



# Mass balance and thermodynamic description of solid state fermentation of lignocellulosics by *Pleurotus ostreatus* for animal feed production

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This study was focused primarily on the degradation of lignin in water hyacinth and barley straw for animal-feed production. The experiment was performed in a 1.5-L Applikon fermenter for 30 days, varying the air flow rate from 0.022 VVM/0.047 VMM to 0.048 VVM/0.102 VMM. A novel approach was introduced for prediction of a kinetic model by using instantaneous respiratory quotient (RQ) measurements and steady state elemental balances. Growth kinetics were determined for the fungus in a 30-day fermentation with a mixture of barley straw and water hyacinth as the substrate. The instantaneous heat-interaction profile was predicted from steady state balances. Fermentation data were checked for consistency using the entropy balance inequality, and thermodynamic efficiency was calculated to show that degradation of lignocellulosics by *Pleurotus ostreatus* followed more than one metabolic pathway during the course of the fermentation. Growth of *P. ostreatus* on lignocellulosics, such as water hyacinth and barley straw, was di-auxic or possibly tri-auxic during the 30 days of fermentation.

**Keywords:** protein to lignin ratio; *in vitro* dry matter enzyme digestibility; entropy balance model

## Introduction

White-rot fungi, such as *Pleurotus ostreatus*, degrade lignin. There are other lignin-degrading fungi, such as *Phanerochaete cryosporium*, that utilize more cellulose and hemicellulose than lignin; however, the rumen can digest cellulose and hemicellulose but not lignin. Lignin limits rumen digestibility of the lignocellulosics. A completely lignin-specific fungus is needed. However, lignin degradation is complicated and not yet completely understood.

In a tropical climate, the water hyacinth is a common agricultural weed that is rich in lignocellulose. The organism could be used for generating animal fodder for developing countries such as Bangladesh, where the water-hyacinth is a problem because it clogs waterways. In this study, attempts were made to convert water hyacinth to a protein-rich animal feed and to give a thermodynamic description of the solid state fermentation process.

## Materials and methods

### Organism and inoculum

*Pleurotus ostreatus*, ATCC 32783, was cultured in slants containing 10 ml of yeast malt agar (3.8 g per 100 ml water) at 30° C. After 10 days of incubation, the fungus was transferred to petri plates containing 10 ml yeast malt agar. Barley grain (10% of the wet weight of the substrate) was sterilized in 10 1-L canning jars (each jar containing equal amounts of grain), and the sample was autoclaved for 30 min at 120° C. Each jar was inoculated with 6–8

plugs (no. 9 cork-borer) of the fungus cultured in the petri plates and incubated for 2 weeks at ambient temperatures (28–29° C).

### Substrate preparation

Water hyacinth plants, excluding roots, were separated into leaves and bulbs. The plants, originally from Florida, were obtained from Raymond E. Smith, Fort Collins, Colorado. The leaves, bulbs and barley straw were chopped according to the following size distribution: leaves (2.5–3.8 × 1.3–2.5 cm), bulbs (2–2.5 × 1.3–2.5 cm) and straw (1.3–2.5 cm). The pH was adjusted to 6.0 using 90% tris hydroxy methyl-amino methane hydrogen chloride. The pH was measured using a pH meter and taking a 10-g sample, diluting it with 90 ml of water and soaking it for 1 h.

### Analytical methods

Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF) and Acid Detergent Lignin (ADL) of the straw were determined according to the procedure of Agricultural Handbook No. 379 [4]. Total protein was estimated by measuring trichloroacetic acid (TCA)-soluble Kjeldahl protein, and *in vitro* dry matter enzyme digestibility (INVDMED) was determined by the procedure reviewed by Rajarathnam and Bano [8]. Analysis of oxygen and carbon dioxide was accomplished using a gas chromatograph made by Gow-Mac Instrument Company (Madison, NJ, USA, model no. 69-550), using a thermal conductivity detector.

### Experimental

Experiments were performed in a 1.5-L Applikon fermenter (model no. Z81305C001, Foster City, CA, USA). The inlet air was humidified by a humidifying column (115-cm length, 5.5-cm OD) containing Rashig rings. The flow rate of water passing through the column was 100 ml min<sup>-1</sup>.

