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Bioresource Technology 93 (2004) 261–268
BIORESOURCE TECHNOLOGY

Thermal denaturation: is solid-state fermentation really a good technology for the production of enzymes?

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Received 8 August 2002; received in revised form 7 July 2003; accepted 2 November 2003

Abstract

The potential for thermal denaturation to cause enzyme losses during solid-state fermentation processes for the production of enzymes was examined, using the protease of *Penicillium fellutanum* as a model system. The frequency factor and activation energies for the first-order denaturation of this enzyme were determined as 3.447×10^{59} h^{-1} and 364,070 $J\,mol^{-1}$, respectively. These values were incorporated into a mathematical model of enzyme deactivation, which was used to investigate the consequences of subjecting this protease to temporal temperature profiles reported in the literature for mid-height in a 34 cm high packed-bed bioreactor of 150 mm diameter. In this literature source, temperature profiles were measured for 5, 15 and 25 liters per minute of air and enzyme activities were measured as a function of time. The enzyme activity profiles predicted by the model were distributed similarly, one relative to the other, as had been found in the experimental study, with substantial amounts of denaturation being predicted when the substrate temperature exceeded 40 °C, which occurred at the lower two airflow rates. A mathematical model of a well-mixed bioreactor was used to explore the difficulties that would be faced at large scale. It suggests that even with airflows as high as one volume per volume per minute, up to 85% of the enzyme produced by the microorganism can be denatured by the end of the fermentation. This work highlights the extra care that must be taken in scaling up solid-state fermentation processes for the production of thermolabile products.

Keywords: Solid-state fermentation; *Penicillium fellutanum*; Enzyme production; Thermal denaturation

1. Introduction

Solid-state fermentation (SSF) has often been proposed for the production of enzymes, especially fungal enzymes (Pandey et al., 2000). Much of the work has been done at laboratory scale, where it is easy to control the temperature at the optimum value for growth. The promising results obtained at laboratory scale often lead researchers to believe that their processes would work well at large scale (Pandey et al., 2000). However, in large scale SSF bioreactors, the substrate bed often reaches temperatures well above the process optimum, due to the difficulties of removing heat from solid beds of low water content (Mitchell et al., 2000). The possibility that the temperatures reached might cause significant thermal denaturation of the enzyme during the fermentation has not been investigated.

We investigate this potential in the context of protease production by *Penicillium fellutanum*. A mathematical model is used to reproduce temperature profiles similar to those observed during SSF processes in bioreactors. This model is used, in combination with experimentally-determined denaturation kinetics, to explore the extent to which high temperatures might affect large-scale enzyme production processes.

2. Methods

2.1. Model development

The bioreactor modeled is cylindrical, with diameter $\varnothing$ and height L, and its sides are water-jacketed (Fig. 1).

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doi:10.1016/j.biortech.2003.11.007

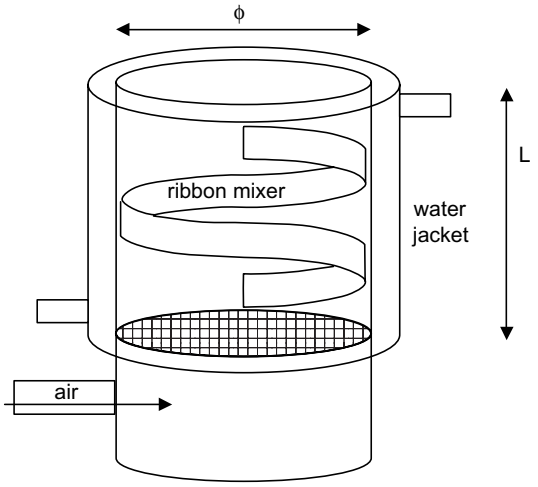

Fig. 1. The well-mixed bioreactor described by the mathematical model.

Since the main aim of the heat transfer part of the model is to reproduce temporal temperature profiles similar to those found in the literature, to maintain simplicity it is assumed: that the substrate bed is well-mixed, with the air leaving the bioreactor being in equilibrium with the solids; that growth does not alter the properties of the bed; and that water is added as necessary to maintain an optimal water activity for growth. Therefore a water balance is not done, even though evaporation is described in the energy balance. Such replenishment of water is not difficult in a well-mixed bioreactor (Nagel et al., 2001).

