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Optimization of Solid-state Fermentation Parameters for the Production of Xylanase by *Trichoderma longibrachiatum* on Wheat Bran in a Forced Aeration System

E. R. Ridder, S. E. Nokes, B. L. Knutson

Abstract. *The effect of aeration on the production of xylanase by Trichoderma longibrachiatum on wheat bran in a solid-state fermentation (SSF) system has not been investigated. This study was conducted to investigate the interactive effects of aeration, initial moisture content of the substrate, and depth of the substrate on xylanase activity produced in a tray fermenter. The experiment was conducted as a split plot experiment with factorial treatments and three replications of each treatment combination. The whole plot treatment was aeration rate (0, 2.9, 5.7 L/min/kg bran). Initial moisture content (45, 55, 65% w.b.) and depth of substrate (1.0 and 2.5 cm) were investigated factorially. Trays of wheat bran were assayed after fermentation by Trichoderma longibrachiatum to determine the production of xylanase activity. Aeration rate had a significant nonlinear effect on enzyme activity with highest yields obtained at an aeration rate of 2.9 L/min/kg bran (738 U/g, averaged over all initial moisture contents and depth of substrates). Initial moisture content of the substrate also had a significant nonlinear effect on enzyme activity with the highest yields at 55% (556 U/g, averaged over all airflow rates and depths of substrate). Depth of substrate had no significant statistical effect on enzyme activity. The treatment combination of 2.9 L/min/kg airflow rate and 55% moisture content resulted in the highest yields (948 U/g, averaged over depth of substrate).*

Keywords. *Aeration, Substrate depth, Trichoderma longibrachiatum, Solid-state fermentation, Xylanase, Wheat bran.*

Aeration, whether by forced air or agitation of the substrate, can significantly increase microbial growth in solid-state fermentation (SSF). Aeration reproduces those conditions under which the microorganism grows naturally, by providing oxygen to the microorganism while removing carbon dioxide. Aeration can also remove heat from the substrate (Pandey, 1992; Hesseltine, 1972; Ramana Murthy et al., 1993). If static aeration systems are used in SSF, the decrease in oxygen and the increase in carbon dioxide can both be growth limiting (de Reu et al., 1995). Excessive agitation can break the mycelia thus hindering microbial growth. Agitation may also crush the interparticle spaces necessary for growth and diffusion of gases (Fung and Mitchell, 1995; Rao et al., 1993; Mitchell et al., 1988). On the other hand, aeration may stimulate excessive heat production because the increase in oxygen availability increases the metabolic rate of the organisms (Ramana Murthy et al., 1993; de Reu et al., 1995). Therefore, the impact of aeration is complex and dependent on fermenter configuration, substrate, and organism interactions.

Oxygen is vital for the growth of a microorganism such as *Trichoderma longibrachiatum,* and the presence of oxygen in bioreactors may result in a higher enzyme production (Feijoo et al., 1995). As the metabolic heat in the SSF system increases due to microbial oxygen consumption, changes in the substrate occur such as shrinkage of the bed and a decrease in porosity (Ramana Murthy et al., 1993; Rathbun and Shuler, 1983). These changes prevent heat transfer to the environment thus increasing temperature in the bed (Nagai and Nishio, 1980; Ramana Murthy et al., 1993). At first this increase of temperature promotes growth and production of enzyme by the microorganism, but at higher temperatures the process becomes inhibited (Finstein and Hogan, 1993). Aeration is the most effective mode of temperature control in SSF (Ramana Murthy et al., 1993).

