

Effects of Culture Conditions on the Production of Inulinase by *Kluyveromyces marxianus*.

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ABSTRACT

The present study was conducted to investigate the influence of initial sucrose concentration, pH and aeration rate on biomass and inulinase production by *Kluyveromyces marxianus* var. *bulgaricus* in a stirred batch reactor. Maximum inulinase activity (15.29 U mL^{-1}) was obtained at a sucrose concentration of 10 g L^{-1} , pH 5.0 and aeration rate of 1 vvm. The 20 g L^{-1} sucrose concentration was suitable for cell growth; however, enzymatic activity at this concentration was inhibited due to catabolic repression. The increase in aeration rate caused a reduction in enzyme activity with no relevant biomass increase.

Key words: *Kluyveromyces marxianus* var. *bulgaricus*, sucrose, inulinase, biomass, batch fermentation

INTRODUCTION

Inulinase is classified as a hydrolase and is designed as a 2,1- β -D-fructan fructanohydrolase (EC 3.2.1.7). Unlike an invertase, an inulinase is a non-specific β -fructofuranosidase and can hydrolyze 2,1-linked and 2,6-linked β -D-fructofuranose residue in fructan with the release of β -D-fructose. Natural inulinase substrates include inulin, sucrose and levan (Nagem *et al.*, 2004). This enzyme has advantages over the use of invertase in food industries.

Interest in inulinase was aroused by with the discovery that this enzyme has the capability of hydrolyzing the inulin in practically pure fructose. This represents a great advantage over the conventional production of fructose from starch (potato or corn), which requires three enzymatic

stages, including the action of α -amylase, amyloglucosidase and glucose isomerase (Vandame and Derycke, 1983). This process produces 45% of the fructose solution due to the thermodynamic equilibrium between fructose and glucose.

Fructose can also be obtained by acid hydrolysis of inulin. However, this is not the method of choice for fructose production, as it results in the formation of difructose anhydrides, which are colored and have no sweetening capacity (Vandame and Derycke, 1983). Fructose synthesis by microbial inulinase, on the other hand, can yield up to 95 percent pure fructose in a single enzymatic step and promises to be a viable alternative (Vandame and Derycke, 1983; Gill *et al.*, 2005; Zhang *et al.*, 2005). Despite providing an adequate tool for overcoming the drawbacks, as the process is carried out under mild pH (4 to 5)

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and temperatures (35 to 0°C), compared to the pH 2 and 75°C used in the chemical process (Kim and Rhee, 1989; Arruda and Vitolo, 1999; Sturm, 1999; Ettalibi and Baratti, 2001), this process has not yet been commercialized. Inulinases is also usually thermostable and commercially available for industrial applications (Chiang *et al.*, 1997; Gupta *et al.*, 1998).

Inulin is described as the most commonly used substrate in inulinase production. Inulin is a fructose polymer that consists of linear chains of β -2,1 linked D-fructofuranose molecules terminated at reduction and by a glucose residue attached through a sucrose-type linkage (Nakamura *et al.*, 1995; Menne *et al.*, 2000). However, due to its non-specificity, other substrates are successfully being used to produce inulinase by *K. marxianus*. Grootwassink and Flemming (1980) obtained high inulinase synthesis in a batch culture with a glucose, fructose and sucrose medium at low concentrations. In this type of process, Hewitt and Grootwassink (1984) found that maximum activity in sucrose was as high as in inulin. Rownhenrost *et al.* (1988) also obtained high inulinase production by *K. marxianus* CBS 6556 in a continuous sucrose-limited culture. The same occurred in experiments performed by Kushi *et al.* (2000) with *K. marxianus* var. *bulgaricus*. Thus, the aim of the present study work was to investigate the influence of culture condition on inulinase production by *K. marxianus* var. *bulgaricus* using sucrose as the carbon source in batch fermentation, as Brazil is an important sucrose producer and the industry that uses inverted sugar has a great interest in this research.

