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Effects of Carbon and Nitrogen Sources and Oxygenation on the Production of Inulinase by *Kluyveromyces marxianus*

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Abstract Cultivations of *Kluyveromyces marxianus* var. *bulgaricus* ATCC 16045 were performed on both minimal and complex media using different carbon and nitrogen sources either in the presence or absence of aeration. The results collected were worked out and compared so as to provide a useful contribution to the optimization of inulinase production. Kinetics of extracellular inulinase release were similar on glucose, fructose, and sucrose. Inulinase was detected at basal level since the beginning of batch runs on these three carbon sources and overproduced after their depletion. The highest inulinase activity in minimal medium containing 10 g/l sucrose (6.4 IU/ml) was obtained at an initial $(NH_4)_2SO_4$ concentration of 5 g/l, whereas it was reduced to about one fourth of this value and detected only at the beginning under nitrogen-limited conditions. The best sucrose concentrations for the enzyme production were 30 and 20 g/l in minimal and complex media, yielding 15.4 and 208 IU/ml, respectively. In general, the enzyme activity was much higher in complex than in minimal medium under all conditions. O_2-enriched air neither improved inulinase production nor prevented ethanol formation.

Keywords Carbon source · Inulinase production · *Kluyveromyces marxianus* · Medium optimization · Nitrogen source · Oxygenation

Introduction

Inulin is a linear biopolymer made up of fructose residues linked by β-2,1 bonds [1] that constitutes the storage carbohydrate in the roots and tubers of different plants [2, 3]. Its hydrolysis by microbial inulinases appears to be an interesting alternative for the production

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of high fructose, calorie-reduced sweeteners, which are presently produced mainly by enzymatic isomerization of pre-hydrolyzed corn starch [1, 4, 5]. Although inulin is the best carbon source for inulinase production [6], sucrose was successfully used as an alternative substrate [7].

Inulinase production has largely been explored in various bacteria, among others *Staphylococcus* sp. [8], *Xanthomonas* sp. [9], and *Pseudomonas* sp. [10], yeasts such as *Kluyveromyces* sp. [8, 11–14], and fungi such as *Aspergillus* sp. [6, 15]; however, the literature information, mainly dealing with optimization of culture media and some operating parameters, such as pH, temperature, agitation, and aeration, is insufficient to get a clear picture of its regulation mechanism.

As a consequence, there are contrasting opinions about the constitutive, repressible, or inducible nature of this enzyme. For example, it was demonstrated that inulinase synthesis is subject to a mechanism of induction/repression in *Kluyveromyces fragilis*, *Kluyveromyces bulgaricus* ATCC 16046, and *Aspergillus niger* [6, 16, 17], of induction without catabolic repression in *Kluyveromyces marxianus* UCD (FST) 55–82 [7], and vice versa in *K. marxianus* CBS 6556 [18], whereas other strains of this microorganism did not exhibit any induction mechanism [11, 19, 20]. Finally, Gupta et al. [21] observed that glucose was responsible for catabolic repression, whereas sucrose and fructose acted as weaker inducers than inulin in *K. fragilis*.

The influence of carbon and nitrogen source concentrations was already investigated to optimize cultivation media for inulinase production by *K. marxianus* [3, 12, 14, 22]; however, the use of experimental factorial design for optimization did not allow evaluating each single effect independently of the others [22, 23]. Other studies reported unclear response of inulinase synthesis to carbon source concentration [11, 18, 21], whereas there is sufficient agreement about the better role of organic (peptone, yeast extract, casamino acids) rather than inorganic (ammonium sulfate and phosphate, sodium nitrate, urea) nitrogen sources [20, 21]. Notwithstanding the contrasting opinions, no systematic study has been performed, to the best of our knowledge, to clarify these effects on the enzyme synthesis. Besides, the mechanism of inulinase production can be quite different even in different strains of the same species, thus requiring separate characterization.

It has been demonstrated that the fluid dynamic aspects responsible for mechanical stress are of great significance for the production of extracellular inulinase [24]; therefore, additional efforts should be made to investigate the influence of other process parameters on this fermentation. Moreover, inulinase was produced in very little amounts in batch cultivation, thereby suggesting catabolic repression by glucose.

Batch cultivations of *K. marxianus* ATCC 16045 have been carried out in this work varying the type and concentration of both carbon and nitrogen sources as well as the oxygenation level, either in minimal or complex medium, to get more information on the release of extracellular inulinase by this strain and to enhance its production yield.

Materials and Methods

Microorganism

The microorganism utilized in this study as inulinase producer, *K. marxianus* var. *bulgaricus* ATCC 16045, was maintained in yeast malt agar at 4 °C. Cryogenic pre-inocula (20% glycerol) were prepared in the medium described by Kalil et al. [12] at 30 °C so as to obtain suspensions with optical density of 0.5.

Batch Fermentations

Batch fermentations were carried out either on minimal [25] or complex medium [12] at the optimum temperature of 30 °C [26], 450 rpm and aeration of 1.0 l of air per liter of medium per minute (vvm) in a 3.0-l fermenter, type BioFlo III (New Brunswick Scientific, Edison, NJ, USA), with 2.0-l working volume, after inoculation with biomass from the below suspensions and addition of little amounts of antifoam, type Aratrop (Alcamo Química, Indústria e Comércio Ltda, Sertãozinho, SP, Brazil). A 2.0 N NaOH solution was added to control the pH at its optimum value (3.5) [26]. Cells maintained at −80 °C in 20% glycerol were firstly transferred into 500-ml baffled Erlenmeyer flasks containing 100 ml of minimal medium, then incubated at 30 °C for 24 h, recovered by centrifugation at 6,000×*g* (10 min, 10 °C), and finally used for inoculum. Samples were withdrawn throughout the fermentations to determine the levels of the main metabolites, inulinase, biomass, and total proteins. Some tests were performed at high oxygenation levels using O_2-enriched air. For this purpose, a system of valves was used so as to ensure a percentage of O_2 with respect to its solubility in water in the range 90–100%. Anaerobic fermentations were also performed substituting air with N_2.