The importance of initial moisture content of the substrate in SSF has been discussed in depth in Ridder et al. (1998). In general, if the moisture content of the substrate is too high, void spaces in the substrate become filled with water and the oxygen is forced out; however, if the moisture content is too low, the growth of the microorganism will be inhibited (Moo-Young et al., 1983). Moisture content of the substrate may be reduced in the process of aerating the SSF system to reduce temperature and dissipate heat (Ramana Murthy et al., 1993). The challenge is to sufficiently aerate the system to promote microbial growth without changing the moisture content from the desired level. Moisture content of the substrate may be maintained by using moist air for aeration (Ramana Murthy et al., 1993) under certain conditions. The direction of mass transfer of water between the moist aeration air and the moist substrate is highly dependent on temperature. Kim et al. (1985) discovered little if any change in moisture content over time with humidified aeration in the SSF production of cellulase by *Trichoderma reesei* on wheat bran. Durand and Chereau (1988), using *Trichoderma viride* on sugar beet pulp in a deep bed

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fermenter, found that it was possible to maintain constant temperature and water content in the substrate by decreasing the inlet air temperature to 26°C from 29°C and increasing the aeration rate from 750 to 1000 m^3/h during the exponential growth phase of the organism. These changes removed the water and heat produced by microbial metabolism.

The effect of the depth of the substrate (wheat bran) on xylanase production by *Trichoderma longibrachiatum* in solid-state fermentation has not been well studied (Ridder et al., 1998). However, depth of substrate is an important factor in composting (Hansen et al., 1993) which is similar to 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. Ridder et al. (1998) found that the depth of substrate was important to the production of xylanase by *Trichoderma longibrachiatum* on wheat bran in a static system. The depth of the substrate can affect the magnitude of temperature and oxygen gradients (Rathbun and Shuler, 1983). The gradients are usually a function of the direction of airflow (Rathbun and Shuler, 1983).

The type of fermenter selected for SSF impacts the possible aeration configuration. In a tray fermenter, the substrate is placed in trays, stacked one over another, and incubated in a controlled atmosphere room or chamber (Ramana Murthy et al., 1993). Humid air is circulated around the trays. Airflow rates are usually moderate (4 to 6 L/min) with low pressures (less than 138 kPa) to ensure good aeration without major disruption of the substrate (Kim et al., 1985).

The required airflow rate, as well as initial substrate moisture content, must be determined for each process (Hesseltine, 1972). The objective of this research was to determine the effect of humidified aeration rate, at differing initial substrate moisture contents and substrate depths, on the production of xylanase by *Trichoderma longibrachiatum* on wheat bran in a tray fermenter.

Materials and Methods

Organism

Trichoderma longibrachiatum was maintained on potato dextrose agar (PDA) slants at 4°C and subcultured at monthly intervals (Chen et al., 1997).

Substrate

Wheat bran, bought in bulk from a local retailer, was used as the SSF substrate. The wheat bran was thermally pretreated for sterilization at 121°C for 15 min at 137.8 kPa (20 psi). Initially, air dried wheat bran (approximately 12% water content wet basis) was placed in the trays to the desired treatment depth and weighed. To achieve a depth of 1 cm in the trays, 177.8 g of wheat bran was required, and 442 g of wheat bran was required to achieve a depth of 2.5 cm in the tray. Subsequent trays were filled based on wheat bran weight. Initial water content was adjusted by adding sterile magnesium sulfate/potassium phosphate buffer mixed with the substrate before sterilization. The pH of the initial substrate/buffer mixture was 6.2.

Inoculum

Trichoderma longibrachiatum 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 $10^{6\text{-}7}$ spores in a magnesium sulfate/potassium phosphate buffer (Biswas et al., 1988; Wiacek-Zychlinska et al., 1994). Spore numbers were determined using a hemacytometer (Bright Line, Hausser Scientific, Horshaw, Pa.). The suspension, when necessary, was diluted with sterile magnesium sulfate/potassium phosphate buffer to obtain a spore count within the desired range. One milliliter of inoculum was added for each 5 g of bran in the tray. Inoculation was performed in a sterile hood by pipetting the liquid spore inoculum onto the bran and mixing with a sterile spoon.