MATERIAL AND METHODS

Microorganism

Kluyveromyces marxianus var. *bulgaricus* ATCC 16045 was obtained from the Department of Food Engineering of Campinas University – UNICAMP. The stock culture was preserved frozen in cryogenic tubes with 20% glycerol. The culture was transferred to Petri dishes and maintained at 4°C until use

Cell growth

Biomass was determined by measuring the turbidity of the diluted sample at 600 nm using a standard absorbance curve against the dry cell mass.

Enzymatic activity

The enzymatic activity of the supernatant was determined following the procedures described by Suzuki *et al.* (1988), through the determination of reducing sugars formed by the incubation of 1 mL of enzyme in 2% sucrose, 0.05 M citrate-phosphate buffer in pH 4.0 at 50°C, using 3,5-dinitrosalicylic acid (Miller, 1959). Glucose (1 g L⁻¹) was used for the standard curve. One unit of inulinase activity is defined as the amount of enzyme that hydrolyses 1 μ mol of sucrose per min under the above conditions.

Total reducing sugars (TRS)

Total reducing sugars (TRS) were determined following the hydrolysis of sucrose with 2 M HCl and neutralization with 2 M NaOH through 3,5-dinitrosalicylic acid methods (Miller, 1959).

Batch culturing

Fermentations were carried out in 5-L fermenter containing 2 – L of culture medium. The fermentation medium was made up of g/L sucrose (5, 10 and 20), 5 yeast extract, 10 peptone, 5 KH₂PO₄, 1.5 NH₄Cl, 1.2 KCl and 0.7 MgSO₄.7H₂O. The medium was sterilized in an autoclave at 121°C for 30 min. The sucrose solution was sterilized separately and added aseptically to the culture medium. The pH was adjusted by adding orthophosphoric acid. Temperature was controlled at 30°C and the agitation rate was 300 rpm. The inoculum was prepared using the fermentation medium with 10 g L⁻¹ of sucrose and maintained under agitation overnight at 180 rpm and 30°C. Ten percent of this was transferred to the fermentation medium.

RESULTS AND DISCUSSION

Influence of initial sucrose concentration

In order to determine the effect of carbon source concentration on biomass and inulinase production, three sucrose concentrations were studied (5, 10 and 20 g L⁻¹) in a 0.5 vvm aerated reactor at 300 rpm of agitation, 30°C and a working volume of 2 L. The initial pH of the medium was adjusted to 3.5. At 5 g l⁻¹ of initial sucrose concentration, the cell mass yield was 2.76 g l⁻¹. At 10 g l⁻¹ of sugar concentration, the cell growth reached 3.11 g l⁻¹ after 24 h of culture (Fig.

1A). The biomass yield ($Y_{x/s}$), however was higher in the 5 g l⁻¹ sucrose concentration (0.491 g g⁻¹). In

the 10 g l⁻¹ sucrose concentration, biomass yield was 0.318 g g⁻¹ (Table 1).

Table 1 - Effect of sucrose concentrations on cell growth and enzymatic activity by *K. marxianus* var. *bulgaricus* at 30⁰C, 0.5 vvm and pH 3.5.

Parameters	Sucrose concentration (g l ⁻¹)		
	5	10	20
Specific growth rate (h ⁻¹) Growth phase	0.336	0.318	0.298
$Y_{x/s}$ (g g ⁻¹)	0.491	0.301	0.231
$Y_{p/s}$ (U ml ⁻¹ g ⁻¹)	0.642	0.615	0.070
$Y_{p/x}$ (U ml ⁻¹ g ⁻¹)	1.309	2.044	0.302
Q_p (U ml ⁻¹ h ⁻¹)	0.142	0.245	0.054

