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An Unstructured Mathematical Model for Growth of *Pleurotus ostreatus* on Lignocellulosic Material in Solid-State Fermentation Systems

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ABSTRACT

Inedible plant material, generated in a Controlled Ecological Life Support System (CELSS), should be recycled preferably by bioregenerative methods that utilize enzymes or micro-organisms. This material consists of hemicellulose, cellulose, and lignin with the lignin fraction representing a recalcitrant component that is not readily treated by enzymatic methods. Consequently, the white-rot fungus, *Pleurotus ostreatus*, is attractive since it effectively degrades lignin and produces edible mushrooms. This work describes an unstructured model for the growth of *P. ostreatus* in a solid-state fermentation system using lignocellulosic plant materials from *Brassica napus* (rapeseed) as a substrate at three different particle sizes. A logistic function model based on area was found to fit the surface growth of the mycelium on the solid substrate with respect to time, whereas a model based on diameter, alone, did not fit the data as well. The difference between the two measures of growth was also evident for mycelial growth in a bioreactor designed to facilitate a slow flowrate of air through the 1.5 cm thick mat of lignocellulosic biomass particles. The result is consistent with the concept of competition of the mycelium for the substrate that surrounds it, rather than just substrate that is immediately available to single cells. This approach provides a quantitative measure of *P. ostreatus* growth on lignocellulosic

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biomass in a solid-state fermentation system. The experimental data show that the best growth is obtained for the largest particles (1 cm) of the lignocellulosic substrate.

Index Entries: Lignocellulose; SSF; *Pleurotus ostreatus*; growth model; logistic equation

INTRODUCTION

A large fraction of the carbon fixed by photosynthesis of plants is deposited as lignocellulose, an abundant renewable resource on earth, as well as a significant source of fixed carbon in a Controlled Ecological Life Support System (CELSS) *(1)*. The main organic constituents of lignocellulose are cellulose, hemicellulose, and lignin. Polysaccharides in lignocellulosic material can be enzymatically hydrolyzed to sugars that then could be used directly for human food or as a nutrient source for fermentation processes *(2)*. Lignin's association with cellulose protects it from enzymatic hydrolysis. Pretreatment methods must be found to modify the lignocellulosic structure, and to increase the bioavailability of cellulose by removing the protective effect of lignin.

White-rot basidiomycetes degrade lignin more extensively and rapidly than any other known group of micro-organisms, and play a key role in the recycling of carbon from lignin and plant polysaccharides *(3)*. In this study, a white-rot fungi, *Pleurotus ostreatus*, was grown on lignocellulosic fractions of *Brassica napus* (rapeseed) plant materials. *P. ostreatus* could be used in a CELSS to produce fruiting bodies (oyster mushroom) that could potentially improve the quality of the diet as well as recycling the inedible portions of the crops. Consideration of the metabolism of this fungus and special attention given to its nutritional requirements can result in extensive utilization of a lignocellulosic substrate *(2)*.

The biodegradation of lignin and growth of fungus on an insoluble, lignocellulosic material is achieved by a solid-state fermentation. Direct estimation of the growth in a solid-state fermentation is not possible as it is difficult to separate residual solid substrate from the fungal mycelium. A survey of the literature indicates that headspace gas composition, chitin content, ergosterol content, and linear extension of the mycelium have been used to measure the fungal growth on solid substrates *(4,5)*.

Growth kinetics and mathematical modeling of microbial growth in solid substrate fermentations have received little attention, which is perhaps partly because of the difficulties associated with its study. Growth is initially limited to the surface of the solid particles. A solid-state fermentation system has a nonhomogenous distribution of the components, and stucturally and nutritionally heterogenous substrates. The penetration of fungal hyphae into the solid substrates prevents the direct recovery and determination of biomass *(6)*.

