

Acid Protease Production by Fungi Used in Soybean Food Fermentation

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Growth conditions for maximum protease production by *Rhizopus oligosporus*, *Mucor dispersus*, and *Actinomucor elegans*, used in Oriental food fermentations, were investigated. Enzyme yields by all three fungi were higher in solid substrate fermentations than in submerged culture. The level of moisture in solid substrate must be at about 50 to 60%. Very little growth of these fungi was noted when the moisture of substrate was below 35%, whereas many fungi including most storage fungi generally grow well on solid substrate with that level of moisture. Among the three substrates tested—wheat bran, wheat, and soybeans—wheat bran was the most satisfactory one for enzyme production. The optimal conditions for maximum enzyme production of the three fungi grown on wheat bran were: *R. oligosporus*, 50% moisture at 25 C for 3 to 4 days; *M. dispersus*, 50 to 63% moisture at 25 C for 3 to 4 days; *A. elegans*, 50 to 63% moisture at 20 C for 3 days. Because these fungi are fast growing and require high moisture for growth and for enzyme synthesis, the danger of contamination by toxin-producing fungi would be minimal.

Aspergillus, *Rhizopus*, *Mucor*, and *Actinomucor* have long been used in Oriental food fermentations (3). Extensive studies have been made on soy sauce and miso fermentations carried out by *Aspergilli*, but not on those food processes involving *Rhizopus*, *Mucor*, and *Actinomucor*. During the last decade, pure culture fermentation methods for making tempeh from soybeans by *Rhizopus oligosporus* (2, 8) and sufu from soybeans by *Mucor dispersus* and *Actinomucor elegans* (12) were developed. We have also expanded our effort to explore the enzymes produced by these fungi, partly to find out more about the role of such enzymes in these fermentation processes and partly to discover commercially useful enzymes.

Our previous studies revealed that *R. oligosporus* (14), *M. dispersus* (13), and *A. elegans* (unpublished data) all produce acid type proteases, whereas most fungal proteases have a pH optimum around neutral or alkaline. Although these fungi grew abundantly in submerged culture containing soybeans, wheat, or wheat bran, their enzyme yields were unsatisfactory. The production of proteases by *R. oligosporus* decreased as the concentration of substrate increased (16). The protease produced by *M. dispersus* NRRL 3103 (formerly *M. hiemalis*) was bound to the mycelial surface (13). Addition of sodium chloride or other

ionizable salts in the growth medium for this fungus increased the enzyme in the culture filtrate. The total enzyme yield, however, was limited because fungal growth was suppressed by the added salt.

The low enzyme production by these fungi in submerged culture was not unexpected, because fermentation processes carried out by these fungi are usually in solid state. Before World War II, solid state fermentation, generally known as the "bran process," was almost universally employed for the production of fungal enzymes. Deep-tank fermentation, however, has since replaced this technique in the West. Success in obtaining high yields of secondary metabolites by some fungi on solid substrates (4) and difficulties encountered in increasing enzyme yields of the fungi under investigation in submerged culture prompted us to return to our work with solid substrates. The present study was undertaken to find a set of conditions for protease production by *R. oligosporus*, *M. dispersus*, and *A. elegans* on solid substrate.

MATERIALS AND METHODS

Cultures. *R. oligosporus* NRRL 2710, *M. dispersus* NRRL 3103, and *A. elegans* NRRL 3104 were maintained on slants of potato-dextrose agar at 4 C. Before each experiment, the organisms were transferred to slants that then were incubated at 25 C for 7 days.

Spore suspensions for inoculation were prepared by adding 3 ml of sterilized distilled water to each slant and vigorously shaking the culture for 1 min.

Fermentation. Each 300-ml Erlenmeyer flask containing 10 g of wheat bran and 4, 8, or 15 ml of water was mixed and allowed to stand for 1 h at room temperature with frequent shaking. The flasks were autoclaved at 120 C for 20 min, cooled, and inoculated with 0.2 ml of inoculum. The cotton plugs were covered with aluminum foil to prevent evaporation, and flasks were incubated stationary at 15, 20, 25, or 32 C for varying lengths of time. The fermentation mass was extracted with 100 ml of 2% sodium chloride solution at room temperature for 1 h with frequent stirring, followed by centrifugation. The supernatant was used as the source of protease. Two experiments were carried out for each set of conditions. The average values of two runs will be presented.