The outlet gas was dried by passing it through a silica-gel dryer (22 cm length, 4 cm outer diameter). The fermenter was insulated with 1.25-cm thick fiber glass insulation, and the temperature in the fermenter was maintained at 28–30° C. Figure 1 illustrates the experimental set-up. The fermenter was loaded as follows: wet water hyacinth weighing 450 g (36% leaves and 64% bulbs/stems) was mixed with 22.5 g of dry barley straw. Eighty milliliters of 90% buffer solution (tris-hydroxy methyl-amino methane hydrogen chloride in water) and 105 ml of water were added to the mixture. The mixture was then placed in an autoclavable bag and autoclaved for 30 min at 121° C. Thirty-five grams of mycelium and 16.4 g of barley grain with *Pleurotus mycelium* were added to the bag and thoroughly mixed. Pure mycelium was obtained by inoculating the canning jars which contained 14.8 g of barley grain, incubating the jars for 30 days, and scratching the mycelium from the glass surface. The mixture was then transferred to the fermenter. Initially, the moisture content in the substrate was 75.9%, and the air flow rate in the fermenter varied from 33.5–72.5 ml min<sup>-1</sup> (0.022 VVM/0.047 VMM to 0.048 VVM/0.102 VMM). VVM is defined as the volumetric air flow rate (ml min<sup>-1</sup>) divided by the volume of the fermenter (ml). Similarly, VMM is defined as the volumetric air flow rate (ml min<sup>-1</sup>) divided by the total mass of the wet material (g) in the fermenter.

### Modeling

Solid state fermentation (SSF) is a complex system, and it is difficult to model a solid state fermentation process via the chemical reaction engineering approach due to the heterogeneity of the substrates, the discrepancy in composition because of its agricultural origin, and the very poor mixing environment in the fermenter. A novel approach was proposed that requires only the measurement of gaseous components from the fermenter by using a black-box approach. Thermodynamic analysis of biological systems is an active field because [1,9]:

- Biological systems are often not understood from the microbial physiology point of view.
- If metabolic pathways are known, the physical and chemical properties must be assumed because pertinent information is not available in the literature.
- Understanding the microbial system is important in

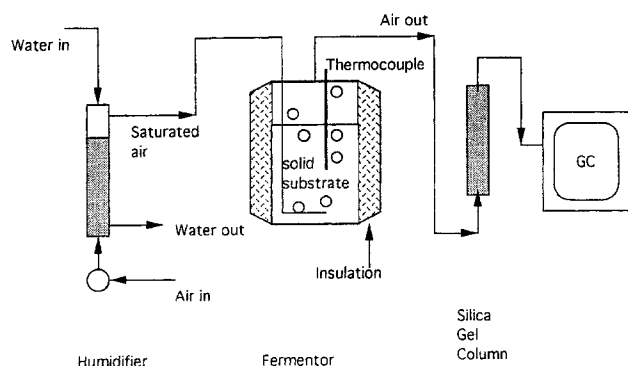


Figure 1 Experimental set-up for 30-day fermentation

developing a generalized tool for the analysis of biochemical reactions without knowing the complex intracellular processes. The thermodynamic approach is well suited to this type of analysis because the system can be modeled from a macroscopic point of view. All biological systems proceed via non-equilibrium processes; thus, non-equilibrium thermodynamics must be used instead of equilibrium thermodynamics.

For these reasons, researchers use the black-box approach to model metabolic processes [6,11]. Among the various black box parameters cited in the literature [1], degree of reduction was taken into consideration in this study. As mentioned earlier, solid state fermentation is a complex system, and the analysis of the solid samples from the fermenter does not provide precise information of the reaction. Attempts have been made in this study to obtain kinetic information from stoichiometric information incorporated with the instantaneous respiratory quotient (RQ) measurements.

### Prediction of growth kinetics from steady state balances

The fermenter was assumed to be a black-box containing an initial amount of substrate. Saturated air is then pumped into it. Fermentation produces biomass and carbon dioxide. A schematic of the fermenter system as well as the black-box model are shown in Figures 1 and 2, respectively. The fermenter mass balances are determined via elemental analysis, based on one mole of carbon, and assuming the chemical formula of the substrate and the biomass as CH<sub>a</sub>O<sub>b</sub>N<sub>c</sub> and CH<sub>x</sub>O<sub>y</sub>N<sub>z</sub>, respectively.