All the fermentations were performed in triplicate and the results expressed as average values.

Analytical Methods

Cell mass concentration was determined using a UV–vis spectrophotometer, model DU 640 (Beckman Coulter, Fullerton, CA, USA), using standard curves relating the optical density at 600 nm to biomass dry weight either in minimal or complex medium. Carbon, hydrogen, and nitrogen contents of dry biomass were determined by elemental analysis with a CHN analyzer, Series II 2400 (Perkin Elmer, Norwalk, CT, USA), while oxygen content was calculated by difference.

The concentrations of glucose, fructose, acetate, ethanol, acetaldehyde, glycerol, and pyruvate were determined by high-performance liquid chromatography (HPLC) as previously described [24]. When the carbon source was sucrose, its concentration was determined by the colorimetric 3,5-dinitrosalicylic acid method [27] after hydrolyzing samples with 2.0 N HCl at 100 °C for 5 min [28]. In this case, the above concentrations of glucose and sucrose determined by HPLC were corrected by taking into account the actual sucrose concentration detected in the same sample.

The dissolved oxygen (DO) level was on-line measured using a polarographic probe (Mettler Toledo, Greifensee, Switzerland).

Inulinase activity was assayed according to Santos [29]. The enzyme solution (0.5 ml) was mixed with 4.5 ml of 2% sucrose solution in 0.1 M acetate buffer, pH 4.8. The mixture was maintained at 50 °C for 8 or 40 min depending on the enzyme concentration. To ensure zero-order kinetics during the inulinase activity determination, four samples were taken during the selected period of time, and the initial rate of reducing sugar release was determined by the above 3,5-dinitrosalicilic acid method [27]. One unit of inulinase activity was defined as the amount of enzyme able to release 1 μmol of reducing sugar per minute under the above conditions. The total protein was determined according to the method of Lowry et al. [30] to know the fraction of inulinase with respect to the overall released proteins.

The activities of extracellular, cell wall, and cell-bound inulinase were determined by the extraction method described by Rouwenhorst et al. [18]. The activity of extracellular

inulinase was assayed in the broth supernatant obtained by centrifugation. The cell-wall-associated inulinase was released from biomass using an enzyme-releasing buffer with the following composition: 50 mM potassium phosphate, 10 mM 2-mercaptoethanol, 10 mM dithiothreitol, 2 mM $MgSO_4$, pH 7. Finally, the remaining biomass was resuspended in a buffer consisting of 50 mM potassium phosphate, 10 mM $MgSO_4$, pH 7. Cell-bound inulinase was obtained by mechanical disruption of biomass in a cold-jacketed mill (0.5 °C) containing glass microspheres with 600-μm diameter by successive milling and cooling every 10 s for 5 min.

The yield of inulinase on biomass was calculated as the ratio of total inulinase activity to biomass concentration.

Results and Discussion

Batch cultivations of *K. marxianus* ATCC 16045 were performed either in minimal or complex medium using different sources of carbon (sucrose, glucose, and fructose) at 10–40 g/l and nitrogen (ammonium sulfate, peptone, and yeast extract) at 0.5–10 g/l, and different aeration conditions (natural air, O_2-enriched air, or N_2 at 1.0 vvm). Tables 1 and 2 list the main results of these fermentations obtained in minimal and complex media, respectively, in terms of maximum total saccharolytic activities during either the initial 8 h of fermentation (growth-associated basal level, A_b) or during the whole fermentation (A_m), as well as of maximum concentrations of biomass (X_m), ethanol (E_m), and total proteins (TP). In all the fermentations, X_m was reached after about 8 h, whereas the maximum enzyme activity was reached after about 72 h.

Table 1 Experimental schedule and main results of batch *K. marxianus* ATCC 16045 cultivations performed under different conditions in minimal medium.

C source	S_o (g/l)[a]	$(NH_4)_2SO_4$ conc. (g/l)	Air conditions (1.0 vvm)	X_m[b] (g/l)	A_m[c] (IU/ml)	TP[d] (g/l)	A_b[e] (IU/ml)	E_m[f] (g/l)
Sucrose	10	5.0	Air	3.7	6.40	0.89	0.31	0.49
Sucrose	20	5.0	Air	6.2	5.03	0.41	0.91	2.9
Sucrose	30	5.0	Air	7.5	15.4	0.45	0.38	2.8
Sucrose	40	5.0	Air	9.1	7.20	1.22	2.35	7.3
Sucrose	10	0.5	Air	3.4	1.70	0.10	1.70	0.20
Sucrose	10	2.0	Air	3.9	2.84	0.27	1.07	0.97
Sucrose	10	10	Air	3.8	7.09	0.74	0.72	0.79
Glucose	10	5.0	Air	3.5	7.41	0.30	0.50	0.68
Fructose	10	5.0	Air	3.4	6.01	0.21	0.62	0.95
Sucrose	10	5.0	N_2	1.0	1.30	–	0.08	7.8
Sucrose	10	5.0	Air+O_2	3.5	5.33	–	0.58	0.50
Sucrose	40	5.0	Air + O_2	10.7	6.54	–	1.60	2.5

[a] Initial carbon source concentration

[b] Maximum biomass concentration

[c] Maximum saccharolytic activity

[d] Total protein concentration

[e] Maximum basal level of saccharolytic activity

[f] Maximum ethanol concentration

Table 2 Experimental schedule and main results of batch *K. marxianus* ATCC 16045 cultivations performed under different conditions in complex medium.