In cultures with a lower initial sucrose concentration (5 g L⁻¹), enzymatic activity was 3.42 U mL⁻¹ and at 10 g L⁻¹ 6.51 U mL⁻¹ after 24 h (Fig. 1B). This signified a higher inulinase yield ($Y_{p/x}$) for cultures at 10 g L⁻¹ (2.044 U mL⁻¹ g⁻¹) compared to 1.309 U mL g⁻¹ at a sucrose concentration of 5 g L⁻¹). Inulinase yield for sucrose ($Y_{p/s}$) was similar for both cultures and productivity was also higher in the 10 g L⁻¹ sucrose concentration (Table 1). When the sugar concentrations increase from 10 to 20 g L⁻¹, the cell growth increased to 4.5 g L⁻¹ after 24 h, which

represented an increase of 30 %. Nevertheless, the higher initial sucrose substrate concentration, three-fold decrease (1.92 U mL⁻¹) (Fig. 1B). Cazetta *et al.* (2005) observed the same results using yacon extract as substrate, in which *K. marxianus* show a decrease of about 14% in enzyme activity at concentrations above 40% (v/v) of the extract. Repression by substrate is common in the metabolism of many microorganisms. Wee *et al.* (2004) also observed a decrease in lactic acid yield produced by *Enterococcus faecalis* with an increase in sugar concentration in the medium.

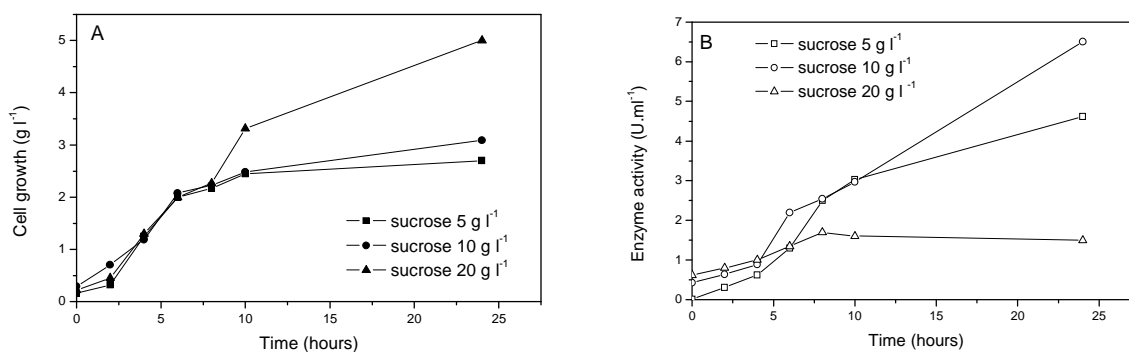


Figure 1 - Sucrose concentration effect on (A) cell growth and (B) enzymatic activity by *K. marxianus* var. *bulgaricus*: ■□ 5 g l⁻¹; ●○ 10 g l⁻¹; ▲△ 20 g l⁻¹.

The substrate was completely consumed after 6 h in the 5 and 10 g L⁻¹ sucrose concentrations. At 20 g L⁻¹ of sucrose concentration, substrate was spent after 8 h of culturing (Fig. 2). Inulinase synthesis started together with cell growth and increased after 6 to 8 h, when the amounts of total carbohydrate and reducing sugars were low, suggesting that the inulinase synthesis was

suppressed by the high concentration of reducing sugars, as observed by Wei *et al.* (1998). According to Parekh and Margaritis (1986), inulinase synthesis is controlled by catabolic repression and higher productions are reached at the end of the growth phase, apparently demonstrating that low concentrations of the carbon source are a prerequisite for inulinase

synthesis. Pinheiro *et al.* (2000) also found that, at low concentrations, *K. marxianus* ATCC 10022 completely consumed the substrate, producing biomass and ethanol. However, in higher concentrations, this yeast only consumed 30% of the initial substrate, resulting in less productivity. Similar to the inulinase of *Aspergillus niger* A 42 (Öngen-Baysal *et al.*, 1994), *A. fumigatus* (Kauer

et al., 1999) and *Streptomyces sp.* (Gill *et al.*, 2003), *K. marxianus* var. *bulgaricus* inulinase also appeared to be regulated by a double mechanism: increase by the substrate and repression by the product (glucose and fructose), as the activity decreased with sucrose concentrations above 10 g L⁻¹, probably suppressed by reducing sugars.