Trinci defined the growth unit of mycelium as total length of mycelium divided by number of tips. Hyphal length and number of tips possessed by a mycelium increased exponentially at approximately the same specific growth rate. The maximum rate at which hyphae can extend may be limited either by transportation of the precursors to the tips or assembling of the tip wall from these precursors *(7,8)*. Indirect methods for measuring microbial biomass, such as counting the number of tips, can be tedious. Hence, we sought an alternate approach. The diameter and the area of the fungal growth on a solid substrate as a function of time was found to represent the fungal growth rate. When combined with a logistic function model, values of mycelial growth constants can be determined. This paper reports the application of the logistic function model to a solid substrate fermentation of *Pleurotus ostreatus* on lignocellulose consisting of *Brassica napus* stems, pods, and leaves.

EXPERIMENTAL

Plant Growth and Substrate Preparation

Rapeseed plants were grown hydroponically in the Horticulture Department at Purdue University *(9)*. Plants were harvested at maturity after 55 d of growth. Stems, leaves, and pods of the plants were separated, air dried for several days, then oven dried for 2 d at 70–75°C. The plant materials were then ground to obtain three different particle sizes: 20–40 mesh (0.84–0.42 mm), smaller than 40 mesh (<0.42 mm), and hand ground (~1.0 cm). These materials were prepared from a mixture of equal parts by weight of brassica leaves, pods, and stems at an initial moisture content of 6%. The 10 mm particle size material was hand ground using a mortar and pestle. To obtain the various particle size fractions, the brassica material was passed through a mill (Arthur H. Thomas Co., Scientific Apparatus, Philadelphia, PA, RPM:1725, HP: 1/4). The 0.42 and 0.42–0.84 particle size fractions were obtained by seiving the machine-ground leaves and stems through 20 and 40 mesh stainless steel screens, which had corresponding seive openings of 0.84 and 0.42 mm, respectively. The ground material was stored in sealed containers at 4°C until use.

Culture Handling

Pleurotus ostreatus (NRRL 2366), growing on a PDA slant in the mycelial form, was obtained from the Northern Regional Research Laboratory (NRRL) in Peoria, IL. Initial transfers were made by adding 1–2 mL of deionized distilled water to the original slant and then using this liquid to prepare streak plates on PDA plates. The plates were incubated at 28°C. New slants were prepared from individual colonies to inoculate the liquid nutrient media in order to produce submerged cultures. The procedures of Kaneshiro, and of Linderfelser et al. were followed to prepare nutrient solutions *(10,11)*.

Flasks, each containing 50 mL of the nutrient solution, were sterilized and cooled down. Mycelial growth from PDA slants was transferred to the nutrient flasks. The inoculated flasks were placed in an incubator/shaker, New Brunswick Scientific Co., Inc., Edison, NJ, at 25 rpm and 28°C. In approx 7 d, fungal pellets were clearly visible, and were ready to inoculate the plant material.

Solid State Fermentation

Sealed Glass Jars

Fermentations were carried out in glass jars of 9 cm diameter, incubated at 28°C. An amount of 7.5 g of plant material, ground at three different particle sizes, were put into the jars, and water was added to each jar at a water:solids weight ratio of 5 to 1. The jars were sealed and autoclaved at 121°C for 20 mins, then allowed to cool to room temperature. These were inoculated by *P. ostreatus* pellets that were growing in the nutrient solution. A pellet was used to aseptically inoculate each jar at the center of the substrate. The diameter of the mycelial growth was measured every day for each jar.

A schematic representation of the jar is shown in Fig. 1. Since the dimensions of the jar are 9 cm (inside diameter) by 9.5 cm high, accurate measurement of the area of fungal growth required that the jar be removed from the incubation chamber, and opened in a sterile hood using aseptic procedures. The diameter of the mycelial mat was then measured using a previously sterilized compass. On a daily basis for each of the jars, three measurements were taken in a sterile hood as indicated in Fig. 1, and then averaged to obtain a value of the diameter of the mycelial growth. At the same time that the jar was opened, 0.5 mL of water was distributed on top of the biomass in order to keep the material sufficiently moist to support fungal growth and at the same time avoiding mushroom formation that would otherwise occur if the material was too wet. Each experiment consisted of three jars. The growth data were based on averaging measurements carried out daily over 12 d for three replicate experiments.