Logistic growth kinetics are assumed in terms of the biomass content per kg of initial dry substrate (IDS)

$$\frac{dX}{dt} = \mu X\left(1 - \frac{X}{X_m}\right) \tag{1}$$

The specific growth rate constant is assumed to be unaffected by changes in temperature. Such behavior has been suggested for other fungi by modeling work (Saucedo-Casteneda et al., 1990) and experimental work (Raimbault and Alazard, 1980; Ikasari et al., 1999; Stuart et al., 1999). This is not surprising. Typically the effects of temperature on growth kinetics are estimated based on experiments in which a number of different cultures are incubated at different temperatures. However, in typical SSF processes the culture is only subjected to higher temperatures after it has been growing for some time at the optimum temperature for growth. In this case delayed effects can be expected (Ikasari et al., 1999).

The experimental protease activity profile is described empirically

$$\frac{dE}{dt} = \alpha(E+D)\left(1 - \frac{(E+D)}{E_m}\right) - kE \tag{2}$$

where E represents active enzyme, this term being used to denote enzyme that has not denatured and therefore still retains its catalytic ability, and D represents denatured enzyme. Each term has units of protease units (PU) per gram of initial dry solid. The specific enzyme production rate is assumed to be unaffected by temperature. In contrast, the rate constant of the denaturation reaction is expressed as a function of temperature

$$k = A_0 \exp\left(\frac{-E_a}{R(T+273.15)}\right) \tag{3}$$

Denatured enzyme is produced by a first-order denaturation reaction

$$\frac{dD}{dt} = kE \tag{4}$$

An energy balance over the bioreactor gives an equation for the bed temperature (T)

$$\frac{dT}{dt} = \frac{F_a C_{pa}(T_a - T) + F_a(\lambda(H_a - H) + C_{pv}(H_a T_a - HT)) - hA(T - T_w) + BY_Q\frac{dX}{dt}}{C_{pb}B(1+W)} \tag{5}$$

where the humidity of the outlet air (H) is calculated in two steps. First the vapor pressure in mm Hg (P_{vap}) is calculated

$$P_{vap} = \exp\left(18.3036 - \frac{3816.44}{(T+273.15) - 46.13}\right) \tag{6}$$

Then the humidity is calculated as

$$H = 0.6246\frac{P_{vap}}{P - P_{vap}} \tag{7}$$

where the factor 0.6246 is the ratio of the molecular weight of water (18.0 g mol^{-1}) to the average molecular weight of dry-air (28.82 g mol^{-1}). For the humidity at the air inlet, T is replaced with T_a, allowing the calculation of P_{vapa} and H_a with the same equations. The quantity of dry solids in the bioreactor is given by

$$B = \rho_b \cdot L \cdot \pi \cdot (\phi/2)^2 \tag{8}$$

It is assumed that only the sides of the bioreactor in contact with the bed are used for heat removal. Therefore the area for heat transfer is calculated as

$$A = L \cdot \phi \cdot \pi \tag{9}$$

When the temperature-control strategy is used, the following equation adjusts the temperature of the cooling water in response to the bed temperature

$$T_w = T_s - F \cdot (T - T_s) \quad (10)$$

The parameter values used in the model are given in Table 1 (Nagel et al., 2001; Himmelblau, 1982; Sweat, 1986; Mitchell and von Meien, 2000; Gumbira-Sa'id et al., 1992; Stuart, 1996). The heat transfer coefficient used is for a well-mixed SSF bioreactor filled with moisturized oats and equipped with a stainless steel water jacket (Nagel et al., 2001). The equations were integrated using the subroutine DRKGS within a program written in FORTRAN (Ralston and Wilf, 1960).

2.2. Microorganism

The fungus *P. fellutanum* was isolated from Brazil nuts in the Enzyme Technology Laboratory of the Universidade Federal do Paraná, Brazil, and deposited as IOC4229 in the Oswaldo Cruz Foundation Institute, Rio de Janeiro. Spore suspensions of approximately 10^9 spores ml^{-1} were prepared by addition of a 1 $g\,l^{-1}$ solution of Tween-80 in water to cultures that had been growing on potato dextrose agar (Biobras) for 5 days at 28 °C.

2.3. Substrate preparation

Sunflower seeds with hulls were ground and sieved. The fraction between 0.8 and 2.0 mm was retained and dried at 60 °C. The substrate for each flask was prepared with 20 g of this dried meal and 24 ml of a mineral solution containing, per l: 2.0 g KNO_3; 0.5 g $MgSO_4 \cdot 7H_2O$; 1.0 g K_2HPO_4; 0.44 g $ZnSO_4 \cdot 7H_2O$; 1.12 g $FeSO_4 \cdot 7H_2O$ and 0.20 g $MnSO_4 \cdot 7H_2O$. The moistened substrate was autoclaved at 121 °C for 15 min.