Introducing  $\Delta\phi_j = \phi_j - \phi_j^i$ , where  $j$  is any species other than water and  $i$  is any stream in the fermenter (Figure 2) and defining  $\Delta\phi_w = \phi_w + \phi_{wv} - \phi_w^i$  for water, elemental balances for carbon, oxygen, hydrogen and nitrogen can be obtained as follows:

$$Y_{bo} - Y_{so} + RQ = 0 \quad (1)$$

$$y \cdot Y_{bo} + 2 \cdot RQ + Y_{wo} = 2 + b \cdot Y_{so} \quad (2)$$

$$a \cdot Y_{so} = x \cdot Y_{bo} + 2 \cdot Y_{wo} \quad (3)$$

$$c \cdot Y_{so} = z \cdot Y_{bo} \quad (4)$$

The yield coefficients can be defined as follows:

$$Y_{bo} = \frac{\text{moles biomass produced}}{\text{moles O}_2 \text{ consumed}}$$

$$Y_{so} = \frac{\text{moles substrate consumed}}{\text{moles O}_2 \text{ consumed}}$$

$$Y_{wo} = \frac{\text{moles moisture produced}}{\text{moles O}_2 \text{ consumed}}$$

$$RQ = \frac{\text{moles CO}_2 \text{ produced}}{\text{moles O}_2 \text{ consumed}}$$

The compositional formula of the substrate (dry water hyacinth and barley straw mixture) and the fungus (biomass)

