AMYLOLYTIC ACTIVITY OF KLUYVER-POSITIVE *DEBARYOMYCES OCCIDENTALIS* CELLS IMMOBILIZED IN FOAMED ALGINATE GEL

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ABSTRACT

This study investigates the amylolytic activity of Kluyver-positive yeast strain *Debaryomyces occidentalis* immobilized in foamed alginate gels. Encapsulation was performed through the traditional process of droplet formation from a foamed alginate solution. The beads were coated with a layer of 3% alginate. Amylolytic enzyme activities were determined in the presence of different carbon sources – glucose, maltose, starch or dextrin – in both complex and minimal culture media. The yeast was capable of producing inducible alpha-amylase (EC 3.2.1.1) and glucoamylase (EC 3.2.1.3). The highest level of cell proliferation was observed in the complex medium with glucose. Immobilized cells showed the highest amylolytic activities in culture media with maltose. Both alpha-amylase and glucoamylase activities were higher in minimal media than in complex media.

Keywords: *Debaryomyces occidentalis*; encapsulation; foamed alginate; Kluyver effect; alpha-amylase; glucoamylase; ethanol

INTRODUCTION

Microbial amylases began to be produced on an industrial scale during the last century. They are the most important group of enzymes, in terms of biotechnological applications. In particular, α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) and glucoamylase (exo-1,4-α-D-glucan glucanohydrolase, EC 3.2.1.3) have found a wide variety of uses in a number of industrial processes, including fermentation, food production and textile and paper manufacturing (**Monteiro de Souza and de Oliveira e Magalhães, 2010**). Other amylolytic enzymes can bypass the α-1,6 branch points in the starch molecule (debranching activity) (Hii et al., 2012). Amylases are produced by a wide range of organisms, including yeasts (**Yalçın and Çorbacı, 2013**). The main industrial use of amylases is in the starch saccharication process (**Hostinová and Gašperík, 2010**).

The unconventional yeast *Debaryomyces occidentalis* (syn. *Schwanniomyces occidentalis*) was identified as early as the 1980s as a 'super yeast' because of its ability to hydrolyze whole starch to glucose completely without prior hydrolysis (**Wang *et al*., 1999**). The ability of *Debaryomyces* spp. to tolerate extreme stress could be an additionally advantage in low-cost fermentation processes (**Johnson and Echavarri-Erasun, 2011**). However, *D. occidentalis* exhibits the Kluyver effect for maltose and starch (**Weusthuis *et al*., 1994**). Fermentation of maltose and starch is blocked under anaerobic conditions. This effect may be caused by the rapid down-regulation of sugar carrier capacity, which occurs under oxygen-limited conditions, since certain yeasts require oxygen to transport sugars into their cells and to produce extracellular amylases (**Barnett and Entian, 2005**). Kluyver effect positive yeasts play an important role in the study of immobilized cells, as they provide a specific indicator for the availability of oxygen within alginate beads.

This study is the continuation of previous research on yeast immobilization, in which *Debaryomyces* spp. cells were grown inside foamed alginate beads without undergoing irreversible structural changes to their Ca-alginate networks (**Kregiel *et al*., 2013**). These results suggested a further avenue of enquiry, into amylolytic activity and ethanol formation in Kluyver-positive yeast cells encapsulated in foamed alginate. This manuscript describes the continuation of previous research, with a special focus on two enzymes: alpha-amylase and glucoamylase. The formation of ethanol, as the end product of yeast fermentation, is also discussed.

MATERIAL AND METHODS

Yeast strain and culture conditions

The study used amylolytic yeast *Debaryomyces occidentalis* Y500/5 from the LOCK105 Culture Collection (Lodz University of Technology, Poland). The strain was maintained on wort agar slants (MERCK-MILLIPORE) and cultured in 50 mL of complex medium [yeast extract (DIFCO) 2.5 g/L, peptone Bacto (DIFCO) 5 g/L] with 10 g/L of D-glucose (SIGMA), maltose (SIGMA), corn starch (SIGMA) or corn dextrin (SIGMA) in 500 mL round bottom flasks at 25 °C on a rotary shaker (HEIDOLPH) at 220 rpm. For the purposes of the experiment, after cultivation the cells were washed twice using Ringer solution (MERCK-MILLIPORE) with centrifugation (2000×g, 10 min, 4 oC). The concentration of yeast cells was determined using a haemocytometer, BX41 (OLYMPUS) microscope and a digital camera.

