Production of glucoamylase using *Aspergillus phoenicus* immobilized in calcium alginate beads

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Summary. As a means of better exploiting the growth-dissociated nature of the synthesis of glucoamylase, a production process where the growth phase was separated from the enzyme synthesis phase was developed. Immobilized mycelia arising from a 6-day culture of conidia immobilized in calcium alginate beads could be subsequently used repeatedly to produce glucoamylase in a second step using a Dextran T-10 medium. Glucoamylase production was sustained over 5 sequential batches in a 19 day period and immobilized mycelia remained confined to the subsurface of the beads.

Introduction

Studies on *Aspergillus niger* have shown that the rate of glucoamylase production is inversely proportional to the specific rate of mycelial growth and is maximal only after completion of the growth phase (Okazaki *et al*., 1965; Okazaki and Terui, 1966 a,b). It is therefore likely that all *Aspergillus* species generally exhibit synthesis of glucoamylase which is "growth-dissociated" (Enatsu and Shinmyo, 1978). The current industrial practice of producing glucoamylase in batch culture (Frost and Moss, 1987) does not fully exploit this pattern of growth and synthesis.

The potential advantages of dividing batch production processes for growth-dissociated products into two phases *i.e.* the accumulation of biomass (growth phase), followed by the repeated use of the same biomass for synthesis of the growth-dissociated product (synthesis phase) have been discussed (Kuek, 1986).This phase separation, particularly when applied to fungi, is a novel approach because it is yet to be routinely used in production processes where product synthesis is growth-dissociated e.g. in the production of secondary metabolites such as antibiotics. It is likely that phase separation has not been incorporated in current batch production practice because of the difficulty in recovering biomass in a state which is fit for reuse. While this problem can nowadays be overcome by immobilization of cells, there are still few papers on growth-dissociated synthesis using fungal mycelia. It is speculated that this paucity is related to the fact that the most usual result of immobilizing fungi is that the mycelia ends up growing on the surface of the immobilized aggregations (Kuek, 1986). This causes bioengineering problems and reduces the advantages of immobilization.

Living mycelia of *Aspergillus phoenicus* has been previously reported to be immobilized in such a fashion that all the biomass was confined to the subsurface of alginate beads (Kuek and Armitage, 1985). This paper extends the previous study towards the use of immobilized *Aspergillus phoenicus* R4M5.10 in phase separated batch production of glucoamylase. The potential of pre-grown, immobilized mycelia for repeated use in the production of glucoamylase by batch culture was investigated in this study, firstly in shake flasks and later in a bubble column. Since synthesis of the enzyme is growth-dissociated, the glucoamylase productivity of the beads will be determined not only by the amount of biomass immobilized but also the physiological age of that biomass. For this reason, the effect of physiological age of the biomass on yield of enzyme was examined.

Whether glucoamylase synthesis is subject to catabolite repression is not clear, largely because of the difficulty in interpreting results from studies where the growth and enzyme synthesis phases are not separated, and because there have been few studies on either induction or catabolite repression (see Fogarty and Kelly, 1980). For this reason, and since catabolite repression is minimized when substrates which are metabolized slowly are used in a medium (Reese *et al.*, 1969), Dextran T-10 rather than starch was used as the polysaccharide substrate in this study. Being 95% alpha-1,6 linked (Van Cleve *et al.*, 1956), dextran is hydrolysed more slowly than starch. The predominance of the latter linkage may have another advantage in that it could serve as an inducer for glucoamylase if indeed an inducer is required.

MATERIALS AND METHODS

Fungus. The culture used was *Aspergillus phoenicus* R4M5.10, an isolate improved in glucoamylase productivity

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produced by the treatment of *Aspergillus phoenicus* ATCC 15556 with N-Methyl-N'-Nitro-N-Nitrosoguanidine (Kuek and Kidby, 1984).