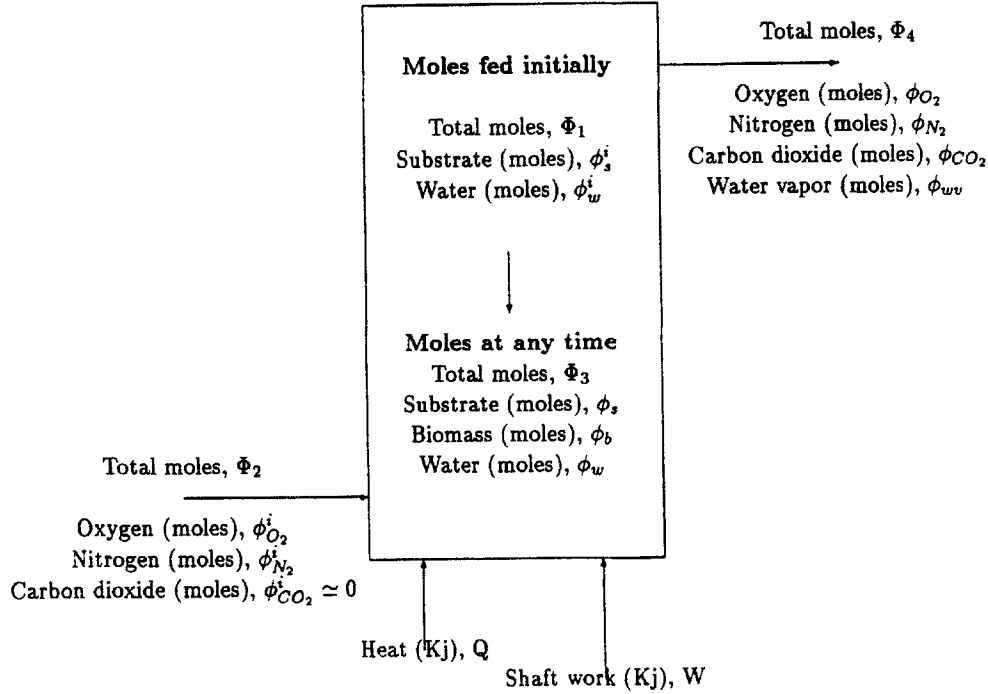


Figure 2 Black-box representation of a fermenter

has been obtained from elemental analysis of the dry substrate and the fungus. A large quantity of substrate (200 g) and fungal mycelium (100 g) was vacuum-dried at 40° C and milled before performing the elemental analysis. The total dry matter used for the elemental analysis of the substrate and biomass was 10 g for each case. The chemical composition of the fungus varies depending on the maturity of the fungus; however, there is no way to obtain the instantaneous biomass composition. Similarly, the substrate (lignocellulosic mixture) chemical formula depends on the maturity of the plant and the proportion of leaves and stems used in the substrate. The best approximation is to assume a constant chemical formula for the biomass and the substrate. Coefficients of substrate ( $\text{CH}_a\text{O}_b\text{N}_c$ ) and biomass ( $\text{CH}_x\text{O}_y\text{N}_z$ ), on the basis of 1 mole of carbon, are:  $a = 1.692$ ,  $b = 0.72$ ,  $c = 0.05$ ,  $x = 0.17$ ,  $y = 0.64$ , and  $z = 0.08$ . Solving Equations (1–4) simultaneously and substituting the values of  $a$ ,  $b$ ,  $c$ ,  $x$ ,  $y$  and  $z$  in Equations 1–4, provides the following relationships:

$$Y_{\text{so}} = 2.667 \text{ (RQ)} \quad (5)$$

$$Y_{\text{bo}} = 1.667 \text{ (RQ)} \quad (6)$$

$$Y_{\text{wo}} = 2.115 \text{ (RQ)} \quad (7)$$

Thus, measuring the oxygen consumed and the carbon dioxide evolution rate, different yield coefficients can be calculated. From these yield coefficients, the concentrations of biomass and substrate can be calculated.

#### Energy and entropy balances

The generalized degree of reduction is the number of equivalents of oxygen required for the complete oxidation of that quantity of organic compound contained in 1 gram atom

of carbon. This parameter can be used advantageously to simplify the fermenter energy and entropy balances [11]. To perform this simplification, assume: (i)  $\text{CO}_2$  and  $\text{N}_2$  are in the gaseous state, and  $\text{H}_2\text{O}$  is in the liquid state at standard temperature and pressure; (ii) heat capacity remains constant in the experimental temperature range; (iii) the inlet temperature equals the fermenter temperature; and (iv) the heat of combustion is expressed as  $\Delta h_{\text{c},i}^0 = -109.04 \cdot \gamma_i \text{ kJ mol}^{-1}$  [11]. These assumptions give the simplified energy balance of the fermenter. Equation 8 represents the final expression for energy balance of the fermenter with these assumptions.

$$Q + W = 109.4 \cdot (\Delta\phi_s\gamma_N + \Delta\phi_N\gamma_N + \Delta\phi_b\gamma_b) \quad (8)$$

Here,  $h_{\text{c},i}^0$  is heat of combustion of species  $i$  at 298 K,  $\gamma_i$  is the degree of reduction of species  $i$ ,  $Q$  and  $W$  are the fermenter heat input and the shaft work input, respectively. Division by  $(-\Delta\phi_{\text{O}_2})$ , substitution of  $\Delta\phi_i$ 's in terms of yield coefficients as defined earlier and insertion of the  $\gamma_i$ 's ( $\gamma_s = 4.25$  and  $\gamma_b = 2.89$ ) into Equation 8 gives:

$$Q/(-\Delta\phi_{\text{O}_2}) = 109.04 \cdot (2.89 \cdot Y_{\text{bo}} - 4.25 \cdot Y_{\text{so}}) \quad (9)$$

Equation 10, which is the entropy balance on the fermenter, can be obtained from the following assumptions: (i) all the assumptions from which Equation 7 were obtained; (ii) the substrate components behave as an ideal mixture, and (iii) the Gibb's free energy of combustion can be described as:  $\Delta g_{\text{c},i}^0 = -110.23 \cdot \gamma_i \text{ kJ mol}^{-1}$  [11].

$$\gamma_s \cdot Y_{\text{so}} - \gamma_w \cdot Y_{\text{wo}} + \gamma_{\text{O}_2} - \gamma_{\text{CO}_2} \cdot \text{RQ} + \gamma_b \cdot Y_{\text{bo}} \leq 0 \quad (10)$$

With the insertion of  $Y_{so}$ ,  $Y_{bo}$  and  $\gamma_i$  values into Equation 10, Equation 11 is obtained.

$$T \cdot S_{generation} / (-\Delta\phi_{o_2}) = 6.517 \cdot RQ + 4.0 \geq 0 \quad (11)$$

Here,  $S_{generation}$  is entropy generated in the fermentation process. Another important thermodynamic parameter,  $\eta$ , is the thermodynamic efficiency of the system. Roels and Suijdam [10] proposed a definition of the thermodynamic efficiency (Equation 12).

$$\eta = 1 - \frac{T \cdot S_{generation} / (-\Delta\phi_{o_2})}{\sum_j^{products} \gamma_j Y_{j_o} - \sum_j^{reactants} \gamma_j Y_{j_o}} \quad (12)$$

The relationship between the thermodynamic parameters derived in this section with the fermentation experimental results are established later.

