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MODELS FOR SOLID-STATE CULTIVATION OF *RHIZOPUS OLIGOSPORUS*

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ABSTRACT

Physical and mathematical models for solid-state fermentation were developed, using cultivation of *Rhizopus oligosporus* on soy beans as an example. A simple mathematical model was developed which predicts the dynamic behaviour of an adiabatic homogeneous packed-bed reactor accurately during the first 40 hours. Biomass measurements and experimental verification of the substrate composition are required to improve model validation. After 40 hours, the simple kinetic model gives very poor predictions. Axial gradients and bed shrinkage make the packed bed with soy beans an unsatisfactory physical model. Based on the problems encountered, the experimental set-up has been modified: agar beads with an oleic acid emulsion are a suitable model substrate; a scraped-drum reactor offers better perspectives than the packed bed for studies of solid-state fermentation.

INTRODUCTION

Solid-state fermentations have been used for ages in Asia and Africa for production of fermented foods, starter cultures, enzymes, etc. (Lonsane *et al.*, 1992). Despite their long history, the design and scale-up of these systems is still more an art than a technology. Several models combining black-box descriptions of process kinetics with physical transport phenomena have been published recently, but their use is hampered by erroneous assumptions and lack of independently determined parameter val-

ues or poor validation (Saucedo-Castañeda *et al.*, 1990; Raghava Rao *et al.*, 1993; Sargantanis *et al.*, 1993; Rajagopalan and Modak, 1994).

This paper describes our research on modelling of the dynamic behaviour of an adiabatic packed-bed bioreactor. Axial gradients in the bed were minimised by off-gas recirculation, in order to reduce the complexity of the experimental set-up and the model. Growth of *Rhizopus oligosporus* on soy beans was used as model fermentation. Our aim was not to address all scale-up problems involved in packed-bed SSF systems, but to predict the behaviour of a simple physical model with a simple mathematical model based on independently determined kinetics and conservation laws, using only on-line temperature and CO_2 measurements. Based on the problems encountered, the experimental set-up has been modified. The first results obtained with the new set-up are reported.

MATERIALS AND METHODS

Micro-organism. Sporangiospore suspensions of *Rhizopus oligosporus* NRRL 5905 were prepared as described previously (De Reu *et al.*, 1993).

Soy beans. Yellow seeded soy beans (*Glycine max*) were prepared as described previously (De Reu *et al.*, 1993).

Defined media. Agar solutions containing either glucose or oleic acid as sole carbon and energy source (Table 1) were sterilised (121 °C, 20 min.) and allowed to solidify in layers (glucose medium) or beads (4-6 mm diameter, oleic acid medium).

Incubations. Soy beans or agar media were incubated isothermally in closed serum flasks or small aerated packed columns (Raimbault and Alazard, 1980), or in the adiabatic packed-bed or scraped-drum reactor. All flasks and bioreactors were sterilised (121 °C, 20 min.) before they were filled with inoculated soy beans or agar. The spore suspension was either mixed through the liquid agar medium (40 °C, all experiments

with glucose except one, see results) or evenly distributed on the soy beans or the agar surface (one experiment with glucose, all experiments with oleic acid). All manipulations took place in a laminar flow cabinet.

Table 1. Composition of defined media (g/dm^3)

compound	glucose medium	oleic acid medium
glucose ($C_6H_{12}O_6.H_2O$)	30	0
oleic acid	0	40
Tween 80	0	1
Gibco Bacteriological Agar	15	0
Oxoid Technical Agar	0	30
$ZnSO_4.7H_2O$	1	1
$MgSO_4.7H_2O$	1	1
$(NH_4)_2SO_4$	7.5	20
urea (CH_4ON_2)	2.5	7
KH_2PO_4	1	1
K_2HPO_4	1	1
KCl	1.5	0
pH	7.0	7.0

Analyses. CO_2 and O_2 in serum flasks were determined by GC. Biomass dry matter was determined gravimetrically or by Kjeldahl nitrogen analysis. Agar samples were melted and filtered over Schleicher & Schuell filters (520B). Filters were rinsed with boiling water and dried at 80 °C for 48 hours or subjected to Kjeldahl analysis. Dry matter was corrected for agar retained on the filter. Kjeldahl nitrogen was determined using Gerhardt equipment and Thompson and Capper, Ltd. special Kjeldahl tabs no. 4.

Respiration quotient. The respiration quotient was determined in closed serum flasks and calculated from the cumulative amounts of CO_2 produced and O_2 consumed from the start of the incubation.

