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OPTIMIZATION OF SOLID-STATE FERMENTATION PARAMETERS FOR THE PRODUCTION OF XYLANASE BY *TRICHODERMA LONGIBRACHIATUM* ON WHEAT BRAN

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ABSTRACT. *Solid-state fermentation has the potential to produce inexpensive enzymes for use in high-volume industrial applications. Process parameters such as substrate moisture content and length of fermentation can have a significant effect on the amount and timing of enzyme production. This study was conducted in two stages, a screening stage and an optimization stage, to determine the effects of moisture content of the substrate, surfactant addition upon inoculation, depth of the substrate, and duration of fermentation on xylanase activity produced by Trichoderma longibrachiatum. Screening fermentations were conducted at 25°C, 50 and 75% wet basis moisture content (w.b.), 0.0 and 0.2% v/v surfactant addition, 0.5 and 1.5 cm depth of wheat bran, and 5 and 10 days of fermentation. Optimization fermentations were conducted at 25°C, 45, 55, and 65% moisture content (w.b.), 1.0, 1.5, and 2.0 cm depth of wheat bran, and three and five days of fermentation. Experiments were conducted as full factorial experiments with three replications of each treatment. The optimal values of the process variables were selected based on the units of xylanase activity produced per gram of wheat bran (U/g). Moisture content, depth of substrate, and duration of fermentation had significant main effects on the production of enzyme activity. Surfactant addition upon inoculation had interaction effects with moisture content, and the duration of fermentation by moisture content interaction also was significant. The treatment of 55% moisture content, 1.5 cm depth of substrate, and five days of fermentation resulted in the highest average xylanase activity (716 U/g wheat bran).*

Keywords. *Xylanase, Trichoderma longibrachiatum, Solid-state fermentation, Process parameters, Wheat bran.*

Industrial enzymes are used in numerous areas from food processing to the paper and pulp industry. Xylanase is capable of hydrolyzing the xylosidic linkages in xylan (Jain, 1995). Xylan is a major component of hemicellulose, which is second only to cellulose in abundance in plant matter (Jain, 1995; Biely, 1985). The industrial uses of xylanase includes paper and pulp processing (Biely, 1985), petrochemical production (Jain, 1995), and as a supplement to animal feed (Wong et al., 1988). In paper and pulp processing, xylanase is used in the pre-bleaching of kraft pulps where it reduces the use of chlorine (Viikari et al., 1994). As a supplement to animal feed, xylanase may improve hemicellulose accessibility to ruminal digestion and thus improve the nutritional value of the feed (Wong et al., 1988). Uses of xylanase are expanding to include processing plant fiber sources, such as flax and hemp (Biely, 1985), clarification of juices along with pectinases, and for the preparation of dextrans for use as food thickeners (Wong et al., 1988). The U.S. market for industrial enzymes was an estimated $390.9 million in 1997. By 2000 the market is expected to increase to $465.8 million and $685.2 million by 2006. The industrial enzyme market for paper and pulp processing, with xylanases claimed to be the major enzyme used, is believed to have a growth rate of 15% per year (Wrotnowski, 1997).

The majority of the industrial enzymes on the market are produced by submerged liquid fermentation (SmF). Solid-state fermentation (SSF) is an attractive alternative to SmF because of increased enzyme yield and decreased production costs. Solid-state fermentation refers to the growth of microorganisms on solid materials without the presence of free liquid (Cannel and Moo-Young, 1980). Solid-state fermentation mimics the natural environmental conditions and habitat for some microorganisms (Kim et al., 1985). Filamentous fungi are the most commonly used microorganisms in SSF because they are able to grow on solid materials with low water content (Cannel and Moo-Young, 1980; Pandey, 1992).

The major advantage of SSF over SmF is the potential to produce a larger amount of enzyme at a lower cost. *Aspergillus niger*, for example, can produce 10 times more amyloglucosidase by SSF than SmF at a lower cost (Ghildyal et al., 1985). Solid-state fermentation typically uses agricultural residues such as wheat straw, wheat bran, and rice straw; whereas, SmF requires more expensive media (Biswas et al., 1988, 1990). Other advantages of SSF include simpler product recovery and reduction of bacterial contamination due to low substrate moisture levels (Shuler and Kargi, 1992; Durand and Chereau, 1988).

