0273-2289/91/3003—0285$02.20

High Cell Density Cultures of *Schizosaccharomyces pombe* in a Cell-Recycle Reactor

Growth Kinetics and Metabolic Status

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Received July 2, 1990; Accepted October 8, 1990

ABSTRACT

Cultivation of the fission yeast *Schizosaccharomyces pombe* in a cell-recycle fermentor with cross-flow filtration using mineral membranes to recycle the biomass is described. Total cell retention resulted in high cell density cultures with high productivities.

The dependence of both the growth kinetics and metabolic status on the operating conditions was identified and quantified. Growth was controlled by the inhibitory effect of ethanol so long as glucose was in excess as might be expected for fermentative metabolism. Under oxygen excess conditions, a partly oxidative catabolism of glucose occurred due to growth limitation by the glucose feed flow. The cells displayed a purely oxidative metabolism when ethanol was not present in the broth but a respiro-fermentative metabolism when ethanol was present as was the case when oxygen supply to the culture was limiting.

These physiological observations accounted for the expected response of the glucose-sensitive yeast *S. pombe* to its environment and gave basic information enabling growth to be predicted in concentrated cultures.

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Index Entries: Cell-recycle reactor; cross-flow filtration; high cell density cultures; *Schizosaccharomyces pombe* (biomass production of; growth kinetics of)

INTRODUCTION

Maloalcoholic fermentation, which occurs mainly in certain yeasts of the genus *Schizosaccharomyces,* has been suggested as a way of reducing the acidity of musts and wines by removal of malic acid. The ability of these yeasts to convert malic acid to ethanol has stimulated several studies on the technological applications (*1–6*) and physiological and kinetic aspects of malate degradation (*7–10*).

However, few studies have been performed on the growth kinetics and mass production of *Schizosaccharomyces* strains. This lack of information is detrimental to the development of the commercial use of these yeasts for biological deacidification.

In a previous paper we reported the effect of various culture conditions on batch culture growth kinetics of the fermentative malate-ethanol yeast *S. pombe* (*11*). The present study describes the cultivation of the same strain at high cell concentrations in a cell-recycle fermentor with total cell retention, with a view to producing biomass as starter cultures. The influence of the dilution rate on growth kinetics and physiological behavior of the culture is reported.

MATERIALS AND METHODS

Microorganism and Medium

All experiments were performed with a strain of *Schizosaccharomyces pombe* supplied by the Institut Coopératif du Vin (Montpellier, France). The defined synthetic culture medium, the composition of which is representative of a standard grape must, was that described by Sablayrolles and Barré (*12*), except that no malate was added and a 50 g/L glucose concentration was used.

Pre-cultures were propagated in Erlenmeyer flasks and incubated on an orbital shaker (100 rpm) at 30°C.

Analytical Methods

Biomass was estimated after dilution of samples and measuring the absorbance at 640 nm using a Kontron model Uvikon 810 spectrophotometer. Measurements were calibrated to cell dry wt determinations. Cell mass concentrations were determined by harvesting the cells by centrifugation, washing twice with distilled water, and gentle drying (60°C under

reduced pressure) to constant wt. Cell viability was estimated using the methylene blue test as described by Lee et al. (*13*).

Residual glucose concentration was measured either in the culture broth immediately after sampling or in the permeate, using an YSI glucose analyzer. Ethanol concentration was determined by GLC on Porapak Q-column using a FID detector (temperature 190°C; Nitrogen flow rate 30 mL/min) with isopropanol as internal standard.

The respiratory quotient, expressed as the ratio mol of produced carbon dioxide/mol of consumed oxygen, was determined by gas analysis. Outflow gas rate was measured volumetrically using a wet gas meter and carbon dioxide production and oxygen consumption were determined by measurement of their concentration in the outlet gas using a gas chromatograph (Porapack Q+molecular sieve 5 Å, 80–100 mesh columns; temperature 40°C; Helium flow rate 20 mL/min). Oxygen partial pressure in the culture was measured using an Ingold probe coupled to a dissolved oxygen meter.

