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Activities of β-glucanases and β-glucosidases during blastospore formation in *Saccharomycopsis fibuligera*

C.E. Nwoguh and D.R. Berry

Department of Bioscience and Biotechnology, University of Strathclyde, Glasgow, U.K.

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SUMMARY

The activities of three glycosidases, β-glucosidase and β(1,3)- and β(1,6)-glucanases have been monitored during growth and blastospore formation in *Saccharomycopsis fibuligera*. The assays were carried out on the cell-free culture and in a cell-free extract and a wall autolysate preparation from the growing cells. In complex medium containing 1% glucose an increase in the level of all three enzymes was associated with the transition from mycelium to blastospores. When the level of glucose was increased to 5% blastospore formation was repressed and the level of β-glucanases only increased at the end of the fermentation. The β-glucosidase activity increased during the growth phase. In a defined medium in which slow growth in a wholly yeast-like form was observed, growth was not associated with a high level of β-glucanase activity.

INTRODUCTION

Saccharomycopsis fibuligera is an ascosporogenous yeast which grows in a mycelial form and also produces yeast-like cells or blastospores [23]. It has been described as dimorphic by Sadova [20] and Necas and Svoboda [10] but there is little information available on the morphological development of this organism.

A considerable body of evidence exists indicating that wall growth and morphogenesis in both mycelial and yeast-like fungi is associated with changes in β-glucanase activities [1,11,16,18]. β-Glucanases have been implicated in both cell wall extension and bud initiation and it has been suggested that both these processes are dependent upon the opening up of β-glucan bonds by β-glucanases before new material is inserted in the cell wall [17]. During blastospore formation in *Saccharomycopsis fibuligera*, mycelial cells are replaced by yeast-like cells in the culture. This transition appears to involve not only the formation of blastospores but the complete dissolution of the mycelial biomass [13,14]. In this paper we have investigated the possible role of glucan-degrading enzymes during blastospore formation. Three enzymes have been examined; β-glucosidase and β(1,3)- and β(1,6)-glucanases. Although exoglucanases have been reported to be the most abundant glucan hydrolysing enzymes in yeast, the assay technique used measures both exo- and endoglucanase activity [9,11].

Correspondence: D.R. Berry, Department of Bioscience and Biotechnology, University of Strathclyde, 204 George Street, Glasgow G1 1XW, U.K.

MATERIALS AND METHODS

Growth and maintenance of organism

Stock cultures of *Saccharomycopsis fibuligera* strain 485 were obtained from the National Collection of Yeast Cultures, Norwich, and maintained on MYGP slopes containing (g/l): malt extract, 3.0; yeast extract, 3.0; glucose, 10; peptone, 5.0; and agar, 20.0. Subcultures were prepared every month to maintain the culture. Inoculum preparation was carried out in either 250-ml or 1-l flasks which were shaken on a Gallenkamp orbital shaker at 30 °C.

Experiments were carried out with yeast minimal medium [19] (Difco) supplemented with 0.34% w/v methionine. Yeast extract, peptone, casein, casamino acids, starch, etc. were also obtained from Difco. Complex media were autoclaved at 121 °C, 15 psi, for 20 min, but sugar, vitamin and trace element solutions were sterilized by membrane filtration through a Millipore 0.14 μm membrane.

All the experiments reported in this paper were carried out in a 3-l LHE fermenter (L.H. Engineering Ltd.) model 1/1000 with a working volume of 2.5 l. Agitation was set at 300 rpm, pH 5.0, and air flow was maintained at 1 l/V/min. The fermenter was inoculated with 200 ml of stationary phase cells grown on an appropriate medium in a 1-l flask.

Analytical techniques

Total dry weights were determined by filtering duplicate samples through a Whatman GF3C filter paper. The samples were washed three times with equal volumes of distilled water, then dried in an oven at 105 °C and left in a desiccator for 30 min before being weighed. Percentage mycelium was determined by filtering the same volume of samples as in total dry weight through a 45-μm preweighed nylon mesh filter (Henry Simon Ltd.) using a gravity filter. Subsequent washings (three times) of the trapped cells generally gave satisfactory preparations of the M-form while the Y-cells pass through the mesh devoid of contaminating M-form cells as confirmed by microscopic examination. The cells were dried as above. The dry weight remaining on the filter was considered to represent mycelial dry weight [3]. The ratio of the mycelial dry weight to the total biomass is expressed as percentage mycelium. Cell number was determined using a Thoma haemacytometer.

