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ORIGINAL PAPER

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Production of β-glucosidase using immobilised *Piromyces* sp. KSX1 and *Orpinomyces* sp. 478P1 in repeat-batch culture

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Abstract Two anaerobic fungi, one a monocentric strain (*Piromyces* sp. KSX1) and the other a polycentric strain (*Orpinomyces* sp. 478P1), were immobilised in calcium alginate beads and cultured in sequential batches where spent medium (containing 0.25% cellobiose) was repeatedly drained and replaced. β-Glucosidase production with KSX1 was maintained for 45 days over six repeated batch cultures yielding a maximum level of 107 mIU/ml. For 478P1, β-glucosidase production was maintained for 30 days over four repeated batches yielding a maximum level of 34 mIU/ml. Although repeat-batch cultures of KSX1 produced more β-glucosidase than strain 478P1, the maximum specific β-glucosidase produced from these immobilised cultures was similar. The immobilised polycentric strain proved to be operationally superior to strain KSX1, as strain 478P1 did not produce any growth in the culture liquor.

Keywords Anaerobic fungi · Immobilisation · β-Glucosidase · Repeat-batch culture

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Introduction

Anaerobic fungi isolated from the digestive tract or faeces of herbivorous mammals, both ruminant and non-ruminant, produce all of the cellulolytic enzyme activities needed for complete cellulose degradation, i.e. endoglucanases, exoglucanases and β-glucosidases [11]. The finding that the cellulose solubilisation capabilities of *Neocallimastix frontalis* and *Piromyces communis* are better than that of *Trichoderma reesei* [12, 13] provides confidence that the potential for converting cellulose to fermentable sugar may lie in the superior cellulolytic activity of anaerobic fungi.

The most studied method of cellulase production by both aerobic and anaerobic fungi has been batch culture, which can be coupled with the use of immobilised cells. This approach has been little exploited in cellulase production but appears to hold considerable potential for improving it since immobilisation enables the microbial cells to be used repeatedly and continuously [3]. The first report on fungal enzyme production by an immobilised biocatalyst was that of Frein et al. [2], who showed that the fungus *T. reesei* entrapped in a κ-carrageenan gel matrix can produce extracellular cellulolytic enzymes. In another study, glucoamylase production by immobilised, pre-grown mycelia of *Aspergillus phoenicus* was sustained over five repeated batches for a total time of 19 days [6].

In a previous investigation we achieved colonisation of alginate beads using either monocentric or polycentric types of anaerobic fungi [7]. The two types had different growth habits in calcium alginate gel, providing further insight into the life cycle and growth patterns particular to monocentric and polycentric fungi. This current study is the first to report on the use of these immobilised anaerobic fungi in repeat-batch fermentations and relates these differences in colonisation mode to subsequent enzyme productivity.

Materials and methods

Organisms and culture media

The isolation and description of *Piromyces* sp. strain KSX1 and *Orpinomyces* sp. strain 478P1 have been described previously [7]. Cultures were maintained at 39°C in a semi-defined ruminal-fluid-free medium [8, 9]. A detailed description of the modifications to this medium (complete medium 10×) and the method for preparation were as reported previously [7].

Cellobiose medium (CM) was used in β-glucosidase production using immobilised cultures and consisted of basal medium 10 (BM; complete medium 10× without carbohydrates) containing 0.25% cellobiose, which was separately sterilised at 110°C for 20 min in concentrated solutions under a N_2 atmosphere and added aseptically to sterile BM. Glucose medium (GM) was prepared by substituting 0.25% glucose for cellobiose.

Production of immobilised rhizomycelium

Batches of approximately 150 calcium alginate beads (2.5–3.0 mm in diameter) were produced as reported previously [7] using zoospore and partially homogenised rhizomycelial suspensions of strains KSX1 and 478P1, respectively. Sodium alginate (Manugel GMB; Kelco A.I.L., Melbourne, Australia) was prepared as a 3% (w/v) solution dissolved in GM and all washes were performed in BM at 39°C. In immobilised KSX1 cultures, alternate repeat-batch and fed-batch techniques using GM were used until fungal biomass was maximised in the beads. Immobilisation of the polycentric fungus was similar to that described for the monocentric isolate but without the need for a culture establishment phase. After 24 h static incubation at 39°C, the immobilised 478P1 cultures were shaken on a reciprocating shaker at 80 oscillations per minute.