Process Equipment and Culture Conditions

The cell-recycle fermentor was based on the fermentation vessel and control instrumentation of a conventional chemostat fermentor. A line diagram has been presented in a previous paper (*14*). The fermentor consisted of an instrumental 3-L vessel (working vol 1 L). The separation device was a double cross-flow filtration unit consisting of two tube-type ceramic membranes (SFEC M14, surface 0.018 m^2, nominal pore size of 0.14 μm). The total working vol including pumping was 2.8 L when functioning with one filtration module. A variable-speed pump (SEW-ENRODRIVE, F.R.G.) was used to withdraw the culture broth from the fermentor and provide a circulation rate sufficient to avoid membrane fouling and to maintain a suitable permeate flow rate (superficial liquid velocity 4.5 m/s, pressure 2–3 bar). A part of the permeate stream was removed at a desired rate using a peristaltic pump, thus fixing the dilution rate D. The excess of permeate was recirculated into the fermentor, allowing the dilution rate to be maintained constant over the entire experiment. The supply of fresh medium was provided via a peristaltic pump connected to a level controller, keeping the working vol constant.

The temperature was 30°C. The pH of the culture was maintained at 3.0 by automatic addition of ammonium hydroxide. The fresh medium was acidified by orthophosphoric acid to pH 2.0, this arrangement enabling the nitrogen nutrient needs to be satisfied. Dissolved oxygen was monitored to a minimal level of 20% of saturation (except where mentioned otherwise). If necessary oxygen-enriched air was used as inlet gas.

After inoculation of the cell-recycle fermentor, the cultures were grown batchwise until a sufficient biomass concentration was obtained before starting the cell-recycle operation.

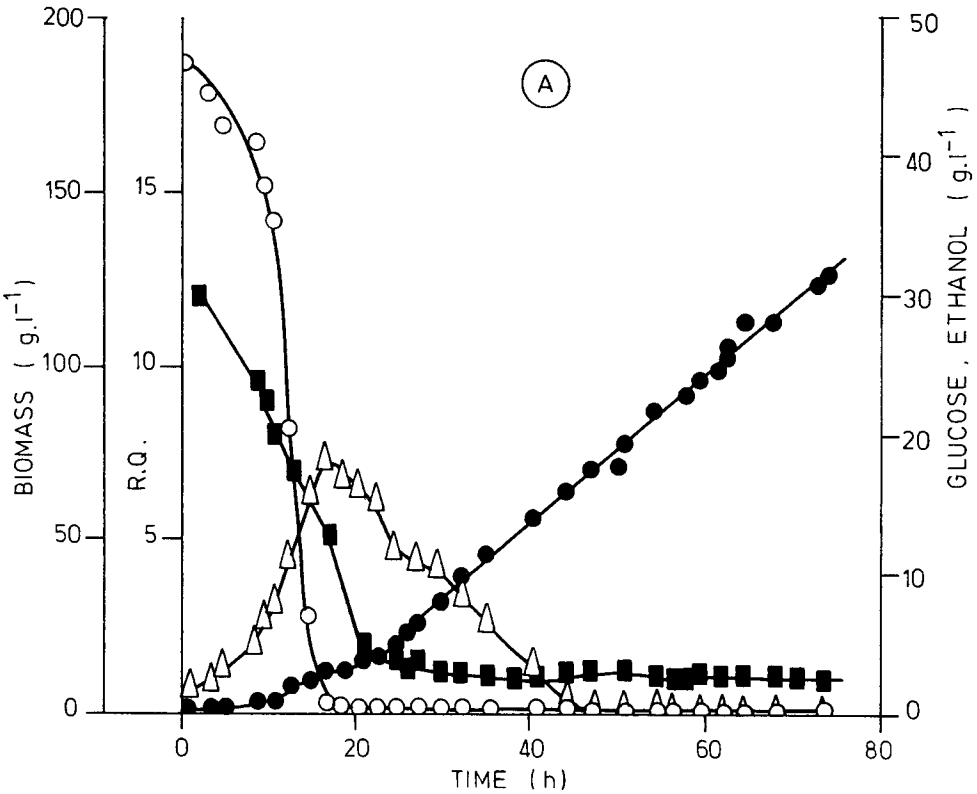
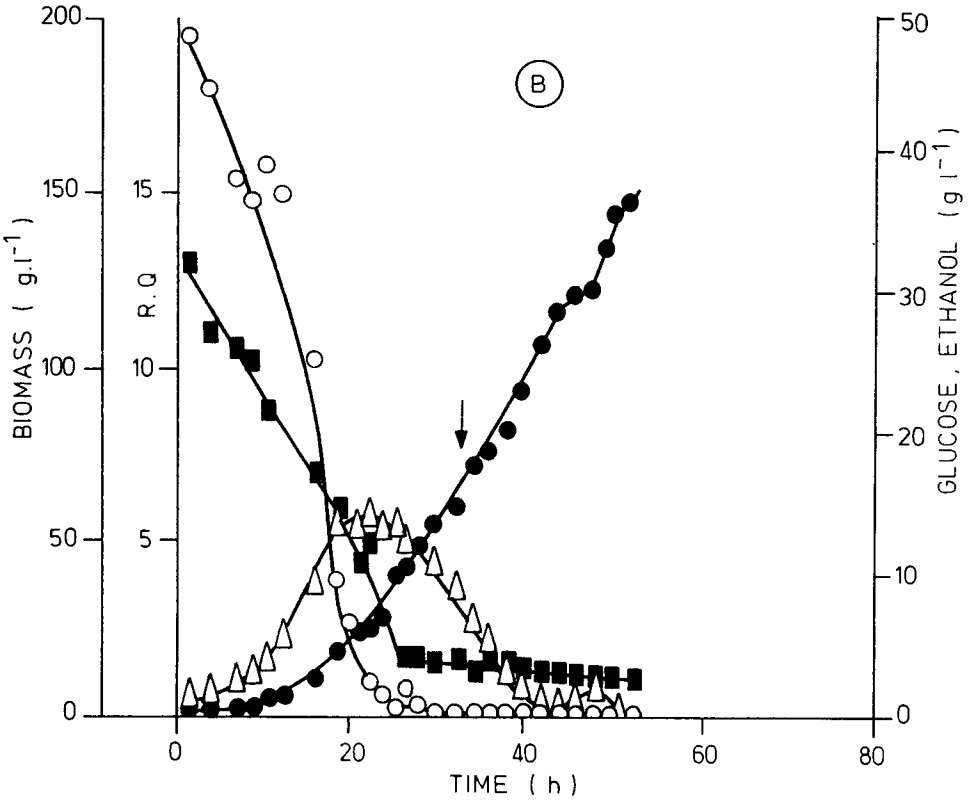