2.4. Fermentation and protease extraction

Each flask was inoculated with 1 ml of spore suspension and then incubated at 28 °C. At each sampling time duplicate flasks were removed. One hundred milliliters of a solution consisting of 2% (w/v) NaCl was added to each flask, which was subsequently agitated for

Table 1
Symbols used in the model and their values

Symbol	Description	Value and units	Source
A_0	Frequency factor denaturation	3.447×10^{59} h^{-1}	Fig. 4
A	Bed/cooling water jacket contact area	m^2	Eq. (9)
B	Total mass of solids in the bed	kg	Eq. (8)
C_{pa}	Dry-air heat capacity	1007 J kg-dry-air^{-1} $°C^{-1}$	Himmelblau (1982)
C_{pb}	Moist substrate bed heat capacity	2500 J kg-$solid^{-1}$ $°C^{-1}$	Sweat (1986)
C_{pv}	Water vapor heat capacity	1867 J kg-$vapor^{-1}$ $°C^{-1}$	Himmelblau (1982)
D	Denatured enzyme	$D_0 = 0$ PU g IDS^{-1}	Chosen
E	Solid substrate active enzyme content	$E_0 = 1$ PU g IDS^{-1}	Fig. 2
E_a	Denaturation reaction activation energy	364,070 J mol^{-1}	Fig. 4
E_m	Solid substrate maximum enzyme content	141.3 PU g IDS^{-1}	Fig. 2
F	Gain factor temperature control strategy	0 or 2, as indicated (–)	Mitchell and von Meien (2000)
F_a	Dry-air flow rate	60 or 120 (kg-dry-air h^{-1})	Chosen
h	Bioreactor wall heat transfer coefficient	360,000 J h^{-1} m^{-2} $°C^{-1}$	Nagel et al. (2001)
H	Air outlet air humidity	kg-vapor kg-$dry\text{-}air^{-1}$	Eq. (7)
H_a	Air inlet air humidity	kg-vapor kg-$dry\text{-}air^{-1}$	Eq. (7)
k	Denaturation reaction rate constant	h^{-1}	Eq. (3)
L	Bed height	m	Chosen
P	Total pressure in the system	760 mm Hg	1 atm.
P_{vap}	Water vapor pressure at the air outlet	mm Hg	Eq. (6)
P_{vapa}	Water vapor pressure at the air inlet	mm Hg	Eq. (6)
R	Universal gas constant	8.314 J mol^{-1} $°C^{-1}$	Himmelblau (1982)
t	Time	$t_0 = 0$ h	
T	Bed temperature	$T_0 = 30$ °C	Eq. (5)
T_a	Temperature of the air at the air inlet	30 °C	Chosen
T_s	Temperature control strategy setpoint	30 °C	Chosen
T_w	Temperature of the cooling water	°C	Eq. (10)
W	Water content of the bed	1 kg-water kg-IDS^{-1}	Chosen
X	Biomass content of the solid substrate	$X_0 = 0.002$ kg-biomass kg-IDS^{-1}	Gumbira-Sa'id et al. (1992)
X_m	Maximum possible biomass content	0.250 kg-biomass kg-IDS^{-1}	Gumbira-Sa'id et al. (1992)
Y_Q	Heat yield from growth	8.366×10^6 J kg-$biomass^{-1}$	Saucedo-Casteneda et al. (1990)
α	Enzyme production specific rate constant	0.0782 h^{-1}	Fig. 2
ϕ	Diameter of the bed	m	Chosen
λ	Enthalpy of evaporation of water at 0 °C	2,500,900 J kg-$vapor^{-1}$	Himmelblau (1982)
μ	Specific growth rate constant	h^{-1}	Chosen
ρ_b	Bed packing density	178.5 kg-dry-solids m^{-3}	Stuart (1996)

1 h at 110 rpm at 28 °C. The solution was then filtered through cheesecloth, with the excess water held in the solids being squeezed out manually. The pH of the crude extract was measured as 6.2 with a pH meter (Corning, USA).

2.5. Temperature stability studies

Samples of crude extract were incubated at various temperatures. Samples were removed at intervals and the protease activity determined as described below. Residual activity was calculated as the ratio of this activity to the activity of the crude extract before incubation. Several replicates of these assays were done, although only one set of results is presented.