S_o (g/l)[a]	Air conditions (1.0 vvm)	X_m[b] (g/l)	A_m[c] (IU/ml)	TP[d] (g/l)	A_b[e] (IU/ml)	E_m[f] (g/l)
10	Air	5.5	97.2	17.5	1.56	0.73
20	Air	8.0	208	18.0	9.10	3.2
30	Air	8.9	60.6	18.0	5.05	6.3
40	Air	9.7	29.5	15.8	4.68	8.9
40	Air + O_2	10.5	31.9	–	0.91	5.2

The nitrogen source was a mixture of peptone (20 g/l) and yeast extract (10 g/l). The carbon source was sucrose.

[a] Initial carbon source concentration

[b] Maximum biomass concentration

[c] Maximum saccharolytic activity

[d] Total protein concentration

[e] Maximum basal level of saccharolytic activity

[f] Maximum ethanol concentration

Cultivations on Minimal Medium

Effect of the Carbon Source

The results of batch fermentations on 10 g/l sucrose, glucose, or fructose are illustrated in Fig. 1 in terms of X_m, A_m, TP, and A_b.

Comparable values of maximum total inulinase activity (6.0–7.4 IU/ml), biomass concentration (3.4–3.7 g/l), and yield of inulinase on biomass (1.7–2.1 IU/g biomass) were

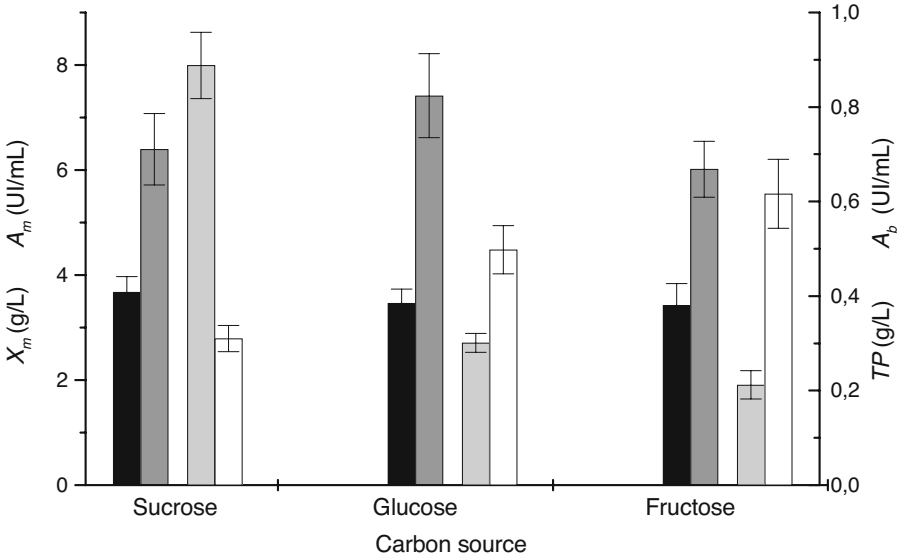

Fig. 1 Effect of the type of carbon source on the cultivation of *K. marxianus* ATCC 16045 performed on minimal medium at 450 rpm and aeration of 1.0 vvm. X_m (*black bar*); A_m (*gray bar*); TP (*medium gray bar*); A_b (*white bar*)

detected with the three sugars under investigation, which suggests that inulinase could be, as previously proposed, constitutively present in the selected strain [11, 19]. Nevertheless, some authors observed appreciably higher enzyme production on sucrose than on glucose or fructose [7, 18, 20], whereas others observed higher production on fructose than on glucose [11]. Moreover, some studies reported the existence of only one gene responsible for inulinase expression in *K. marxianus*, with glucose acting as a repressor [31–33] or even as a competitive inhibitor of inulin [34]. Finally, it was demonstrated that other substrates (inulin, lactose, glycerol, and ethanol) could act as inulinase inducers with different effectiveness [7, 11, 18, 21], which suggests the strain-dependent nature of this enzyme.

The results of Fig. 1 also show that inulinase was produced at basal level (A_b) at the start of fermentation not only from glucose but also from fructose. Moreover, inulinase overproduction (A_m), which was observed after total depletion of the carbon source, was similar with the three carbon sources. This behavior could be explained with a derepression mechanisms. The results of Fig. 2 point out that inulinase was actually oversynthesized along the fermentation (A_m), rather than released a posteriori as a simple cell-wall-bound enzyme, a behavior that seems to be a sort of defense response to the lack of carbon source.

The production of a variety of metabolites, such ethanol, acetate, glycerol, pyruvate, and acetaldehyde, was detected during the first 10 h of a typical cultivation of *K. marxianus* ATCC 16045 (Fig. 3). These compounds reached appreciable levels already in the initial phase (up to 8 h) and then were totally consumed throughout the fermentation.