Fig. 1. Time-course of biomass (●), glucose (○), ethanol (△) and respiratory quotient (■) in total cell-recycle cultures; glucose concentration $So = 50$ g/L. A. Dilution rate $D = 0.1$/h; normal air supply during the full-time of the experiment. B. Dilution rate $D = 0.3$/h; ↓: from 50% to 90% oxygen-enriched air. C. Dilution rate $D = 0.6$/h; ↓: 50% oxygen-enriched air.

RESULTS AND DISCUSSION

High Cell Concentration Cultivation

Cultures with total cell-recycle were performed at three dilution rates, $D = 0.1$, 0.3, and 0.6/h, using a 50 g/L glucose concentration in the feeding medium. The time-courses of biomass, ethanol, and glucose concentrations and respiratory quotient for each run are given in Fig. 1. Increasing the dilution rate resulted in gaster growth. After a brief rapid growth phase, growth slowed down as the ethanol concentration increased, and an almost linear increase of biomass was then observed, in parallel with the depletion of glucose in the broth (down to a 0.15–0.25 g/L residual concentration). During the last growth period, when no ethanol was detected in the permeate (except for the $D = 0.6$/h condition), biomass productivities reached 2 and 5.9 g/L/h for $D = 0.1$ and $D = 0.3$/h respectively. This growth behavior was accompanied by the evolution of the respiratory quotient, which was found to vary between 12 and 1.06. The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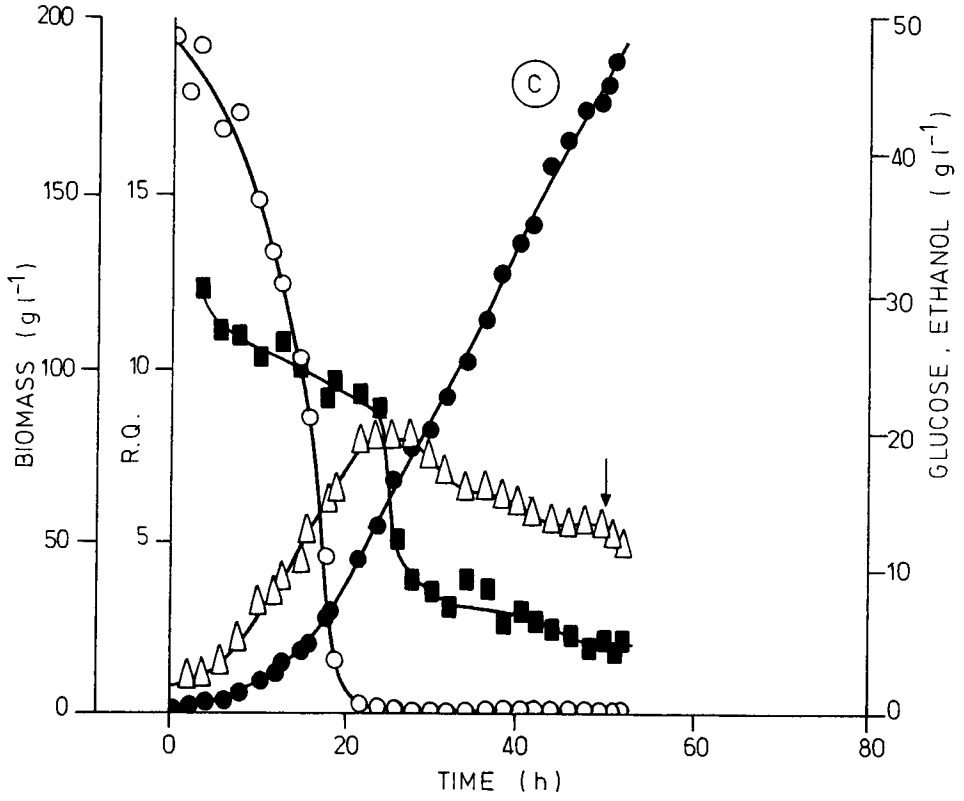
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viability counts revealed that the cell viability remained higher than 80–85%, whatever the biomass concentration.

In a general manner, three growth periods could be distinguished, the first characterized by an excess of glucose and production of ethanol, the second by glucose depletion and ethanol release, and the third by glucose depletion and no ethanol formation. Moreover, the transition between the different growth periods occurred at increased biomass concentrations as the dilution rate was increased. Note that a purely oxidative regime was not achieved at $D=0.6$/h because of a limiting oxygen supply. In spite of a global oxygen transfer rate k_La of approx 1200/h, the oxygen demand of the culture was not satisfied using normal air, when the biomass concentration reached 120 g/L. This oxygen demand was estimated to be in the range of 3 g/L/h assuming a specific oxygen uptake rate of 25 mg/g/h for the strain grown under aerobic and glucose excess conditions (unpublished observations), equivalent to a maintenance oxygen requirement for *Saccharomyces cerevisiae* strains (*14,15*).

Growth Kinetics and Metabolic Status

The time courses of the cultures and the physiological changes regarding the metabolic status of the cells could be analyzed if separate information on both the effect of ethanol concentration (inhibition by the end-product) and the influence of dilution rate (carbon source feed flow and ethanol wash-out) on the growth kinetics were available.

The inhibitory effect of ethanol was quantified during the culture period in which glucose was in excess. As shown in Fig. 2, plotting $1/\mu$ vs P gave a straight line, and the inhibition was expressed by the equation

$$\mu = \mu_m K_P / (K_P + P) \quad (1)$$

where

μ_m is the maximum specific growth rate of the strain (μ when $P \rightarrow 0$),
μ and P are the actual specific growth rate and ethanol concentration in the culture, and
K_p is a constant representative of the inhibition.