Aerated Growth Chamber

A stacked tray fermenter (fig. 1) was designed to sit inside a 1.1 m × 0.5 m × 0.6 m (inner dimensions) constant humidity, constant temperature chamber (CHCT). The stacked tray fermenter was enclosed in a 0.5-cm thick plexiglas chamber with aluminum framing. The plexiglas

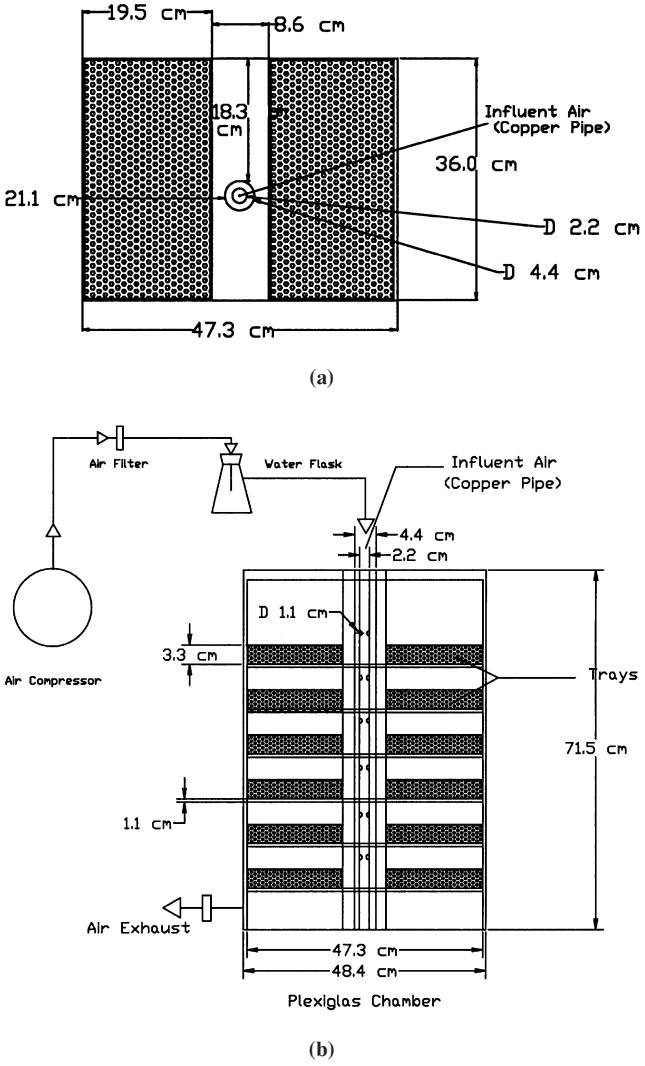

Figure 1–Stacked tray fermenter diagram: (a) top view, (b) side view of fermentation chamber with aeration system.

chamber was used to reduce the potential for microbial contamination pf the trays, as the plexiglas chamber could be cleaned more thoroughly than the CHCT chamber. The tray structure was constructed of aluminum framing. Removable trays (12 trays; 19.0 cm × 36.0 cm each, two trays per layer) were constructed of perforated aluminum sheeting (McNichols, Tampa, Fla.). Air was filtered using a 0.3 μm HEPA-VENT filter (Whatman) and humidified by bubbling the air through a flask of water. The air was then passed through the plexiglas chamber at the desired airflow rate (liters per minute per kilogram bran) which varied by treatment. Air entered the chamber at the top and was directed down the middle of the tray structure to be dispersed over the trays by a perforated copper pipe (2.2 cm in diameter) located in the center of the tray structure (fig. 1). Outlet air was filtered at the bottom of the chamber.

Before cultivation, the plexiglass growth chamber was cleaned by wiping the inside with 20% bleach and water solution and rinsing with DI water. The tray structure was cleaned with isopropyl alcohol and the air distribution tube was autoclaved at 121°C for 15 min. The trays were autoclaved with the bran (described in Substrate section).