Effect of Ammonium Sulfate Concentration

As nitrogen availability is notoriously one of the limiting factors of inulinase synthesis, four tests were carried out varying the ammonium sulfate level in the range 0.5–10 g/l, but

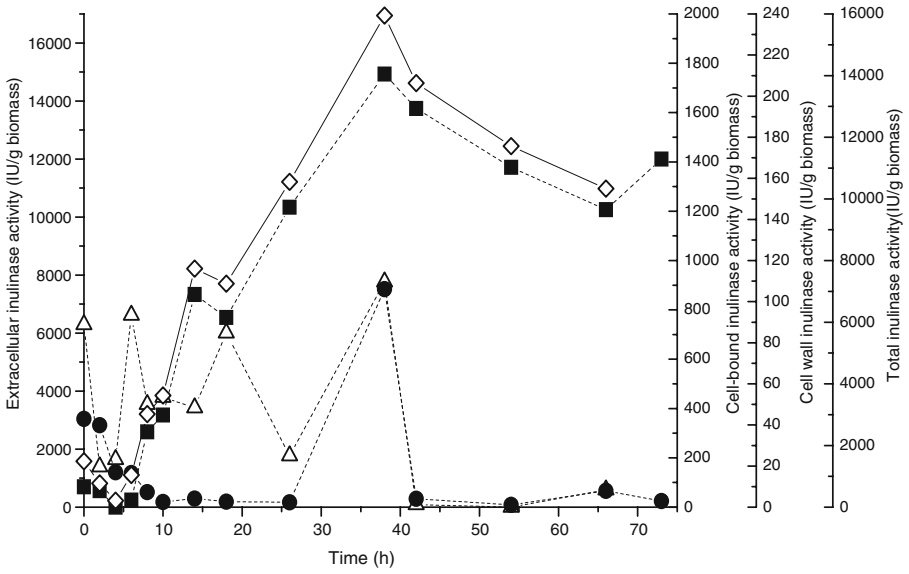

Fig. 2 Localization and distribution of inulinase related to structure of the cell throughout a typical fermentation on sucrose-based complex medium. Extracellular inulinase activity (*filled square*); cell-bound inulinase activity (*open triangle*); cell wall inulinase activity (*filled circle*); total inulinase activity (*open diamond*)

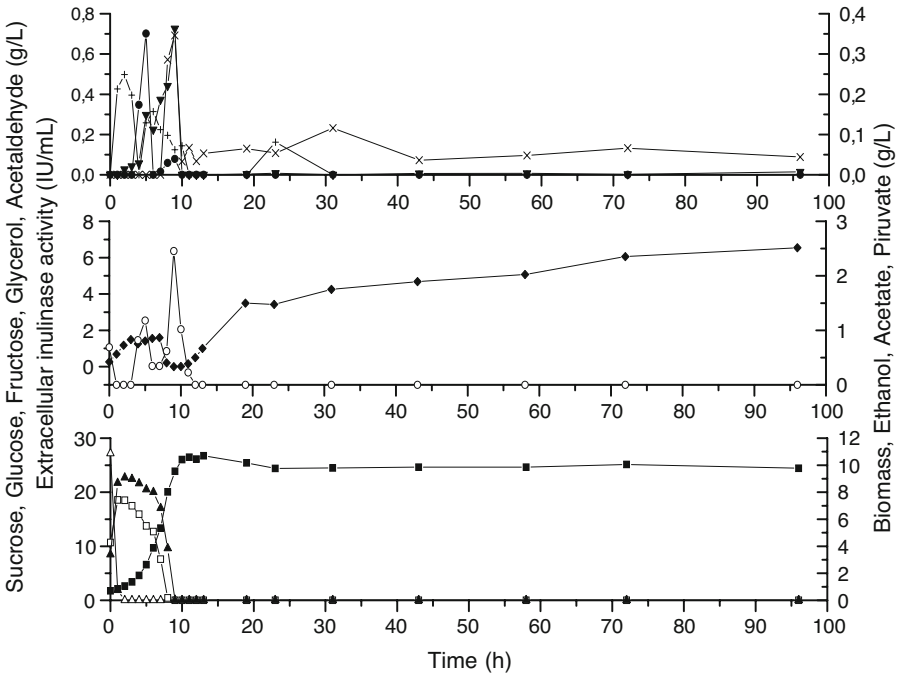

Fig. 3 Inulinase activity and metabolites production during *K. marxianus* ATCC 16045 cultivation on minimal medium containing 40 g/l sucrose and 5 g/l ammonium sulfate performed supplying 1.0 vvm oxygen-enriched air. Extracellular inulinase activity (IU/ml) (*filled diamond*); concentrations of sucrose (*open triangle*), glucose (*open square*), fructose (*filled triangle*), glycerol (*filled inverted triangle*), acetate (*cross*), ethanol (*open circle*), pyruvate (*ex*), acetaldehyde (*open inverted triangle*), and biomass (*filled square*)

keeping constant that of sucrose at 10 g/l. Figure 4 shows that the overall enzyme activity progressively increased to 6.4 IU/ml with increasing ammonium sulfate concentration up to 5 g/l, beyond which it kept almost constant (7.0 IU/ml at 10 g/l). These results suggest a nitrogen optimal level of about 1 g/l for inulinase synthesis, corresponding to an N/C molar ratio of about 0.2. On the contrary, the observed decrease in A_b related to the increment of the nitrogen source level is quite difficult to explain. Additionally, with ammonium sulfate concentrations higher than 2.0 g/l, the productions of inulinase and biomass occurred simultaneously during the whole fermentation, while at 0.5 g/l, the extracellular enzyme activity was observed only at the beginning of the run.