Respective values of 9.65 g/L and 0.32/h were obtained for K_p and μ_m, the latter being close to that previously observed in batch cultures (*11*). Based on the aforementioned results, potential growth rates, μ^*, obtained after deduction of the contribution of inhibition by ethanol, were determined according to the equation

$$\mu^* = \mu . (K_P + P) / K_P \quad (2)$$

and they were compared to the actual specific growth rates observed during the times-course of the cultures. Increased dilution rates resulted in

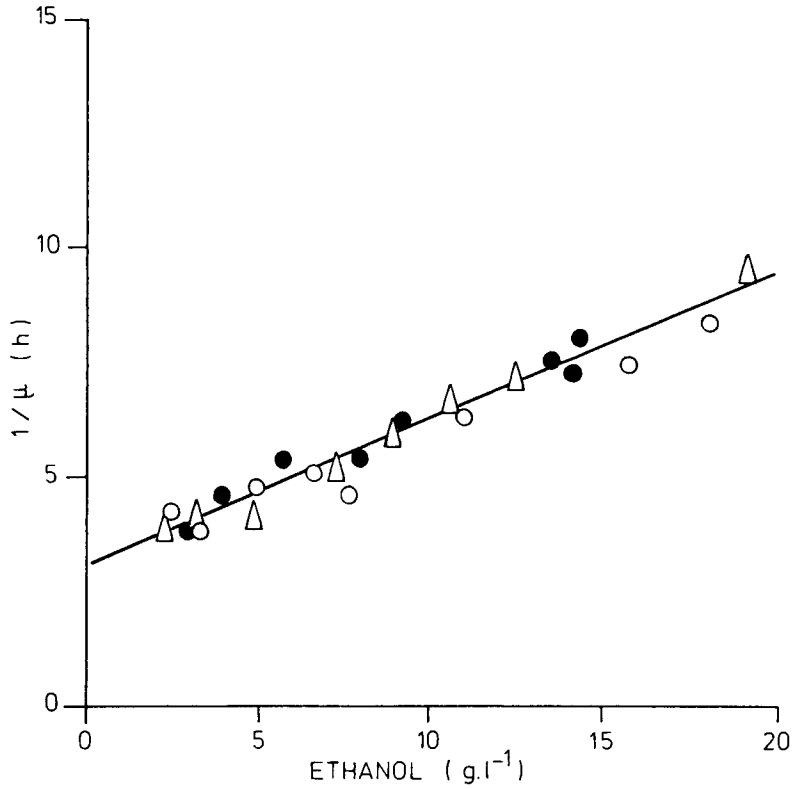

Fig. 2. Representation of $1/\mu$ versus ethanol concentration during glucose excess growth periods; ○: D=0.1/h; ●: 0.3/h; △: D=0.6/h.

constant potential growth rates over longer culture periods and increased biomass concentrations, as the consequence of increased glucose feed flows (Fig. 3). Furthermore, the decrease of the observed specific growth rates was slowest for the highest dilution rate, as the result of a more efficient ethanol wash-out. It was assumed that growth followed a Luedeking-Piret type equation

$$\gamma_P = \alpha\mu + \beta \quad (3)$$

during glucose-excess periods. As shown in Fig. 4 a linear relationship between the specific ethanol production and growth rates was obtained, with the slope α=3.41 g/g and the intercept close to zero. These results accounted for the control of growth solely by the product concentration during the first culture period, i.e., glucose excess and ethanol formation, with a fermentative metabolism. However, this cannot explain the further decrease of the specific growth rates observed for the three experiments (*see* Fig. 3).