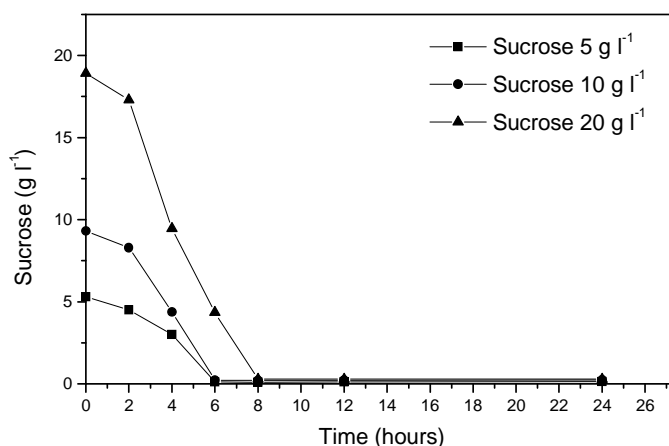


Figure 2 - Sucrose consumption by *K. marxianus* var. *bulgaricus*: ■: sucrose 5 g l⁻¹; ●: sucrose 10 g l⁻¹; ▲: sucrose 20 g l⁻¹.

Influence of pH

Figures 3A and 3B illustrate the effect of different culture pH values (3.5, 5.0 and 6.0) on cell growth and inulinase activity by *K. marxianus* var. *bulgaricus*. The experiments were carried out in a reactor at 30°C, 0.5 vvm aeration and agitation of 300 rpm. As seen in Fig. 3A, pH did not significantly influence cell growth, achieving 3.13 g L⁻¹ at pH 3.5; 3.82 g L⁻¹ at pH 5.0 and 3.22 at pH 6.0. However, the specific growth rate (μ) increased concomitantly with pH and gave the highest yield at pH 6.0 (0.814h⁻¹). For enzyme

activity, however, the best pH was 5.0, reaching 13.14 U ml⁻¹ after 10 h of fermentation, followed by pH 6.0, with 11.75 U ml⁻¹. pH 3.5 was an unfavorable condition, as enzyme activity was 4.27 U mL⁻¹ after 12 h (Fig. 3B). Inulinase yield for sucrose ($Y_{p/s}$) and inulinase yield for biomass ($Y_{p/x}$), were similar for pH 5.0 and pH 6.0 (Table 2). Productivity (Q_p) was higher at pH 5.0 (1.31 U ml⁻¹ h⁻¹) (Table 2). Pessoa and Vitolo (1999) obtained better results in a batch culture with inulin as the substrate (26 U ml⁻¹) in the pH range from 3.5 to 5.0 for *K. marxianus* DMS 70106.

Table 2 - Effect of culture pH on cell growth and enzymatic activity by *Kluyveromyces marxianus* var. *bulgaricus* at 30°C, 300 rpm and 0.5 vvm.

Parameters	pH		
	3.5	5.0	6.0
Specific growth rate (h ⁻¹) Growth phase	0.318	0.473	0.814
$Y_{x/s}$ (g g ⁻¹)	0.330	0.452	0.413
$Y_{p/s}$ (U ml ⁻¹ g ⁻¹)	0.711	1.573	1.547
$Y_{p/x}$ (U ml ⁻¹ g ⁻¹)	2.155	3.781	3.743
Q_p (U ml ⁻¹ h ⁻¹)	0.271	1.315	1.176