Gas-Flow Bioreactors

This concept was extended to a specially designed microbial bioreactor through which humidified, temperature controlled air could be passed in order to more closely control the environmental conditions to promote extensive fungal growth as well as avoiding premature formation of fruiting bodies (i.e., mushrooms). The reactor, developed during the course of this research, consists of a glass vessel which had a 9 cm diameter. A 4 cm layer of 5 mm diameter glass beads was placed upon a ceramic frit located at the bottom of the vessel. A weight of 7.5 g (wet weight basis) of plant material, consisting of a ground mixture of brassica stems, leaves, and pods with particle size of 0.42–0.84 mm, was layered on top of the glass beads to give a 1.5 cm depth. A layer of cheese cloth was placed between the glass beads and

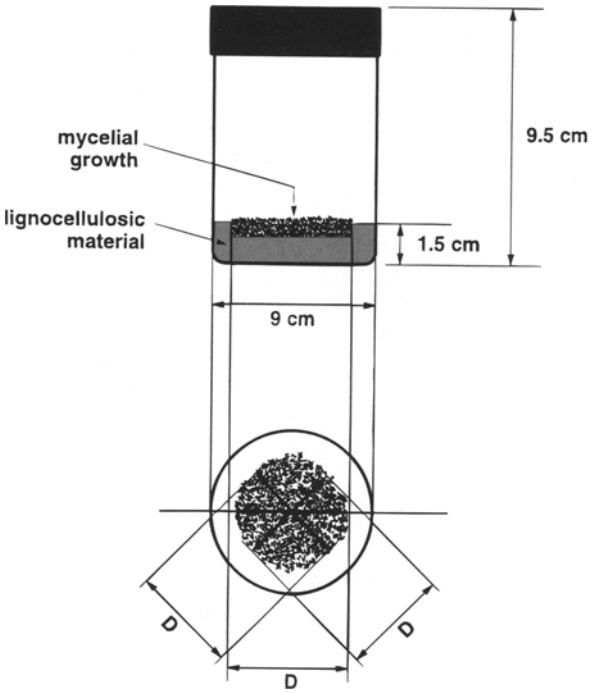

Fig. 1. Schematic representation of the measurement of mycelial growth for a solid-state fermentation performed in a jar.

plant material. Once loaded, the entire apparatus (shown in Fig. 2) was placed into an autoclave (Sterilmatic Sterilized Chamber, Model 95-2678, Market Forge) for 20 min at 121°C. Upon cooling the apparatus was placed into the thermo-regulated enclosure illustrated as part of Fig. 3.

The temperature of the bioreactors was controlled by placing the vessel in a glass aquarium (92 cm wide, 50 cm high, 30 cm deep). Three sides of the aquarium were covered with 2 cm thick styrofoam to insulate the incubation system. The incubation temperature was kept at 28°C by a heating mantle controlled with a variac. The air inside the incubator was circulated with a small cooling fan.

Each reactor was connected to a gas washing bottle filled with 400 mL water. The entire saturator apparatus, together with the water, was sterilized prior to use. Air from a gas cylinder was passed through a 0.20 micron filter and then to the saturator through a 13 mm OD (7 mm ID) tygon tube. The outlet of the saturator was connected to the bottom of the bioreactor using a 70 cm length of 7 mm ID tygon tubing. All the tygon tubing was also sterilized prior to use. The saturator apparatus was placed in the thermostatted enclosure together with the bioreactor as illustrated in Fig. 3.