2.6. Determination of protease activity

Protease activity was determined by the method of Leighton et al. (1973). Extracts were diluted to give final absorbances in the assay between 0.2 and 0.7. 150 μl of diluted extract was added to 250 μl of a 10 mg ml^{-1} solution of azocasein in 0.1 M phosphate–citrate buffer at pH 6.0. The tubes were sealed and incubated at 47 °C for 30 min. The reaction was stopped by addition of 1.2 ml of 10% (w/v) trichloroacetic acid. Tubes were centrifuged at 14,000*g* for 10 min. The 1.2 ml of the supernatant was added to 1.4 ml of 1 M NaOH. The A_{440} was read against a blank that had been processed identically except that the trichloroacetic acid was added to the extract prior to the azocasein in order to denature the enzyme. A unit of protease activity (PU) was defined as the amount of enzyme that produced a difference in A_{440} of 1.0 absorbance unit per minute in this assay.

3. Results

3.1. Determination of the parameters of the denaturation reaction

The profile of protease production by *P. fellutanum* is shown in Fig. 2. The logistic equation was fitted, with P_0 restricted to a maximum of 1 PU g IDS^{-1}, to give an empirical equation describing protease production. This yielded values for α and E_m of 0.0782 h^{-1} and 141.3 PU g IDS^{-1}, respectively (Table 1).

Straight lines were obtained on the log-linear plot for thermal denaturation of the protease, indicating a first-order reaction (Fig. 3). The Arrhenius plot in Fig. 4 gives the values of A and E_a for Eq. (3), being 3.447×10^{59} h^{-1} and 364,070 J mol^{-1}, respectively (Table 1).

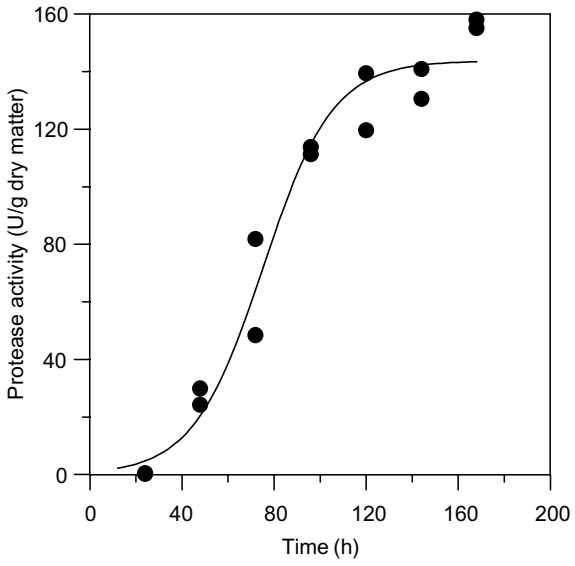

Fig. 2. Profile of enzyme production by *Penicillium fellutanum* with Eq. (2) fitted.

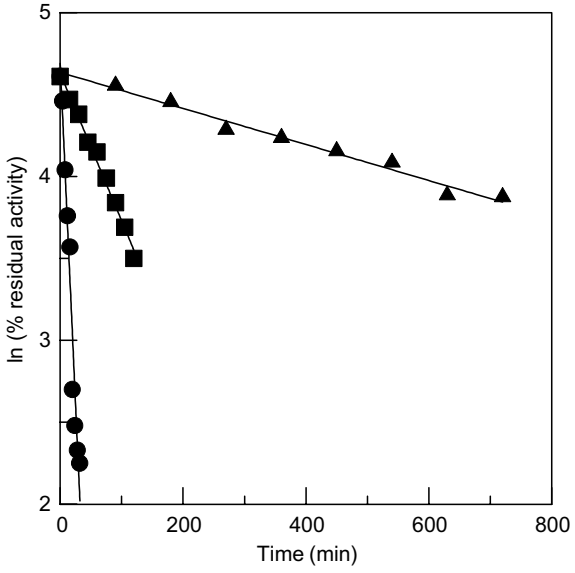

Fig. 3. Residual activity of the *Penicillium fellutanum* protease as a function of time during incubation at various temperatures, plotted on a log-linear scale: (▲) 40 °C; (■) 45 °C and (●) 50 °C. The lines of best fit were obtained by linear regression, in all cases the value of R^2 was 0.96 or higher.