Assay of proteolytic activity. Proteolytic activity was measured according to the hemoglobin digestion method described by Anson (1). Reaction mixture containing 1 ml of 1% denatured hemoglobin in 0.05 M citrate buffer of pH 2.5 and 1 ml of properly diluted culture extract was incubated at 38 C for 20 min. The reaction was stopped by the addition of 3 ml of 5% trichloroacetic acid. The undigested hemoglobin was removed by filtration, and the acid-soluble products were determined spectrophotometrically at 280 nm. One unit of protease is defined as the amount of enzyme that yields a change in optical density at 280

nm equivalent to 1 μ mol of tyrosine per h at 38 C.

RESULTS

Moisture content of wheat bran medium.

The initial moisture content of autoclaved wheat bran media containing 10 g of bran and 4, 8, or 15 ml of water was determined by drying at 110 C for 24 h. The average values obtained from three experiments were 35, 50, and 63%, respectively.

Visual growth as affected by incubation temperature and moisture of wheat bran. Quantitative determination of growth on solid substrates, such as wheat bran, is difficult. Therefore, only subjective observations on growth are presented in Table 1.

Of the three wheat bran media studied, the medium having the lowest moisture, 35%, did not support good growth of *R. oligosporus* or *A. elegans* at any of the temperatures investigated, although growth usually was noticeable after 2 weeks of incubation. *M. dispersus* grew fairly well on wheat bran of 35% moisture at 25 and 32 C, but it grew slower at 20 C. On the other hand, wheat bran media containing 50 and 63% moisture provided all three fungi with an environment for luxuriant growth. Rates of growth,

TABLE 1. Effect of incubation temperature, time, and percentage moisture of wheat bran on visual growth^a of fungi

Days	15 C			20 C			25 C			32 C		
	35%	50%	63%	35%	50%	63%	35%	50%	63%	35%	50%	63%
<i>R. oligosporus</i> NRRL 2710												
1				-	-	-	-	-	T	-	++	+++
2				-	T	T	-	++	+++	-	+++	++++
3				-	T	+	-	+++	+++	-	++++	++++
4				-	++	++	-	+++	+++	+	++++	++++
7				-	+++	+++	-	+++	++++	+	++++	++++
14				T	+++	+++	T	++++	++++	+	++++	++++
<i>M. dispersus</i> NRRL 3103												
1				-	-	T	-	T	+	-	+	+
2				-	T	++	T	++	++	T	+	++
3				-	++	+++	+	++++	++++	++	++++	+++
4				-	+++	++++	++	++++	++++	++	++++	++++
7				+	+++	++++	++	++++	++++	+++	++++	++++
14				+++	++++	++++	+++	++++	++++	+++	++++	++++
<i>A. elegans</i> NRRL 3104												
1	-	-	-	-	-	T	-	T	+			
2	-	T	+	-	++	++++	-	++	++++			
3	-	+	++	-	++	++++	-	++	++++			
4	-	+	+++	-	+++	++++	-	++	++++			
7	-	+	+++	-	+++	++++	-	++	++++			
14	+	+++	++++	+	+++	++++	+	++	++++			

^a Symbols: -, no visible growth; T, trace of visible growth; +, scattered colonies; ++, mycelial growth almost covered the surface; +++, luxuriant growth covered the entire surface; and +++, heavy growth filled the flask.

however, were affected by the incubation temperatures and also varied between the three fungi.

R. oligosporus grew rapidly on moist media of 50 and 63% moisture. Abundant growth was observed after 1 day at 32 C, 2 days at 25 C, and 4 days at 20 C. *M. dispersus* did not grow as rapidly as *R. oligosporus* at the highest temperature, 32 C, but it surpassed the growth of *R. oligosporus* at the lower temperature of 20 C. *A. elegans* also grew well at low temperature, and it preferred to grow on the medium with 63% moisture.

Production of acid protease by *R. oligosporus* NRRL 2710. The amounts of acid protease produced by *R. oligosporus* grown on wheat bran containing 50 and 63% moisture and at three different temperatures are summarized in Fig. 1. The data on enzyme production by the fungus on 35% moisture bran are not presented, because the activities were low throughout the 2 weeks of incubation.