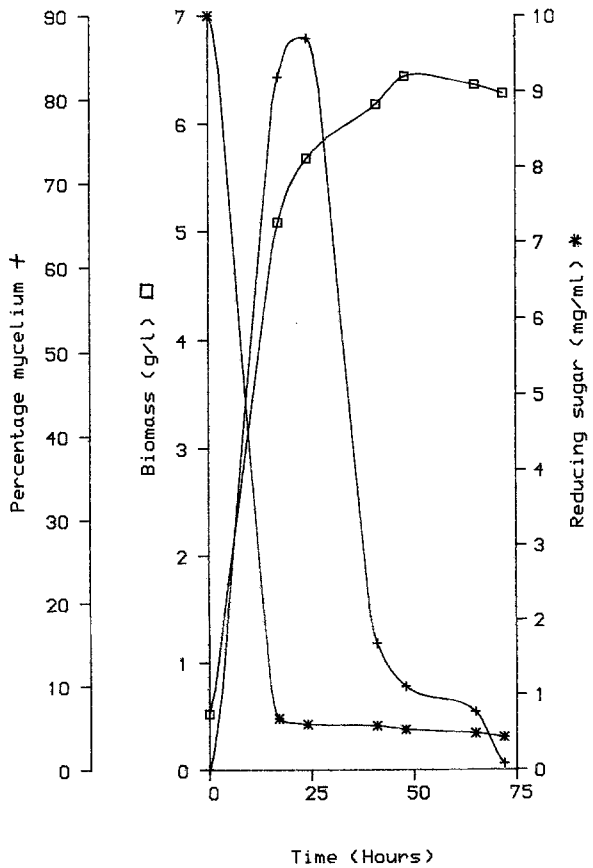


Fig. 1. Growth of *S. fibuligera* in complex medium containing 1.0% (w/v) glucose and 0.25% (w/v) peptone; (+) percentage mycelium, (□) biomass and (*) reducing sugar.

Preparation of cell free extract and cell wall autolysate

Cell-free extract and cell wall autolysate were prepared according to Fleet and Phaff [5]. After homogenization in a Braun Homogenizer, the crude homogenate was separated from the glass beads by suction. The cell wall debris was sedimented by centrifugation at 3000 rpm for 5 min at 4 °C. The supernatant fraction was further centrifuged at 10000 rpm for 30 min (4 °C) to remove particulate material and the supernatant was used as cell-free extract. The cell-free extract was stored at −20 °C till required.

The cell wall debris from the above protocol was washed three times by centrifugation at 3000 rpm for

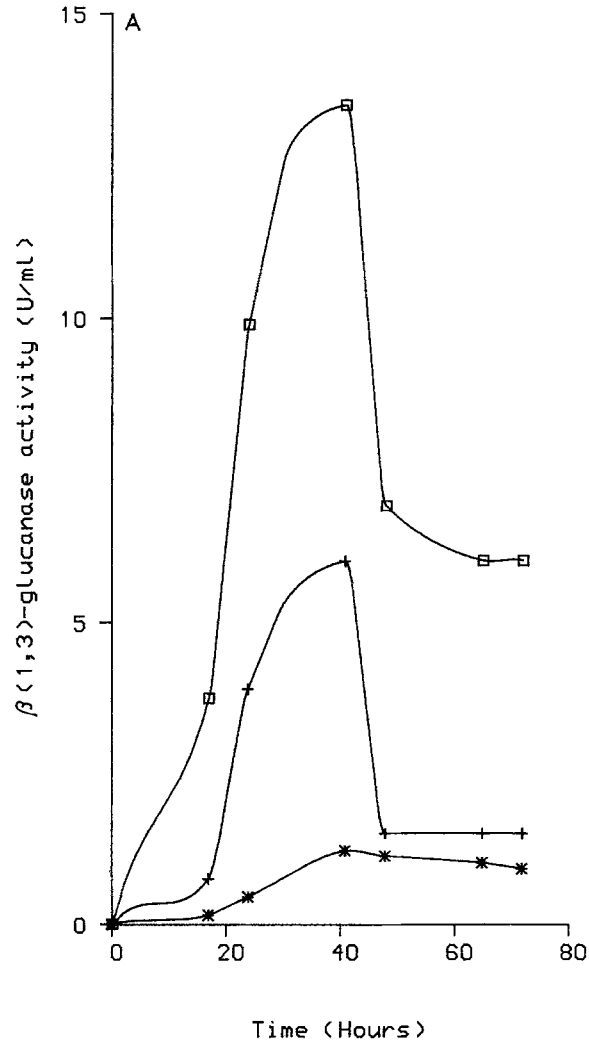


Fig. 2. **A.** β(1,3)-glucanase activities in (□) cell-free extract, (+) cell wall autolysate and (*) culture fluid in *S. fibuligera* grown on 1% glucose and 0.25% peptone. **B.** β(1,6)-glucanase activities in (□) cell-free extract, (+) cell wall autolysate and (*) culture fluid. **C.** β-Glucosidase activities in (□) cell-free extract, (+) cell wall autolysate and (*) culture fluid.