Airflow Rate

An initial estimate for the required aeration rate was obtained using the following equation from Rodriguez Leon et al. (1991):

$$W_g = \frac{0.39\,\mu e^{\mu t} - 0.58\,(T_2 - 25)}{0.24\,(T_2 - 25) + 560\,(H_2 - 0.036)} \qquad (1)$$

where

W_g = mass velocity of dry air (kg dry air/h)
μ = maximum specific growth rate (h^{-1})
t = time since fermentation initiation (h)
T_2 = temperature of outlet air (°C)
H_2 = absolute humidity of outlet air (kg water/kg dry air)

The specific growth rate for *Trichoderma longibrachiatum* was assumed to be equal to that of *Trichoderma reesei* (μ = 0.123/h) (Smits et al., 1996). Using equation 1, it is possible to estimate airflow rate needed to maintain the desired temperature of the outlet air during the course of fermentation (Rodriguez, Leon et al., 1991). In the current study, the airflow rate was constant over the duration of fermentation. Therefore, the time of maximum temperature and the measured outlet air temperature, determined from temperature probe data for a non-aerated experiment (data not shown), were used to estimate the needed airflow rate. Once the mass velocity of dry air (W_g) was calculated, it was converted to liters per minute (L/min) using the specific volume of conditioned air. The second airflow treatment was determined by halving the calculated airflow rate. Aeration rates of 2.9 and 5.7 L/min were supplied by an air compressor (DeVilbiss, GFAC1025) operating at 137.8 kPa (20 psi).

Cultivation

Once inoculated, trays were placed inside the plexiglas chamber, and the plexiglas chamber was placed in the CHCT chamber which maintained an average temperature of 28.5°C (standard deviation = 0.88°C) throughout all fermentations, which is within the optimal temperature range for this organism (Bailey et al., 1993; Chahal, 1985; Chen et al., 1997). Relative humidity in the CHCT was maintained at 90%. The trays were incubated for 5 d.

Temperature Measurement

Temperature sensors (Campbell Scientific Type No. 108, Logan, Utah) were sterilized with a 20% bleach and water solution and rinsed with DI water. The probes were placed in the center of the wheat bran in randomly selected trays within each airflow experiment. Resource limitations prevented temperature measurements from all trays to be recorded, therefore only a partial data set was collected. The data were stored in a Campbell Scientific 21X datalogger (Logan, Utah), and downloaded after each experiment. Temperatures were read every 10 min, and averaged to generate recorded values every half hour for the length of the fermentation.

Extraction

Enzyme was extracted by adding the entire tray contents to a solution of 0.1% v/v Tween 80 and tap water (100 mL solution for every 5 g of bran in the tray). The contact time was approximately 30 min. The mixture was stirred and approximately 100 mL was removed for further analysis. This sample was vacuum filtered on a 90-mm glass fiber filter paper, then centrifuged at 2900 rpm for 30 min. 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. Frozen samples were compared to fresh samples and no evidence of decreased enzyme activity was observed.

Assay

Total reducing sugars were estimated using the method of Bailey et al. (1992). A 10 g/L xylan suspension was made by suspending 1 g birchwood xylan (Sigma No. 232-760-6) in 80 mL of sodium citrate buffer at pH 5.3 and 60°C then heating until boiling. The suspension was mixed overnight and brought to a volume of 100 mL. From the 10 g/L xylan 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. Enzyme activities were expressed in international units (U), where one unit is the amount of enzyme which liberates one micromole of product (xylose) per minute under assay conditions, per gram of substrate (Ridder et al., 1998).