3.2. Denaturation of the protease subjected to literature temperature profiles

Ghildyal et al. (1994) measured temperatures and the activities of the enzyme glucoamylase at various different heights during growth of *Aspergillus niger* in a packed-bed with various different airflows. Simulations were done with Eqs. (2)–(4) to investigate the active enzyme levels that would be expected if an enzyme with

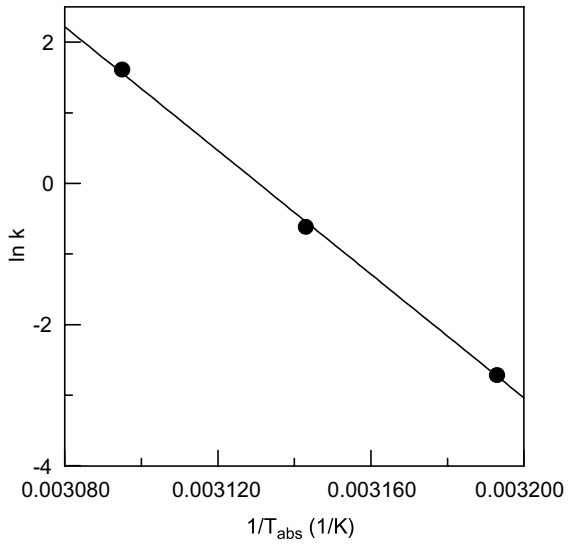

Fig. 4. Arrhenius plot for the denaturation reaction of the *Penicillium fellutanum* protease.

the properties of the protease characterized in the previous section were produced according to Eq. (2) and subjected to these temperature profiles. Given that the process of Ghildyal et al. (1994) took only 50 h, the parameter α in Eq. (2) was changed to 0.2 h^{-1}, which allows the protease production to occur within this time period. The temperature profiles, shown in Fig. 5A, were simply described empirically.

Fig. 5B shows the results obtained by Ghildyal et al. (1994) for the glucoamylase levels in the substrate whereas Fig. 5C shows the active enzyme levels that would be expected for the protease subjected to the same temporal temperature profile. Given that the results are for different enzymes, the absolute values of the activities in the two graphs are not important. What is important is that the distribution of the lines within the two graphs is similar. In both Fig. 5B and C the final enzyme levels for the airflow of 15 litres per minute (lpm) is approximately three times greater than the final value for 5 lpm. The major difference between the two graphs is that in Fig. 5B the last two values for the airflow of 25 lpm are little different from those for 15 lpm whereas in Fig. 5C there are large differences between these two lines at the end of the process. This could be due to the drying out of the bed that occurred at 25 lpm, which limited enzyme production Ghildyal et al. (1994), but is not described in the model. Fig. 5B shows the enzyme activity profile obtained at 25 lpm for the lower bed height of 33 mm, where the bed did not dry out. In this case the final enzyme levels are much closer to the pattern predicted by the model for mid-height of the bioreactor at 25 lpm. However, Ghildyal et al. (1994) did not provide a temperature profile for the 33 mm bed height, so the model predictions cannot be directly compared with the results for this bed height.