Media. The basal nutrients used were (mg l^{-1}): K_2HPO_4, 70; KH_2PO_4, 130; $MgSO_4.7H_2O$, 200; $Na_2SO_4.10H_2O$, 200; $FeSO_4.7H_2O$, 20; $MnSO_4.H_2O$, 10; $ZnSO_4.7H_2O$, 2; $CuSO_4.5H_2O$, 2; monosodium glutamate, 550; and yeast extract (Difco Laboratories), 500.

Growth Medium consisted of basal nutrients plus soluble starch (BDH Chemicals), 20 g $l.^{-1}$ and $CaCl_2.2H_2O$, 3.68 g l^{-1} (0.025 M).

Wash medium consisted of basal nutrients plus $CaCl_2.2H_2O$, 3.68 g l^{-1}.

Production Medium consisted of basal nutrients plus Dextran T-10 (Pharmacia Fine Chemicals), 5 g l^{-1} and $CaCl_2.2H_2O$, 3.68 g l^{-1}, adjusted to pH 2.5 with 0.1 M HCl before autoclaving, so that it had a similar value to the final pH of culture liquors at the completion of the growth phase. Dextran T-10 was only added after pH adjustment.

Sterilization. Media and apparatuses were autoclaved at 121°C for 15 min.

Production of immobilized mycelia. Batches of 4000 and 16000 beads (1.5 - 1.6 mm in diameter) were produced according to the method reported previously (Kuek and Armitage, 1985) where conidia immobilized in beads of calcium alginate were incubated in Growth Medium at 35°C. Incubation time was varied as required for the different experiments. After incubation, the beads were drained and rinsed with Wash Medium equal to twice the volume of culture liquor. The beads were then ready for use in the production of glucoamylase in either shake flasks or the bubble column (see below). For determination of the growth curve for the immobilized fungus, nine 2.8 l Fernbach flasks (with cotton sheik closure) each containing 16000 beads and 200 ml of Growth Medium were incubated at 35°C and 250 r.p.m. in a Gallenkamp cooled-orbital incubator (orbit diameter = 28 mm). At daily intervals, a flask was removed and the beads assayed for nitrogen content (see below).

Shake flask production of glucoamylase. To test the effect of age of mycelia on the production of glucoamylase, separate 4000-bead batches were incubated in Growth Medium for 2, 3, 4, or 6 days. For glucoamylase production, each batch of beads were placed in 250 ml Erlenmeyer flask (cotton sheik topped) with 50 ml of Production Medium. Incubation in a Gallenkamp cooled-orbital incubator was at 35°C and 250 r.p.m. (orbit diameter = 28 mm). Samples of culture liquor (1.5 ml) were aseptically collected each day. At the end of each batch culture, liquor was drained and the beads were washed in Wash Medium equivalent to twice the volume of liquor. The next batch culture was then begun with the addition of 50 ml of fresh Production Medium.

Bubble column production of glucoamylase

Bioreactor. A glass bubble column reactor (3.0 cm internal diameter; 200 ml working volume) and ancillary parts were custom blown. The air used was filtered on-line with a gas purifier (Type 451, Matheson Inc.) and a 0.2 micrometer cellulose acetate filter (Millipore Corp.). Air was humidified prior to entry in the bubble column by passage through a jacketed vessel containing water. The sparger was downward pointing (0.5 cm from the bottom of the reactor) with a single orifice 0.5 mm in diameter. Culture pH was maintained by the automatic addition of 0.1 M HCl or 0.1 M NaOH regulated by a pH controller. The electrode was sterilized by immersion in 70% (v/v) ethanol for 15 min. before use. Electrode drift was corrected daily by comparisons of pH controller readings against an external pH meter. Culture temperature was maintained by water flow from a recirculating bath through the water jacket of the reactor. Fresh medium was added to the reactor when required by aseptic transfer from reservoirs.

Each batch culture consisted of 200 ml of Production Medium and 16000 beads which had been previously incubated for 6 days in Growth Medium. Air was supplied at 1.25 l $min.^{-1}$. Temperature was maintained at 35°C. Samples (1.5 ml) were withdrawn each day with a sterile hypodermic needle through a sampling port which was sealed with a septum. At the end of each batch culture, liquor was drained, and the beads were washed within the reactor with Wash Medium equivalent to twice the volume of liquor. The next batch culture was begun with the addition of 200 ml of Production Medium.