Experimental Design and Statistical Analysis

This experiment was designed as a split plot with factorial treatments (fig. 2). The whole plot treatment was the airflow rate. The factorial treatments included three levels of initial substrate moisture content and two levels of substrate depth. Twelve trays, allowing for two subsamples of each treatment combination, were randomly assigned to the six treatments varying the factors of initial moisture content (45, 55, and 65% w.b.) and depth of substrate (1.0 and 2.5 cm) then placed in the plexiglas chamber. The

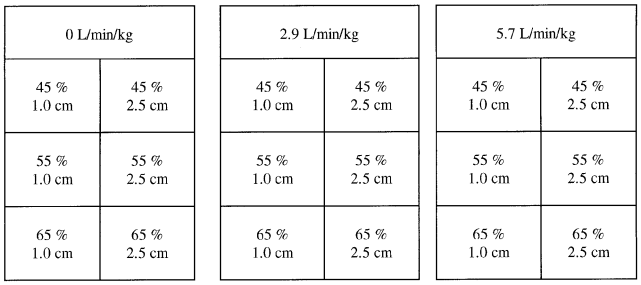

Figure 2–Diagram of the split plot with factorial treatments experimental design. Whole plot treatments were airflow rates (1, 2.9, and 5.7 L/min/kg bran). Factorial treatments were combinations of moisture content of the substrate (45, 55, and 65% w.b.) and depth of substrate (1.0 and 2.5 cm).

chamber was then assigned to one of three airflow rates (0, 2.9, and 5.7 L of air per minute per layer of trays). Three replications of each airflow rate were performed. Trays were placed in the plexiglas chamber at random locations and the plexiglas chamber was then placed inside the CHCT. After 5 d, the plexiglas chamber was removed from the CHCT chamber and the enzyme extracted and assayed. Data were statistically analyzed as a split plot with a factorial treatment structure using SAS®.

Results and Discussion

A summary of the xylanase activity and the temperature achieved in the trays (both maximum and average temperature) by treatment is presented in table 1. The reader may note that the standard deviation between enzyme activities in replications of identical treatments is sometimes large. Since the replications were conducted at different times, the larger standard deviation may be due to slight differences in the microorganism used. The spore inoculum for the replications was obtained from different plates of the same culture. Also, since the fermentations are conducted in a semi-sterile environment, differing amounts of contamination occurred randomly throughout the treatments and may have affected xylanase levels.

Table 1. Summary of xylanase activity and temperature in trays (both maximum and average temperature) by treatment*

Moisture Content (%)	Depth of Substrate (cm)	Airflow Rate (L/min)	Activity (U/g wheat bran)	Std Dev (Activity)	Max Temp (°C)	Std Dev (Max Temp)	Avg Temp (°C)	Std Dev (Avg Temp)
45	1.0	0	192	52	NE†	NE	NE	NE
45	1.0	2.9	659	695	32.4	NE	29.6	NE
45	1.0	5.7	368	285	30.4	NE	28.9	NE
45	2.5	0	147	45	30.1	0.33	24.3	3.2
45	2.5	2.9	686	142	35.6	1.7	31.2	0.3
45	2.5	5.7	396	88	33.3	1.6	29.6	0.9
55	1.0	0	307	133	NE	NE	NE	NE
55	1.0	2.9	1082	311	29.1	NE	27.4	NE
55	1.0	5.7	521	398	30.1	NE	25.8	NE
55	2.5	0	222	167	31.5	NE	28.7	NE
55	2.5	2.9	713	438	29.2	NE	26.0	NE
55	2.5	5.7	390	134	40	2.4	29.3	3.2
65	1.0	0	132	34	30.8	0.06	27.9	0.23
65	1.0	2.9	847	142	31.3	2.4	28.3	0.9
65	1.0	5.7	453	335	36.8	2.7	30.3	3.2
65	2.5	0	86	114	35.5	2.7	28.2	0.4
65	2.5	2.9	554	292	NE	NE	NE	NE
65	2.5	5.7	284	99	44.5	7.9	33.0	0.9

* The standard deviation is calculated from three replications for the activity and two replications for the temperature.
† NE stands for non-estimable because data were not recorded.