The production of high enzyme yields using SSF requires optimal environmental conditions to promote maximum growth of the microorganism and increased production of the enzyme. The growing environment includes many variables such as temperature, moisture content (Smits et al., 1996), depth of substrate, and the

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length of time for fermentation (Biswas et al., 1988; Bailey et al., 1992). The productivity of SSF may also depend on additives upon inoculation such as surfactant (Sternberg, 1976; Reese and Maguire, 1971). Aeration and oxygen levels during SSF can also have a significant impact on growth and metabolite production. It is difficult to predict the optimal values for these factors due to the heterogeneity of the system and the interdependency of the factors (Mitchell et al., 1988; Moo-Young et al., 1983). Thus, optimal SSF conditions are currently determined experimentally.

Substrate moisture content is an important factor for both cell growth and enzyme production in SSF (Kim et al., 1985). High moisture contents cause swelling of the substrate and facilitates utilization of the medium by the organism. If conditions are too moist, the substrate porosity decreases which prevents oxygen penetration and promotes bacterial growth, while at very low moisture levels no growth of the organism will occur (Pandey, 1992; Hesseltine, 1972). In the production of cellulase by *Trichoderma reesei* in SSF, cell growth and oxygen consumption rate increased in conjunction with an increase in moisture content from 36% to 65% (Kim et al., 1985). However, the enhancement of enzyme production leveled off after 47% moisture content. A reduction of 30 to 78% in enzyme production was also seen when the moisture content was higher than 65% w.b. in the SSF production of alpha-amylase on wheat bran by *Bacillus licheniformis* M27 (Ramesh and Lonsane, 1990).

Optimal moisture contents for maximum cell growth and for enzyme production may sometimes be different (Kim et al., 1985). The optimal moisture content (w.b.) for xylanase production by *Aspergillus ochraceus* on wheat bran was 55% (Biswas et al., 1988). Xylanase yield by *Aspergillus niger* was optimal at 60% w.b. moisture content on a one to one mixture of straw and wheat bran (Deschamps and Huet, 1985). Maximum cellulase production for *Trichoderma reesei* on wheat bran was found to occur at 47% w.b. moisture content (Kim et al., 1985).

The effect of the depth of the substrate on enzyme production for solid-state fermentation has not been well studied. Hesseltine (1972) reported the depth of substrate which he observed in researching koji preparation (60 cm), and a few authors such as Wiacek-Zychlinska et al. (1994) reported the depth of the substrate in their experiments, but there is a lack of published information on the effects of depth of the substrate on enzyme production. In the examination by Ramana Murthy et al. (1993) of Rathbun and Shuler's (1983) investigation of tray type fermentors, the importance of depth was discussed. Experiments were initially performed on thin beds (0.5 cm) to eliminate heat and mass transfer effects while studying the kinetics of growth because transport effects were observed to be more prominent in deeper beds. It was also noted that in the final stage of fermentation there existed the possibility of new growth in the bottom layers of a bed 6.35-cm deep due to increased gas exchange because of channeling resulting from shrinkage of the top layers of the bed.

Depth of substrate is an important factor in composting (Hansen et al., 1993) which is a form of SSF. The rates of oxygen diffusion and thermal diffusion through a compost pile are affected by depth (Lynch and Cherry, 1996). Depth may also be important in the production of an industrial enzyme by solid-state fermentation. The depth of the substrate can affect the magnitude of temperature and oxygen gradients. The gradients are usually a function of the direction of airflow.

Solid-state fermentation of lignocelluloses, such as wheat straw, corn stover, and aspen wood give higher yields of xylanases than liquid fermentations, but the required times of incubation may be longer (Dubeau et al., 1986). The maximum enzyme production for SSF required a 14-day fermentation for xylanase production by *Aspergillus ochraceus* on wheat bran (Biswas et al., 1988). The yield of xylanase by *Chaetomium cellulolyticum* on wheat straw (Dubeau et al., 1986) and *Penicillium funiculosum* on wheat bran reached a maximum at 10 days (Mishra et al., 1985). *Trichoderma reesei* on sugarcane bagasse had a maximum xylanase production after 130 h or roughly five days (Dekker, 1983) and *Melanocarpus albomyces* IIS-68 on wheat straw and bagasse after four days (Jain, 1995). Growth and enzyme synthesis were maximal after three days of fermentation for *Aspergillus niger* on a variety of substrates (Deschamps and Huet, 1985).