Analysis of growth kinetics associated with the glucose depleted periods was attempted with the assumption of a glucose-limited growth

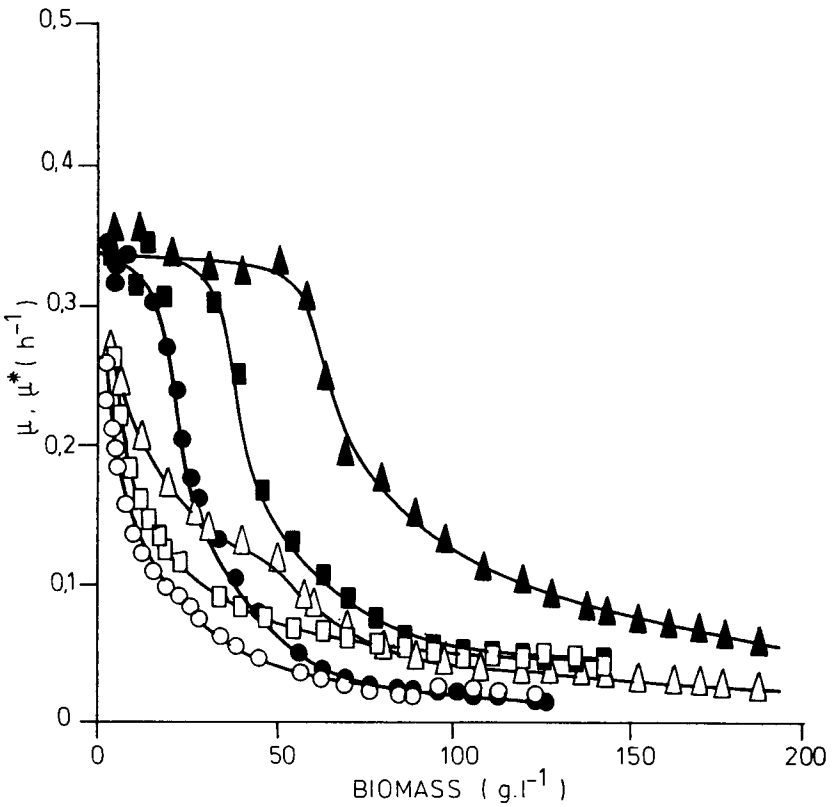

Fig. 3. Actual (μ, open symbols) and potential (μ^*, closed symbols; after deduction of the contribution of inhibition by ethanol) specific growth rates for increasing biomass concentrations; ○, ●: D=0.1/h; □, ■: D=0.3/h; △,▲: D=0.6/h.

following a Monod's type equation and substitution of μ by μ^* to take into account the inhibition by the product when present.

From the corresponding equation

$$\mu^* = \mu_m . (DSo - \mu X/R_{SX}) / [D(K_S + So) - \mu X/R_{SX}] \quad (4)$$

plotting μ_m/μ^* vs $\mu X/DSo$ ($\mu_m/\mu^* - 1$) gave straight-lines with the slopes $1/R_{SX}$ and the intercept $K_S/So + 1$ (Fig. 5). The yields of biomass on glucose were 0.417 and 0.12 g/g for D=0.1 and D=0.6/h respectively, whereas it was found to vary continuously between these extremes for the D=0.3/h experiment. The apparent K_S was approximated at 0.025 g/L. As demonstrated by others (*16,17*), this low value suggested that glucose uptake probably proceeded by high affinity facilitated diffusion system, resulting in a much lower residual concentration in the broth than that effectively present in the cells. These results were consistent with the control of growth by both the dilution rate, D, acting on the substrate feed and the ethanol wash-out, and the oxygen transfer rate, $k_L a$, fixing the oxygen supply level to the culture. The cells presented a purely oxidative catabo-