Packed-bed reactor (PBR). The PBR set-up is shown in Figure 1. A glass cylinder (internal diameter 0.06 m, height 0.26 m) with stainless steel flanges was used. The packed bed (0.1 kg wet beans) was supported by a wire mesh. Two Pt-100Ω sensors were introduced in the bed and one in the off-gas above the bed. The PBR was located in an incubator; the incubator setpoint temperature was maintained at 30 °C initially and at 0.1 °C below the measured temperature of the PBR as soon as this exceeded 30 °C. Fresh air was introduced through a sterile membrane filter; the flow rate (0.42 dm^3/min) was controlled with a mass flow controller. The fresh air was saturated with water vapour at 30 °C in a humidifier with independent temperature control. A membrane pump recycled off-gas to the bottom of the bed (2 dm^3/min). CO_2 in the off-gas was measured on-line using an infrared analyser.

Scraped-drum reactor (SDR). A glass cylinder (internal diameter 0.176 m, length 0.44 m) with stainless steel flanges, equipped with a rotating scraper (not operated during the first 12 hours, thereafter rotation frequency 0.02 Hz) was used in the same set-up as described for the PBR. The SDR was aerated (1 dm^3/min) through the hollow scraper. Agar beads (335 g wet weight) containing oleic acid medium were used

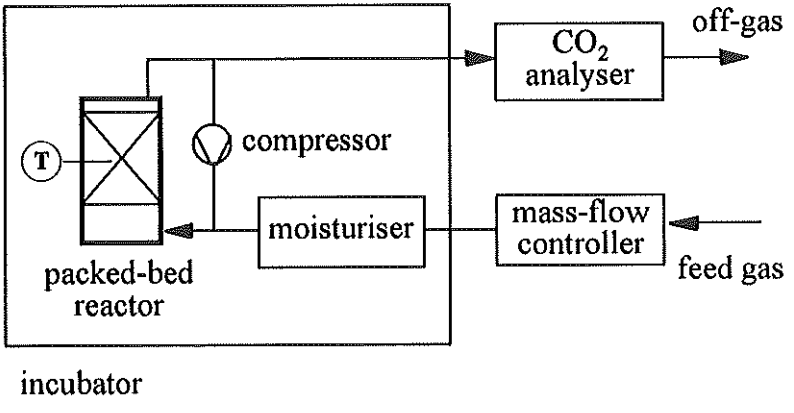

Figure 1. Experimental set-up adiabatic packed-bed reactor with off-gas recycle.

as model substrate. Two Pt-100Ω sensors were introduced in the bed.

RESULTS AND DISCUSSION

Soy bean fermentation in an adiabatic PBR

The main results of our work on cultivation of *R. oligosporus* on soy beans are summarised here; details are described elsewhere (De Reu, 1995; De Reu *et al.*, submitted). The effect of temperature on the specific growth rate of *R. oligosporus* was determined by measuring CO_2 accumulation in serum flasks (Figure 2). The very steep decline in specific growth rate above 40 °C indicates that severe temperature control problems may occur during SSF.

Using these independent measurements of the specific growth rate, mass and enthalpy conservation laws, elemental balances, and empirical relations for the saturated water vapour pressure, a mathematical model for the packed bed reactor was developed. The major assumptions underlying this model are: (1) growth of the fungus is only limited by tempera-

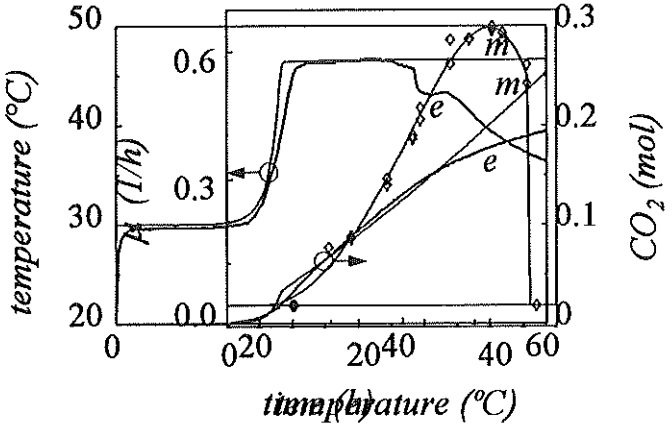

Figure 2. Effect of temperature on specific growth rate of *R. oligosporus* (*e* experimental, *m* model) on soy beans.

ture, (2) the PBR is adiabatic and homogeneous, (3) the off-gas is at equilibrium with the packed bed, (4) lipids are the sole carbon and energy source, NH_3 liberated by proteolytic enzymes is the nitrogen source, biomass, CO_2 and H_2O are the only fermentation products, sulphur and phosphorous are neglected, biomass composition for *R. oligosporus* equals that reported by Sargantanis *et al.* (1993), (5) the true yield is $Y_{x/s} = 0.65$ Cmol biomass per Cmol substrate (Roels, 1983), (6) the maintenance requirements of the fungus and heat capacities and densities of all materials are independent of temperature.