Enzyme production may be affected by adding surfactant to the substrate before beginning fermentation. Increased yields may be related to increased permeability of the cell membrane, allowing for more rapid secretion of enzymes which in turn leads to greater enzyme synthesis (Sternberg, 1976). Surfactant addition increased xylanase production 1.6-fold from *Chaetomium cellulolyticum* in SmF when 0.05% Tween 80 was added to the liquid basal medium with 1% xylan before inoculation (Dubeau et al., 1987). Tween 80 at 0.2% (v/v) added to the cellobiose in SmF before inoculation doubled cellulase yield by *Trichoderma viride* (Reese and Maguire, 1971).

The values of fermentation parameters which maximize enzyme production are highly dependent on the microorganism and the substrate. Smits et al. (1996) found for *Trichoderma reesei* on wheat bran, in a static environment, a maximum yield of xylanase activity of approximately 1100 U/g at a fermentation temperature of 26°C and 96% relative humidity. Determination of the effect of process parameters on the production of xylanase by *Trichoderma longibrachiatum* on wheat bran is needed for further development of this process for industrial scale SSF. The objective of this research was to determine the effects of moisture content, depth of the substrate, surfactant addition upon inoculation, and length of fermentation on production of xylanase using SSF in a statically aerated system.

Materials and Methods

Organism

Trichoderma longibrachiatum was maintained on potato dextrose agar (PDA) slants at 4°C and subcultured at monthly intervals.

Substrate

Wheat bran, bought in bulk from a local retailer, was the SSF culture substrate. The wheat bran was thermally pretreated by sterilization at 121°C for 15 min with steam pressure at 137.8 kPa (20 psi).

Inoculum

Sporulation was heavy on PDA streak plates after seven days incubation at room temperature. The spores were harvested from the streak plate using a flame sterilized loop. The inoculum contained 10^{6-7} spores per milliliter of a magnesium sulfate/potassium phosphate buffer (Biswas et al., 1989; Wiacek-Zychlinska et al., 1994). Spore numbers were determined using a hemacytometer. The suspension, when necessary, was diluted with sterile magnesium sulfate/potassium phosphate buffer to obtain a spore count within the predetermined range. One milliliter of inoculum was added for each 5 g of bran in the container using a sterile pipette and stirred with an aseptic glass rod. Sterile magnesium sulfate/potassium phosphate buffer was mixed with the substrate before sterilization to obtain the desired moisture content in the wheat bran.

Cultivation

Fermentation was carried out in 250 mL working volume (300 mL total volume) polypropylene containers with lids (Nalgene No. 2118-0008). Wheat bran (5 g for 0.5 cm depth, 10 g for the 1.5 cm depth, and 20 g for the 2 cm depth treatment) was added to each container which resulted in a head space between 291 cm^3 (for 0.5 cm depth of bran) and 246 cm^3 (for 2.0 cm depth of bran). Once inoculated, the sealed containers (statically aerated conditions) were placed in an environment (constant humidity, constant temperature chamber—CHCT) maintained at 80% relative humidity and at 25°C.

Extraction

Enzyme was extracted by adding 100 mL of a 0.1% v/v solution of Tween 80 and deionized (DI) water to each container for every 5 g of substrate. The contents of the containers were stirred and the containers were placed in an oscillatory shaker for 30 min at 25°C and 200 rpm. After shaking, the contents of each container were vacuum filtered on 90-mm glass fiber filter paper. The extractant was centrifuged at 900 g for 30 min. Subsequently, the supernatant was decanted using a sterile pipette, placed in 2-mL plastic vials, and frozen at –13°C until assayed. Vials were stored approximately seven days before assaying. This method of storage was checked by comparing frozen samples to fresh samples and no evidence of decreased enzyme activity was observed.

Assay

Total reducing sugars were determined using the method reported by Bailey et al. (1992). A xylan suspension was made by suspending 1 g birchwood xylan (Sigma No. 232-760-6) in 80 mL of sodium citrate buffer, pH 5.3, 60°C and heating until boiling. The suspension was mixed overnight and brought to a volume of 100 mL. From the 10 g/L xyaln suspension, 1.8 mL was taken, pre-heated at 50°C and incubated for exactly 5 min with 0.2 mL suitably diluted enzyme. The reaction was stopped by adding dinitrosalicyclic acid (DNS). Color was measured spectrophotomically at 540 nm and compared to a standard curve (Bailey et al., 1992).