The immobilized yeast cells were cultivated in 50 mL of complex medium with a carbon source (D-glucose, maltose, corn starch or corn dextrin). The incubation was conducted on a laboratory shaker (HEIDOLPH) under aerobic conditions with constant gentle agitation (120 rpm) at 25 ºC for 5 days. After 5 days of incubation inside foamed cores, the immobilized cells (100 beads) were gently washed using sterile distilled water and transferred into 100 mL of new rich medium YP [yeast extract (DIFCO) 2.5 g/L, peptone (DIFCO) 5 g/L] or fermentation performance of the immobilized cells [(NH$_4$)$_2$SO$_4$ 3 g/L; KH$_2$PO$_4$ 1 g/L, MgSO$_4$•7H$_2$O 0.5 g/L, yeast extract (DIFCO) 0.5 g/L, CaCO$_3$ 3 g/L] with a carbon source (1%): glucose (G), maltose (M), natural starch from wheat (S) with 80% amylopectin and 20% amylose (MERCK-MILLIPORE) or dextrin from corn (D) (SIGMA). They were then incubated at 25 ºC for 5 days on a rotary shaker (120 rpm). Samples were collected from each culture media, centrifuged (2000×g, 10 min, 4 ºC) and the supernatants analyzed to determine both amylolytic activity and ethanol production.

Encapsulation

The traditional liquid-droplet-forming method was used, but from foamed alginate solution. Alginic acid salt from the brown algae was purchased from FLUKA (art. no. 71238). The specifications of this product are the most suitable for immobilization of microorganisms: molecular weight 100 000 ÷ 200 000, pH (1% in water) 6,0 ÷ 7,5 (**Blandino *et al.,* 2001**). The inoculum cells were centrifuged and suspended in 0.85% NaCl solution. To this solution was added (1:1) sodium alginate gel (4% w/w, FLUKA), which had been foamed by adding

0.1% Triton X-100 (MERCK-MILLIPORE). Triton X-100 was used as the foaming agent in a novel procedure. The toxicity of this compound was verified at the beginning of this research on foamed alginate (unpublished data). This compound at a concentration of 0.01% did not show any toxicity towards yeasts. Only chemicals of the highest purity were used. The solutions and materials were sterilized before use at 121 °C, except for the sodium alginate solution which was sterilized using tyndallization. The mixture, which contained around 1×10^4 cells per 1 mL of gel, was dropped through a 1.8 mm needle into 0.15 M $CaCl_2$ solution. The beads (ϕ ~3 mm) were hardened in 0.15 M $CaCl_2$ solution for 60 minutes and then washed in distilled water (5 min). To study the internal morphology beads were frozen (-20°C) and cut with a sharp razor blade. Slices of alginates were observed under a light microscope CX41 (OLYMPUS). The procedure for creating the foamed gels was described in Polish Patent No. 210458 (**Ambroziak *et al.*, 2012**). The resultant cores of foamed alginate consisted of micro-spheres approximately 50-200 microns in diameter and had a specific internal bubble structure (Fig. 1). The foamed beads were vented inside a sterile syringe under a mild vacuum at 10 °C for 24 hours in a liquid complex medium with glucose, maltose, starch or dextrin, in order to introduce nutrients into the bead spheres. To increase mechanical stability, the foamed beads were given an additional alginate coating by placing them in a solution of 3% alginate (FLUKA) for 15 minutes. They were then washed in sterile water (**Kregiel *et al.*, 2013**).
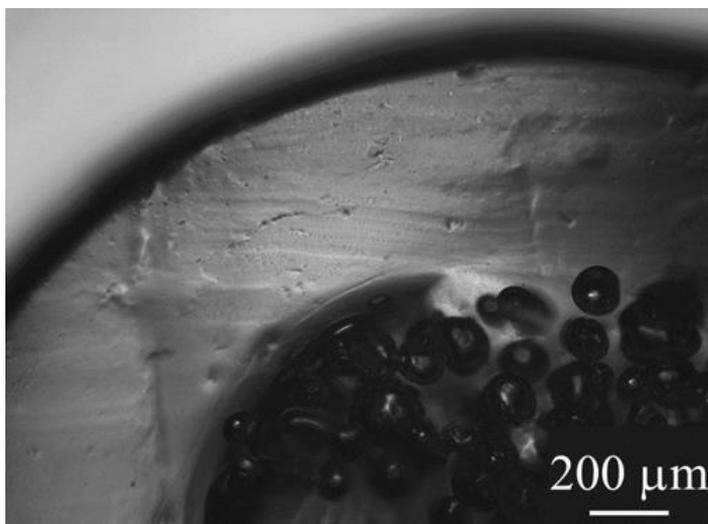

Figure 1 Internal foamed structure of alginate bead and additional alginate coating

A quantitative assessment of the immobilization was performed by dissolving the alginate beads in 0.2 M Na_2HPO_4 (in a ratio of 1 bead per 1 mL). The number of immobilized cells was determined using the microscopic method. The number of cells per single tested bead was calculated and presented as a logarithmic function.