5 min from 0.1 M sodium succinate buffer, pH 5.0, at 4 °C. It was then washed another three times in 0.01 M Na-succinate buffer (pH 5.0) at 4 °C.

The sediment was then suspended in a known volume of 0.01 M succinate buffer containing 0.1% sodium azide, as an antimicrobial agent. Autolysis was carried out by incubating the suspension at 30 °C with gentle agitation in a rotary shaker for 24 h. The autolysate was recovered as the supernatant after centrifugation at 10000 rpm for 30 min.

Enzyme assays

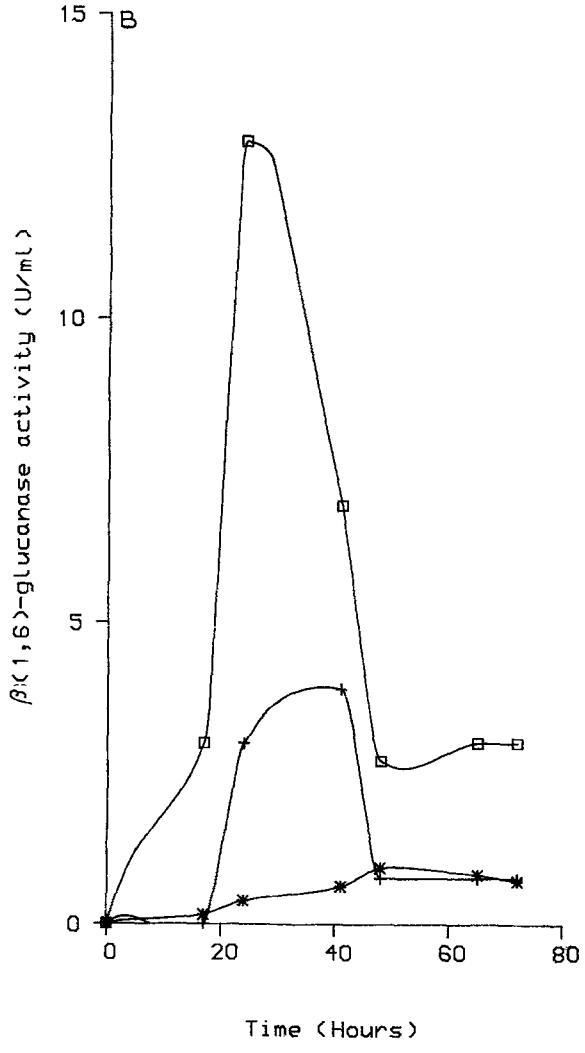
$\beta(1,3)$- and $\beta(1,6)$-glucanase activities were determined as described by Fleet and Phaff [5]. Pustulan and laminarin were used for $\beta(1,6)$-glucanase and $\beta(1,3)$-glucanase assays respectively. The amount of reducing sugar produced was subsequently determined by the DNS methods [22]. One unit of enzyme was defined as the amount of enzyme which produced 1.0 mg of reducing sugar equivalent, expressed as glucose per min under the assay conditions.

The β-glucosidase activity was determined using *p*-nitrophenyl-β-D-glucoside as the substrate [7,8].

The measure of enzyme activity is the amount of enzyme liberating 1.0 μmol *p*-nitrophenyl per min under the conditions of the assay.