Assays. For residual glucose the culture filtrate was inactivated with an equal volume of 6 % (w/v) trichloroacetic acid after sampling. Glucose concentrations were determined (see later) after appropriate dilution.

For glucoamylase, substrate solution was prepared by bringing to the boil, soluble starch (1.0 g l^{-1})(BDH Chemicals) in McIllvaine buffer (Dawson *et al.*, 1969) made to pH 5.0 at 35°C. Both substrate solutions and culture liquors were clarified by centrifugation before assay. For hydrolysis, reaction tubes consisted of 0.5 ml aliquots of culture liquor and 2.5 ml of substrate solution. The blank consisted of 0.5 ml of culture liquor and 3.0 ml of 6% (w/v) trichloroacetic acid. The tubes were incubated for 1 h at 35°C after which 3.0 ml of 6% trichloroacetic acid was added to the reaction tube and 2.5 ml of substrate solution was added to the blank. After appropriate dilution, the glucose concentrations of hydrolysates and blank solutions were determined using a glucose oxidase-peroxidase-chromogen kit (Sigma Chemical Co.; Kit No. 510). The amount of glucoamylase in 1 ml of culture liquor which will produce 1 micromole of glucose in the reaction tube in 1 min. is defined as 1 Glucoamylase Unit (U).

For biomass, samples consisting of 16000 beads were rinsed clean of culture liquor with Wash Medium equivalent to twice the volume of the liquor and then dehydrated in three changes of absolute methanol (200 ml each change) over 24 h. After draining the methanol, the beads were brought to surface dryness by blowing with an airstream. The beads were fragmented to fine particles with a rotary blender and dried at 35°C for 24 h. Nitrogen content was determined by the Kjeldahl method (Williams, 1984).

Results

Production of beads in shake flasks During growth, the beads changed in color from an opaque grey (the color just after bead manufacture), to light brown, and finally to dark brown by Day 7. The morphology of the bead interior and exterior have been previously described (Kuek and Armitage, 1985). The peaking of residual glucose level and biomass content, and the attainment of final liquor pH occurred around Days 2 and 3 (Fig. 1). The growth-dissociated nature of glucoamylase synthesis can be clearly seen by a comparison of the biomass and glucoamylase curves which shows a rapid increase in enzyme titre after Day 2 and peaking at Day 4-5 by which time biomass accumulation had already peaked.

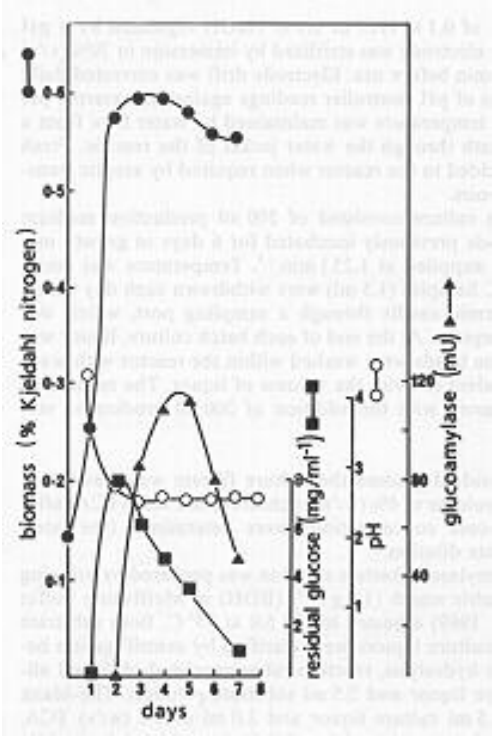

Fig. 1. The production of glucoamylase by immobilized *Aspergillus phoenicus* R4M5.10, with concomitant measurement of biomass, residual glucose and pH with time: U, units.

