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Full Length Research Paper

Influence of nitrogen source and sucrose concentration on inulinase production by *Kluyveromyces marxianus* in fed-batch fermentation

Cazetta, M. L.[1,2]* and Contiero, J.[1]

[1]Departamento de Bioquímica e Microbiologia, Instituto de Biociências, Universidade Estadual Paulista (UNESP)-São Paulo-Brasil, Av. 24A, 1515, CEP13506-900, Rio Claro, São Paulo, Brasil.
[2]Centro de Ciências Exatas e Tecnológicas, Universidade Federal do Recôncavo da Bahia (UFRB), Rua Rui Barbosa, 710, Campus de Cruz das Almas, CEP:44380-000, Cruz das Almas, Bahia-Brasil.

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This work studied the influence of nitrogen source and sucrose concentration in the feeding medium for biomass and inulinase production by *Kluyveromyces marxianus* var. *bulgaricus.* The results show that the best nitrogen source was a combination of 5 g/L of yeast extract and 10 g/L of peptone. Both cellular growth and enzymatic activity increased with sucrose concentration in the feeding medium (from 200 to 500 g/L). When the sucrose concentration reached 600 g/L, both cellular growth and enzymatic activity decreased.

Key words: *Kluyveromyces marxianus*, nitrogen, sucrose, inulinase, fed-batch fermentation.

INTRODUCTION

Fed-batch culture is characterized by a process in which one or more nutrients are continuously added to the fermentor (Kleman et al., 1996). The fed-batch process is often used to achieve high cell density and improve productivity, while minimizing undesired metabolic effects such as substrate inhibition, inhibitory byproduct formation and dissolved oxygen limitations in aerobic cultures. The fed-batch process is effective for the fermentation in which substrate inhibition, catabolic repression and product inhibition are important (Modak et al., 1986; Liu et al., 2000). For *Kluyveromyces marxianus,* yeast fed-batch fermentation has been described as a very efficient process in the control of the substrate concentration, avoiding catabolic repression. It is thus possible to obtain high cell density and enzyme activity in fermentations in which the carbon source is limiting (Grootwassink and Fleming, 1980). They observed that high inulinase synthesis in batch fermentation on glucose, fructose and sucrose at low concentrations. Using this kind of process, Hewitt and Grootwassink (1984) stated that inulinase activity in sucrose was as high as in inulin. Rouwenhorst et al. (1988) also achieved a high production of inulinase by *K. marxianus* CBS 6556 in a continuous culture with the limited addition of sucrose. Kushi et al. (2000) observed similar results in the experiments with *K. marxianus* var. *bulgaricus*. Therefore, the aim of this study was to study the influence of the initial substrate concentration and nitrogen sources on the cell growth and the production of inulinase by *K. marxianus* var. *bulgaricus* through fed-batch fermentation using sucrose as the carbon source.

MATERIALS AND METHODS

Microorganism

The yeast *K. marxianus* var. *bulgaricus* ATCC 16045 was obtained from the Food Engineering Department, Campinas University, Brazil.

Inoculum

The inoculum was composed by (g/L): sucrose, 10; $MgSO_4$, 0.7; KH_2PO_4, 5; KCl, 1.2; NH_4Cl, 1.5; yeast extract, 5 and peptone, 10. The medium was sterilized at 121°C for 30 min. The sucrose was sterilized separately. The inoculum was carried out "overnight" at

*Corresponding author. E-mail: malulz@yahoo.com.br.

30°C and agitation of 180 rpm. Later, 10% of culture volume was transferred into the reactor.

Fed-batch fermentation

The fermentation occurred in two stages: first, the fermentation was carried out on bath fermentation with working volume of 2 L until all sucrose was completely consumed. After, the feeding medium was injected into the reactor through peristaltic pumps. The feeding rate (F) was defined by Equation (1):

$$F = \mu . X_0.V_0.\exp(\mu.t) /S_0.Y_{x/s} \quad (1)$$

In which μ = specific growth rate; X_0 = initial biomass; V_0 = initial reactor volume; t = time; S_0 = initial substrate and $Y_{x/s}$ = biomass yield. Aeration rate was gradually increased from 0.2 to 1 vvm and the agitation rate was increased from 200 to 600 rpm.