The flowrate of the saturated air at 28°C to the bioreactor, measured with a digital flow meter (Digital flow check, New Seven-Gas Model, Altech, IL), ranged from 0.2–0.4 mL/min. This gave a moisture content in

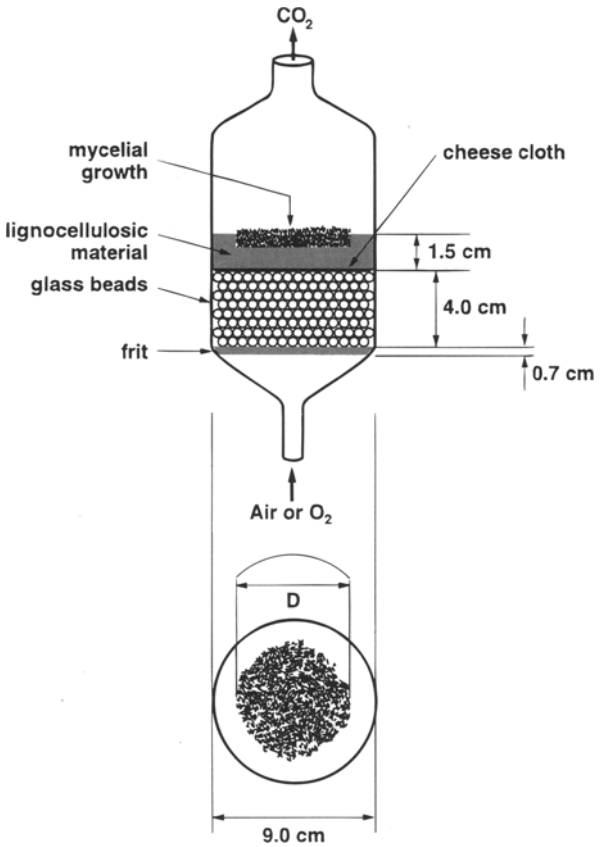

Fig. 2. Schematic representation of the solid-state fermentor used for *P. ostreatus* growth on inedible rapeseed material.

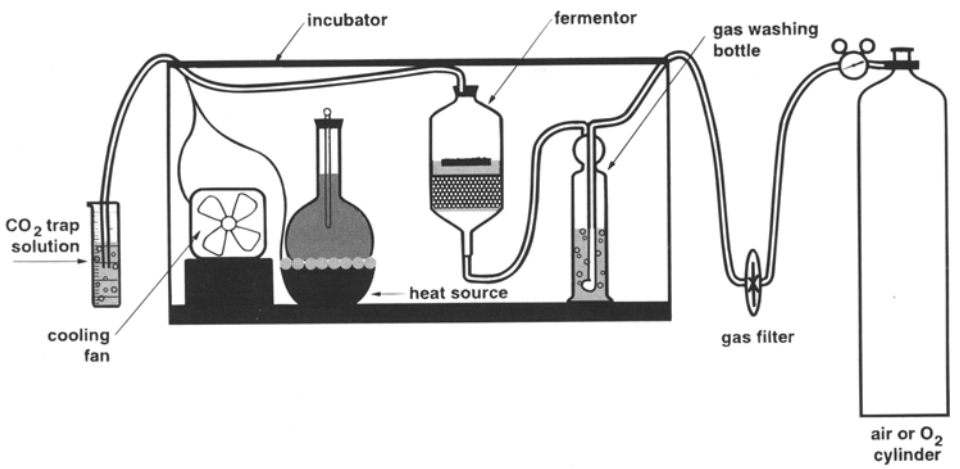

Fig. 3. Schematic representation of the solid-state fermentation system.

the bioreactor chamber which promoted fungal growth, but kept the moisture content low enough to control the onset of mushroom formation.

Mushroom formation was obtained by lowering the incubation temperature to 20°C, and increasing the moisture content by spraying with sterilized water, approx 2 mL/day, after the substrate was covered by white colored mycelial growth. Approximately 10 d after changing the incubator conditions, fruiting body formation began.

The initial growth of the fungal biomass was measured by the increase in the diameter of the mycelial mat. An indirect method of measuring growth was used since the bioreactor was closed and kept in the thermostatted enclosure throughout the entire experiment. A rule marked in 1 mm increments was placed against the wall of the bioreactor as illustrated in Fig. 4. The diameter of the mycelial mat was measured indirectly by lining up the respective marks of the plastic ruler and then using the mensuration formula (1) to calculate the diameter, D, of the mycelial mat from the length of the arcs:

$$D = 2x = r \sin \theta = r \sin (s/r) \quad (1)$$

where r is the radius of the fermentor, and θ is the angle (in radians) determined from the length of the arc, s ($\theta = s/r$). Three measurements, taken at approx 120° increments, were averaged to obtain a reading. Readings were taken on a daily basis. The method of measurement is illustrated in Fig. 4.