Enzyme activities were expressed in international units (U), where one unit is the amount of enzyme that liberates one micromole of product (xylose, determined as reducing sugar) per minute under assay conditions. The calculation used is as follows:

$$\frac{\mu\text{mole}}{\text{mL enzyme}} = \left[(\text{absorbance} - \text{blank@540 nm}) \times (\text{slope std curve}) \right] \times (\text{dilution factor}) \quad (1)$$

$$\frac{\text{U}}{\text{gram bran}} = \frac{\mu\text{mole}}{\text{mL enzyme}} \times \frac{1}{\text{time of reaction}} \times \frac{100\ \text{mL}}{5\ \text{g dry bran}} \quad (2)$$

Experimental Design and Statistical Analysis

In each of two screening experiments, 24 containers, allowing for three replications, were randomly assigned to eight treatments varying the factors of moisture content of the substrate, surfactant addition upon inoculation, depth of the substrate, and length of fermentation. Experimental factor levels (table 1) for these experiments were chosen from reviewing similar systems in the literature. The first screening experiment investigated the effects of moisture content of the substrate, surfactant addition upon inoculation, and length of fermentation. The second screening experiment studied the effects of moisture content, depth of the substrate, and length of fermentation. Containers were placed in the CHCT chamber at random locations. After specified lengths of time, containers were removed from the CHCT chamber, the enzyme was extracted, and enzyme activity (U/g) was determined for each container.

Based on the results of the screening experiment, treatment variables and levels were selected for an optimization experiment (table 1). In the optimization experiment, 54 containers, allowing for three replications, were randomly assigned to 18 treatments varying the factors of moisture content of the substrate, depth of the substrate, and length of fermentation. Containers were placed in the CHCT chamber at random locations. After specified lengths of time, containers were removed from the CHCT chamber, the enzyme was extracted, and enzyme activity (U/g) was determined for each container.

Data were statistically analyzed using a completely randomized full factorial treatment structure for each set of experiments using SAS®. Containers with no visible growth were considered to be outliers and were excluded from analysis. Xylanase production is known to be growth-associated for *Trichoderma longibrachiatum*, and at three days of fermentation *Trichoderma longibrachiatum* growth is observable to the naked eye. Therefore, containers with

Table 1. Experimental factor levels for the three full factorial experiments used to determine optimal process parameters for the production of xylanase activity by *Trichoderma longibrachiatum* on wheat bran

	Experiment One	Experiment Two	Optimization Experiment
Moisture content (%)	50, 75	50, 75	45, 55, 65
Surfactant addition (% v/v)	0.0, 0.2	0	0
Depth of substrate (cm)	0.5	0.5, 1.5	1.0, 1.5, 2.0
Duration of fermentation (days)	5, 10	5, 10	3, 5

no visible growth were assumed to be the result of experimental error (e.g., no inoculum added) and not due to treatment effects.

Results and Discussion

The treatment means are presented in table 2 for a screening experiment investigating the effects of moisture content of the substrate, surfactant addition upon inoculation, and length of fermentation. An analysis of variance (ANOVA) for this experiment is presented in table 3.

Two containers (one at 75% moisture content, 0% v/v surfactant addition and five days of fermentation, and the other at 75% moisture content, 0.2% v/v surfactant addition and five days of fermentation) were not included in the analysis because there was no visible growth of the organism at harvest.

Moisture content was the only main treatment effect that was significant. Initial moisture content of 50% resulted in a 40% increase in average xylanase activity over the 75% initial moisture content treatment. A possible interaction between moisture content and surfactant addition was observed. For the 75% initial moisture content treatment, surfactant addition increased xylanase production compared to the 75% initial moisture content treatment with no surfactant when averaged over the fermentation duration. This increase in xylanase production due to surfactant was not observed for the 50% moisture content treatment. Since 50% moisture content produced much more xylanase than the 75% moisture content treatment, investigation into surfactant addition was not pursued.