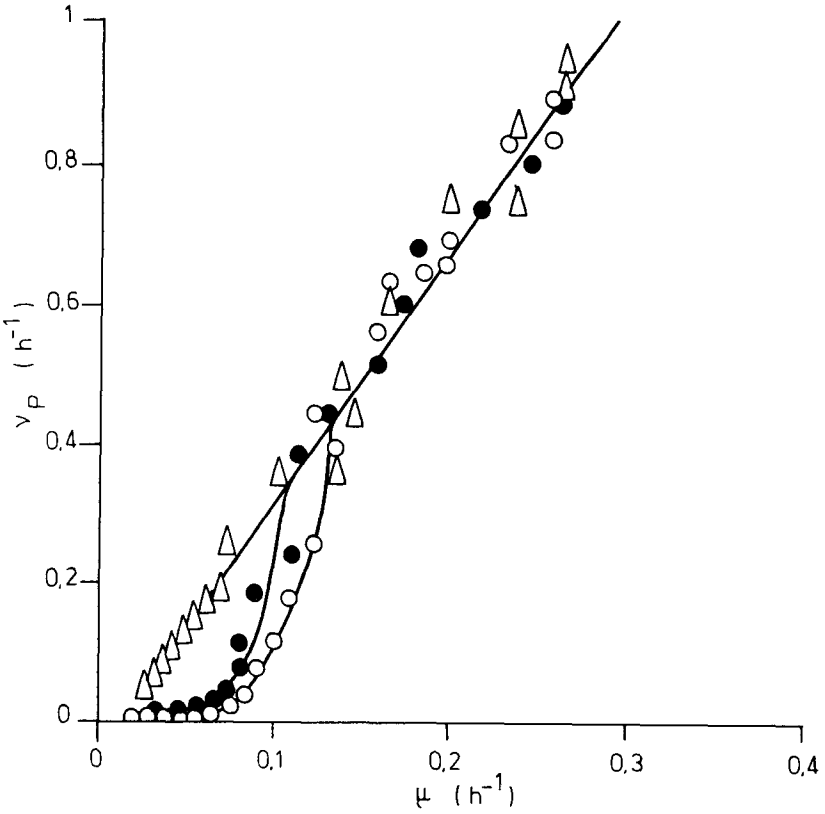

Fig. 4. Relationship between the specific ethanol production rate and the specific growth rate; symbols as in Fig. 3.

lism of glucose under sugar limitation and oxygen nonlimited conditions, which became an oxido-reductive metabolism when respiration was reduced by oxygen limitation. This oxidoreductive behavior occurred when growth was not limited by the oxygen availability but limited by the sugar feed and inhibited by ethanol, as was the case in glucose-excess conditions.

All the observations presented above underlined the concordance between the metabolic status of the cells and the physiological behavior and growth kinetics of the *S. pombe* cultures. They were in good agreement with the expected response of the aerobic fermenting yeast to its environment, and revealed obvious similarities with *Saccharomyces cerevisiae* strains (*15,18*).

CONCLUSION

Cultivation of *S. pombe* in a cell-recycle fermentor using cross-flow filtration allowed high cell density cultures with high biomass productivities to be obtained, up to 5.9 g/L/h at a more than 150 g/L of dry biomass

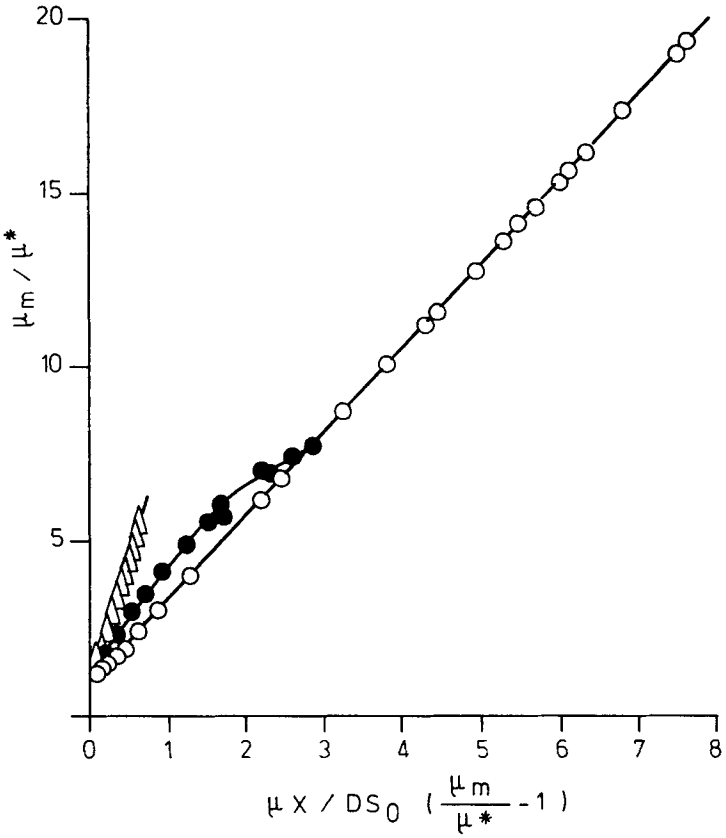

Fig. 5. Representation of μ_m/μ^* versus $\mu X/D/So$ $[(\mu_m/\mu^*-1)]$ during glucose limiting growth periods; symbols as in Fig. 3.