Figure 3 compares the measured and predicted values of the cumulative CO_2 production and the temperature in the centre of the packed bed. After 24 hours, the PBR temperature approached the maximum level that allows growth (Figure 2). Model predictions are accurate during the first 40 hours.

The predictions of CO_2 production are strongly affected by the chosen substrate composition, but the temperature prediction is hardly affected (results not shown). *R. oligosporus* uses lipids during growth on soy beans (Paredes-Lopez *et al.*, 1987; Nout and Rombouts, 1990; De Reu *et al.*, 1994), but we have not yet verified that this is the only substrate. Measurement of O_2 in the off-gas may alleviate the uncertainty about the stoichiometry, but this is extremely difficult because of the high gas flow rate required for cooling.

The point in time where temperature and CO_2 increase is determined by the initial amount of biomass, which was estimated by fitting. In this experiment, the estimated value (2×10^{-6} Cmol) agreed reasonably with four independent measurements of dry weight in a spore suspension ($(4.6 \pm 0.5)\times10^{-6}$ Cmol), but this was not the case in all experiments. There was also no clear relation between the estimated initial amount of biomass and viable spore counts. Biomass measurements are required to get better validation of the model.

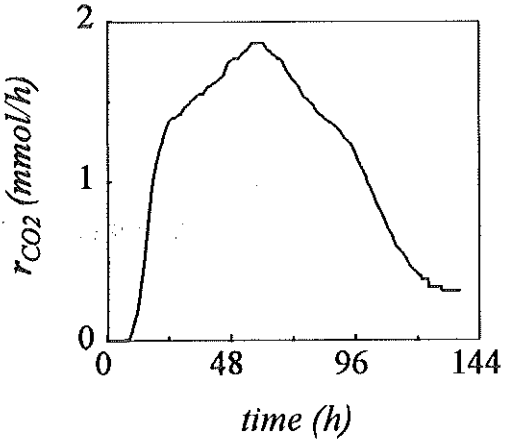

Figure 4. CO_2 production rate during cultivation of *R. oligosporus* on soy beans (29 g wet beans, 30 °C).

After ca. 40 hours, the measured CO_2 production rate and temperature decrease, while the model predicts no such decline. This must be attributed to the very simple kinetic model. During the independent growth rate measurements, only 0.27 mol CO_2 was produced per kg of wet beans, compared to 2 mol/kg in the PBR experiment. A more extensive CO_2 production rate measurement conducted in an isothermal PBR (Raimbault and Alazard, 1980) shows that assumption (1) above is not valid for extended incubations (Figure 4). Probably, substrate, water or space limit fungal growth. The frequently used logistic law also cannot describe the abrupt changes in rate observed after ca. 24 and 96 hours. More experimental information and a more complex kinetic model will be required to improve model predictions.

Two important complications were observed in the physical model: (1) Axial temperature gradients up to 0.5 °C/cm occurred when the bed temperature reached 45 °C. Theoretical calculations show that an ex-

tremely high recirculation flow rate would be required to decrease these gradients. (2) Bed shrinkage occurred after ca. 24 hours. This causes channelling of the gas, which undermines assumption (3) above.

In order to circumvent experimental problems related to substrate composition, stoichiometry and biomass measurements in the soy bean system, and to the packed bed, we decided to develop a simpler physical model. Agar beads with defined media using glucose or oleic acid as sole carbon and energy source, and a scraped-drum fermentor with a mixed bed were studied.

Defined glucose medium

The respiration quotient increased steadily in three experiments with

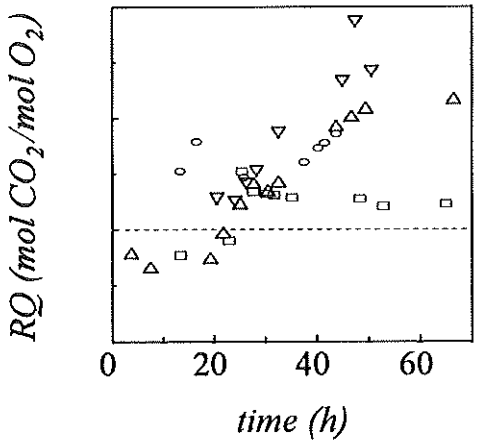

Figure 5. Respiration quotient during cultivation of *R. oligosporus* on agar beads with glucose as sole substrate. Spores distributed in (Δ,∇) and on (○) several millimetres thick agar layers, and in (□) 10 μm thick agar layers.