RESULTS AND DISCUSSION

The activities of β-glucosidase and $\beta(1,6)$-glucanases were measured throughout the growth cycle in *S. fibuligera* grown on medium containing 1% glucose and 0.25% peptone. Each enzyme was measured in cell-free extract, in the wall autolysate preparation and in the culture filtrate. In the growth conditions used, maximum mycelial biomass was obtained at 20 h at which time the



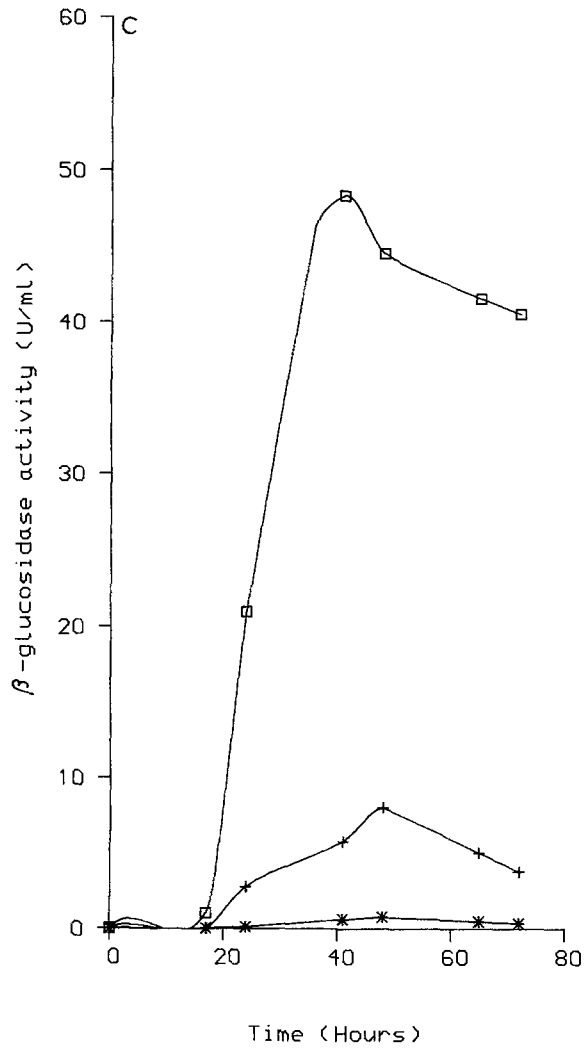


Fig. 2. (continued).

glucose was depleted and 80% of the biomass existed in the mycelial form. Between 20 and 40 h there was a transition from a predominantly mycelial form to predominantly blastospores (Fig. 1).

Throughout the growth cycle, the majority of the $\beta(1,3)$- and $\beta(1,6)$-glucanase activity detected was present in the cell-free extract. However, there was also some activity in the cell wall fraction. Both glucanase enzyme activities increased in cell-free extract and wall autolysate during the transition phase then decreased after 40 h. The levels of the two glucanase enzymes in the culture fluid was much lower, but also increased during the transition phase (Fig. 2A,B). However, the activity in the culture fluid did not decrease after the period of blastospore formation. The β-glucosidase activity exhibited a similar pattern but the maximum activity was achieved somewhat later than the peak glucanase activity and did not fall so rapidly after the transition phase (Fig. 2C).

The same three enzymes were monitored in the cell-free extract during growth on 5% glucose and 0.25% peptone. In these conditions the glucose was not fully utilized, the percentage mycelium remained high and the levels of $\beta(1,3)$- and $\beta(1,6)$-glucanase only increased after 60 h. In contrast, β-glucosidase increased with the biomass increase (Figs. 3 and 4).

In an attempt to establish whether the increase in glucanases was always associated with a yeast-like growth form, whether it was simply a response to reduced glucose levels or whether it was associated with some other phenomenon, *S. fibuligera* was grown on defined medium containing only ammonium sulphate and methionine as the nitrogen source. In these conditions, growth is slow and always occurs in the yeast form [13–15] (Fig. 5). The levels of the glucanase enzymes in the cell-free extract increased only when the glucose had been utilized. Some indication of a decrease in their levels was obtained after 60 h (Fig. 6). It seems that yeast-like growth can occur without high levels of these enzymes.

β-Glucanase enzymes are widespread in the fungi. Many are soluble but some are tightly bound to the cell-wall. The association between β-glucanases and the cell-wall is very tight so that, even after washing in strong salt