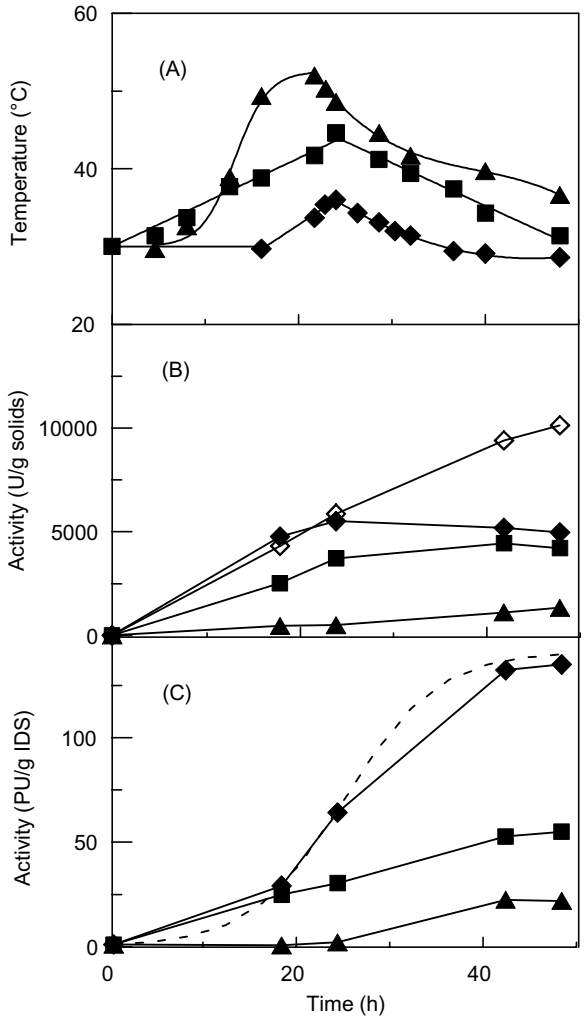

Fig. 5. (A) Temperature profiles obtained by Ghildyal et al. (1994) at 170 mm height for a packed-bed bioreactor, with aeration rates of (▲) 5; (■) 15 and (◆) 25 lpm. The solid lines represent the equations used to describe these curves. (B) Glucoamylase activities measured by Ghildyal et al. (1994) at 170 mm height with aeration rates of: (▲) 5; (■) 15 and (◆) 25 lpm and (◇) glucoamylase activities measured by Ghildyal et al. (1994) at 33 mm height with an aeration rate of 25 lpm. (C) Protease activity profiles predicted for protease production according to Eq. (2) with $\alpha = 0.2$ h^{-1} and being subjected to the temperature profiles shown in part A, with the same symbol, for: (▲) 5; (■) 15; (◆) 25 lpm and (- - -) total enzyme produced, as described by Eq. (2).

The model predicts that denaturation will be almost negligible at the airflow of 25 lpm, which can be seen by comparing the predicted curve with the curve for total enzyme production (Fig. 5C). This is not surprising, because the maximum temperature reached is less than 36 °C. At 15 lpm, the maximum temperature reached is 45 °C, and, at the end of the fermentation, two-thirds of the enzyme that was produced is denatured. At 5 lpm the maximum temperature reached is 52 °C, and 85% of the enzyme produced is predicted to have denatured by the end of the fermentation. Ghildyal et al. (1994) obtained the temperature profiles shown in Fig. 5A in a

laboratory-scale bioreactor. Even greater denaturation is likely to occur in large-scale bioreactors where temperature control is even more difficult. The next section explores this.

3.3. Simulation of the performance of a large scale bioreactor

The model was used to explore the magnitude of the temperature profiles that might be expected in a large scale process for protease production and the effect of these profiles on active enzyme levels obtained in a fermentation. Simulations were done for a bioreactor with a bed of 1 m diameter and 1 m height. Two situations were compared: an organism having specific growth rate (μ) and specific protease production rate (α) constants equal to 0.2 h^{-1}, to match the rate of heat production expected in the scale up of a process with *A. niger*, and an organism having both these values equal to 0.0782 h^{-1}, which is equal to the value of α for *P. fellutanum*, to match the rate of heat production that would be expected with a slower growing organism. Fig. 6 shows the bed temperatures that can be expected in these two situations, as well as the active enzyme levels, or, in other words, the enzyme activities that would be measured if samples of the substrate were removed and assayed for protease.

With the fast growing organism, temperatures as high as 48 °C will be reached when the airflow is 60 kg-dry-air h^{-1} and the cooling water temperature is maintained at 30 °C (Fig. 6). Note that 60 kg-dry-air h^{-1} represents approximately one volume of air per total bioreactor volume per minute, an aeration rate that is typical of submerged liquid fermentations. Substantial losses of the enzyme are predicted, with only 25% of the total enzyme being active at 50 h. Increasing the airflow to 120 kg-dry-air h^{-1} is not very effective since the temperature still reaches 44 °C, with substantial enzyme losses. It is more effective to control the temperature of the cooling water in response to the bed temperature according to Eq. (10), with $F = 2$. Even with an airflow of only 60 kg-dry-air h^{-1}, this strategy enables reasonable temperature control, with the maximum temperature reached being 38 °C and the final active enzyme level reaching 123 PU g IDS^{-1}, which represents a loss of only 13%. However, this approach needs cooling water temperatures as low as 14.5 °C, which may necessitate refrigeration, especially in tropical countries.