## Results

Solid state fermentation is a very heterogeneous system, and it was pointed out above that it is very difficult to collect precise information by measuring solid samples from the fermenter. In order to circumvent this problem, only gaseous components were measured. The implication of these measurements will be described in this section.

The fermenter is aerated from the top via a tube with an outer diameter of 0.5 cm (Figure 1). When aeration is performed in one section of the fermenter, oxygen diffusion to other sections is minimal; hence, the sections can be considered independent from one another. Moreover, no stirring is performed because the fungal mycelium of the *P. ostreatus* is extremely sensitive to shear force [7]. The performance of the fermenter is determined by day-to-day measurements of the respiratory quotient (RQ). The RQ is calculated from the ratio of evolved carbon dioxide and consumed oxygen. Figure 3 illustrates the RQ values *versus*

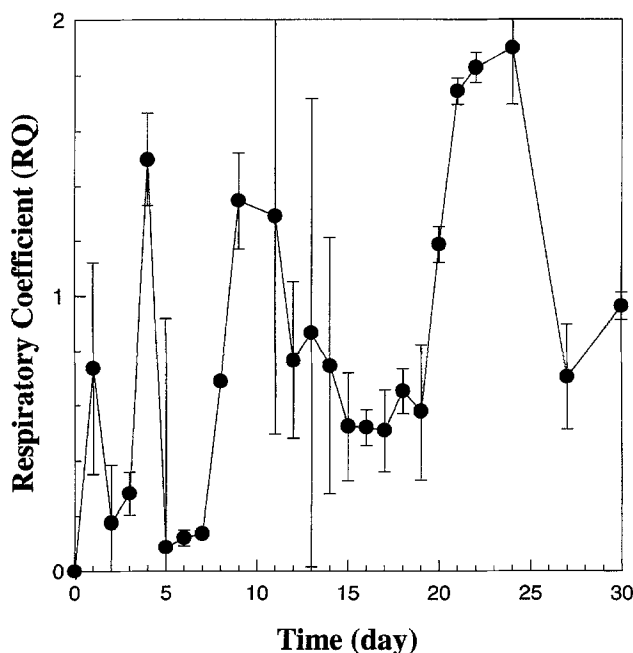


Figure 3 Respiratory coefficient profile for the Applikon fermenter

sampling time. Each point is the average of two fermented samples that were analyzed in duplicate via gas chromatography. The error bar is calculated by dividing the standard deviation by the mean of the four measurements. Generally, Figure 3 shows a relatively large error for a given RQ measurement, which indicates that the biological fermentation varies during the growth due to inhomogeneities in the fermented material. Even if no solid samples are withdrawn and analyzed during the fermentation period of 30 days, one assumes that both anaerobic and aerobic fermentation are operative in some regions of the fermenter. This statement is not unreasonable because thermodynamic efficiencies vary with time, and as mentioned by Dawes [3], this can only be explained by the presence of an anaerobic metabolic process. Three maxima can be observed from the RQ profile in Figure 3. These peaks in fermentation activity are in the vicinity of 4, 10, and 25 days. The presence of these peaks indicates that more than one component is present in the growth substrate. Inhomogeneities in the substrate can also be invoked to rationalize this behavior. Due to the heterogeneity of the fermentation environment, anaerobic processes will be dominant in some regions of the fermenter, and because the fermentation proceeds readily this implies that in the oxygen-poor regions the fungus adapts and survives, and carries the reaction via a secondary metabolic pathway.