Taking into account the dry biomass composition of *K. marxianus* ATCC 16045 experimentally determined under non-limiting nitrogen conditions ($CH_{1.94}O_{0.76}N_{0.17}$), we calculated a minimum ammonium sulfate concentration for growth of 1.53 g/l. However, Fig. 4 did not show any significant difference between the growth with 0.5 and 2 g/l ammonium sulfate, which can be explained with a less nitrogen content of biomass grown under nitrogen-limited conditions [35]. Besides, there was a direct dependence of the released total proteins and inulinase on the nitrogen level up to an ammonium sulfate concentration of 5.0 g/l, even though the released inulinase was only a small fraction of total proteins, being its intrinsic specific activity only 879 IU/mg inulinase [36].

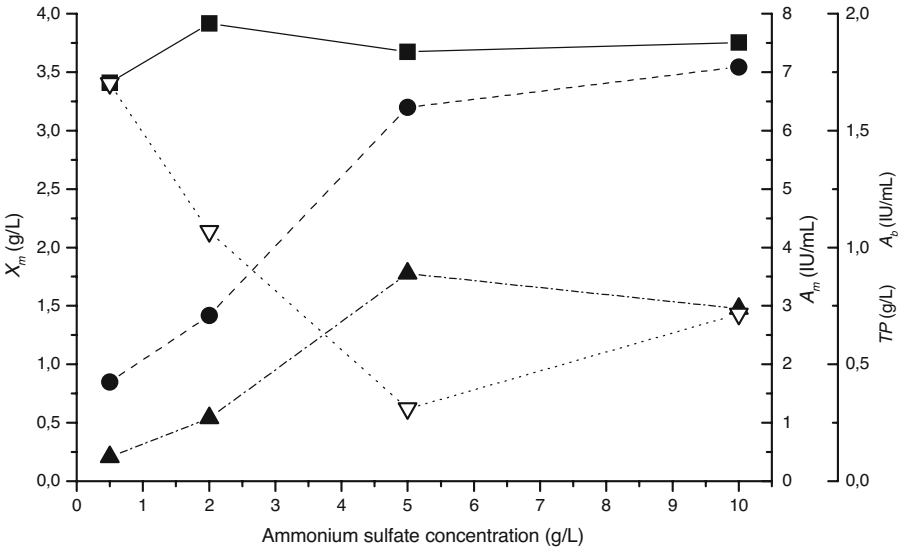

Fig. 4 Effect of ammonium sulfate concentration on the inulinase production in minimal medium. X_m (*filled square*); A_m (*filled circle*); TP (*filled triangle*); A_b (*open inverted triangle*)

Effect of Sucrose Concentration

Inulinase production was then investigated by a set of four cultivations performed on minimal medium at variable initial sucrose level ($10 \leq S_o \leq 40$ g/l), while ammonium sulfate concentration was kept constant at 5.0 g/l. The highest enzyme activity (15.4 IU/ml) was obtained using 30 g/l sucrose (Fig. 5). At S_o=40 g/l, biomass concentration and biomass

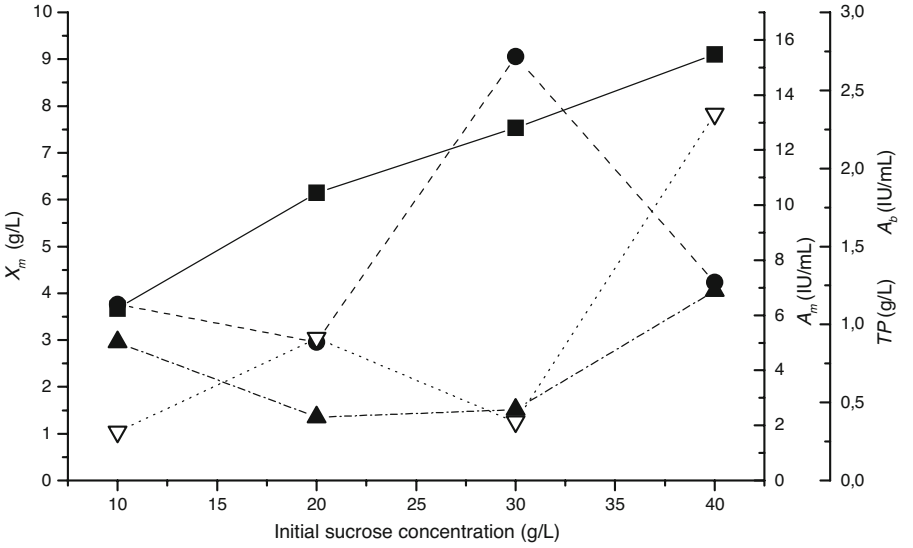

Fig. 5 Effect of sucrose concentration on the inulinase production in minimal medium. X_m (*filled square*); A_m (*filled circle*); TP (*filled triangle*); A_b (*open inverted triangle*)

yield on substrate were 9.1 g/l and 0.23 g biomass per gram sucrose, respectively. These values are about 2.5 times higher and about 40% lower than those obtained at S_o=10 g/l, respectively, likely owing to carbon source limitation at the lowest level. A carbon source overflow through the glycolytic pathway or the occurrence of oxygen-limited conditions could have addressed an appreciable fraction of pyruvate to the production of ethanol (7.3 g/l) and other metabolites.

On the other hand, the release of total proteins in the medium did not show any noticeable dependence on sucrose concentration level. Unexpectedly, A_b increased by 7.5 times with increasing S_o from 10 to 40 g/l, which denotes a complex regulation of inulinase synthesis.

These results are comparable with those obtained by Gupta et al. [21] who detected a maximum inulinase activity of 7 IU/ml when *K. fragilis* cultivation was carried out at a fructan concentration of 15 g/l. On the other hand, the specific activity of *K. marxianus* CDBB-L-278 inulinase decreased from 4.33 to 0.78 IU/mg and from 8.06 to 0.59 IU/mg when glucose and fructose concentrations were increased from 2.5 to 10 g/l, respectively [11]. However, it should be stressed that those results were obtained with different strains, cultivation media, and carbon sources.