concentration. With total cell retention, growth kinetics and physiological behavior of the cultures were shown to be directly controlled by the operating conditions, especially the dilution rate and the oxygen supply. Hence, a strategy for producing concentrated starter cultures having a defined physiological status is possible by fixing the substrate concentration in the feeding medium, and the permeate flow and oxygen transfer rates; obviously such a strategy cannot be applied if the limits of the filtration capacity of the membranes or the oxygen transfer capability of the reactor are exceeded. In addition, by varying the operating conditions, all physiological variations of the cultures and corresponding metabolisms of the cells were observed, making identification and quantification of essential biochemical phenomena of this glucose sensitive yeast possible.

From experiments with controlled growth rates using a biomass bleed (they are now in progress), more complete information on glucose catabolism is expected. A mechanistic model should then be possible, similar to those proposed by Sonnleitner and Käpelli (*19*) or Alexander and Jeffries (*20*) for various yeast strains, making possible prediction of growth of *S. pombe*.

ACKNOWLEDGMENTS

The authors wish to thank Dr. N. Lindley for his linguistic assistance. The financial support of the D.G.Al., French Ministry of Agriculture is acknowledged.

REFERENCES

1. Ribereau-Gayon, J. and Peynaud, E. (1969), *C. R. Acad. Agric. F.* **48,** 558.
2. Peynaud, E., Domerq, S., Boidron, A. M., Lafon-Lafourcade, S., and Guimberteau, E. (1964), *Arch. Mikrobiol.* **48,** 156.
3. Benda, I. and Schmitt, A. (1969), *Werberg Keller* **16,** 71.
4. Yang, H. Y. (1973), *Am. J. Enol. Vitic.* **24,** 1.
5. Snow, P. G. and Gallander, J. F. (1979), *Am. J. Enol. Vitic.* **30,** 45.
6. Charpentier, C., Feuillat, M., Gerbeau, V., and Arther, R. (1985), *C. R. Acad. Agric. F.* **71,** 425.
7. Mayer, K. and Temperli, A. (1963), *Arch. Mikrobiol.* **46,** 321.
8. Maconi, E., Manachini, P., Aragozzini, F., Gennari, C., and Ricca, G. (1984), *Biochem. J.* **217,** 585.
9. Osothsilp, C. and Subden, R. E. (1986), *J. Bacteriol.* **168,** 1429.
10. Taillandier, P., Riba, J. P., and Strehaiano, P. (1988), *Biotechnol. Lett.* **10,** 469.
11. De Queiroz, H. and Pareilleux, A. (1990), *Appl. Microb. Biotechnol.* **33,** 578.
12. Sablayrolles, J. M. and Barre, P. (1986), *Sciences Alim.* **6,** 373.
13. Lee, S. S., Robinson, F. M. and Wang, H. Y. (1981), *Biotechnol. Bioeng.* **11,** 641.
14. Uribelarrea, J. L., Winter, J., Goma, G., and Pareilleux, A. (1990), *Biotechnol. Bioeng.* **35,** 201.
15. Roels, J. R. (1983), *Energetics and Kinetics in Biotechnology,* Elsevier, North Holland, pp. 75–98.
16. Does, A. L. and Bisson, L. F. (1989), *J. Bacteriol.* **171,** 1303.
17. Van Urk, H., Postma, E., Scheffers, A., and Van Dijken, J. P. (1989), *J. Gen. Microbiol.* **135,** 2399.
18. Rieger, M., Käpelli, O., and Fiechter, A. (1983), *J. Gen. Microbiol.* **129,** 653.
19. Sonnleitner, B. and Käpelli, O. (1986), *Biotechnol. Bioeng.* **28,** 927.
20. Alexander, M. A. and Jeffries, T. W. (1990), *Enzyme Microb. Technol.* **12,** 2.