Feeding medium

Nitrogen source

In order to define the best nitrogen source in the feeding medium, the compositions were tested as follows: medium A (g/l): Yeast extract 5, and peptone 10; medium B (g/L): yeast extract 5, and peptone 10; supplied with mineral salts (mg/L) ($CoCl_2.6H_2O$, 15; $FeSO_4.7H_2O$, 150; $ZnSO_2.7H_2O$, 225; $CuSO_4.5H_2O$. 15; H_3BO_3, 50; $Na_2MoO_4.2H_2O$, 20); medium C (g/L): yeast extract 10, and peptone 20 and medium D (g/L): ammonium sulfate 5, and peptone 5. The initial sucrose concentration was of 200 g/L for all the experiments.

Sucrose concentration on the feed medium

The feeding medium was composed of (g/L): sucrose, 200 to 600; peptone, 10; yeast extract, 5; KH_2PO_4, 10; KCl 1.2; $MgSO_4$, 5; NH_4Cl, 5. The medium was sterilized at 121°C for 30 min. The sucrose was sterilized separately and later added to the medium. In order to control the foam, a silicone-based chemical anti-foaming agent, FG 10 Dow corning, was added in the feeding degree.

Cellular growth

Biomass was determined through turbidimetry with a spectrophotometer at λ = 600 nm. Biomass (g/L) was calculated by the correlation between dry cell weight and optic density (OD) to a standard curve. All experiments were conducted in triplicate.

Total reducing sugars (TRS)

Following hydrolysis with 2 M HCl and neutralization with 2 M NaOH, total TRS were determined through the 3, 5-dinitrosalisylic acid method, according to Miller (1959).

Enzymatic activity

Enzymatic activity of the supernatant was determined according to Suzuki et al. (1988) through the determination of reducing sugars formed through incubation of 1 ml of enzyme in 2% sucrose, 0.05 M citrate-phosphate buffer with pH 4.0 at 50°C, using the reagent 3,5-dinitrosalisylic acid, according to Miller (1959). One unit of inulinase activity is defined as the amount of enzyme that hydrolyses 1 µmol of sucrose per min under the aforementioned conditions.

RESULTS AND DISCUSSION

The runs were carried out at 30°C and pH 5.0. First, we carried out bath fermentation with sucrose at 10 g/L until substrate exhaustion, when the feeding was initiated. The feeding inlet control was performed by the determination of TRS through withdrawal of periodic samples from the reactor. The increase of pH was also utilized as an indication of substrate exhaustion, because in the nutrient starvation, the pH increased, showing that it necessarily increased the pump rate. This fact occurs probably because of the release of ammonium ions by the cells (Suzuki et al., 1990). Lee and Chang (1993) also utilized the pH values to monitor the growth of *Escherichia coli*, obtaining a final dry biomass of 88 g/L. The sugar concentration on the feeding medium was carefully controlled to avoid the substrate excess into the reactor, because that could cause a metabolic repression over the yeast cells. The substrate control into the reactor is a technique spread between different authors. Lee et al. (2000), controlling the glucose concentration in the medium, avoided the ethanol synthesis, which is inhibitory to xylitol production by the recombinant *Saccharomyces cerevisiae*. Minning et al. (2001) controlled the feeding with ethanol and methanol to avoid the substrate repression by recombinant *Pichia pastoris*. Different nitrogen sources and nitrogen concentrations on the feeding medium were utilized to study the influence both over growth and inulinase production. The sucrose concentration was 200 g/L (Table 1 and Figure 1). The A medium composed by 5 g/L of yeast extract and 10g/L of peptone, offered the best condition to the enzyme production, achieving 98.04 U/ml (Figure 2), followed by B medium, which reached 72.20 U/ml, after 72 h. The best condition to cellular growth was observed in the D medium (5 g/L of ammonium sulfate and 5 g/L of peptone) with 16.32 g/L. Therefore, it was unfavorable to enzymatic activity, which reached 46.55 U/ml. The C medium, which had 5 g/L of yeast extract, and 10 g/L of peptone and was supplied with salts showed unsatisfactory results both in the cellular growth and enzymatic activity, 11.8 g/L and 39.08 U/ml, respectively. According to Kalil et al. (1999), the inulinase production is affected by the changes in the peptone and yeast extract concentrations. These authors observed that the major inulinase production is related to the high peptone and yeast extract concentrations and low sucrose concentration. In their experiments, the optimum inulinase production (91 U/ml) occurred at 20 g/L of peptone, 10 g/L of yeast extract and sucrose at 10 g/L.

