Immobilization of Monocentric and Polycentric Types of Anaerobic Chytrid Fungi in Ca-Alginate

Bernadette K. M[c]Cabe[*1], Clem Kuek, Geoffrey L. R. Gordon[2] and Michael W. Phillips[2]

School of Science, Food and Horticulture, University of Western Sydney, Campbelltown Campus, Locked Bag 1797, Penrith South DC N.S.W. 1797, Australia

[2]CSIRO Livestock Industries, Locked Bag 1, Delivery Centre, Blacktown, N.S.W. 2148, Australia

A procedure for the immobilization of rhizomycelia of two strains of anaerobic fungi, namely Piromyces sp. KSX1 and Orpinomyces sp. 478P1 using Ca-alginate beads has been developed. Zoospores were found to be suitable propagules for the immobilization of the monocentric strain KSX1 giving rise to colonization in 100% of beads. For polycentric strain 478P1, which produced very few zoospores, partially homogenized rhizomycelia proved a suitable inoculum for the beads. The distribution of rhizomycelial growth inside the beads and on the surface depended on which fungal strain was immobilized. Strain 478P1 grew as a mycelial network inside the beads whereas strain KSX1 grew in microcolonies throughout the bead and on the surface and was unable to evenly colonize the entire Ca-alginate matrix.

* Corresponding author
[1] Present address: Department of Biological and Physical Sciences, University of Southern Queensland, Toowoomba, QLD, 4350, Australia.

Keywords: Anaerobic fungi; immobilization; Ca-alginate, cellulase production; *Piromyces*; *Orpinomyces*

Abbreviated title for use as a running headline: Immobilization of Anaerobic Fungi.

Introduction

Anaerobic fungi isolated from ruminant and non-ruminant herbivores have a high digestion capacity for plant structural polysaccharides, particularly cellulose[1,2] and the cellulolytic enzymes of anaerobic fungi have made them the subject of a number of studies over recent years. These studies have focused on the optimization of production of cellulolytic and xylanolytic enzymes by screening anaerobic fungi grown in batch culture on a range of cellulosic substrates.[1,3,4,5]

Most anaerobic fungal research in the laboratory has been conducted with cultures growing in closed batch cultures and only a few reports have investigated semi-continuous and continuous-flow cultures of anaerobic fungi.[6,7] Those studies have shown that it is a difficult task to set up continuous-flow cultures with filamentous fungi which possess a complex life cycle. Therefore, the development of efficient methods for enzyme production using anaerobic fungi is essential for the development of the biotechnological potential of their cellulolytic enzymes.

Processes using immobilized growing cells seem to be more promising than traditional fermentations with free cells since the immobilization enables the microbial cells to be used repeatedly and continuously.[8] Many aerobic fungi have been immobilized for production of extracellular enzymes and metabolites.[9,10] This paper describes the production of immobilized anaerobic fungi as a first step in the development of an alternative production system for cellulolytic enzymes. The objective was to optimise the immobilization conditions in order to maximize fungal biomass within the alginate matrix, while at the same time confining fungal growth to the bead. Thus, the amenability of two morphologically different strains of anaerobic fungi toward this procedure was evaluated.

Materials and Methods

Isolation and maintenance of fungal cultures.

Piromyces sp. strain KSX1 was isolated from fresh faeces of a red kangaroo (*Macropus rufus*) collected at Western Plains Zoo, Dubbo, (New South Wales, Australia) using a roll tube method.[11,12] This monocentric fungus produced monoflagellated zoospores. The polycentric strain, *Orpinomyces* sp. strain 478P1, was isolated from ruminal digesta obtained from a fistulated cow housed at CSIRO Tropical Crops and Pastures, Samford (Queensland, Australia). Zoospore production by this strain has not been observed so it was tentatively classified as an *Orpinomyces* species on the basis of rhizomycelial appearance and rapid growth rate.[13]

Anaerobic fungi were grown at 39°C in semidefined ruminal-fluid-free medium.[11,12] Complete medium 10X, used in the isolation of fungi, contained glucose and cellobiose (each at 0.03% [w/v]) and xylan (oat spelt; Sigma Chemical Co., St Louis, Mo.) and starch (each at 0.07%[w/v]) in basal medium 10 (BM).[11] Glucose medium (GM) contained 0.25% (w/v) glucose in basal medium 10, and cellobiose medium (CM) contained 0.25% (w/v) cellobiose. Strain KSX1 was maintained by subculturing every 4-5 days in medium 10X using an inoculum of 4% (v/v). Strain 478P1 was maintained on BM broth (7 ml) in a screw-capped tube containing ten to twenty 1 cm pieces of sisal (*Agave* sp.) fibre and grass leaf.[14] A piece of sisal fibre containing the actively growing fungus was transferred to fresh medium every 4-5 days. Manipulation of these cultures was performed in an anaerobic cabinet (Coy Laboratory Products Inc. Ann Arbor, MI, USA) which contained an atmosphere of 95% CO_2 and 5% H_2 (v/v).