The xylanase activity data were summarized by aeration rate and averaged over the other two treatments (moisture content and depth). These data are presented in table 2. An

Table 2. Treatment means table for optimization experiment evaluating the effects of moisture content of the substrate, depth of the substrate, and aeration rate on xylanase activity (U/g) production by *Trichoderma longibrachiatum* on wheat bran at 25°C

				Xylanase Activity Average (U/g) for Given Moisture Content
Airflow (L/min/kg)		0		
Depth (cm)		1.0	2.5	
Moisture Content (%)	45	176 151 250	99 152 189	170
	55	216 246 460	57 390 219	265
	65	105 170 120	22 217 18	109
Xylanase activity average (U/g) for given substrate depth		210	152	

				Xylanase Activity Average (U/g) or Given Moisture Content
Airflow (L/min/kg)		2.9		
Depth (cm)		1.0	2.5	
Moisture Content (%)	45	636 736 605	524 789 745	673
	55	905 899 1442	1099 802 237	948
	65	727 811 1004	613 811 237	596
Xylanase activity average (U/g) for given substrate depth		793	684	

				Xylanase Activity Average (U/g) for Given Moisture Content
Airflow (L/min/kg)		5.7		
Depth (cm)		1.0	2.5	
Moisture Content (%)	45	130 290 684	336 497 356	382
	55	153 944 466	390 524 255	456
	65	125 794 441	207 396 248	369
Xylanase activity average (U/g) for given substrate depth		447	357	

Table 3. ANOVA table evaluating the effects of airflow rate, initial moisture content of the substrate, and depth of the substrate for xylanase production in SSF by *Trichoderma longibrachiatum* on wheat bran

Source of Variation	df	Mean Square	F	F_{crit}*
Airflow rate	2	1418600	20.09†	5.14
Error for whole plot treatment	6	70603		
Moisture content	2	191190	4.09†	3.32
Depth	1	100104	2.14	4.17
Airflow × moisture content	4	32292	0.69	2.69
Airflow × depth	2	2827	0.06	3.32
Moisture × depth	2	31290	0.67	3.32
Airflow × moisture × depth	4	9799	0.21	2.69
Error	30	46783		

* Probability of a larger F = 0.05.
† Significant treatment effects.

analysis of variance (ANOVA) for this experiment is presented in table 3.

Airflow rate and initial substrate moisture content had a highly significant effect on the production of enzyme activity. Contrasts were performed using SAS to determine which treatments differed significantly for treatments of airflow rate and moisture content. There were significant differences between all airflow rates with the highest mean (738 U/g) at 2.9 L/min/kg bran, averaged over all substrate depths and initial moisture contents. Enzyme activity increased 46% with an airflow rate of 2.9 L/min/kg bran over 5.7 L/min/kg bran and 75% with an airflow rate of 2.9 L/min/kg bran over a static environment. Both the linear and quadratic orthogonal comparisons were significant indicating the response of enzyme activity to airflow rate is nonlinear.

The treatment of 55% initial substrate moisture content had a mean significantly greater than the 45 and 65% moisture content treatments (27 and 36% higher, respectively) whose means were statistically equal. Quadratic orthogonal comparisons were significant for moisture content. The treatment combination of 2.9 L/min/kg bran airflow and 55% moisture content had the highest enzyme activity mean (947 U/g, averaged over substrate depth) of all treatments. There was no significant effect of depth of substrate on the production of enzyme activity. No significant interactions were observed between the treatment factors.

A contour plot (fig. 3) was constructed by interpolating between experimental data to graphically present the relationship between airflow rate and initial moisture content for xylanase activity produced by SSF using *Trichoderma longibrachiatum* on wheat bran, averaged over substrate depth. The contour plot has a closed contour in the center indicating that our experimental conditions bracketed the optimal conditions, at least locally. From visual evaluation of the contour plot, the maximum xylanase production occurs at 2.9 L/min/kg airflow and 55% initial moisture content.