At the end of the fermentation period (30 days), the total fermented matter was dried overnight at 100° C. The determination of the residual components (lignin, cellulose, hemicellulose, and soluble sugars) are summarized in Table 1. After fermentation, reduction in all the residual components was observed (Table 1). This decrease indicates an effective fermentation process. Fermentation led to consumption of 63% of the lignin, 85% of the cellulose, 65% of the hemicellulose, and 43% of the soluble sugars on the basis of the original amount of each component (Table 1). One of the objectives of this study was to find a fungus that would specifically degrade lignin. The results were not satisfying because the *P. ostreatus* is not lignin-specific. Nevertheless, the results show that the fungus degrades twice the amount of lignin relative to the total amount of cellulose and hemicellulose. The reduction of the protein content relative to the control is a bit surprising, but this may be due to the loss of soluble protein during analysis and the production of volatile nitrogenous fermentation by-products.

In view of the objectives, the fermented matter also should exhibit a protein enrichment. It is clear that this objective was not fully achieved (Table 1), but if the protein to lignin ratio is calculated (Table 2), it is shown that this ratio increased by a factor of 2 relative to the control. Although after fermentation the absolute content in proteins decreased, the second objective was met because the *P. ostreatus* led to an effective increase of the proteins relative to the lignin. The *in vitro* enzyme digestibility test, INVDMED, is a convenient way to characterize how ruminants will digest the feed. However, these determinations are fraught with experimental uncertainties. The determination of the INVDMED from the fungus-treated feed was usually lower relative to the control (Table 2).

**Table 1** Fiber components on a wet basis after 30 days of fermentation

Sample	Lignin (%)	Cellulose (%)	Hemicellulose (%)	Soluble sugar (%)	Proteins (%)
Control	0.60	3.4	2.8	13	4.4
After 30 days	0.22	0.50	0.96	7.2	3.4

**Table 2** Relevant parameters determined after 30 days of fermentation

Sample	INVDMED <sup>a</sup> (%)	Proteins to lignin ratio (%)
Control	88	7.4
After 30 days	83	15

<sup>a</sup>The INVDMED abbreviation represents *in vitro* dry matter enzyme digestibility

**Modeling**

Using Equations 5 and 6, the biomass and substrate concentrations can be calculated, respectively, from the RQ values illustrated in Figure 3. The biomass and the substrate profiles obtained from the application of both equations are illustrated in Figure 4. Figure 4 suggests that a di-auxic (or possibly a tri-auxic) growth occurred, which implies that more than one component is degraded by the fungus. Preliminary in-house results on a similar system exhibited the same type of behavior [2]. A typical fermentation leads to the utilization of soluble sugars in the first stage, while less favorable components such as hemicellulose, cellulose and lignin are degraded in the second stage. In Figure 4, it is shown that after 30 days, the model evaluates the final substrate content to be approximately 1.8 mol. This compares relatively well with the determined experimental

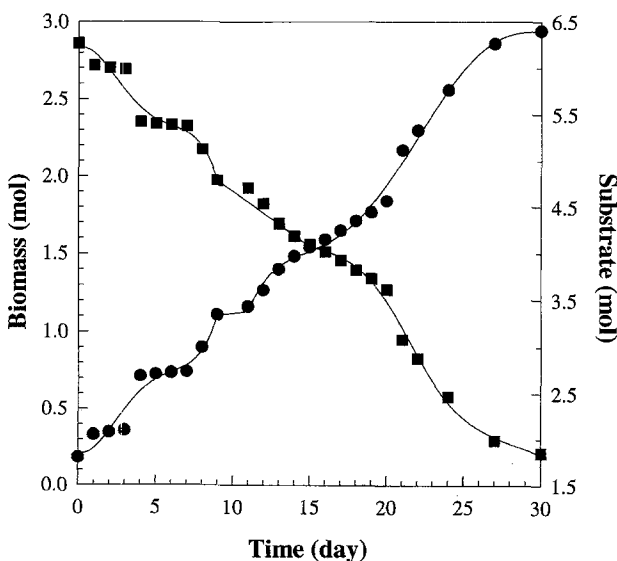
substrate content (2 mol), and suggests the model is insensitive to the heterogeneity of the substrate.

