG. 6. *Effect of incubation temperature on changes in pH during protease synthesis.*

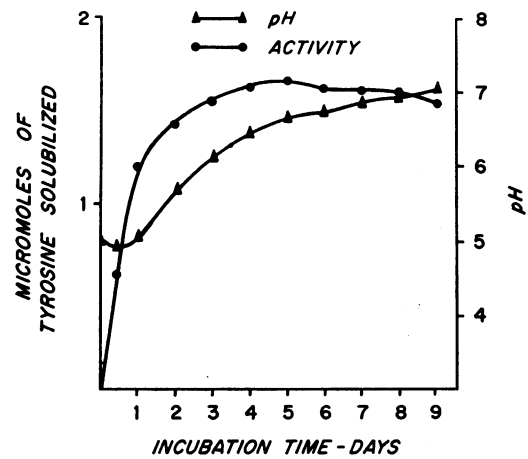

FIG. 7. *Protease synthesis by Mucor pusillus Lindt under optimal conditions as the function of time.*

(Fig. 3). After 72 hr of incubation, maximal enzyme titers were detected in flasks that had been adjusted to *p*H 5.0 prior to sterilization. The largest amount of mycelia was produced at an initial *p*H of 6.0. The enrichment of the medium with 1% glucose resulted in an approximately twofold increase in the maximal mycelial yield (initial *p*H 7.0), whereas the initial *p*H required for optimal enzyme synthesis shifted from *p*H 5.0 to 7.0 (Fig. 4). After 72 hr, the *p*H values in the media not containing and containing glucose, initially adjusted to *p*H 5.0, were *p*H 5.5 to 6.1 and *p*H 3.9 to 4.2, respectively, whereas the corresponding values for media initially adjusted to *p*H 7.0 were *p*H 6.5 to 6.8 and *p*H 5.5 to 5.7. It is believed that the higher initial *p*H of the glucose-containing medium compensated for the *p*H-lowering effect of the acid end products of glucose

metabolism. In the glucose-enriched medium, the maximal protease activity was about 15% less than that attained in unsupplemented wheat bran medium.

The milk-clotting activity of culture filtrates at 72 hr also showed that an initial *p*H of 5.0 was the most desirable for protease synthesis (Table 1).

Effect of incubation temperature. The enzyme production medium was prepared as described previously. The medium was adjusted to *p*H 5.0 with 1 N HCl, and 200-ml amounts were dispensed in 1,000-ml Erlenmeyer flasks. Each flask was inoculated with the equivalent of 1.9 mg (dry weight) of mycelia, and incubated at 30, 35, 40, and 45 C. After 24, 48, and 72 hr, 10-ml samples were withdrawn aseptically, filtered through Whatman no. 42 filter paper, diluted 1:2 with 0.1 M acetate buffer (*p*H 5.45), and tested for protease activity by method II. Values reported are averages of four replicate determinations. A temperature of 35 C provided the best results (Fig. 5). Although a greater initial increase (at 24 hr) in protease activity was detected when incubation was at 40 C, the activity at 72 hr failed to reach the level of the flasks incubated at 35 C. With an incubation temperature of 45 C, there was considerable enzyme production early in the incubation period, followed by a drastic decline in activity of the culture filtrate. The loss in activity may have been the result of the prolonged exposure of the enzyme to the relatively high incubation temperature or the substantially higher *p*H of the medium (Fig. 6).

Protease synthesis as the function of time. The medium was prepared as described previously, adjusted to *p*H 5.0 with 1 N HCl, dispensed in 350-ml amounts in 2-liter Erlenmeyer flasks, and sterilized. Each flask was inoculated with the equivalent of 4.2 mg (dry weight) of mycelia. The flasks were agitated at 35 C. At 12 hr, 24 hr, and every 24 hr thereafter, 10-ml samples were withdrawn aseptically, filtered through Whatman no. 42 filter paper, and diluted 1:5 with 0.1 M acetate buffer (*p*H 5.45). Protease activity was measured according to method II.

The synthesis of the milk-clotting protease under optimal conditions, i.e., initial *p*H 5.0, 35 C incubation temperature and 4% wheat bran medium, was followed for a period of 9 days (Fig. 7). Maximal enzyme activity was detected after 5 days of incubation, followed by a slight decrease in activity during the subsequent days. During the incubation period, the *p*H of the medium decreased slightly in 12 hr and then increased to about neutrality.

In summary, the results indicate that the production of the milk-clotting acid protease of *M. pusillus* Lindt, in quantities required for the preparation of experimental cheese, or for further studies of the enzyme, may be carried out most satisfactorily in a 4% wheat bran medium initially adjusted to near *p*H 5.0, with incubation at 35 C for 4 to 5 days.

ACKNOWLEDGMENT

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