As indicated in Fig. 1, enzyme activities reached a maximum after 2 to 3 days at 32 C, 3 to 4 days at 25 C, and 5 to 7 days at 20 C. After that, the activities decreased. The rate of inactivation, however, seemed to be slower at 20 C than at the other two temperatures. A large shift in culture pH values was noted. An initial pH value of 5.7 usually rose to above 7 as

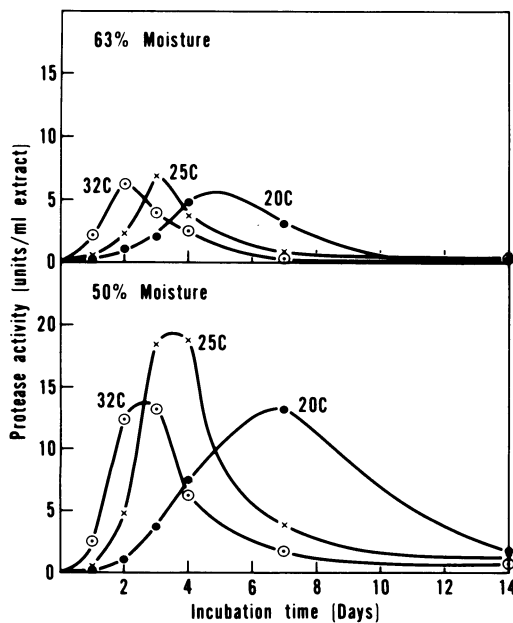


FIG. 1. Acid protease production by *R. oligosporus* as related to incubation time, temperature, and moisture content of wheat bran. All values are the average of two separate experiments.

enzyme production reached its maximum and continued to rise gradually. Although the fungus appeared to grow as well on wheat bran containing 63% moisture as on that of 50%, the amount of enzyme recovered from media of 50% moisture was much greater than that from media of 63% moisture. Under the conditions investigated, *R. oligosporus* grown on wheat bran of 50% moisture for 3 to 4 days at 25 C yielded the highest amount of protease. Our results also indicated that the moisture content of the medium was a more important factor than incubation temperature.

Production of acid protease by *M. dispersus* NRRL 3103. As stated before, *M. dispersus* grew fairly well on bran containing 35% moisture; the amount of enzyme produced, however, was insignificant during the 2 weeks of incubation. Thus, only the results obtained from the growth media containing 50 and 63% moisture are presented in Fig. 2.

When *M. dispersus* was grown on either 50 or 63% moisture bran, it produced equally impressive amounts of enzyme at growth temperatures of 20 or 25 C, but significantly less at 32 C. Production of enzyme by the fungus grown on 50% moisture bran reached a steady maximum after 4 to 6 days at 25 C and 7 to 9 days at 20 C,

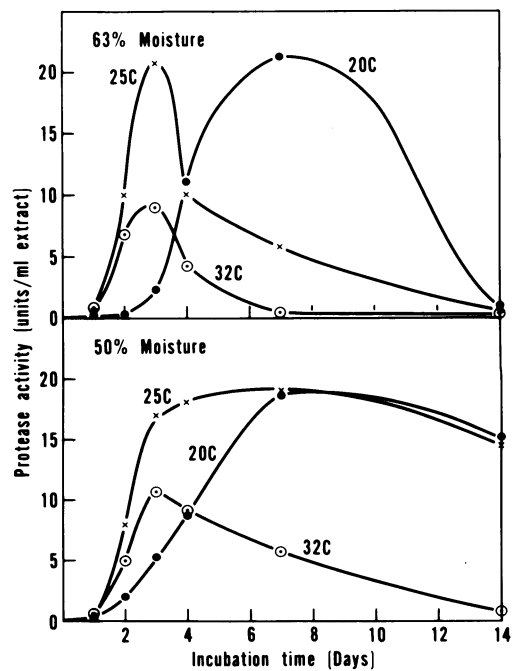


FIG. 2. Acid protease production by *M. dispersus* as related to incubation time, temperature, and moisture content of wheat bran. All values are the average of two separate experiments.

and then gradually decreased. At 63% moisture, the protease yield was maximum at about 3 days at 25 C and 7 to 8 days at 20 C, and then rapidly decreased to about zero. It is apparent that *M. dispersus* can tolerate a broad moisture range for enzyme synthesis. The enzyme, however, was increasingly susceptible to denaturation as the percentage of moisture increased. Therefore, excellent yields of enzyme by *M. dispersus* can be recovered from bran containing 50 to 63% moisture after 3 to 4 days of growth at 25 C, or for longer incubation time at lower temperatures.