RESULTS AND DISCUSSION

The most appropriate particle size for promoting growth of the *Pleurotus* was chosen based on experiments carried out with material having particle sizes in three particle size ranges; smaller than 0.42 mm, 0.42–0.84 mm, and 1 cm. The particle sizes of 0.42–0.84 mm and 1.0 cm gave better results than particle sizes of smaller than 0.42 mm where extension in diameter of the mycelial growth was highest for the largest particle size substrate. This is believed to be because of the larger interparticle spaces associated with the largest particle size material, which allowed better penetration of the mycelia as well as enhanced distribution of O_2 concentration. The smaller particle substrate, when wet, is subject to channeling at the low pressure drops (approx 1 inch water) of the gas flow across the layer of plant material.

The mycelial growth was close to circular. Three different diameter measurements at 120° rotation were carried out daily for each jar and for the bioreactor, and then averaged. The area of the mycelial growth was calculated directly from the average diameter:

$$A = \pi D^2/4 \quad (2)$$

where A is the area, and D is the diameter of the mycelial mat.

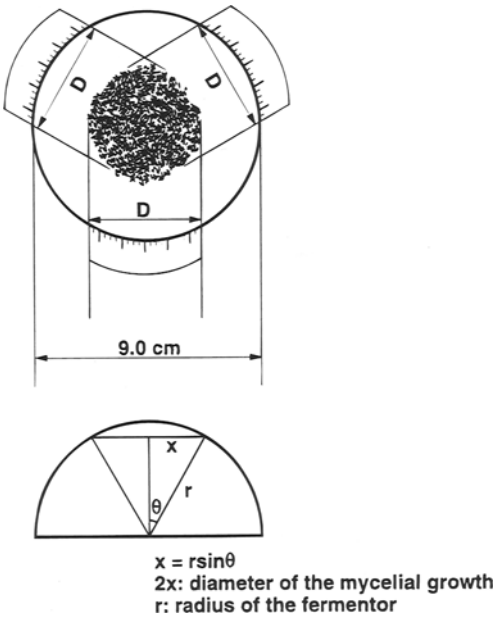

Fig. 4. Schematic representation of the method used to measure mycelial growth in the solid-state fermentor.

In the simplest approach, the rate of increase in biomass covering the surface of the substrate, as measured by the diameter of the fungal growth, was assumed to be independent of the amount of biomass initially present. The change in area, A, can therefore be represented by:

$$dA/dt = \mu_2 A \tag{3}$$

where μ_2 is the specific growth rate, A is the area of the substrate covered by the fungus, and t is time. When the mycelial population becomes large, an equation of the form of Eq. 3 cannot be very accurate because of competition of the microbial population for limited resources. Verhulst addressed this by adding a competition term to the exponential growth rate equation to obtain the logistic law of population growth *(12,13)*. For the equation based on area, the form is:

$$dA/dt = \mu_2 A - k_2 A^2 \tag{4}$$

where the constant k_2 represents an inhibition effect. Development of a growth equation based on the diameter of the mycelial growth gives a form which is analogous to equation 4:

$$dD/dt = \mu_1 D - k_1 D^2 \tag{5}$$

Equations 4 and 5 represent fundamentally different growth rates, with Eq. 4 giving representation of growth that considers the effect of competition of adjacent areas of growth. Equation 5 represents competition along a line. Both formalisms, whereas analogous, have different meaning. At a small value of D, the k_1D^2 or k_2A^2 terms can be negligible whereas the microbial population is in early stages of exponential growth. But if coverage of the surface by microbial biomass is large, the rapid mycelial growth rate will slow down as a result of competition of a growing population for limited substrate. In the case of equation 5, the competition effect would be related to hyphal length alone, compared to the area covered by the mycelial mat as represented in equation 4. It is expected that the model using area as a measure of mycelial growth would give a better fit than the model using diameter as a measure of growth, since the mycelial growth resembles a sheet rather than a porous network of fibrous hyphae. Further comparison is given by integration of the Eq. 4 and 5:

$$\int_{A_0}^{A} dA/(\mu_2 A - k_2 A^2) = \int_{t_0}^{t} dt \tag{6a}$$

and

$$\int_{D_0}^{D} dD/(\mu_1 D - k_1 D^2) = \int_{t_0}^{t} dt \tag{6b}$$

where A_0 is the initial area of the mycelia and D_0 is the initial diameter at time zero (t_0). The respective integrated equations are:

$$A = \mu_2 A_0 / [k_2 A_0 + (\mu_2 - k_2 A_0) e^{-\mu_2 (t - t_0)}] \tag{7a}$$

and

$$D = \mu_1 D_0 / [k_1 D_0 + (\mu_1 - k_1 D_0) e^{-\mu_1 (t - t_0)}] \tag{7b}$$

Measurements of D at the initial stages of exponential growth were used to obtain an initial estimate of μ_1.

$$D = D_0 e^{\mu_1 (t - t_0)}, \text{or} \quad \log (D/D_0) = (\mu_1 / 2.302)(t - t_0), \text{and} \tag{8}$$

$$A = A_0 e^{\mu_2 (t - t_0)}, \text{or} \quad \log (A/A_0) = (\mu_2 / 2.302)(t - t_0) \tag{9}$$

During exponential growth phase, when competition terms were negligible, both equations gave the same mycelial doubling time, t_d:

$$t_d = 0.693/\mu \tag{10}$$

The slope of log (A/A_0) vs t gave an estimate for the value of μ_2. The estimated value of μ_1 was obtained with the same method by using Eq. 8.

As t approaches long times, the whole surface of the substrate will be covered by mycelia, and Eq. 7 becomes:

$$D^{\infty} = \mu_1/k_1 \text{ and } A^{\infty} = \mu_2/k_2 \tag{11}$$

Since values of A^{∞} and D^{∞}, the cross-sectional area and diameter of the jar or bioreactor, are known, the values of k_1 and k_2 can be calculated.

Initial estimates of μ_1, μ_2, k_1, and k_2 were required for carrying out the regression analysis. We obtained values of μ_1 and μ_2 by plotting the measured diameters and areas as functions of elapsed time $(t - t_0)$ between 0 and 4 d. Equations 8 and 9, respectively, fitted to the data, gave straight lines whose slopes corresponded to values of μ_1 and μ_2. The time that had elapsed after the entire surface was covered with mycelia was used to estimate k_1 and k_2, according to Eq. 11. These values were obtained by using a statistical fitting software such as Sigma Plot (Jandel Scientific, San Rafael, CA), and aided the selection of the most appropriate model.

Table 1 compares values of the specific growth rates calculated from the exponential growth rate (equation 3) as initial estimates, and the values obtained from the nonlinear regression analysis using logistic equations 7a and 7b.

Table 2 shows that standard error and coefficient of variation values are lower for specific growth rates and inhibition constants when area is used rather than diameter. Figures 5 and 6 also show that the model and the data fit better when area is used for the measurement of mycelial growth. Even though the sigmoidal, logistic function model works well for both cases, we chose area to describe the mycelial growth for our system since area accounts for neighboring effects in competition of the mycelium for substrate. Figures 5 and 6 also show that the controlled moist atmosphere provided throughout the fermentation by the bioreactor (illustrated in Fig. 2) considerably shortened the time required for the mycelia to cover the surface of the substrate. The specific growth rate is higher for the gas-flow bioreactor than for fermentation in jars. Therefore, for the bioreactor, D_{∞} was reached in a very short time compared to the fermentation carried out in the sealed jar.