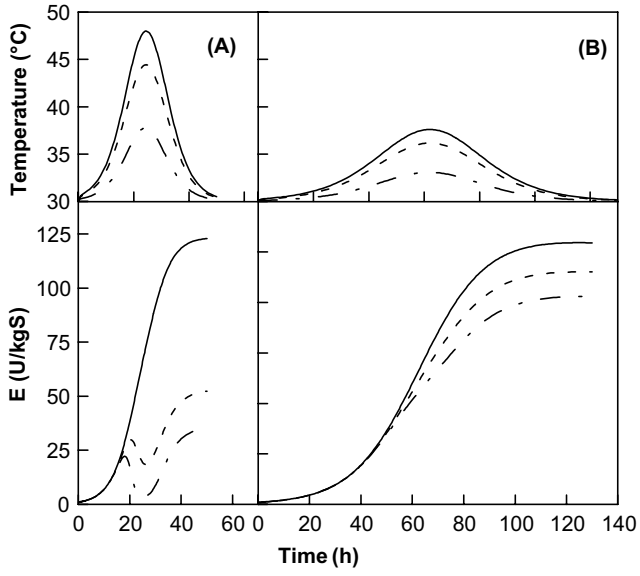

Fig. 6. Simulated performance of the bioreactor shown in Fig. 1, with a bed of 1 m diameter and 1 m height: (A) temperature and active enzyme profiles for an organism with both μ and $\alpha = 0.2$ h^{-1}; (B) temperature and active enzyme profiles for an organism with both μ and $\alpha = 0.0782$ h^{-1}. Key: (—) 60 kg-dry-air h^{-1} and $F = 0$; (- - -) 120 kg-dry-air h^{-1} and $F = 0$; (– – –) 60 kg-dry-air h^{-1} and $F = 2$.

With the slow growing organism, an aeration rate of 60 kg-dry-air h^{-1} gives a maximum temperature of 38 °C, the same as that attained in the third simulation with the fast growing organism (Fig. 6). However, since the temperature peak is broader, there is more enzyme loss, the active enzyme at 130 h being only 103 PU g IDS^{-1}. Increasing the airflow to 120 kg-dry-air h^{-1} increases final active enzyme levels, but only slightly. Again, maintaining the aeration rate at 60 kg-dry-air h^{-1} but varying the cooling water temperature according to Eq. (10) with $F = 2$ is more effective: The temperature does not exceed 33 °C and the active enzyme at 130 h reaches 130 PU g IDS^{-1}. The minimum cooling water temperature required is 23.9 °C, which is quite reasonable.

4. Discussion

4.1. The importance of denaturation in SSF processes for enzyme production

This work has highlighted a point that has not previously been raised for the production of enzymes by SSF: the time–temperature profiles that occur can denature a substantial fraction of the enzyme produced by the microorganism, even for relatively slow growers. This point applies to any product that is not fully stable at the temperatures that will be reached during an SSF process. It does not apply to all enzymes, only those that show significant instability at the temperatures reached during the fermentation.

In fact, the deleterious effects on enzyme production may be even greater than those predicted by the model. If enzyme synthesis by the microorganism were negatively affected by high temperatures, then the results would be even worse. Furthermore, a well-mixed bioreactor represents a best case situation. In a real process

the mixing might not be ideal, not only decreasing the overall heat removal rate, but also leading to temperatures higher than the bed average in localized regions of the bioreactor.

The potential for thermal denaturation of enzymes has not previously been raised because many of the studies into enzyme production in SSF are done at laboratory scale, where temperature control is less problematic. Our analysis suggests that it is crucial to investigate the thermal stability of the enzyme, and to take this into account when scaling-up the process. The need to control the temperature may impose upper limits on the scales that can be used, and lower limits on the aeration rates that are necessary.

Optimal temperature control is theoretically possible at large scale through the use of air–solid fluidized beds (ASFBs). The present work suggests that optimal temperature control was the reason that Kikkoman obtained the same productivity of enzymes in an 8000 l air-solid fluidized bed as in a small scale bioreactor (Matsuno et al., 1993). However, the economic viability of ASFBs will depend on the trade-off between increased enzyme yields and the increased operating costs associated with the high airflow needed for fluidization. Furthermore, some microorganisms may be adversely affected by the constant agitation in ASFBs, necessitating the use of bioreactors in which the bed is unmixed during long periods, such as packed-beds or intermittently-stirred bioreactors. In such bioreactors it is impossible to prevent high temperatures from being reached in some regions of the bed (Mitchell et al., 2000), which will result in low productivity in these regions.

4.2. Challenges for future work

This work raises interesting points for further study. First of all, the kinetics of denaturation may differ between an aqueous solution and the environment provided within an SSF process, since the presence of various compounds can increase the thermal stability of enzymes. Secondly, to validate the predictions of the model it would be necessary to follow the total amount of protease produced, during a fermentation in which the culture was subjected to temporal temperature variations. This is not a simple matter. Typically enzyme levels are measured in terms of activity, but this gives only the active enzyme concentration, and provides no information about the amount of denatured enzyme present. For example, to validate the predictions for the protease in the present work, a method would be needed that was specific for proteases but that was able to detect both active and denatured forms of the enzymes. An insight into how this might be achieved is given by the work of Negroni et al. (1998), who produced two monoclonal antibodies, one specific for native beta-lactoglobulin and the other specific for denatured beta-lactoglobulin. If such antibodies could be raised against an enzyme of interest, they could be used to measure native and denatured enzyme concentrations. Such studies have not yet been undertaken in enzyme production systems, which means that there are currently insufficient data to model how enzyme production will be affected by temporal temperature variations.