The pH of extract from fermented wheat bran at 63% moisture was above 7 after 2 weeks of incubation; whereas at 50% moisture, it was around 6 except when the culture was incubated at 32 C. Here the pH also reached 7 after 2 weeks.

Production of acid protease by *A. elegans* NRRL 3104. Of the three fungi studied, *A. elegans* required the lowest temperature for growth and enzyme production. Like *R. oligosporus* and *M. dispersus*, this fungus did not grow well on 35% moisture bran nor did it produce meaningful amounts of enzyme. However, when the moisture of bran was high enough for good growth, the organism showed a marked degree of moisture tolerance for enzyme production as indicated in Fig. 3. The enzyme yield, on the other hand, was greatly affected by temperature. The fungus grew luxuriantly at 25 C yet produced low enzyme yield. In this respect, *A. elegans* behaved similarly to *M. dispersus*. Unlike *M. dispersus*, a marked pH shift from 5.6 to 7.9 of *A. elegans* culture extracts was observed under all the conditions investigated, and the enzyme decreased rapidly regardless of the moisture content of the media.

Based on this study, the optimal conditions for protease production by *A. elegans* grown on wheat bran were 50 to 63% moisture with 3 days of incubation at 20 C or 4 to 5 days of incubation at 15 C.

Effect of substrate and culture method on protease production. Like wheat bran, soybeans or wheat did not provide a good growth condition for *R. oligosporus*, *M. dispersus*, and *A. elegans* unless the moisture level of these materials was around 50%. The protease yield per unit substrate produced by these fungi grown on solid state and in submerged culture is presented in Table 2. Wheat bran generally was a superior substrate for enzyme production by these fungi regardless of the culture method employed. Solid culture fermentation was a better method than the submerged culture fermentation.

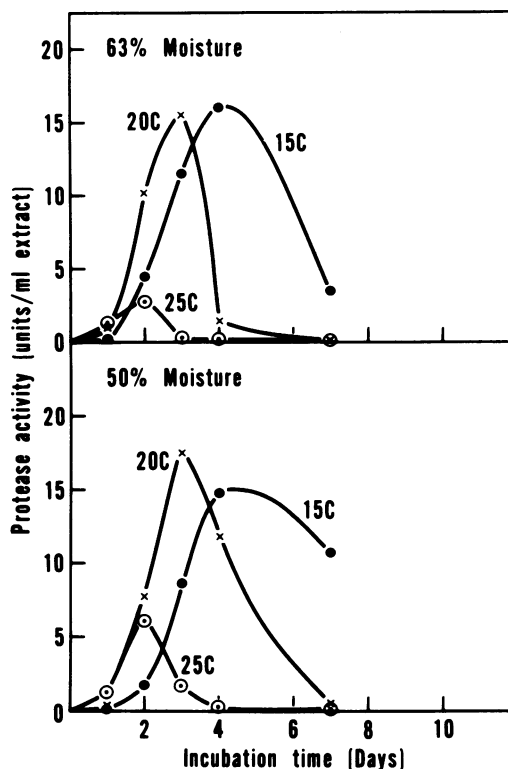


FIG. 3. Acid protease production by *A. elegans* as related to incubation time, temperature, and moisture content of wheat bran. All values are the average of two separate experiments.

DISCUSSION

Many members of the order Mucorales are known to be hydrophytes. They require a relative humidity of 90% or greater for growth and grow best at humidities near 100%. However, little is known regarding the relationship between their growth conditions and enzyme yields. The three fungi investigated in this study, representing three genera in Mucorales, were found to be hydrophytes and fast-growing. *R. oligosporus* is used for tempeh fermentation. *M. dispersus* and *A. elegans* are used for sufu fermentation. The substrates for these fermentation processes have moisture contents of about 55 and 80%, respectively. Therefore, the high moisture requirement by those fungi for growth is expected.