Repeat-batch production of β-glucosidase using immobilised cells

Repeat-batch fermentation was conducted using duplicate sets of immobilised fungal cultures contained in the same 100 ml serum bottles used in the preparation of immobilised rhizomycelium.

After culture establishment, the spent medium in immobilised KSX1 cultures was removed using aseptic anaerobic procedures with 23 gauge needles on 50 ml syringes and the beads were washed with 25 ml BM. CM (25 ml) was added and the beads were re-incubated statically in repeat-batch mode at 39°C. Immobilised cultures of strain 478P1 were grown in 25 ml CM as described above and subsequently cultured repeatedly in batch mode for enzyme production using the same substrate at the same concentration. All cultures were incubated in a reciprocating water bath at 39°C and shaken.

During repeat-batch fermentation, samples of culture liquor (1.0 ml) were removed by aseptic anaerobic procedures at regular intervals and filtered using 0.2 μm syringe filters (Minisart, Sartorius, Australia). The pH of the filtered culture liquor was measured immediately and also assayed for residual cellobiose. β-Glucosidase in the culture filtrate was assayed on the day of sampling and when enzyme levels reached a maximum, the medium was aseptically removed. After washing the beads with 25 ml BM, 25 ml sterile CM was added to start another batch fermentation with subsequent sampling.

At the end of each batch fermentation duplicate sets of bottles were sacrificed and chitin estimations were performed as an indicator of fungal biomass. The beads were washed and the alginate was dissolved in 10% hexametaphosphate (BDH, Poole, Dorset, England). The free fungal rhizomycelium was sedimented by centrifugation at 2,000 *g* for 15 min, and the pellets were washed and stored frozen for later chitin analysis.

Free rhizomycelium batch cultures

Free rhizomycelium batch cultures of strains KSX1 and 478P1 were performed in duplicate using 100 ml sealed serum bottles containing 25 ml CM using inocula as for immobilised cultures. Samples of culture liquor (1.0 ml) were taken aseptically at regular intervals for the determination of β-glucosidase and chitin.

Assays

Cellobiose in the culture liquor was determined after enzymatic hydrolysis with β-D-glucosidase glucohydrolase (Sigma) based on the method of Russell and Baldwin [10].

Aryl-β-glucosidase assays were performed using a *p*-nitrophenol derivative of β-D-glucose (5 mM; Sigma) [4]. One β-glucosidase unit (IU) is defined as the amount of β-glucosidase that produced 1 μmol glucose/min.

For biomass determinations, fungal chitin of immobilised and free cultures was estimated colorimetrically as the total hexosamine content after acid hydrolysis [4].

Microscopy

The surface of the immobilised fungal cultures was observed by scanning electron microscopy (SEM) at the end of the repeat-batch operation as reported previously [7].

Results

Repeat-batch production of β-glucosidase using immobilised rhizomycelium

β-Glucosidase production by immobilised cultures of KSX1 was sustained over six repeat-batch cultures (Fig. 1), although free rhizomycelium was present in the