5. Conclusion

The study aimed to evaluate whether SSF is the best system for producing enzymes. Certainly SSF is appropriate for the production of enzymes and other thermolabile products, especially when higher yields can potentially be obtained than in submerged liquid fermentation. However, in the production of thermolabile products by SSF it becomes even more crucial to control the temperature.

Acknowledgements

This work was supported by the Brazilian National Council for Scientific and Technological Development (CNPq, Conselho Nacional de Desenvolvimento Científico e Tecnológico). Marcelo Muller dos Santos, Alexandre Souza da Rosa, Nadia Krieger and David Mitchell thank CNPq for research scholarships.

References

Ghildyal, N.P., Gowthaman, M.K., Raghava Rao, K.S.M.S., Karanth, N.G., 1994. Interaction between transport resistances with biochemical reaction in packed bed solid-state fermentors: effect of temperature gradients. Enzyme and Microbial Technology 16, 253–257.

Gumbira-Sa'id, E., Mitchell, D.A., Greenfield, P.F., Doelle, H.W., 1992. A packed bed solid-state fermentation system for the production of animal feed: cultivation, drying and product quality. Biotechnology Letters 14, 623–628.

Himmelblau, D.M., 1982. Basic Principles and Calculations in Chemical Engineering. Prentice Hall, Englewood Cliffs, NJ.

Ikasari, L., Mitchell, D.A., Stuart, D.M., 1999. Response of *Rhizopus oligosporus* to temporal temperature profiles in a model solid-state fermentation system. Biotechnology and Bioengineering 64, 722–728.

Leighton, T.J., Doi, R.H., Warren, R.A.J., Kelln, R.A., 1973. The relationship of serine protease activity to RNA polymerase modification and sporulation in *Bacillus subtilis*. Journal of Molecular Biology 76, 103–122.

Matsuno, R., Adachi, S., Uosaki, H., 1993. Bioreduction of prochiral ketones with yeast cells cultivated in a vibrating air–solid fluidized bed fermentor. Biotechnology Advances 11, 509–517.

Mitchell, D.A., Berovic, M., Krieger, N., 2000. Biochemical engineering aspects of solid state bioprocessing. Advances in Biochemical Engineering/Biotechnology 68, 61–138.

Mitchell, D.A., von Meien, O.F., 2000. Mathematical modeling as a tool to investigate the design and operation of the Zymotis

packed-bed bioreactor for solid-state fermentation. Biotechnology and Bioengineering 68, 127–135.

Nagel, F.J.I., Tramper, J., Bakker, M.S.N., Rinzema, A., 2001. Temperature control in a continuously mixed bioreactor. Biotechnology and Bioengineering 72, 219–230.

Negroni, L., Bernard, H., Clement, G., Chatel, J.M., Brune, P., Frobert, Y., Wal, J.M., Grassi, J., 1998. Two-site enzyme immunometric assays for determination of native and denatured beta-lactoglobulin. Journal of Immunological Methods 220, 25–37.

Pandey, A., Soccoll, C.R., Mitchell, D.A., 2000. New developments in solid-state fermentation: I—Bioprocesses and products. Process Biochemistry 35, 1153–1169.

Raimbault, M., Alazard, D., 1980. Culture method to study fungal growth in solid fermentation-state fermentation: I—Bioprocesses and products. European Journal of Applied Microbiology and Biotechnology 9, 199–209.

Ralston, A., Wilf, H.S., 1960. In: Mathematical Methods for Digital Computers. Wiley, New York, pp. 110–120.

Saucedo-Casteneda, G., Gutierrez-Rojas, M., Bacquet, G., Raimbault, M., Viniegra-Gonzalez, G., 1990. Heat transfer simulation in solid substrate fermentation. Biotechnology and Bioengineering 35, 802–808.

Stuart, D.M., Mitchell, D.A., Johns, M.R., Litster, J.D., 1999. Solid-state fermentation in rotating drum bioreactors: operating variables affect performance through their effects on transport phenomena. Biotechnology and Bioengineering 63, 383–391.

Stuart, D.M., 1996. Solid-state fermentation in rotating drum bioreactors. Ph.D. Thesis, Department of Chemical Engineering, The University of Queensland, Brisbane, Australia.

Sweat, V.E., 1986. Thermal properties of foods. In: Rao, M.A., Rizvi, S.S. (Eds.), Engineering Properties of Foods. Marcel Dekker, New York, pp. 49–132.