The relationship between enzyme yield and the three environmental factors investigated varied with the organism, but they all yielded greater amounts of enzyme at temperatures lower than their optimum growth temperatures. Similar findings with respect to protease production by other fungi were reported by Max-

TABLE 2. *Protease yield per unit of substrate as affected by substrate and culture method*

Culture	Substrate	Protease yield (U per g of substrate)	
		Solid culture ^a	Submerged culture ^b
<i>R. oligosporus</i> NRRL 2710	Soybeans	25.4	23.0
	Wheat	56.6	45.8
	Wheat bran	154.1	39.4
<i>M. dispersus</i> NRRL 3103	Soybeans	113.8	1.9
	Wheat	28.8	10.7
	Wheat bran	210.7	17.2
<i>A. elegans</i> NRRL 3104	Soybeans	12.1	9.7
	Wheat	8.8	5.2
	Wheat bran	105.1	20.6

^a Solid culture: soybean grits, pearled wheat, and wheat bran of 50% moisture, *R. oligosporus* at 25 C, 3 days; *M. dispersus*, 25 C, 4 days; *A. elegans*, 20 C, 3 days.

^b Submerged culture: 10 g of substrate, soybean meal, wheat flour, and wheat bran in 100 ml of water for *R. oligosporus*, and in 100 ml of 0.5 M NaCl for *M. dispersus* and *A. elegans*. Incubated on a reciprocating shaker at temperature and time same as for solid culture.

well (6) using *Aspergillus oryzae*, and Yamamoto (17) using *Aspergillus sojae*. The effect of temperature on enzyme production observed in this study also emphasized the importance of some common practices in solid culture fermentation, i.e., frequent turning of the growth mass or use of thin layers of solid substrates. Otherwise, the heat resulting from active growth will increase the incubation temperature and affect the enzyme production.

As with most extracellular enzymes produced by many microorganisms, maximum yield by the three fungi studied was usually reached about the time of maximum growth. However, the enzymes were rapidly inactivated except when *M. dispersus* was grown on wheat bran containing 50% moisture at 20 and 25 C. Although many enzymes are often destroyed by protease produced in the same culture, the disappearance of protease observed in this study probably was not due to self-digestion. Previously, we have reported (15) that no degraded products of self-digestion acid protease isolated from culture filtrate of *R. oligosporus* could be detected when the enzyme preparation was incubated at 28 C for 25 h. The inactivation of the protease was explained as being caused by an alkaline shift in pH. Acid proteases

produced by *R. oligosporus* and *M. dispersus* are very unstable as the pH approaches 7 (13, 14).

The rapid disappearance of the proteases limited the harvest time. On the other hand, this trait can benefit the yield and purification processes for other useful enzymes produced by the same organisms.

There are conflicting reports on the importance of aeration for the production of enzymes by microorganisms. Richou and Kourilsky (7) found that, in general, aeration was unfavorable for protease formation by various microorganisms. Our study also suggested that aeration may not be an important factor for enzyme production by the three fungi investigated. We noted that the very moist substrate and dense mycelial growth resulted in a tight mass that restricted aeration.

The finding that a solid substrate method for protease production by these fungi was superior to the commonly used liquid culture method was not unexpected, mainly because these fungi have been traditionally used in solid substrate fermentation. There have been few comparisons by these two methods of enzyme yield per unit substrate. Recently, Tsujisaka et al. (11) found that *Aspergillus niger* produced more lipase on solid medium containing wheat bran and calcium carbonate than in liquid media. It is, however, surprising to find that soybeans were not as good a substrate for protease production as was wheat bran.

The fact that these fungi are fast growing and require high moisture levels to grow and synthesize enzymes might eliminate the danger of contamination by such toxin-producing fungi as *Aspergillus flavus* and *Aspergillus ochraceus*, which have been reported (5, 9, 10) to require low moisture levels (calculated at 33%) for toxin synthesis. This should improve the feasibility of using *Mucor* crude enzyme preparation in food and feed industries.

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