Amylolytic and fermentation activities

Alpha-amylase activity was estimated using the 3,5-dinitrosalicylic acid method on the basis of the amount of reducing sugars released during starch hydrolysis (**Damián-Almazo *et al.*, 2008**). The reaction was carried out in 1 ml of 10 mg/mL soluble starch from corn (73% amylopectin and 27% amylose) (SIGMA) dissolved in 50mM KH_2PO_4-NaOH buffer (pH=6.0) at 45 °C for 10 minutes. One unit of enzyme activity was defined as the amount of maltose equivalents (μmol) released per minute and per mL of supernatant (**Moranelli *et al*. 1987; Ryu and Sung, 1993**).
Glucoamylase activity was determined from the reaction of 1 mL supernatant (after cultivation in 1 ml of 10 mg/mL soluble starch from corn (SIGMA) in 50 mM citrate buffer (pH=5) at 50 °C for 20 min. The glucose released was measured using 3,5-dinitrosalicylic acid reagent with glucose as a standard. One glucoamylase activity unit (U) was defined as the amount of enzyme that releases one μmol of glucose equivalent per minute per mL (**Clementi and Rossi 1986; Ryu and Sung, 1993**).
The fermentation performance of the immobilized cells was evaluated based on the ethanol content in the precipitate, determined using an AGILENT 6890 gas chromatograph equipped with headspace autosampler, capillary INNOVAX column (60 m × 0.32 mm) and flame ionization detector (FID) (**Kregiel *et al.*, 2012**).

The experiments were carried out in triplicate, and the standard deviation was calculated.

RESULTS AND DISCUSSION

Yeast cell growth inside gel beads

After a 5-day incubation period in complex medium with glucose YPD, the number of immobilized yeast cells increased from an initial 2×10^4 to 1.2×10^7 per bead, indicating significant colonization of the alginate cores (Fig. 2). Multiplication inside beads incubated in complex media with other carbon sources was slightly lower at $5\div6 \times10^6$ cells/bead.
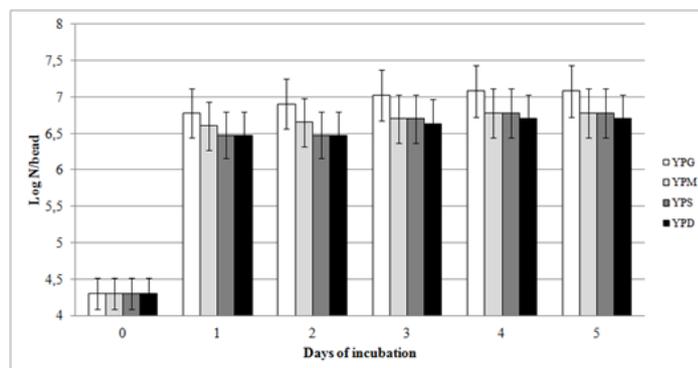

Figure 2 Growth of *Debaryomyces occidentalis* in foamed alginate beads

Oxygen may influence the production of glycolytic enzymes (**Fukuhara, 2003**). **Violle *et al.*** (1992) have shown that amylase and glucosidase are not synthetized in the presence of maltose under anaerobic conditions. However, maltose permease is synthetized in anaerobiosis without its functioning being inhibite. In our studies, foamed alginate cores with special interior spaces provided favorable conditions for the synthesis of amylolytic enzymes.
The alpha-amylase activity in rich culture media gradually increased, reaching the highest value (2.65 U/mL) in YPM medium with maltose as the carbon source (Fig. 3A). No such gradual increase in alpha-amylase activity was observed during the cultivation of immobilized yeast cells in minimal medium Mo, although the highest value of alpha-amylase activity was again in the medium with maltose as the carbon source (3.9 U/mL) (Fig. 3B).
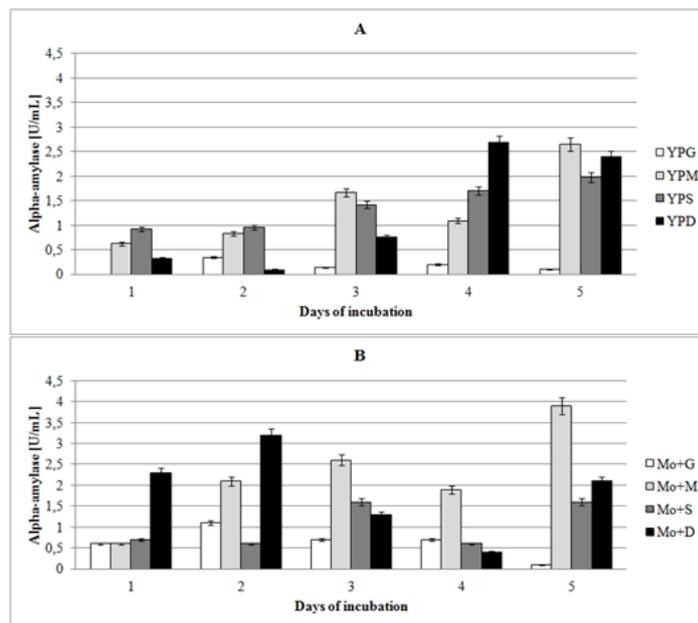