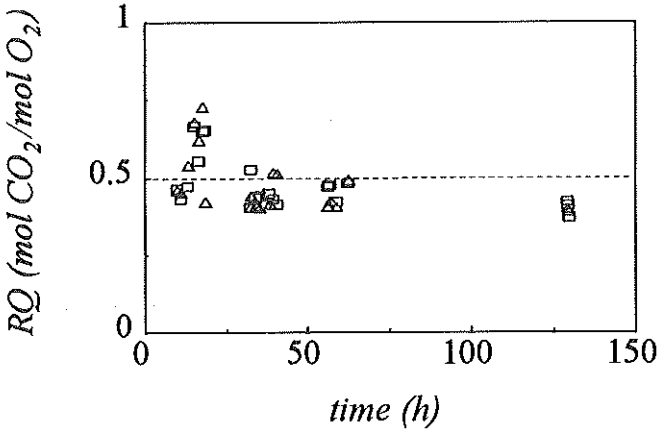

Figure 6. Respiration quotient during cultivation o *R. oligosporus* on agar beads with oleic acid as sole substrate (three experiments).

several millimetres thick agar layers, but not in an experiment with an extremely thin (10 μm) layer (Figure 5). The ratio of accumulated CO_2 and biomass also increased steadily in the flasks with thick agar layers. This indicates that anaerobic glucose conversion occurred, which was confirmed in an experiment under a 100% nitrogen atmosphere. Consequently, agar beads with a defined glucose medium increase the complexity of the physical model, instead of decreasing it.

Defined oleic acid medium

Figure 6 shows the respiration quotient during incubation of agar beads containing an oleic acid emulsion. The observed values agree reasonably well with the theoretical value (0.515 mol/mol, assuming $Y_{x/s} = 0.65$ Cmol biomass per Cmol substrate). During these experiments initially only 0.038 mol O_2 per Cmol oleic acid was present. Fungal metabolism stagnated during 70 hours when the O_2 concentration had dropped to 2-4%. After flushing with air, the O_2 concentration rapidly decreased again to the same level. No anaerobic conversion occurs with oleic acid

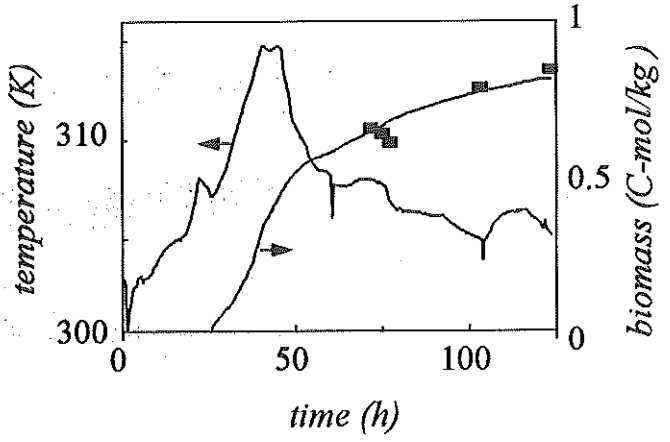

Figure 7. Development of temperature (—) and biomass (calculated from CO_2 production —, from Kjeldahl-nitrogen ■).

as sole carbon and energy source. Therefor, this model system is suitable for further studies.

Scraped-drum reactor

Results obtained with the SDR are shown in Figure 7. The observed peak temperature agrees with the temperature at which no CO_2 production was detected in independent batch experiments. Biomass production was calculated from Kjeldahl nitrogen, using the biomass composition reported by Sargantanis *et al.* (1993), and from CO_2 production, neglecting maintenance and assuming $Y_{x/s} = 0.63$ Cmol biomass per Cmol substrate. Both calculations show reasonable agreement, which indicates a closing carbon balance. Consequently, the scraped-drum reactor with oleic-acid/agar-beads offers good perspectives as a physical model to study solid-state fermentation.

CONCLUSIONS

The simple mathematical model predicts the dynamic behaviour of the adiabatic homogeneous PBR accurately during the first 40 hours. The initial amount of biomass has to be estimated by trial and error for each experiment. Biomass measurements and experimental verification of the substrate composition are required to get better validation of the model. After 40 hours, the simple kinetic model results in very poor predictions. The PBR with soy beans is not fully satisfactory as a physical model: axial temperature gradients cannot be completely suppressed and bed shrinkage causes channelling, which prevents proper equilibration of the off-gas and the bed. In order to circumvent experimental problems related to substrate composition, stoichiometry and biomass measurements in the soy bean system, and to the packed bed, we decided to develop a more defined physical model.

R. oligosporus is capable of anaerobic glucose metabolism. Therefor, agar beads with a defined glucose medium increase the complexity of the physical model, instead of decreasing it. No anaerobic conversion occurs with oleic acid as sole carbon and energy source. Agar with an oleic acid emulsion is a suitable model system. The scraped-drum reactor with oleic-acid/agar-beads offers good perspectives as a physical model to study solid-state fermentation.

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