The treatment means are presented in table 4 for the screening experiment evaluating the effects of moisture content of the substrate, depth of the substrate, and duration of fermentation. An analysis of variance (ANOVA) for these data is presented in table 5. One container (50% moisture content, 1.5 cm depth, and five days of fermentation) was dropped from the analysis because there was no growth of the organism. Moisture content and depth of the substrate had highly significant effects on production of enzyme activity. As in the first

Table 2. Treatment data for the full factorial experiment evaluating the effects of moisture content of substrate (% wet basis), surfactant addition upon inoculation (% v/v), and duration of fermentation (days) on xylanase production (U/g wheat bran) by *Trichoderma longibrachiatum* on 0.5 cm wheat bran at a temperature of 25°C

				Moisture Content Main Effect Avg (U/g)
Duration of fermentation (days)		5		
Surfactant add. (% v/v)		0.0	0.2	
Moisture Content (%)	50	389 415 426	448 409 395	414
	75	247 78 -	323 351 -	250
Surfactant main effect avg (U/g)		311	385	
Duration of fermentation (days)		10		
Surfactant add. (% v/v)		0.0	0.2	
Moisture Content (%)	50	609 377 576	606 249 296	452
	75	129 494 369	292 473 458	369
Surfactant main effect avg (U/g)		426	396	

Table 3. ANOVA for the full factorial experiment evaluating the effects of moisture content of substrate (% wet basis), surfactant addition upon inoculation (% v/v), and duration of fermentation (days) on xylanase production (U/g wheat bran) by *Trichoderma longibrachiatum* on 0.5 cm of wheat bran at a temperature of 25°C

Source of Variation	df	Mean Square	F	Pr > F
Moisture content	1	67833	4.47	0.0530
Surfactant addition	1	1658	0.11	0.7460
Duration of fermentation	1	29942	1.97	0.1821
Moisture × surfactant	1	44592	2.94	0.1087
Moisture × duration	1	8730	0.57	0.4610
Surfactant × duration	1	20597	1.36	0.2637
Moisture × surfactant × duration	1	731	0.05	0.8295
Error	14	15190		

Table 4. Treatment data for the full factorial experiment evaluating the effects of moisture content of the substrate (% wet basis), depth of the substrate (cm), and length of fermentation (days) for xylanase production (U/g wheat bran) by *Trichoderma longibrachiatum* on wheat bran at 25°C and no surfactant

				Moisture Content Main Effect Avg (U/g)
Duration of fermentation (days)		5		
Depth (cm)		0.5	1.5	
Moisture Content (%)	50	350 488 400	489 544 -	454
	75	273 287 354	583 442 468	401
Depth main effect avg (U/g)		359	505	
Duration of fermentation (days)		10		
Depth (cm)		0.5	1.5	
Moisture Content (%)	50	321 352 301	634 559 842	502
	75	272 230 279	362 261 391	299
Depth main effect avg (U/g)		293	508	

Table 5. ANOVA for the full factorial experiment evaluating the effects of moisture content of the substrate (% wet basis), depth of the substrate (cm), and duration of fermentation (days) for xylanase production (U/g wheat bran) by *Trichoderma longibrachiatum* on wheat bran at 25°C with no surfactant

Source of Variation	df	Mean Square	F	Pr > F
Moisture content	1	96743	17.85	0.0007
Depth	1	201401	37.15	0.0001
Duration of fermentation	1	9109	1.68	0.2145
Moisture × depth	1	18479	3.41	0.0847
Moisture × duration	1	22217	4.10	0.0611
Depth × duration	1	4524	0.83	0.3754
Moisture × depth × duration	1	47064	8.68	0.0100
Error	15	5421		

experiment enzyme production by *Trichoderma longibrachiatum* at 50% initial moisture content produced 38% more xylanase activity than 75% initial moisture content. A substrate depth of 1.5 cm resulted in a 56% higher enzyme yield compared to a 0.5 cm depth. Five days versus 10 days fermentation did not have a significant effect on enzyme activity.

The xylanase activity means for the interaction between moisture content and depth, averaged over duration of fermentation, were plotted to investigate the interaction. At 0.5 cm depth of substrate, enzyme activity produced decreased 23% going from 50% to 75% moisture content. Xylanase activity produced at 1.5 cm depth of substrate decreased 30% going from 50% to 75% moisture content. This decrease in production at a higher moisture content may be due to pore space being occupied by water rather than oxygen. At a shallow depth, growth is not hindered as much since there is a higher percentage of substrate exposed to air.