For the three different particle sizes used, the apparent doubling time is longer when diameter rather than area is used as the measure of growth because the time for the diameter to double would correspond to the time required for area to quadruple ($A = \pi D^2$). Therefore, the value for μ_2 (specific growth rate based on area) is larger than the value for μ_1, (based on diameter) and k_2 smaller than k_1 (Tables 1 and 2). The fermentations in jars gave apparent doubling times of 2.48 and 1.50 d for the particle sizes of <0.42 mm and 1 cm, respectively, when diameter was used as the measure of growth. When area was used as the measure, doubling

Table 1
Comparison of Specific Growth Rates Obtained from Exponential Growth at Early Stages of Fermentation and from Nonlinear Regression of the Logistic Equation

	Fermentations performed in the sealed bioreactors Particle size						Gas-flow bioreactor	
Basis	<0.42 mm		0.42–0.84 mm		Hand-ground, ~1 cm		0.42–0.84 mm	
	Exponential growth (Eq. 3)[a]	From regression (Eqs. 7a or 7b)	Exponential growth (Eq. 3)[a]	From regression (Eqs. 7a or 7b)	Exponential growth (Eq. 3)[a]	From regression (Eqns. 7a or 7b)	Exponential growth (Eq. 3)[a]	From regression (Eqs. 7a or 7b)
Diameter (μ_1)	0.275	0.279	0.379	0.342	0.514	0.461	0.526	0.862
Area (μ_2)	0.579	0.466	0.757	0.543	1.030	0.697	1.052	1.286

[a]integrated forms of $D = D_0 \exp(\mu_1 t)$ or $A = A_0 \exp(\mu_2 t)$

Table 2
Estimation of Logistic Equation Constants from Nonlinear Regression by Using Sigma-Plot

		Fermentations performed in the sealed bioreactors									Gas-flow bioreactor		
		Particle size											
		<0.42 mm			0.42–0.84 mm			Hand-ground ~1 cm			0.42–0.84 mm		
Method		Value	Standard error	Coefficient of variance	Value	Standard error	Coefficient of variance	Value	Standard error	Coefficient of variance	Value	Standard error	Coefficient of variance
	μ_1	0.279			0.342			0.461			0.862		
Diameter			0.0116	4.165		0.0131	3.838		0.0233	5.054		0.0440	5.106
	k_1	0.023			0.033			0.048			0.094		
			0.0023	9.926		0.0024	7.132		0.0036	7.497		0.0057	6.008
	μ_2	0.466			0.543			0.697			1.286		
Area			0.0095	2.030		0.0096	1.764		0.0211	3.025		0.0351	2.730
	k_2	0.006			0.008			0.010			0.020		
			0.0004	6.066		0.0003	3.942		0.0005	5.126		0.0007	3.416

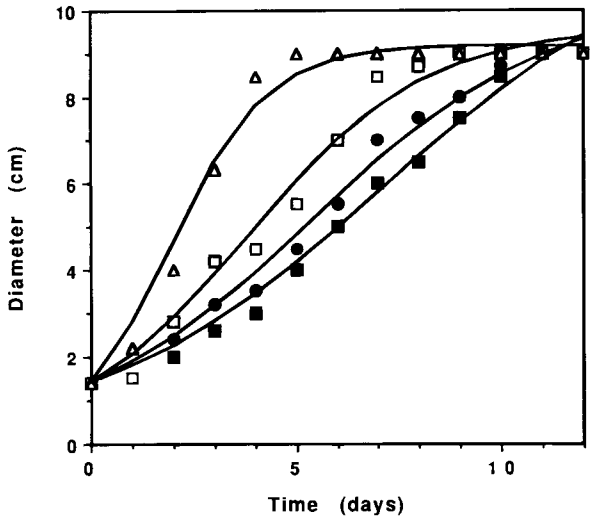

Fig. 5. *P. ostreatus* growth by diameter on lignocellulosic material. Experimental data: fermentations performed in a jar with particle size of (■) <0.42 mm, (●) 0.42–0.84 mm, (□) ~1 cm, and fermentation performed in the controlled bioreactor with particle size of (△) 0.42–0.84 mm. Line given by equation 7b, and constants from Table 2.