Production of immobilized rhizomycelia.

Several procedures were trialed with different combinations of distilled water and culture medium. Sodium alginate (Manugel GMB; Kelco A.I.L., Melbourne, Australia) was prepared as a 3% (w/v) solution dissolved in either distilled water or GM. The mixture was held inside an anaerobic cabinet and thoroughly homogenized with a hand blender (Braun) before 4 ml was dispensed into screw-cap Hungate tubes (Bellco glass, Inc., Vineland NJ). The tubes containing the alginate/GM mixture were allowed to reduce (0.01% resazurin as redox indicator) in an anaerobic chamber before being sealed with black butyl rubber septa and screw caps and then autoclaved at 115°C for 30 minutes.

A 24-hour-old suspension of strain KSX1 (25 ml CM containing about 10^5 zoospores/ml) was aseptically transferred to 4 ml of alginate/medium mixture via a sterile 1 ml syringe. The inoculum and alginate/medium mixture was thoroughly mixed using a vortex and then withdrawn aseptically using a sterile 5 ml syringe fitted with an 18 gauge needle. This suspension was then added dropwise through the same syringe fitted with a 21 gauge needle to 30 ml 0.1 M $CaCl_2$ (prepared and sterilized under N_2 atmosphere contained in a 100 ml serum bottle). Each drop formed a spherical bead (2.5-3.0 mm in diameter) after contacting the electrolyte solution and each 5 ml of alginate-zoospore suspension yielded approximately 150 beads. The beads were allowed to harden in the $CaCl_2$ for about 10 min, after which the $CaCl_2$ solution was removed using a sterile 50 ml syringe and the beads were washed twice with the addition of 25 ml of either sterile distilled water or BM. In the final procedure, the serum bottles containing the beads were held in a 39°C water bath during washing. GM

was added (25 ml) and the immobilized fungus was incubated statically at 39°C. After 48 hours, when the initial glucose in the medium was depleted, the spent culture liquor was removed by syringe. The beads were washed twice *in situ* with BM after which fresh GM was added. After incubation for a further 24 hours, sterile glucose solution was added to reconstitute the initial concentration (0.25%) and the immobilized cell culture was re-incubated. After another 24 hour re-incubation the spent culture liquor was removed and fresh growth medium was added after the beads were washed. This combination of repeat-batch and fed-batch techniques resulted in the addition of growth substrate every 24 hours and was repeated until biomass in the gel bead reached a maximum as measured by cell wall chitin. The entire contents of two bottles were harvested at each 48-hour sample point and the beads from each bottle were washed thoroughly in distilled water before placement at room temperature in 100 ml 10% sodium hexametaphosphate (BDH Chemicals Ltd., England) overnight to dissolve the alginate. The resulting free fungal rhizomycelium was sedimented by centrifugation at 2000 x *g* for 15 minutes at 0°C, and the pellets were washed twice with distilled water and stored frozen for biomass determination (see 'Assays').

Beading of the polycentric fungal strain was similar to that described for the monocentric isolate. CM (25 ml) was inoculated using a piece of sisal fibre containing actively growing strain 478P1 and was incubated for 5 days at 39°C. Throughout the incubation period the serum bottle, which contained 30 to 40 sterile glass beads (3mm in diameter), was shaken vigorously by hand for approximately 1 minute every day to prevent the formation of a rhizomycelial mat during incubation. A 1 ml suspension of this partially homogenized rhizomycelium was used to inoculate the alginate/medium mixture. Beading and washing procedures were performed and CM was added. As a

modification, after 24 hours static incubation, some cultures were transferred to a reciprocating water bath and further incubated at 39°C and shaken at 80 oscillations per minute for 5 days.

Assays.