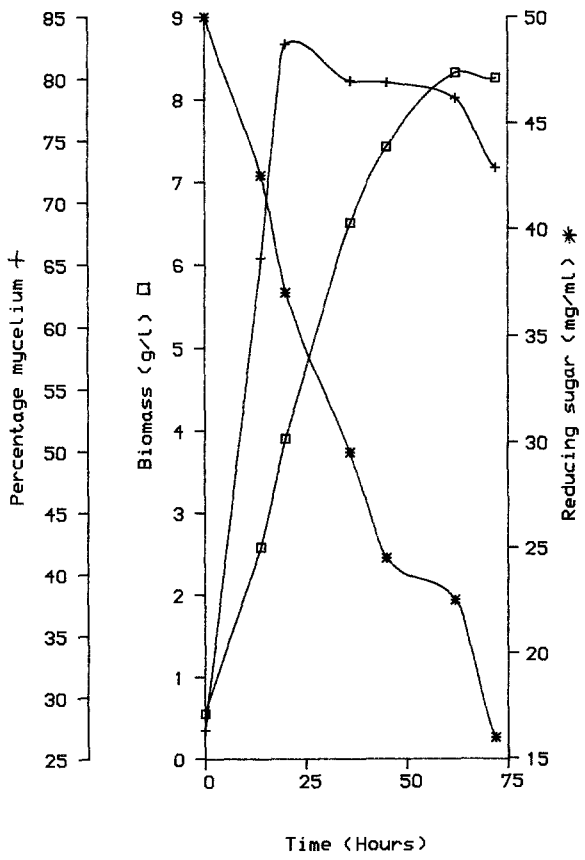


Fig. 3. Growth of *S. fibuligera* in complex medium containing 5.0% (w/v) glucose and 0.25% (w/v) peptone-(+) percentage mycelium (□) biomass and (*) reducing sugar.

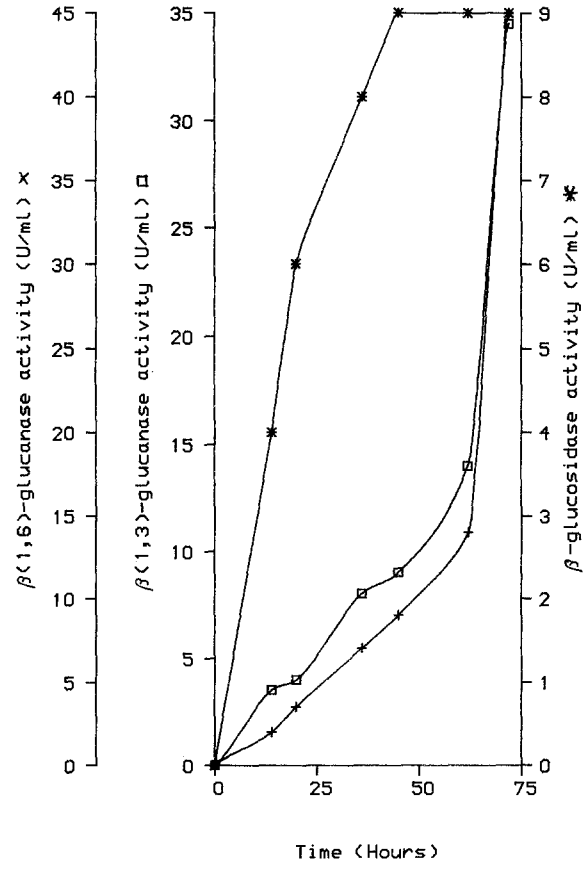


Fig. 4. Enzyme activities in cell-free extract from *S. fibuligera* grown on 5% glucose and 0.25% peptone; (+) $\beta(1,6)$-glucanase. (□) $\beta(1,3)$-glucanase and (*) β-glucosidase.

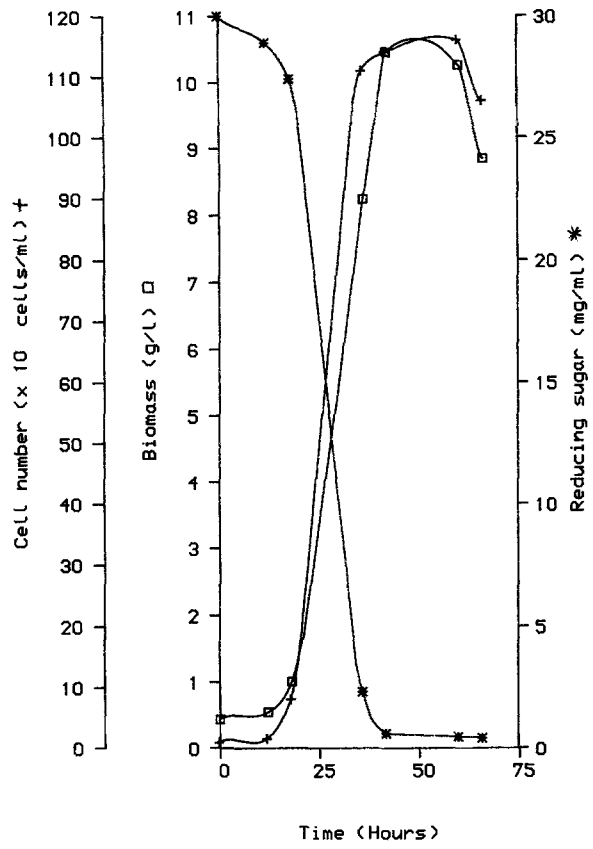


Fig. 5. Growth of *S. fibuligera* in synthetic medium containing 3.0% (w/v) glucose and supplemented with 0.34% (w/v) methionine, (+) cell number, (□) biomass and (*) reducing sugar.