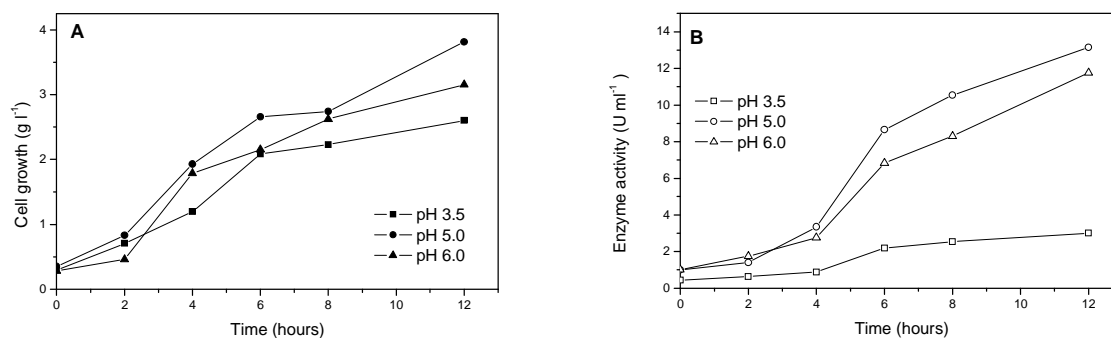


Figure 3 - (A) Cell growth and (B) enzymatic activity of *K. marxianus* var. *bulgaricus* at 30°C and 0.5 vvm and different pH levels. ■ □: pH 3.5 ● ○: pH 5.0 ▲ △: pH 6.0.

Effect of aeration rate

Figures 4A and 4B illustrate the cell growth and enzymatic activity by *K. marxianus* at different aeration rates (vvm): 0.2, 0.5, 1.0, 2.0 and 3.0. Fermentations were performed at pH 5.0, 30°C and 300 rpm. Cell growth was generally positively affected by the aeration increase. The lowest growth was observed at 0.2 vvm (1.83 g l⁻¹). Cell growth was similar at both 0.5 vvm and 1.0 vvm, reaching about 4.0 g l⁻¹. Higher cell growth was obtained at 2 and 3 vvm (5.65 and 5.43 g L⁻¹,

respectively). The increase in aeration rate was accompanied by increase of specific growth rate (μ) from 0.158 to 0.270 h⁻¹. The same occurred for the maximum values of biomass yield ($Y_{x/s}$) (Table 3). Working with two strains of *K. marxianus* (*K. marxianus* ATCC 10022 and CBS 7894), Pinheiro *et al.* (2000) observed that a small 2-bar increase in air pressure led to a three-fold increase in biomass yield, whereas further increase in air pressure did not lead to a significant increase in biomass yield.

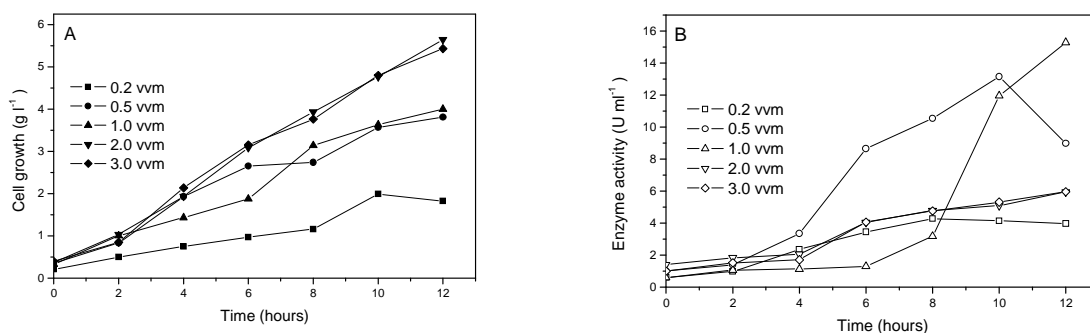


Figure 4 - Cell growth (A) and enzymatic activity (B) of *Kluyveromyces marxianus* var. *bulgaricus* at different aeration rates. ■ □ 0.2 vvm; ● ○ 0.5 vvm; ▲ △ 1.0 vvm; ▼ ▽ 2.0 vvm; ◆ ◇ 3.0 vvm.