Cultivations on Complex Medium

Similar tests were then performed on sucrose-based complex medium containing organic nitrogen (yeast extract and peptone) [12] to study the simultaneous effects of different nitrogen sources and sucrose concentration on inulinase production (Table 2).

The maximum total saccharolytic activity was more than one order of magnitude higher (208 IU/ml; Fig. 6) than the one obtained in minimal medium (15.4 IU/ml), probably because of a fivefold nitrogen source concentration (about 5 g/l) or of the presence of additional carbon source in the form of amino acids. A strong effect of S_o on both enzyme

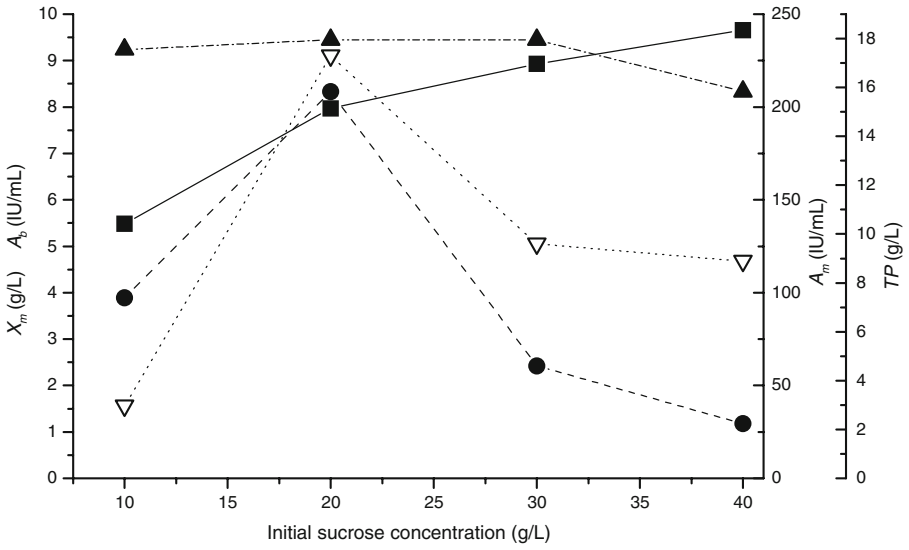

Fig. 6 Influence of sucrose concentration on the cultivation of *K. marxianus* ATCC 16045 on complex medium. X_m (*filled square*); A_m (*filled circle*); TP (*filled triangle*); A_b (*open inverted triangle*)

synthesis and cell metabolism is also evident: inulinase activity fell down to 29.5 IU/ml at 40 g/l, whereas ethanol concentration increased up to 8.9 g/l (Table 2). On the other hand, biomass concentration behaved similarly to the cultivations on minimal medium in that it raised from 5.4 to 9.7 g/l when S_o was increased from 10 to 40 g/l.

The basal inulinase production at the beginning of the fermentation was remarkably higher (1.6–9.1 IU/ml) than in minimal medium. Although this could have been the result of using a richer nitrogen source, it provides further confirmation of the complexity of the mechanism of inulinase synthesis regulation in this yeast.

Effect of Oxygen Availability

Final tests were carried out under different oxygen levels to select conditions able to minimize ethanol formation and then to maximize inulinase production. Previous attempt to improve aeration by mechanical means resulted in mechanical stress to the cells due to high shear stress [24]; therefore, tests were performed supplying O_2-enriched air. As shown in Fig. 7, during conventional tests in minimal medium without air enrichment, the dissolved oxygen level fell rapidly and kept at very low levels for a time depending on the carbon source concentration in the medium.

Figure 8 shows that air enrichment with O_2 had no significant effect on inulinase production either in minimal or complex medium, the little variations observed at the different initial concentrations of sucrose being within the experimental error of the