Singh et al. (2006) and Singh and Bhermi (2008) observed that beef extract was the best nitrogen source to inulinase production. *K. marxianus* YS-1 showed good productions of inulinase when grown in *Asparagus officinalis* root extract supplied with both peptone and

Table 1. Effect of different sources and concentrations of nitrogen on enzyme activity and cellular growth by *K. marxianus* var. *bulgaricus*. pH 5,0, 30°C, 72 h.

Medium (nitrogen source)	Cellular growth (g/L)	Enzymatic activity (U/mL)	$Y_{p/x}$ (U/mL.g)	Qp (U/mL.h^{-1})
A	14.06	98.05	6.39	1.36
B	13.30	72.20	5.63	1.00
C	11.84	39.08	3.38	0.54
D	16.32	46.55	2.92	0.65

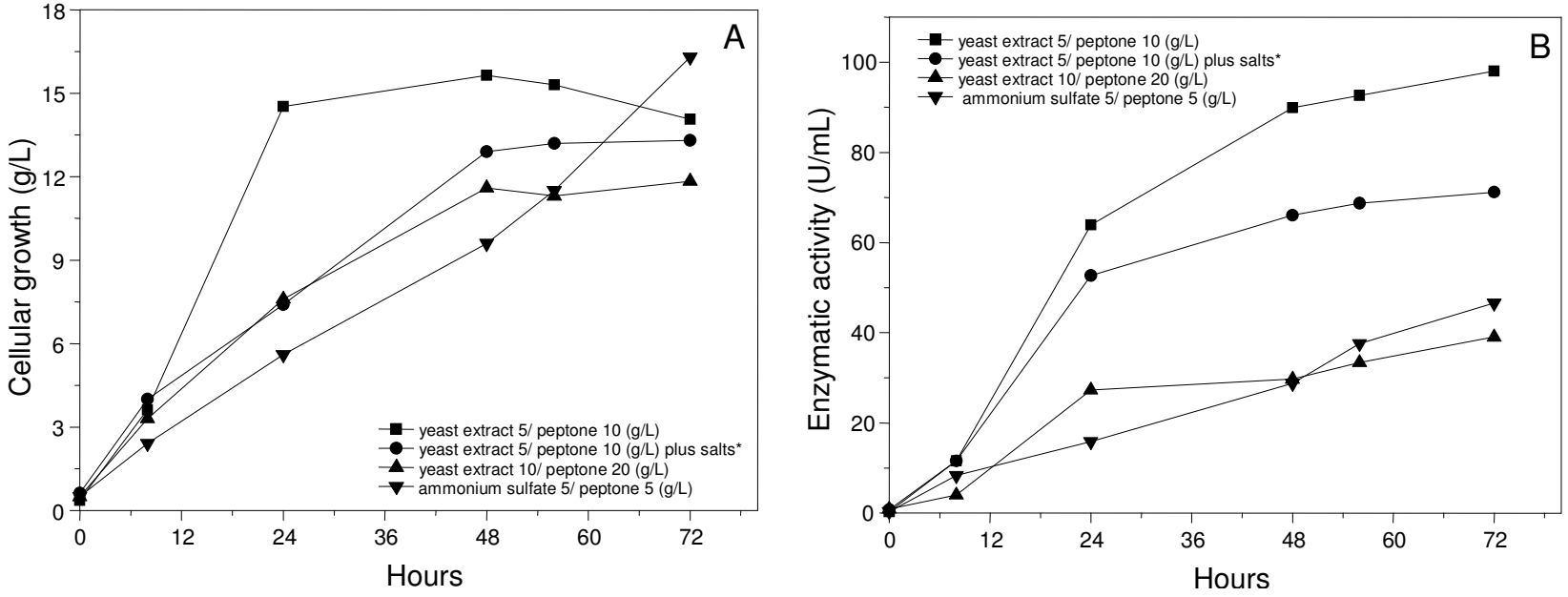

Figure 1. (A) Cellular growth (g/L) and (B) enzymatic activity (U/mL) by *K. marxianus* var. *bulgaricus* at different nitrogen sources.*Salts (mg/L): $CoCl_2.6H_2O$, 15; $FeSO_4.7H_2O$, 150; $ZnSO_2.7H_2O$, 225; $CuSO_4.5H_2O$. 15; H_3BO_3, 50; $Na_2MoO_4.2H_2O$, 20.

beef extract. Singh et al. (2006) observed that beef extract increased the inulinase production.