However, enzyme activity decreased with the increase in aeration. The highest enzymatic activity was achieved at 1.0 vvm (15.29 U ml⁻¹) after 12 h, followed by 0.5 vvm (13.15 U ml⁻¹). At 2 and 3 vvm, enzymatic activity decreased significantly, achieving about 6.0 U ml⁻¹ after 12 h

(Fig. 4B). The highest values for inulinase yield for sucrose ($Y_{p/s}$), inulinase yield for biomass ($Y_{p/x}$) and productivity (Q_p) were obtained at 0.5 and 1.0 vvm. The 0.2 vvm aeration was an insufficient rate, showing about 4.0 U mL⁻¹ of enzyme activity (Table 3).

Table 3 - Effect of aeration rate on cell growth and enzymatic activity by *Kluyveromyces marxianus* var. *bulgaricus* at 30°C, 300 rpm and 0.5 vvm.

Parameters	Aeration (vvm)				
	0.2	0.5	1.0	2.0	3.0
Specific growth rate (h ⁻¹) Growth phase	0.158	0.236	0.227	0.265	0.270
Y _{x/s} (g g ⁻¹)	0.167	0.452	0.370	0.676	0.663
Y _{p/s} (U ml ⁻¹ g ⁻¹)	0.350	1.573	1.479	0.582	0.653
Y _{p/x} (U ml ⁻¹ g ⁻¹)	2.089	3.781	4.003	0.861	0.984
Q _p (U ml ⁻¹ h ⁻¹)	0.331	1.3146	1.274	0.496	0.496

Silva-Santisteban and Maugeri Filho (2005) described a similar behavior in experiments using *K. marxianus* var. *bulgaricus*. The authors did not obtain an increase in inulinase yield with an increase in aeration rate from 1 to 2 vvm (89 and 84 IU mL⁻¹, respectively) after 72 h. In fed-batch fermentation with sucrose as the substrate, Cazetta (2005) also observed higher inulinase production at 1 vvm. Cristiani-Urbina *et al.* (2005) observed cellular metabolism alteration from oxidative to a mix oxidative-fermentative in aerated cultures of some *K. fragilis* strains, resulting in the production of metabolites such as alcohols, aldehydes and esters, which reduced biomass production.

In conclusion, the best condition for inulinase production in batch-reactor fermentation was 10 g l⁻¹ of initial sucrose concentration, pH 5.0 and 1 vvm aeration rate.

NOMENCLATURE

Y_{x/s} - sucrose biomass yield (g g⁻¹)
 Y_{p/s} - sucrose inulinase yield (U mL⁻¹ g⁻¹)
 Y_{p/x} - inulinase biomass yield (U mL⁻¹ g⁻¹)
 Q_p - volumetric enzyme production (U mL⁻¹ h⁻¹)
 μ - specific growth rate (h⁻¹)
 rpm - revolutions per minute (min⁻¹)

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RESUMO

O estudo foi conduzido para investigar a influência da concentração inicial da sacarose, a taxa da aeração e do pH na biomassa e na produção da inulinase pela *Kluyveromyces marxianus* var. *bulgaricus* em um reator em batelada. A máxima

atividade de inulinase, 15.29 UmL⁻¹, foi obtida na concentração de 10 g L⁻¹ de sacarose, no pH 5.0 e na taxa da aeração de 1 vvm. A concentração de sacarose de 20g L⁻¹ foi apropriada para o crescimento celular, porém nesta concentração a atividade enzimática foi inibida, devido a repressão catabólica. O aumento na taxa da aeração propiciou redução da atividade enzimática, ao mesmo tempo em que não houve aumento considerável do biomassa.

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