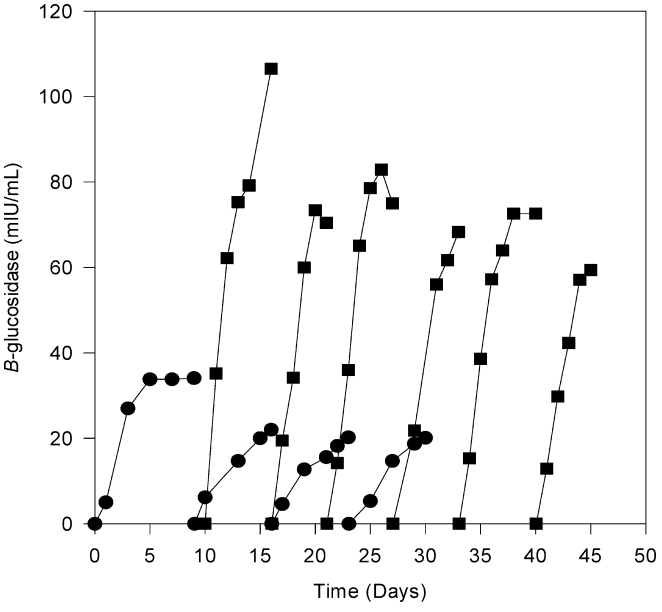


Fig. 1 Production of β-glucosidase by immobilised *Piromyces* sp. KSX1 (■) and *Orpinomyces* sp. 478P1 (●) grown in repeat-batch cultures

culture liquor throughout the duration of the fermentation. Yield of β-glucosidase was highest in the first batch, levelling off to between 56 and 75% of the initial yield in subsequent batches. Cellobiose was consumed by day 2 in the first batch and day 3 in subsequent batches. Culture pH changed little (6.7 to a minimum of 6.2 within 3 days).

Immobilised *Orpinomyces* sp. 478P1 maintained β-glucosidase production for 30 days over four successive batches (Fig. 1). As with KSX1, enzyme yields declined over the batches (to 59% of the initial yield by the fourth batch). Cellobiose in the medium was consumed more slowly by 478P1 compared to KSX1 (depleted by day 3 in the first batch then by days 4–5 in subsequent batches) and culture pH decline was more pronounced (from 6.7 to 5.9 in 4 days).

The amount of immobilised biomass in KSX1 cultures (as indicated by chitin assay) was seven times greater than the amount of free biomass found in the culture liquor (Table 1). The amount of free biomass averaged 0.66 mg chitin and remained constant between the repeat-batches. With 478P1, no viable free biomass was found and, similar to KSX1, the amount of immobilised biomass in the beads did not vary between batches.

Specific β-glucosidase production of immobilised rhizomycelium in repeat-batch culture versus non-immobilised rhizomycelium cultures in a single batch culture

Specific enzyme production of immobilised rhizomycelium is given in Table 1 and was calculated from the β-glucosidase yields and chitin values given in this table. These values were compared with specific enzyme production produced at the end of a single batch culture of non-immobilised rhizomycelium for each strain.

Specific β-glucosidase production of non-immobilised single batch KSX1 cultures was 2.0 IU/mg chitin. This was higher than that obtained in the repeat-batch immobilised cultures. Specific β-glucosidase production in the first batch, calculated from free biomass in the culture liquor was 3.6 IU/mg chitin. This could account for the high β-glucosidase yield in the first repeat-batch of immobilised KSX1 cultures shown in Fig. 1. The specific β-glucosidase production from free biomass in the remaining repeat-batches was approximately equivalent to that found in the single batch non-immobilised culture.

Specific β-glucosidase production of non-immobilised 478P1 cultures was 0.30 IU/mg chitin. In immobilised 478P1 cultures, similar measures of specific β-glucosidase production were obtained at the end of each batch. The exception to this was the increased enzyme activity obtained in the first batch. This is represented as a 42% increase in specific β-glucosidase production using immobilised 478P1 cultures compared to a single batch of free cells. Despite a drop in specific enzyme production in subsequent batches, existing immobilised biomass continued to produce β-glucosidase at levels comparable to free cell cultures.