Influence of sucrose concentration on the feeding medium

Different sucrose concentrations were utilized to study the influence on inulinase production: 200, 300, 400, 500 and 600 g/L. The nitrogen source was peptone 10 g/L and yeast extract 5 g/L. Both cellular growth and enzymatic activity increased at high sucrose concentrations and the optimum was reached at 500 g/L, after 72 h. Sucrose concentration at 600 g/L was unfavorable for both cellular growth and inulinase synthesis, probably due to catabolite repression (Figure 3).

Conclusions

Both kinds and concentration of nitrogen source

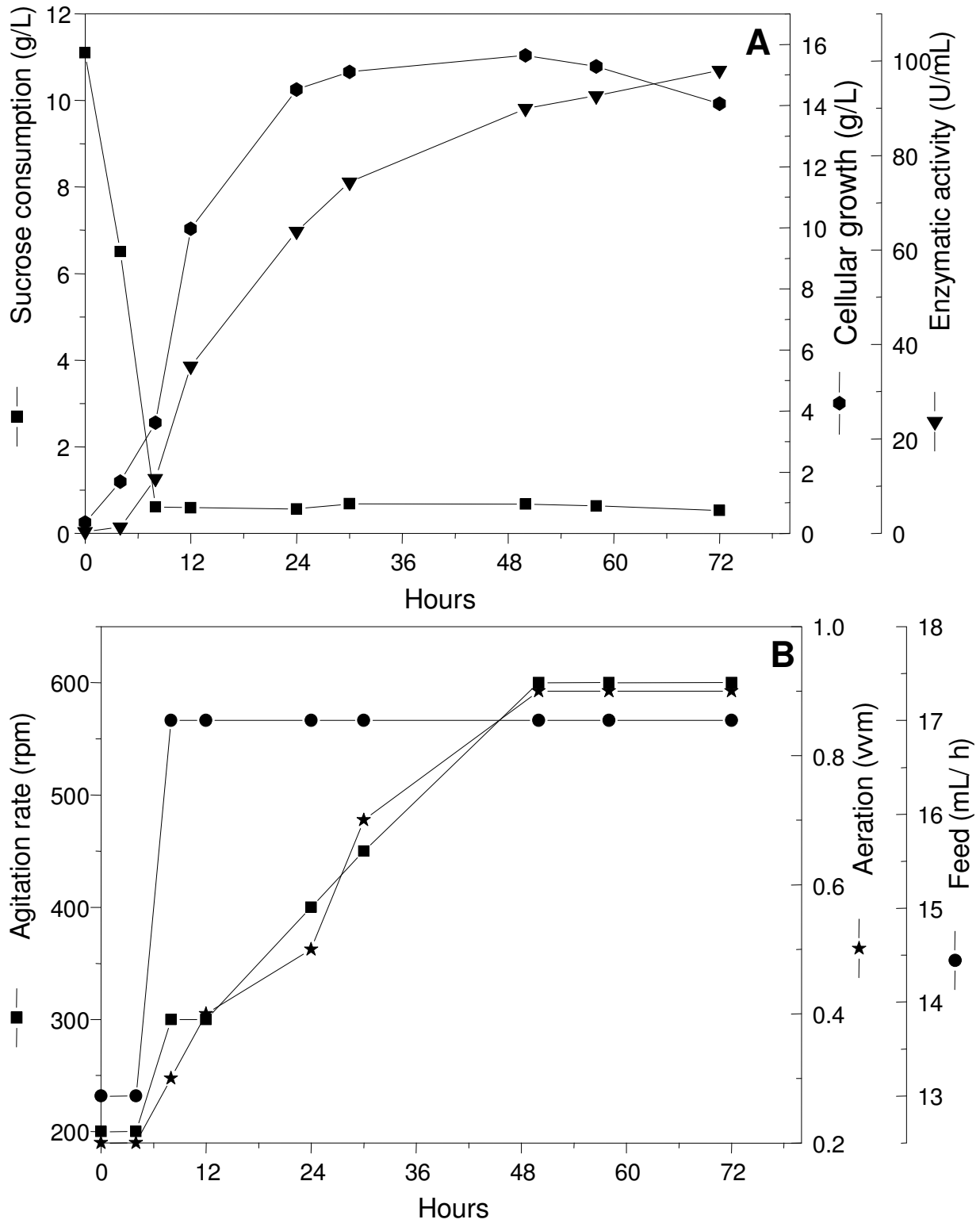

Figure 2: A) Enzymatic activity (U/mL), cellular growth (g/L) and sucrose consumption (g/L) and B) agitation (rpm), aeration rate (vvm) and feed (mL/h) in the best condition (yeast extract 5g/L and peptone 10g/L).