Residual glucose in fermentation broths was measured in triplicate using a glucose oxidase-peroxidase diagnostic kit (Sigma, St Louis, Mo, USA; kit no. 510). A 0.25 ml sample of appropriately diluted culture liquor was added to 2.5 ml aliquots of chromogen solution. In the standard, 0.25 mL of a 0.0667 mg/mL glucose standard solution (prepared from a 1mg/mL glucose standard solution, Sigma Chemicals) was added instead of culture liquor. Following incubation at 37°C for 30 minutes the absorbance was determined at 450 nm in a Bausch and Lomb Spectronic 21 spectrophotometer.

For biomass determinations, fungal chitin of immobilized cultures of KSX1 was estimated as the total hexosamine content after acid hydrolysis.[15] Duplicate cultures were grown under identical conditions in 100 ml serum bottles containing 25 ml of growth medium.

Light microscopy

Colonisation of beads by rhizomycelium was assessed at various intervals throughout the growth phase using an Olympus BH2 phase contrast microscope. The beads were preserved in a solution of formal saline (10 g formalin in 90 g 10% saline).

Preparation of beads for scanning electron microscopy.

Calcium alginate beads were rinsed clean of growth medium with distilled water and were pre-fixed in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C for 2 hours. The beads were then washed three times over 30 minutes in 0.1 M cacodylate buffer solution before being post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 2 hours. Dehydration of the sample was effected through an ethanol series (3 - 100%), then transferred to a critical point drier (Balzers Bal-Tec CPD 030, Liechtenstein) for 2 hours. The dry beads were mounted on an aluminium stub, sputter coated with gold in a modified Edwards coater E306A, and examined using a Jeol (model 35C) scanning electron microscope.

Results and Discussion

Influence of immobilization techniques on the growth of Piromyces sp. KSX1

A 3% aqueous solution of Na-alginate was used in preliminary immobilization experiments. The beading procedure was performed at room temperature and water was used to wash the beads before GM was added. Colonization of the bead by the fungus was localized in a small number of mycelial aggregations (Figure 1a). The addition of GM in the inoculum/alginate mixture resulted in beads which were more evenly colonized by fungi (Figure 1b). The use of BM instead of water for bead washing and performing the immobilization procedure at 39°C resulted in further improvement in the degree of colonization of the fungus (Figure 1c). The modification resulted in a higher proportion (100%) of beads achieving colonization, presumably related to an improved retention of zoospore viability.

Insert Figure 1 (a to c) here

Alternate repeat-batch and fed-batch culture techniques were successful in increasing the biomass of immobilized KSX1 cultures. Figure 2 shows that glucose was exhausted within 36 hours in the first batch and then every 24 hours after substrate addition and maximum growth occurred after 8 days incubation, that is, after 7 additions of glucose. Although the amount of immobilized biomass of KSX1 increased as a consequence of further additions of substrate, the distribution of the biomass within the bead matrix was uneven. After 24 hours of incubation in GM, the zoospores of KSX1 in gel beads germinated and formed rhizomycelial mass which grew in localized areas (see Figure 1c). Zoospores were observed under light microscopy in the growth medium at day 2 and fungal rhizomycelia in the medium was evident from day 3. Changes in the distribution of biomass of immobilized KSX1 on the surface of the gel beads were observed with increasing incubation time as illustrated by the scanning electron micrographs (Figure 3 a-d). By day 10 fungal rhizomycelia was produced to such an extent that the bead was overgrown and the surface could not be observed.

Insert Figures 2 and 3 (a to d) here

There have been other reports of this uneven distribution of growth within beads and the growth of cells on the surface. It has been shown that yeast cell growth mainly occurs in the periphery of the 4 mm diameter bead with some growth also evident within the core,[16] while the mycelia of *Aspergillus niger* covered the outer bead surface

as a fur-like coat after a second incubation of alginate beads.[17] This non-ideal spatial distribution is exemplified by aggregation with mycelial growth on the surface and is a major problem that has to be overcome before immobilized fungi can find wide use in the fermentation industry.[18] There are several consequences of this non-homogeneity in biomass distribution for bioreaction kinetics. It leads to zonation of the microenvironment within the bead which may modify intraparticle growth, metabolism, and product formation.[19] Low concentrations of cells at the particle centre will generate significant unproductive regions and may influence particle density. The accumulation of cells at the surface may rupture the gel surface resulting in outgrowth.[20] Thus, limitation of mycelial growth to the subsurface of beads is desirable, but is not often achieved in studies reported. However, success in this regard with *Aspergillus phoenicus*[21] provides evidence that confinement of mycelial growth is possible.