Bead morphology and containment of rhizomycelia

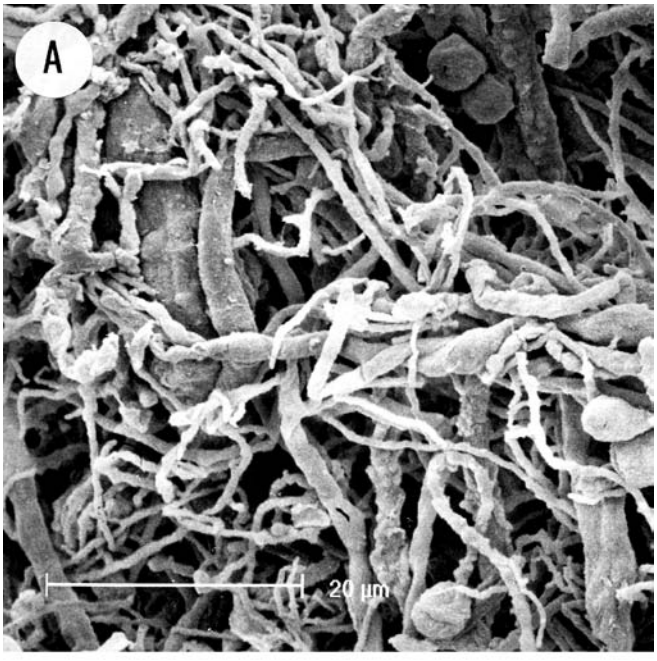
The gel beads of immobilised KSX1 maintained their shape during the 45-day operation. Viable zoospores and rhizomycelium appeared in the culture liquor 24 h into the incubation of each batch of immobilised KSX1 cultures. Closer examination of the bead surface using SEM revealed numerous sporangia associated with an extensive rhizomycelial network (Fig. 2a). It is likely that the zoospores were the source of propagules for the development of free rhizomycelia with each addition of fresh medium.

Short rhizomycelial segments (ca. 0.5 mm in length) appeared in the culture medium in the second batch of immobilised 478P1 cultures and continued to be produced in subsequent batches. These rhizomycelial segments were non-viable when plated on agar-containing M10× medium. SEM of a gel bead at the end of the fermentation period shows peripheral vegetative rhizomycelial growth on the outside of the bead and no sporangia (Fig. 2b).

Table 1 Immobilised biomass, β-glucosidase yields and specific β-glucosidase production in repeat-batch cultures. Each value represents the mean of duplicates. *Piromyces* sp. KSX1 and *Orpinomyces* sp. 478P1

Batch number[a]	Immobilised biomass (mg chitin)		β-Glucosidase production (mIU/ml)		Specific β-glucosidase production (IU/mg chitin)	
	KSX1	478P1	KSX1	478P1	KSX1	478P1
1	3.90 (0.60)[b]	1.32	106.5	34.1	0.50	0.52
2	4.58 (0.67)	1.30	70.4	22.0	0.31	0.36
3	4.21 (0.73)	1.35	75.0	20.2	0.33	0.30
4	4.52 (0.68)	1.36	68.3	20.1	0.31	0.31
5	4.60 (0.61)		72.6		0.30	
6	4.75 (0.69)		59.4		0.25	

[a]Batch time length as indicated in Fig. 1
[b]Free biomass in culture liquor (mg chitin)