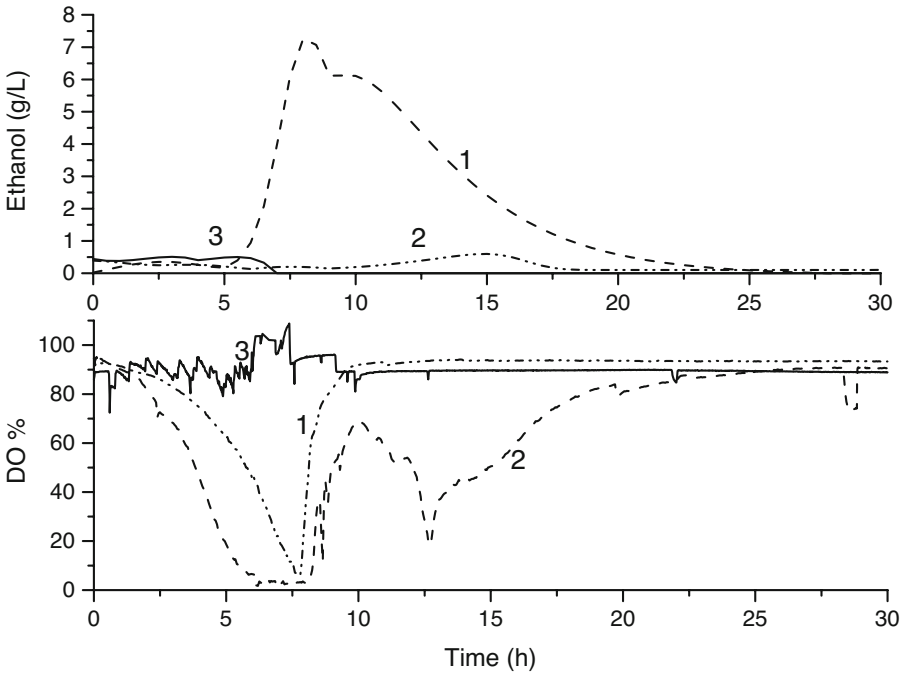

Fig. 7 Time behaviors of dissolved oxygen (DO%) and ethanol concentration during *K. marxianus* ATCC 16045 cultivations on minimal medium performed under different conditions of aeration and carbon source concentration. *1* Sucrose 10 g/l with air supply (*dot dashed line*); *2* sucrose 40 g/l with air supply (*dashed dashed line*); *3* sucrose 10 g/l with oxygen enriched air supply (*solid line*)

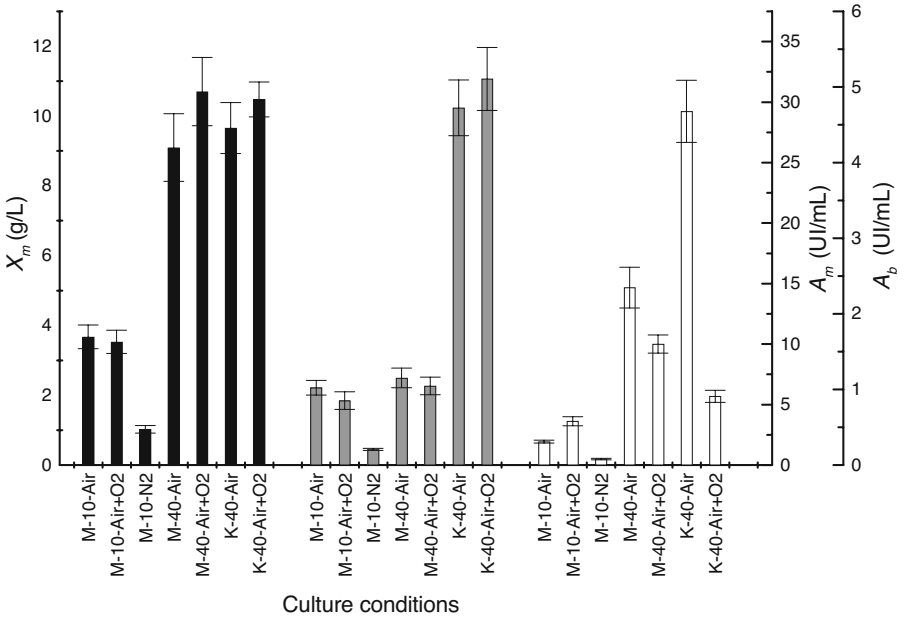

Fig. 8 Effect of air enrichment with oxygen (air + O_2) and anaerobiosis with nitrogen (N_2) on inulinase production at different sucrose concentrations (10 and 40 g/l) either in minimal (*M*) or complex medium (*K*). X_m (*black bar*); A_m (*gray bar*); A_b (*white bar*)

experimental procedure. As a consequence of the increased O_2 level in the medium, ethanol production decreased with respect to fermentations without air enrichment, although it was not completely removed (Tables 1 and 2). These observations are in agreement with the hypothesis of an unbalance between glycolysis and respiration [37, 38] responsible for ethanol formation by yeasts under aerobiosis. Besides, biomass production was 1 to 2 g/l higher than without air enrichment, probably because of the reduction of ethanol formation, while no appreciable effect was detected on the basal inulinase production.

Inulinase production was finally checked in minimal medium under anaerobic conditions ensured by substituting air with nitrogen. Biomass production was about one third of that obtained in aerobiosis, while both A_b and A_m were reduced by about 80% (Fig. 8). Contrary to aerobic conditions, the abundant production of ethanol (7.8 g/l; Table 1) can actually be ascribed in this case to O_2 limitation.

On the basis of these results, we can conclude that 1.0 vvm air supply without any O_2 enrichment can simultaneously sustain the high ATP requirements of this yeast either for aerobic growth or inulinase biosynthesis.

Conclusions

It was shown that *K. marxianus* ATCC 16045 is able to grow and produce extracellular inulinase in minimal medium. The inulinase production on glucose, fructose, and sucrose exhibited similar kinetics and occurred in two phases: a former phase associated to the microorganism growth, during which the enzyme was produced at basal level, and a latter

stationary phase, after total consumption of the carbon source, during which inulinase was likely overproduced by derepression.

The effectiveness of inulinase biosynthesis appeared to depend on the efficient use and the type of carbon and nitrogen sources as well as on their concentrations and oxygen availability in minimal medium. Inulinase activity reached a maximum with an ammonium sulfate level of 5.0 g/l, whereas under conditions of nitrogen limitation, the enzyme was only produced at very low levels at the beginning of the cultivation. The optimum sucrose concentrations for the production of inulinase in minimal and complex media were 30 and 20 g/l, respectively, at which maximum activities of 15.4 and 208 IU/ml were detected. Similar results obtained with natural and oxygen-enriched air suggested that ethanol formed during most of the cultivations was the likely consequence of an unbalance between glycolysis and respiratory chain. The dramatic increase in the inulinase activity on complex medium was the likely result of the presence of additional organic nitrogen and carbon sources in peptone and yeast extract.

The results collected in this work on the effects of medium composition and oxygen level on *K. marxianus* fermentation can provide a significant preliminary contribution to the optimization of nutritional and environmental factors, aiming at overproducing inulinase for industrial purposes.