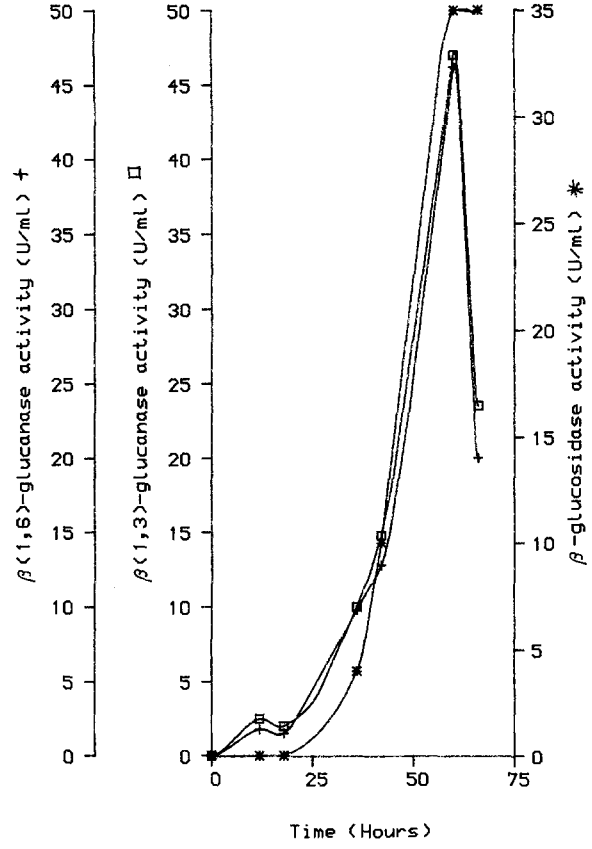


Fig. 6. Enzyme activities in cell-free extract from *S. fibuligera* grown on synthetic medium containing methionine; (+) β(1,6)-glucanase, (□) β(1,3)-glucanase and (*) β-glucosidase.

solution, sufficient enzyme activity remains to induce wall autolysis [4,11]. These wall-bound glucanases have been investigated by incubating the cell-wall fraction for 24 h [5,6,12]. The experiments reported in this paper show that well-washed cell-wall preparations contained enzyme activities, which after incubation were capable of degrading laminarin, pustulan and pNPG and indicates that β(1,3)- and β(1,6)-glucanases and β-glucosidases are present in the cell-wall of *Saccharomycopsis fibuligera* and are probably involved in its autolysis. Although these activities may be important in the formation of blastospores (since glucanases have been implicated in yeast-like growth in *Saccharomyces cerevisiae* [16] and *Candida albicans* [12,17]), they may be more important in the lysis of the mycelial filaments which is associated with blastospore formation. If this is the case, it is important to note that the blastospores are not affected. Either they have a different wall structure or the glucanases present in blastospore cell-walls are not activated under these conditions.

The pattern of enzyme activity is also compatible with their being subject to catabolite repression as are many fungal carbohydrases [21]. This does not preclude them from having a role in morphogenesis since blastospore formation itself appears to occur in conditions of catabolite derepression.

The decrease in β-glucanase activity towards the end of the fermentation may be attributable to a change in fermentation conditions [21] or to the action of specific proteases secreted during lysis. Similar observations have been reported in *Schizosaccharomyces pombe* [2].

High levels of β-glucanase and β-glucosidase have been observed in both the cell-extract and the wall autolysate fractions in our experiments, but it was not possible to evaluate the relative importance of soluble and wall-bound enzymes or even whether these enzymes have an essential role in blastospore formation. The results obtained on defined medium in which only yeast-like growth was observed indicate that this growth is not of necessity associated with high β-glucanase activities. The results reported above indicate that the peaks of glucanase activity observed during blastospore formation are

more important in the concomitant breakdown of the mycelium than in the budding process by which the blastospores are produced. Their absence in the culture suspendant suggests that they are not primarily involved in substrate utilisation.

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