Figure 5 illustrates the instantaneous heat-interaction profile generated via Equation 9. The instantaneous heat interaction profile can be used in the on-line control of the fermenter. Rearrangement of the energy balance (Equation 8) yields the ratio of the generated heat per produced biomass:

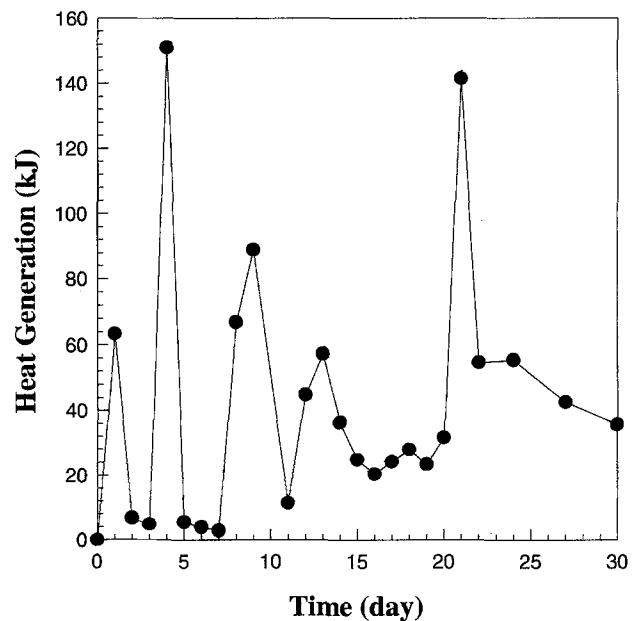
$$\frac{Q}{\Delta\phi_b} = 436.2 \cdot Y_{ob} \tag{13}$$

By use of Equation 13, the biomass production rate can be regulated by controlling the heat-exchange through the fermenter. The process control of the fermenter using Equation 14 is beyond the scope of the current study; however, the heat profile clearly indicates that the fermentation process is exothermic. Moreover, it was observed that the heat generation maxima corresponded relatively well to those previously observed (Figure 3). This correspondence between the peaks provides further evidence that during those days, one specific substrate component gets processed by the fungus which in turn generates the calculated heat excess.

If the fermentation process is operative, the Second Law of thermodynamics dictates that the entropy generation



**Figure 4** Calculated biomass and substrate concentration profile for 30-day fermentation; ● biomass, ■ substrate



**Figure 5** Calculated instantaneous heat generation profile

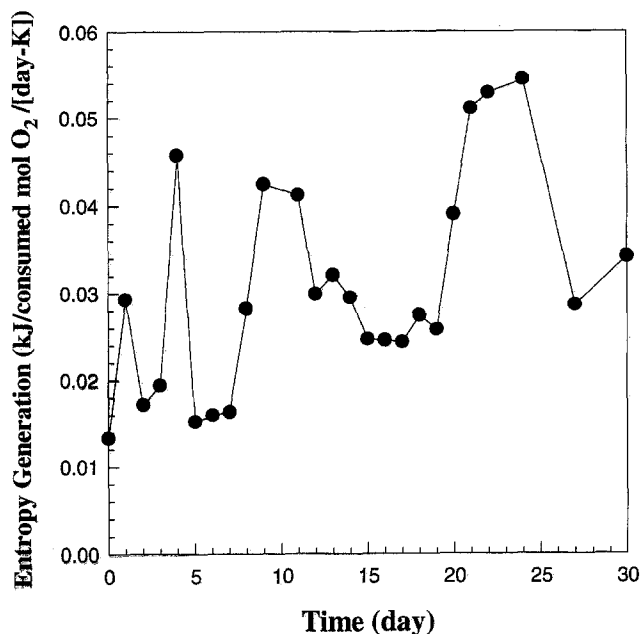


Figure 6 Entropy generation profile for 30-day fermentation

must be positive. As discussed by Schrödinger, microorganisms feed on negative entropy [5]. This is ascertained by the application of Equation 11 to the experimental RQ values. Results from Equation 11 give rise to the entropy generation profile illustrated in Figure 6. All the generated entropy values are positive indicating that the measured values are consistent, and the fermentation process is viable.