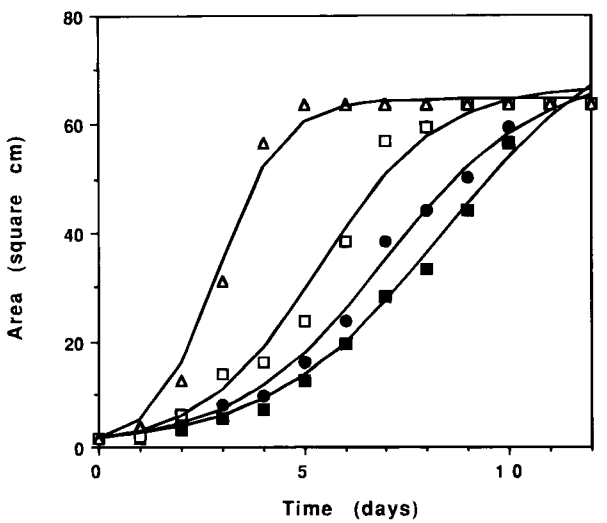

Fig. 6. *P. ostreatus* growth by area on lignocellulosic material. Experimental data: fermentations performed in a jar with particle size of (■) <0.42 mm, (●) 0.42–0.84 mm, (□) ~1 cm, and fermentation performed in the controlled bioreactor with particle size of (Δ) 0.42–0.84 mm. Line given by equation 7a, and constants from Table 2.

times were calculated to be between 1.49 and 0.99 d, respectively. When the bioreactor was used, the doubling time was 0.54 d for the growth measured by area, and 0.80 d for the growth measured by diameter. Therefore, μ values were higher for the bioreactor, possibly because of the controlled humidity of the atmosphere in the bioreactor where a con-

stant flow of air through the biomass was maintained (Fig. 3). For the experiments performed in the jars, air penetration into the substrate and the amount of air accessible to the mycelium was limited, causing the value for specific growth rate to be smaller. The k values for the bioreactor were two to three times more than the k values obtained for jars, since competition effects would be expected to be more pronounced because of more rapid growth in the bioreactor. The substrate becomes truly limiting since oxygen and moisture are provided in an enhanced manner in the bioreactor.

After mycelial growth covered the whole substrate, and the microbial biomass became dense with the mycelia, branches of the fungal growth penetrated the layer of glass beads in the bioreactor (Fig. 2). Hyphae extended toward the gas inlet and on the sides of the glass bioreactor toward the outlet. Fungal growth continued on the tips of the mycelium, with nutrient from the lignocellulose being carried to the tips. Hence, the elongation of mycelium occurred on both sides of the substrate, given sufficient O_2 and space for growth. In the case of fermentations in the jars, the availability of O_2 to the substrate should be highest at the surface of the lignocellulose and decreasing toward the bottom of the jar. Since the bottom is solid, the growth is limited, especially after the initial stages of fermentation.

The thickness of the plant material did not change during the primary growth of *P. ostreatus*, but it started decreasing in secondary growth phase. During fruit-body formation, the thickness of the substrate decreased considerably showing that substrate is being used extensively during mushroom production stage.

CONCLUSIONS

Growth of *P. ostreatus* on rapeseed inedibles of three different particle sizes showed that surface mycelial growth was fastest on a substrate with the largest particle size. Direct measurement of growth in solid state fermentation systems is difficult, and indirect measurements require tedious procedures. This work shows that a simple, unstructured model, based on reading the measured diameter of the mycelial mat, enabled quantitation of *P. ostreatus* growth on lignocellulosic plant material from rapeseed. The best fit of the growth data to an unstructured model was obtained with the logistic equation 7a based on area as the measure of growth. The development of the bioreactor, and demonstration of the utility of the logistic function model will be helpful in designing and monitoring further studies on use of *P. ostreatus* for bioconversion of lignocellulosic materials, whether in a CELSS or for terrestrial applications. Potential applications include treatment of wood using an environmentally friendly method to remove lignin for production of high quality pulps and papers, and enhanced commercial production of edible mushrooms from lignocellulosic waste materials.

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