To investigate the significant interaction between moisture content and duration of fermentation, averaged over depth of substrate, the produced xylanase activity means were plotted. Enzyme activity at five days decreased 40% going from 50% to 75% moisture content. There was a small decrease (14%) in xylanase activity at 10 days going from 50% to 75% moisture content. One possible explanation for the smaller decrease in produced activity at 10 days is that a longer duration allowed the fungal biomass in the 75% moisture content treatment to approach that of the 50% moisture content treatment, although microbial biomass was not measured. It is assumed that the growth rate of the microorganism is lower at 75% moisture content than at 50%, and since xylanase production is growth-associated, five days of fermentation was not sufficient time for the effects of 75% initial moisture content to be overcome.

The three-way interaction between moisture content of the substrate, depth of the substrate, and duration of fermentation was also investigated. Depth of substrate effects were more sensitive to initial moisture content than duration of fermentation. The effects of moisture content were more apparent in the deep layer than the shallow and the deep layer had higher production of xylanase activity. Longer times of fermentation were more sensitive to depth of substrate; whereas, shorter times of fermentation were affected more by initial moisture content.

The reader may note that the first columns in tables 2 and 4 represent identical treatment combinations, however the results were different for some conditions. The difference is most probably due to slight variations in the microorganism used. The spore inocula for the two experiments were obtained from different plates of the same culture.

Because of the significance of the main effects of moisture content and depth of substrate on the amount of enzyme activity in the screening experiments, these factors were included in the optimization experiment. The significant effect of duration of fermentation, as reported in the reviewed literature, was not seen in these experiments at five and 10 days. It was hypothesized that the effect of duration of fermentation on the production of enzyme activity occurred before the five-day sampling period because five- and 10-day samplings were in the stationary phase of the microbial growth curve. Therefore, three and five days of fermentation were investigated in the optimization experiment.

The treatment means are presented in table 6 for the optimization experiment evaluating the effects of moisture content of the substrate, depth of the substrate, and duration of fermentation. An analysis of variance (ANOVA) for this experiment is presented in table 7. Data from two containers (one at 45% moisture content, 1.0 cm depth, and five days of fermentation, and the other at 65% moisture content, 1.0 cm depth, and three days of fermentation) were not included in the analysis because there was no growth of the organism.

The main effects of moisture content, depth of substrate, and duration of fermentation were significant. Orthogonal

Table 6. Treatment data for optimization experiment evaluating the effects of moisture content of the substrate (% wet basis), depth of the substrate (cm), and duration of fermentation (days) for xylanase production (U/g wheat bran) by *Trichoderma longibrachiatum* on wheat bran at 25°C with no surfactant

					Moisture Content Main Effect Avg (U/g)
Duration of fermentation (days)			3		
Depth (cm)		1.0	1.5	2.0	
Moisture Content (%)	45	77	118	228	
		84	236	164	137
		139	75	116	
	55	27	398	158	
		90	144	52	143
		76	247	103	
	65	121	218	114	
		122	249	85	150
		-	294	24	
Depth main effect avg (U/g)		129	219	116	
Duration of fermentation (days)			5		
Depth (cm)		1.0	1.5	2.0	
Moisture Content (%)	45	306	482	438	
		544	599	521	524
		-	494	804	
	55	357	720	846	
		740	682	831	624
		395	745	304	
	65	349	370	84	
		255	233	272	256
		134	289	319	
Depth main effect avg (U/g)		385	513	491	

Table 7. ANOVA for the optimization experiment evaluating the effects of moisture content of the substrate (% wet basis), depth of the substrate (cm), and duration of fermentation (days) for xylanase production (U/g wheat bran) by *Trichoderma longibrachiatum* on wheat bran at 25°C

Source of Variation	df	Mean Square	F	Pr > F
Moisture content	2	138464	8.96	0.0008
Depth	2	74795	4.84	0.0142
Duration of fermentation	1	1381244	89.34	0.0001
Moisture × depth	4	18432	1.19	0.3319
Moisture × duration	2	162180	10.49	0.0003
Depth × duration	2	9930	0.64	0.5323
Moisture × depth × duration	4	2074	0.13	0.9687
Error	34	15460		

contrasts were performed using SAS to determine which treatments differed significantly for treatments of moisture content and depth of the substrate. The contrasts showed a significant difference between 55 and 65% and 45 and 65% moisture content, but no statistically significant difference between enzyme yield at 45 and 55% initial moisture content. Both the linear and quadratic orthogonal comparisons were significant, indicating the response of enzyme activity to moisture content (fig. 1) is nonlinear.