influenced both cellular growth and inulinase production by *K. marxianus* var. *bulgaricus.* The best nitrogen source was 5 g/L of yeast extract and 10 g/L of peptone. The medium with sucrose concentrations at 500 g/L was

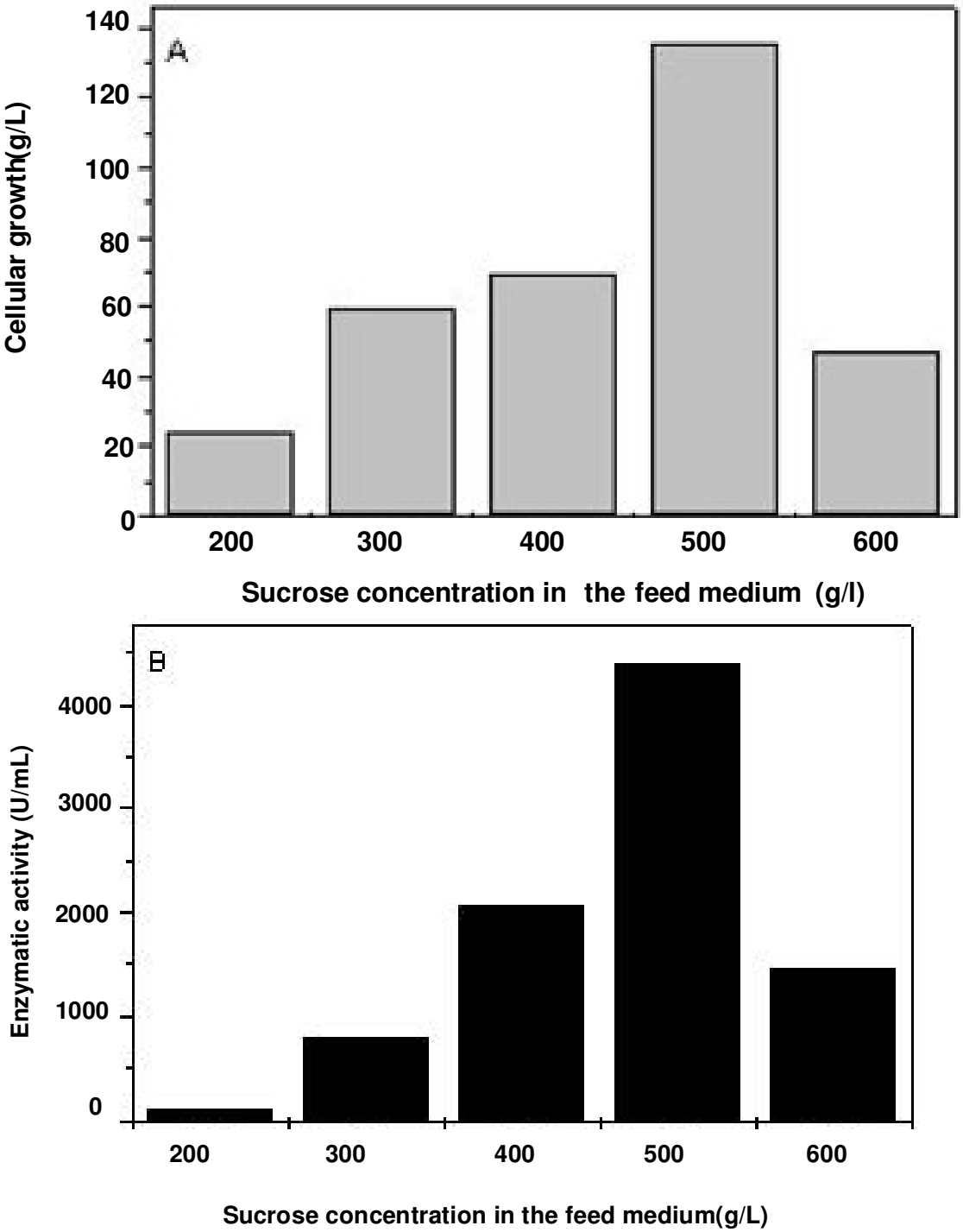

Figure 3. (A) Cellular growth (g/L) and (B) enzymatic activity (U/mL) by *K. marxianus* var. *bulgaricus* under different sucrose concentrations in the feeding medium (g/L), at 30°C, pH 5.0 after 72 h.

the best for both cellular growth and enzymatic activity.

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