Data presented in Figure 4 can be expressed in terms of the specific growth rate. Figure 7 illustrates the specific growth profile for the fermentation. As expected, higher growth rates are observed at the beginning of the fermentation, ie between 0.8 and 1 day<sup>-1</sup>. The low growth rate

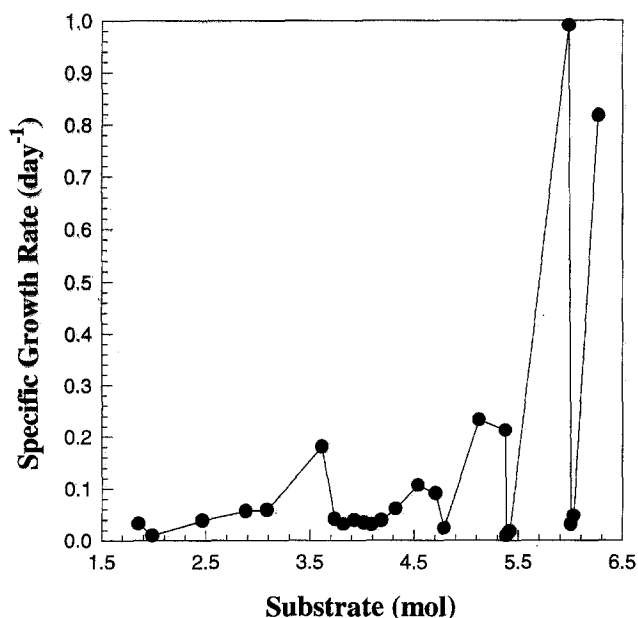


Figure 7 Calculated specific growth rate profile

values centered around 6.2 mol are due to the initial instability of the fermenter. At approximately 5 mol of substrate, the growth rate decreases dramatically, and growth exhibits a plateau. The average value of the plateau oscillates at 0.1 day<sup>-1</sup>. In view of previous discussions and Figures 3 and 5, Figure 7 shows that the soluble sugars are degraded at a higher rate relative to the other substrate components (hemicellulose, cellulose or lignin). Moreover, since the growth rate levels off, this implies that the remaining components are degraded at similar rates.

Figure 8 shows the thermodynamic efficiency profile obtained using Equation 12. In the early stages of the fermentation (up to 10 days), the thermodynamic efficiency profile varied (0.93 to 0.99). After this period, the efficiency levelled off at about 0.985. This behavior is almost identical to that observed in Figure 7 and can be rationalized using the same arguments mentioned above.

## Conclusions

It has been shown how *P. ostreatus* degrades an agricultural substrate. The main objectives of the project were only partially achieved and can be summarized as follows: (i) the fungus did not degrade the lignin specifically, (ii) the *in vitro* digestibility decreased slightly, (iii) the amount of protein increased relative to lignin. The results suggest the degradation process is tri-auxic. Moreover, it was determined that the most probable degradation sequence starts with degradation of soluble sugars, followed by hemicellulose and/or cellulose, and finally, by lignin. It also was shown that from a macroscopic point of view, the thermodynamic approach is well suited for the analysis of the fermentation process. Thermodynamic parameters determined from the experimental data support the proposed degradation scheme.

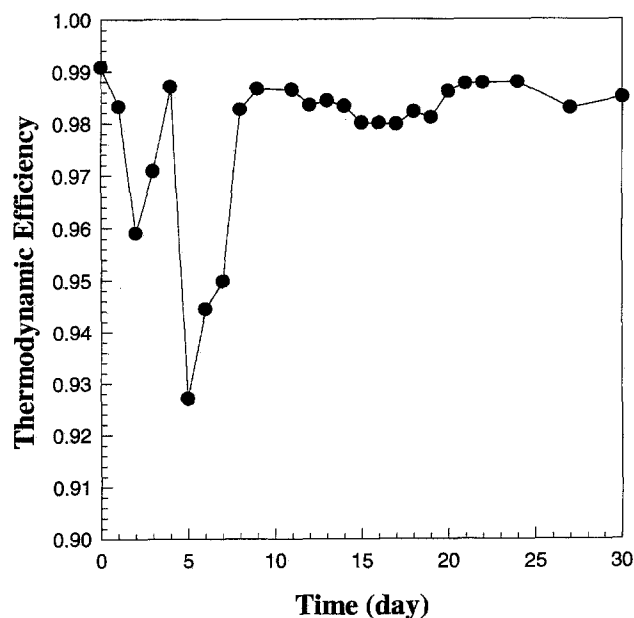


Figure 8 Thermodynamic efficiency profile

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