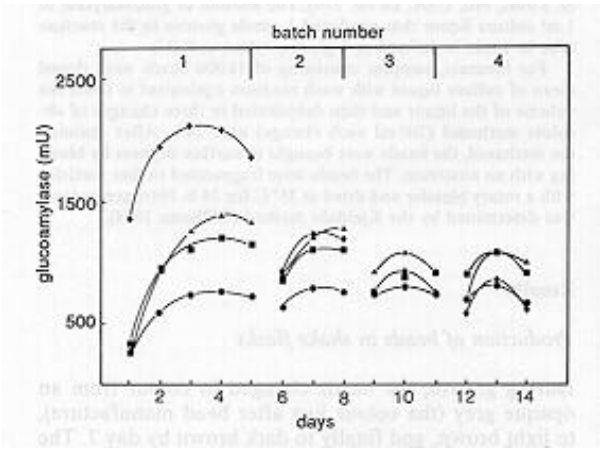

Fig. 2. Glucoamylase production by immobilized mycelia of *A. phoenicus* R4M5.10 of various ages during repeated use in sequential batch cultures in shake flasks. The mycelia used were produced by incubation in growth medium for various periods: 2 days (◆); 3 days (▲); 4 days (■); 6 days (●)

Production of glucoamylase by mycelia of various ages

Immobilized mycelia of *Aspergillus phoenicus* retained the capability to synthesize glucoamylase when the culture liquor of the growth phase was replaced with Production Medium (Fig. 2). Further, batches of beads could be used repeatedly to produce glucoamylase by the drainage of spent and addition new Production Medium. Younger mycelia had higher glucoamylase yields initially but by the fourth batch culture, yields of younger mycelia had fallen to the level of the oldest mycelia. Glucoamylase in the first batch took longer to peak than in subsequent batches. The least amount of glucoamylase produced in phase separated production was about four times greater than the highest amount obtained in the culture with Growth Medium (compare with Fig. 1). Residual glucose levels were below those detectable by the method used. As 6-Day old beads resulted in the least variable yield of glucoamylase over the four sequential batches, it was decided that future studies on production of the enzyme would be based on 6-Day old beads.

Bubble column production with 6-day old beads

Six-day old beads used in the bubble column yielded consistent levels of glucoamylase over five sequential batch cultures (Fig. 3). The yield in the bubble column was about 73% of that obtained with similar age beads using a shake flask. As in shake flask production, the first batch culture was also observed to run longer than subsequent batches. Residual glucose levels were below those detectable by the methods used.

Bead morphology

The internal and external appearance of the beads were as described previously (Kuek and Armitage, 1985). Beads

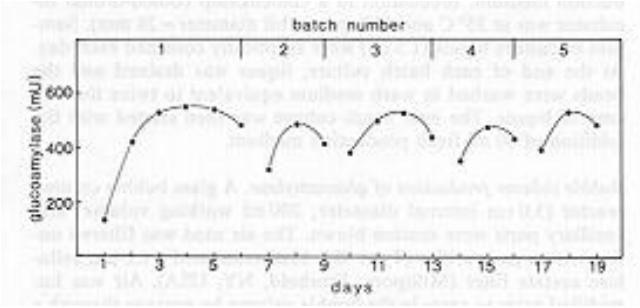

Fig. 3. The glucoamylase yield of immobilized *A. phoenicus* R4M5.10 during repeated use in sequential batch cultures in a bubble column.

used in shake flask and bubble column production of glucoamylase retained their conformation i.e. the mycelia remained confined to the subsurface. Liquors became cloudy as the cultures progressed but this was more of a problemin the shake flask than in the bubble column where cloudiness was minimal. Microscopic examination showed that the cloudiness was caused by gelatinous material and fragmented hyphae with no cellular contents. No free mycelia was observed at any stage of the operations. Electron micrography of beads used for 36 days in the production of glucoamylase in the bubble column confirmed that mycelia remained confined to the subsurface, and revealed wear of the bead surface (Fig. 4a and b).