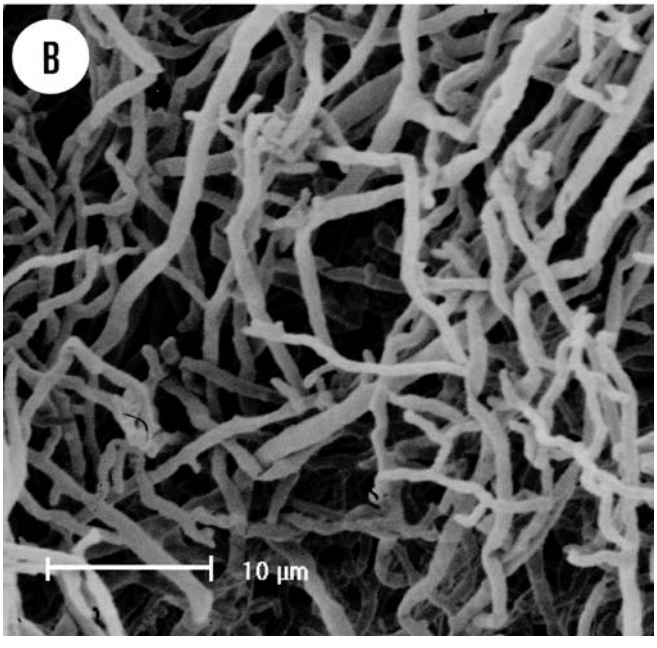


Fig. 2 **a** The surface of immobilised *Piromyces* sp. KSX1 after 45 days incubation, illustrating numerous sporangia and associated rhizomycelial network. **b** The surface of immobilised *Orpinomyces* sp. 478P1 after 30 days incubation. Note the absence of sporangia. *Bars* **a** 20 μm, **b** 10 μm

Discussion

β-Glucosidase can be produced by pre-grown mycelium in repeat-batch cultures of the two anaerobic fungi studied. Although the production of β-glucosidase was sustained throughout repeat-batch cultures of KSX1 and 478P1, an increase in enzyme production in the first batch was a phenomenon observed in all repeat-batch experiments and was possibly due to the higher cell viability in the beads that occurred at this time. In examining the physiological aspects of immobilised cells, Hahn-Hägerdal [5] has suggested there may be an exponential growth phase with concomitant high metabolic activity immediately after immobilisation with only a percentage of the cell population maintaining a high metabolic activity. This population of cells is either in close proximity to the surrounding medium or in a location where space is made available by the release of cells. Although cell viability may have decreased after the first batch, it is likely that peripheral regrowth of both types of anaerobic fungi on the gel bead may be responsible for maintaining enzyme production during the later batches.

Repeat-batch culture of *Piromyces* sp. KSX1 cells immobilised in alginate gel proved to be a novel method in so far as maintaining production of β-glucosidase over an extended period of incubation. However, the merits of repeat-batch culture of the monocentric fungal culture were limited by the confounding problem of rhizomycelial growth in the culture liquor throughout the fermentation. Although repeat-batch cultures of KSX1 produced more β-glucosidase than strain 478P1, the maximum specific β-glucosidase produced from these immobilised cultures was similar. Free mycelia in KSX1 repeat-batch cultures contributed to enzyme production, making strain 478P1 a better candidate for immobilisation since it did not give rise to viable free mycelium.

Long-term viability has been demonstrated for both strains and is another of the advantages encountered when working with immobilised cells. Moreover, the repeat-batch system with immobilised rhizomycelia was operationally more efficient than a single batch culture with free rhizomycelia because immobilisation removed the need to re-grow the cells for every single batch. In this initial study β-glucosidase production in KSX1 and 478P1 cultures was followed for up to 45 and 30 days, respectively. It is possible that the fermentation could have proceeded longer if the incubation was continued considering that the immobilised cultures were still viable.

The alginate beads of both strains of fungi maintained their shape through the repeat-batch cultures. However, beads of KSX1 had lost some strength. Immobilised cultures of 478P1 were comparatively stronger due to their ability to invade the whole alginate matrix. The long-term stability of calcium alginate is a factor limiting the wide application of this gel in immobilisation and may be associated with the release of free mycelial aggregations in immobilised KSX1 cultures. In a study investigating immobilised *Penicillium chrysogenum* strains in penicillin production [1], it was found that maximum instability of alginate beads occurred when a decrease of mycelium in the central layers took place by autolysis and the outer layers reached maximal mycelial capacity. This led to a destruction of the beads and the release of the mycelia into the fermentation medium to form a thick suspension.

This study was performed to investigate the use of immobilisation of fungal rhizomycelia and repeat-batch cultures as a novel and potentially advantageous approach to cellulase production from anaerobic fungi, and has yielded new knowledge on differences between *Piromyces* and *Orpinomyces* in terms of immobilisation. Immobilised polycentric fungi may hold particular promise in scale-up operations due to their operational superiority over monocentric fungi. Future experiments will be required to further define optimal conditions for extracellular enzyme production by immobilised polycentric anaerobic fungi.

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