Figure 3 Alpha-amylase activity in A) complex medium, B) minimal medium

The maximal activities of glucoamylase were also noted in culture media with maltose, at 0.08 U/mL and 0.11 U/mL for rich and minimal medium, respectively (Fig. 4 AB).
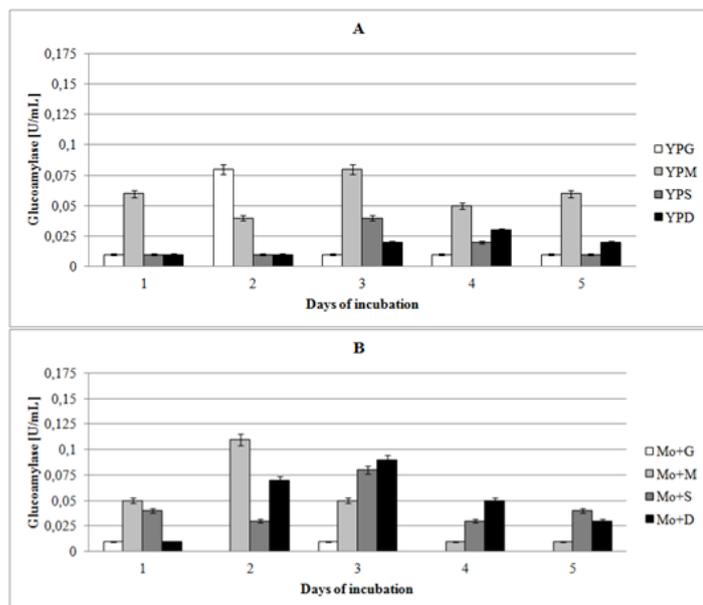
Figure 4 Glucoamylase activity in A) complex medium, B) minimal medium

These results confirm that amylases show substrate specificity, and that the production of amylolytic enzymes is subject to catabolite repression by glucose (Gupta et al., 2003).

Amylolytic activity was detected in the culture media throughout the cultivation period, except in the media with glucose as a carbon source. This may be due to the high stability of these enzymes. **Flores-Maltos *et al.* (2011)** have found that cross-linking the alginate with divalent ions (such as Ca^2+) improves the stability of enzymes. Figure 5 shows that immobilized *D. occidentalis* produced ethanol with different carbon sources.
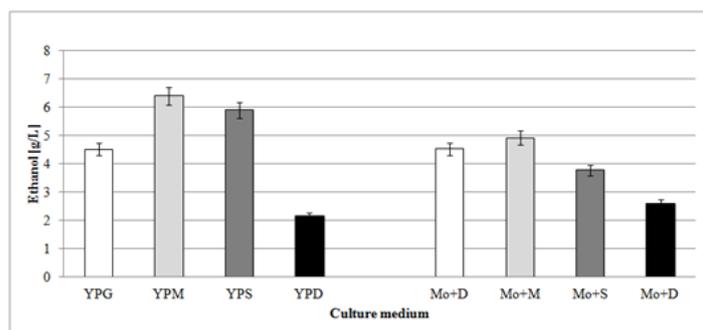

Figure 5 Ethanol formation by encapsulated yeast

The highest levels of ethanol production were obtained in YPM and Mo medium with maltose, at 6.4 and 4.9 g/L, respectively. These results indicate that in the foamed alginate cores with Kluyver-positive cells, takes place not only the synthesis of amylolytic enzymes, but also alcohol fermentation in one-step process. These and previously reported data (**Kregiel *et al.*, 2013**) shows that yeast encapsulation in foamed alginate seems to have an excellent potential for inexpensive, low energy and repeatable process of starch hydrolysis.

CONCLUSION

The method of cell encapsulation in foamed alginate presented in this paper allows not only for good growth of Kluyver-positive yeast within the bead spaces, but also for the production of amylases, using various carbon sources. This immobilization technique may be an effective way to produce stable amylolytic enzymes, which are of great scientific interest and have a broad range of industrial applications.

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