The response of produced enzyme activity to substrate depth was more complicated. Treatment means from 1.0 cm and 1.5 cm depth differed significantly, however no other pair showed a statistical difference. The means for depth main effects averaged over moisture content and length of fermentation were 239, 366, and 303 U/g, respectively, by increasing depth of substrate. The linear effect of substrate depth was not statistically significant over this range, but the quadratic response was highly significant.

The xylanase activity means for the interaction between initial moisture content and duration of fermentation, averaged over depth of substrate, were plotted to investigate the statistically significant interaction. There were no differences in the xylanase activity produced as a function of initial moisture content of the substrate for day 3. This may be due to insufficient time for the microorganism to increase in biomass and reflect the effect of initial substrate moisture content. For day 5, there were differences among the means with 55% moisture content having a 16% higher yield than the 45% moisture content and an 59% higher yield than the 65% moisture content.

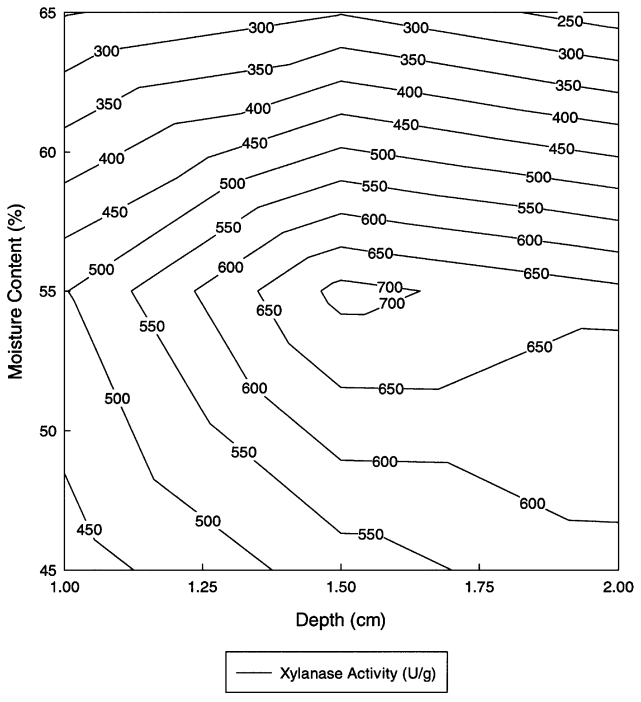

Figure 1–Contour plot with lines of constant enzyme activity for five-day SSF showing the effects of moisture content and depth of substrate on xylanase activity produced by *Trichoderma longibrachiatum* on wheat bran at 25°C. Contours are interpolated from experimental data.

CONCLUSIONS

Environmental conditions affect the ability of *Trichoderma longibrachiatum* to produce xylanase by SSF on wheat bran. Moisture content, depth of substrate, and duration of fermentation are process parameters that have a significant effect on xylanase activity production in a static aeration system at 25°C.

The maximum average yield of xylanase activity was 716 U/g substrate and this occurred for the 55% initial substrate moisture content, 1.5 cm depth of wheat bran, and five days of fermentation treatment combination.

Enzyme activity increased by 40% when an initial moisture content of 50% was used rather than 75%. Surfactant addition upon inoculation increased enzyme activity production at the higher initial moisture content, but no effect of surfactant was seen for the lower moisture content. Interactions between moisture content and depth were suspected to be a result of a decrease in oxygen availability to the microorganism at greater depths and higher moisture contents. Moisture content and duration of fermentation interactions showed an increase in xylanase production at higher moisture content and longer times of fermentation which may have been a result of the microbial biomass increasing over time to a level closer to the 50% moisture content final biomass. Xylanase production is growth-associated, therefore more fungal biomass would result in more xylanase produced. Three-way interactions between moisture content, depth of substrate, and duration of fermentation were due more to the effect of moisture content on depth of substrate than the difference between five and 10 days of fermentation.

Orthogonal contrasts were performed to determine which treatments differed significantly for moisture content and depth of substrate in the optimization experiment. Linear and quadratic orthogonal contrasts for initial moisture content were significant indicating a curvilinear response of xylanase activity to initial substrate moisture content. The linear effect of depth of substrate was not significant, however the quadratic response was highly significant.

These optimal levels of moisture content, depth of substrate, and length of fermentation are for a lab-scale statically aerated fermentation at 25°C. The addition of aeration may further increase xylanase production. Further research is needed to experimentally determine the optimal conditions for an aerated solid-state fermentation system.

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