Influence of immobilization techniques on the growth of Orpinomyces sp. 478P1

In static incubations of strain 478P1 rhizomycelial growth was first visible after 48 hours with the rhizomycelial filaments extending throughout the solid matrix. Static incubation resulted in mycelial outgrowth forming a conglomerate mass containing a number of beads joined by rhizomycelia (Figure 4a), but incubation with shaking resulted in beads that did not aggregate (Figure 4b). The polycentric fungi in this sense are similar to aerobic filamentous fungi where growth occurs by the linear elongation of mycelial filaments. The manner in which shaking limits fungal outgrowth in the bead is probably twofold. Firstly, shaking the culture reduces diffusion gradients making nutrients more readily available to the fungus which in turn reduces the need for mycelial outgrowth to seek nutrients. Secondly, it is possible that with shaking, inter-

bead abrasion broke off hyphae as they appeared at bead surfaces thus allowing little opportunity for surface colonization. Unlike the monocentric strain which colonized all of the beads, approximately 30% of beads were colonized by strain 478P1. An increase in inoculum size from 20 % to 40 % did not increase the percentage of beads colonized by the fungus.

Insert Figure 4 (a and b) here

In contrast to strain KSX1, growing rhizomycelial filaments of strain 478P1 were found to have the potential to colonize the gel matrix in a more uniform manner. Strain KSX1 formed micro colonies inside the matrix and also at the periphery of the matrix. The life cycle of monocentric fungi which consists of an alternation between a motile, zoosporic stage and a vegetative, zoosporangial stage[22] is possibly the reason for the inability of strain KSX1 to colonize the entire bead structure. In the case of immobilized 478P1 cultures it is possible that only sporangia are surviving during the immobilization procedure. Consequently, immobilization may be more suited to the vegetative growth cycle of the polycentric fungi.

In this study, monocentric and polycentric anaerobic fungi, as represented by *Piromyces* sp. KSX1 and *Orpinomyces* sp. 478P1 were found to have colonized alginate gel differently. This can be attributed chiefly to their differences in physical growth characteristics. These preliminary findings have assisted in the evaluation of the potential of immobilized rhizomycelia of anaerobic fungi for repeated use in the production of cellulolytic enzymes in repeat batch culture.

Acknowledgments

The financial support for BKM was received from a joint UWS Macarthur/CSIRO Postgraduate Research Scholarship.

References

1. Teunissen, M.J., De Kort, G.V.M., Op Den Camp, H.J.M., and Vogels, G.D. Production of cellulolytic and xylanolytic enzymes during growth of anaerobic fungi from ruminant and nonruminant herbivores on different substrates. Appl. Biochem. Biotechnol. 1993, **39**, 177-189
2. Dijkerman, R., Bhansing, D.C.P., Op den Camp, H.J.M., van der Drift, C., and Vogels, G.D. Degradation of structural polysaccharides by the plant cell-wall degrading enzyme system from anaerobic fungi: An application study. Enzyme Microb. Technol. 1997, **21**, 130-136
3. Williams, A.G., Withers, S.E., and Orpin, C.G. Effect of the carbohydrate growth substrate on polysaccharolytic enzyme formation by anaerobic fungi isolated from the foregut and hindgut of nonruminant herbivores and the forestomach of ruminants. Lett. Appl. Microbiol. 1994, **18**, 147-151
4. Dijkerman, R., Op den Camp, H.J.M., and van der Drift, C. Cultivation of anaerobic fungi in a 10-L fermenter system for the production of (hemi-) cellulolytic enzymes. Appl. Microbiol. Biotechnol. 1996, **46**, 85-91
5. Dijkerman, R., Ledeboer, J., Op den Camp, H.J.M., Prins, R.A., and van der Drift, C. The anaerobic fungus *Neocallimastix* sp. strain L2: growth and production of (hemi)cellulolytic enzymes on a range of carbohydrate substrates. Curr. Microbiol. 1997, **34**, 91-96