The temperature data shown in table 1 were analyzed statistically to determine if temperature may be used to explain the treatment effects observed. While depth of substrate and airflow rate were significant (Pr > F = 0.05 and 0.04 respectively) in describing the maximum temperature achieved in the treatment, and airflow rate was significant (Pr > F = 0.02) in describing the average fermentation temperature, neither maximum temperature

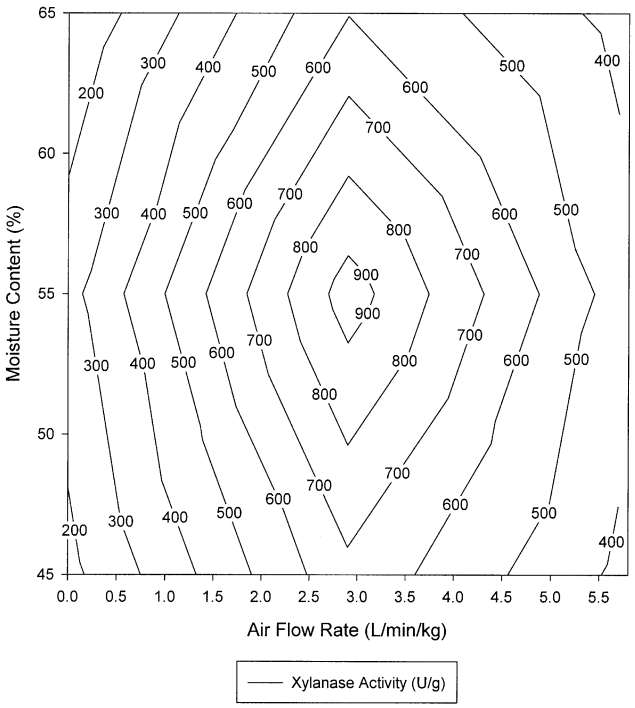

Figure 3–Contour plot with lines of constant enzyme activity showing the effects of moisture content and depth of substrate on xylanase activity produced by SSF using *Trichoderma longibrachiatum* on wheat bran. Contours are interpolated from experimental data.

nor average fermentation temperature showed any relationship with xylanase activity achieved (Pr > F = 0.77 and Pr > F = 0.92, respectively). This result must be interpreted cautiously because of the partial temperature data set available. However, it is interesting to note that a depth of 2.5 cm had a significantly higher maximum temperature than a depth of 1 cm over all airflow rates and moisture contents, and an airflow rate of 5.7 L/min had a significantly higher maximum and average temperature than both 0 and 2.9 L/min when averaged over all moisture contents and substrate depths. Higher airflow rates are expected to cool the fermentation, however the additional oxygen available may have stimulated growth such that the heat generated by the additional metabolism exceeded the heat being removed by the increased airflow. These interactions warrant further study.

CONCLUSIONS

The amount of xylanase produced by *Trichoderma longibrachiatum* in SSF on wheat bran in a tray fermenter is influenced by environmental conditions. Airflow rate and moisture content of the substrate had significant effects on xylanase activity production.

Enzyme activity increased when an airflow rate of 2.9 L/min/kg bran was used rather than 5.7 L/min/kg bran or a static environment. Enzyme activity also increased when an initial moisture content of 55% was used rather than 45 or 65%. The average yield of xylanase activity for all treatment combinations in the experiment was 440 U/g with a maximum yield of 1082 U/g (2.9 L/min/kg airflow, 55% water content, and 1 cm depth treatment) and a minimum of 86 U/g (0 L/min/kg airflow, 65% water

content, and 2.5 cm substrate depth treatment). The optimal treatment was 2.9 L/min/kg bran airflow, 55% moisture content, and 1.0 cm depth of wheat bran (1082 U/g; three replication average).

Orthogonal contrasts were performed to determine which treatments differed statistically for airflow rate and moisture content in the experiment. Linear and quadratic orthogonal contrasts for airflow rate were significant indicating a curvilinear response of xylanase activity to initial substrate moisture content. The linear effect of initial moisture content was not significant, however the quadratic response was highly significant. The treatment effects on xylanase production were not explained by maximum or average temperature in the tray.

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