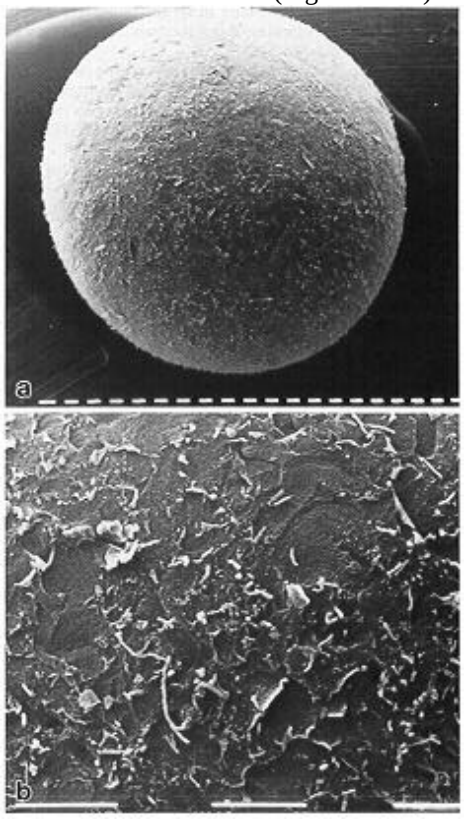

Fig. 4. A. Scanning electron micrograph of a calcium alginate bead immobilizing *A. phoenicus* R4M5.10. The bead was from a batch used for 36 days in the production of glucoamylase in a bubble column reactor. **b.** Surface detail of the bead in **a**. *Bars* = 100 μm

Discussion

This study demonstrates that it is possible to produce glucoamylase via a phase separated process where culture for immobilized mycelial growth is followed by repeated culture for synthesis of the enzyme. Bead conformation, and the synthetic capability of 6-day old beads were retained in the course of repeated batch cultures. A clear advantage of the result achieved is the absence of mycelium either in the medium or on bead surfaces as has been reported in many cases of immobilization of fungi (e.g. Deo *et al.*, 1983; Borglum and Marshall, 1984; Eikmeier *et al.*, 1984; Abdel-Halim *et al.*, 1986; El-Aassar *et al.*, 1990).

Production Medium incorporating Dextran T-10 proved to be suitable for repeated batch-wise production of glucoamylase using pre-grown immobilized mycelia, and resulted in a yield four times greater than that possible with mycelia in the growth phase in a starch medium. This higher yield is unlikely to be due to substrate and pH factors alone as the phenomenon of growth-dissociated synthesis would have had a major contribution.

The observation that the first batch culture of beads of all ages in both shake flasks and the bubble column required longer residence times than subsequent batches is probably due to a requirement for the mycelia to adapt from the growth phase to the enzyme synthesis phase. Age of mycelia when first used for glucoamylase production had an effect on enzyme titre. But this effect diminished as more sequential batches were run. This suggests that a physiological change affecting enzyme synthesis occurred in the mycelia between Day 2 and 4 of the production of immobilized mycelia. This change may in part, be the production of fruiting bodies as the mycelia ages. This is indicated by the color change in the beads and fruiting bodies have been observed with scanning electron microscopy (Kuek and Armitage, 1985). Sporulation would result in a lower proportion of vegetative and thus glucoamylase-synthesizing mycelium. Thus, 2-day old beads could have a higher proportion of vegetative mycelium than 6-day old beads but with time, the younger beads would sporulate and end up with a proportion similar to 6-day old beads. Whilst there were differences in biomass content between the beads of various ages at the time they were first used for enzyme production, neither the trend nor the magnitude of the differences provides a likely explanation for the differences in capability to synthesize glucoamylase.

Glucoamylase production in the bubble column followed the same pattern as that established in shake flasks and confirms a viable production process for glucoamylase. The lower peak level of glucoamylase in the bubble column compared to that obtained in shake flasks was most likely due to the differences in agitation and aeration between the two methods of culture.

Other experiments which extend the results of this study in the number of sequential batches that can be run, and the optimization of the production conditions for

maximization of the yield of glucoamylase will be reported elsewhere.

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