6. Teunissen, M.J., Baerends, R.J.S., Knelissen, R.A.G., Op den Camp, H.J.M., and Vogels, G.D. A semi-continuous culture system for production of cellulolytic and xylanolytic enzymes by the anaerobic fungus *Piromyces* sp. strain E2. Appl. Microbiol. Biotechnol. 1992, **38**, 28-33
7. Zhu, W.Y., Theodorou, M.K., Nielsen, B.B., and Trinci, A.P.J. Dilution rate increases production of plant cell-wall degrading enzymes by anaerobic fungi in continuous-flow culture. Anaerobe. 1997, **3**, 49-59
8. Furusaki, S., and Seki, M. Use and engineering aspects of immobilized cells in biotechnology. Adv. Biochem. Eng/Biotechnol. 1992, **46**, 161-185
9. Kuek, C. Production of glucoamylase using *Aspergillus phoenicus* immobilized in calcium alginate beads. Appl. Microbiol. Biotechnol. 1991, **35**, 466-470
10. Jirku, V. Effect of C/N ratio and cellulose type on the cellulolytic activity of free and immobilized *Trichoderma reesei*. In: Immobilized Cells: Basics and Applications. (Wijffels, R.H., Buitelaar, R.M., Bucke, C., and Tramper, J., eds.). Elsevier Science Publishers, Amsterdam, 1996, 136-139
11. Phillips, M.W., and Gordon, G.LR. Sugar and polysaccharide fermentation by rumen anaerobic fungi from Australia, Britain and New Zealand. BioSystems. 1988, **21**, 377-383
12. Phillips, M.W., and Gordon, G.L.R. Growth characteristics on cellobiose of three different anaerobic fungi isolated from the ovine rumen. Appl. Environ. Microbiol. 1989, **55**, 1695-1702
13. Phillips, M.W., and Gordon, G.L.R. Carbohydrate fermentation of three species of polycentric ruminal fungi from cattle and water buffalo in tropical Australia. Anaerobe. 1995, **1**, 41-47

14. Wubah, D.A., Fuller, M.S., and Akin, D.E. *Neocallimastix:* a comparative morphological study. Can. J. Bot. 1991, **69**, 835-843

15. Gordon, G.L.R., and Phillips, M.W. Degradation and utilisation of cellulose and straw by three different anaerobic fungi from the ovine rumen. Appl. Environ. Microbiol. 1989, **55**, 1703-1710

16. Jamuna, R., and Ramakrishna, S.V. Physiological behaviour of *Saccharomyces cerevisiae* in gel matrix. In: Physiology of Immobilized Cells (De Bont, J.A.M., Visser, J., Mattiasson, B., and Tramper, J., eds.). Elsevier Science Publishers, Amsterdam, 1990, 533-538

17. Gosmann, B., and Rehm, H.J. Oxygen uptake of microorganisms entrapped in Ca-alginate. Appl. Microbiol. Biotechnol. 1986, **23**, 163-167

18. Kuek, C. Immobilized living fungal mycelia for the growth-dissociated synthesis of chemicals. Int. Ind. Biotechnol. 1986, **56:6:4/5**, 123-125

19. Shi, D-J., Brouers, M., Hall, D.O. and Robins, R.J. The effects of immobilization on the biochemical physiological and morphological features of *Anabaena azollae.* Planta. 1987, **172**, 298-308

20. Tanaka, H., and Irie, S. Preparation of stable alginate beads in electrolyte solutions using Ba^{2+} and Sr^{2+}. Biotechnol. Tech. 1988, **2**, 115-120

21. Kuek, C., and Armitage, T.M. Scanning electron microscopic examination of calcium alginate beads immobilizing growing mycelia of *Aspergillus phoenicus.* Enzyme Microb. Technol. 1985, **7**, 121-125

22. Gordon, G.L.R., and Phillips, M.W. The role of anaerobic gut fungi in ruminants. Nut. Res. Rev. 1998, **11**, 133-168

Figure legends

Figure 1 Influence of immobilization conditions on the subsequent growth of *Piromyces* sp. KSX1 in Ca-alginate beads observed at 24 hours:

(a) 3% aqueous Na-alginate preparation.

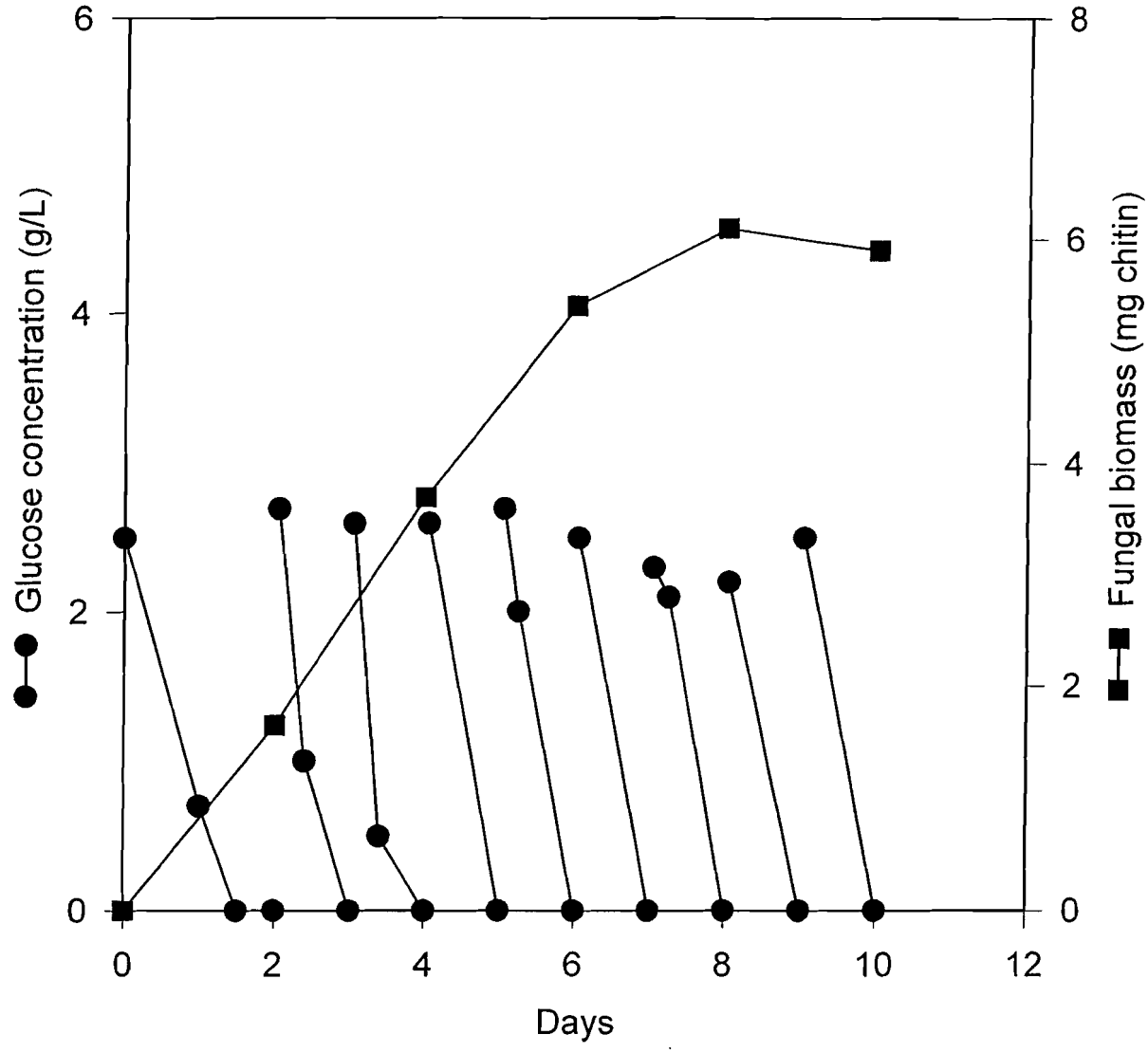
(b) 3% Na-alginate dissolved in basal medium M10 containing 0.25% glucose. Beads were washed with sterile distilled water for (a) and (b).

(c) 3% Na-alginate dissolved in basal medium M10 containing 0.25% glucose with washes in basal medium and maintenance of temperature at 39°C during the washing procedure. Bar = 3 mm for (a), (b) and (c).

Figure 2 Glucose concentration (●) and fungal biomass (cell wall chitin) (■) in sequential batch cultures (25 ml) of immobilized *Piromyces* sp. KSX1 grown statically with 0.25% glucose. Fresh glucose was added daily from day 2.

Figure 3 Scanning electron micrographs of whole Ca-alginate beads containing growing *Piromyces* sp. KSX1 at 2 days (a), 4 days (b), 8 days (c) and 10 days (d) after incubation. Bars = 100μm.

Figure 4 Effect of shaking on the growth of *Orpinomyces* sp 478P1 in alginate beads after 5 days (a) A static culture resulting in aggregates of beads joined by rhizomycelia. (b) Beads from a shaken culture. Bar = 3 mm for (a) and (b).



M^{c}Cabe *et al.*, Figure 2