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References

1. Vandamme, E. J., & Derycke, D. G. (1983). *Advances in Applied Microbiology*, *29*, 139–176.
2. Cazetta, M. L., Martins, P. M. M., Monti, R., & Contiero, J. (2005). *Journal of Food Engineering*, *66*, 301–305.
3. Singh, R. S., Dhaliwal, R., & Puri, M. (2006). *Process Biochemistry*, *41*, 1703–1707.
4. Bajpai, P., & Margaritis, A. (1986). *Process Biochemistry*, *21*, 16–84.
5. Manzoni, M., & Cavazzoni, V. (1992). *Journal of Chemical Technology and Biotechnology*, *54*, 311–315.
6. Poorna, V., & Kulkarni, P. R. (1995). *Bioresource Technology*, *54*, 315–320.
7. Parekh, S., & Margaritis, A. (1985). *Applied Microbiology and Biotechnology*, *22*, 446–448.
8. Selvakumar, P., & Pandey, A. (1999). *Bioresource Technology*, *69*, 123–127.
9. Park, J. P., Bae, J. T., You, D. J., Kim, B. W., & Yun, J. W. (1999). *Biotechnology Letters*, *21*, 1043–1046.
10. Jong, W. Y., Yong, J. C., Chii, H. S., & Seung, K. S. (1999). *Journal of Bioscience and Bioengineering*, *87*, 291–295.
11. Cruz-Guerrero, A., Garcia-Peña, I., Barzana, E., Garcia-Garibay, M., & Gomez-Ruiz, L. (1995). *Journal of Fermentation and Bioengineering*, *80*, 159–163.
12. Kalil, S. J., Suzan, R., Maugeri, F., & Rodrigues, M. I. (2001). *Applied Biochemistry and Biotechnology*, *94*, 257–264.
13. Santos, A. M. P., Oliveira, M. G., & Maugeri, F. (2007). *Bioresource Technology*, *98*, 3142–3148.
14. Singh, R. S., Sooch, B. S., & Puri, M. (2007). *Bioresource Technology*, *98*, 2518–2525.
15. Azhari, R., Szlak, A. M., Ilan, E., Sideman, S., & Lotan, N. (1989). *Biotechnology and Applied Biochemistry*, *11*, 105–117.
16. Grootwassink, J. W. D., & Fleming, S. E. (1980). *Enzyme and Microbial Technology*, *2*, 45–53.
17. Grootwassink, J. W. D., & Hewitt, G. M. (1983). *Journal of General Microbiology*, *129*, 31–41.
18. Rouwenhorst, R. J., Visser, L. E., van Der Baan, A. A., Scheffers, W. A., & van Dijken, J. P. (1988). *Applied and Environmental Microbiology*, *54*, 1131–1137.
19. Schwan, R. F., Cooper, R. M., & Wheals, A. E. (1997). *Enzyme and Microbial Technology*, *21*, 234–244.
20. Noratto, G., & Campos, D. (2000). *Anales Científicos Universidad Nacional Agraria La Molina (Lima, Peru)*, *45*, 193–210.
21. Gupta, A. K., Singh, D. P., Kaur, N., & Singh, R. (1994). *Chemical Technology Biotechnology*, *59*, 377–385.

22. Xiong, C., Jinhua, W., & Dongsheng, L. (2007). *Biochemical Engineering Journal*, *34*, 179–184.
23. Mazutti, M., Bender, J. P., Treichel, H., & Di Luccio, M. (2006). *Enzyme and Microbial Technology*, *39*, 56–59.
24. Yépez-Silva-Santisteban, B. O., & Maugeri, F. F. (2005). *Enzyme and Microbial Technology*, *36*, 717–724.
25. Verduyn, C., Postma, E., Scheffers, W. A., & van Dijken, J. P. (1992). *Yeast*, *8*, 501–517.
26. Vranesick, D., Kurtanjek, Z., Santos, A. M. P., & Maugeri, F. (2002). *Food Technology and Biotechnology*, *40*, 67–73.
27. Miller, G. L. (1959). *Analytical Chemistry*, *31*, 426–428.
28. Kim, M.-K., Lee, I.-Y., Ko, J.-H., Rhee, Y.-H., & Park, Y.-H. (1999). *Biotechnology and Bioengineering*, *62*, 317–323.
29. Santos, A. (1998). *Síntese de Oligossacarídeos por Inulinase de Kluyveromyces bulgaricus*. Master thesis in Food Engineering, Faculty of Food Engineering, State University of Campinas.
30. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951). *Journal of Biological Chemistry*, *193*, 265–275.
31. Laloux, O., Cassart, J.-P., Delcour, J., Beeumen, J. V. E., & Vandenhaute, J. (1991). *FEBS Letters*, *289*, 64–68.
32. Cassart, J. P., Östiling, J., Ronne, H., & Vandenhaute, J. (1997). *Molecular Genetic Genomics*, *255*, 9–18.
33. Georis, I., Cassart, J.-P., Breunig, K. D., & Vandenhaute, J. (1999). *Molecular Genetic Genomics*, *261*, 862–870.
34. Wong, P., Gladney, S., & Keasling, J. D. (1997). *Biotechnology Progress*, *13*, 132–143.
35. Roels, J. A. (1983). *Energetics and kinetics in biotechnology.* Amsterdam: Elsevier Biomedical Press.
36. Yépez-Silva-Santisteban, B. O., Converti, A., & Maugeri, F. (2006). *Food Technology and Biotechnology*, *44*, 479–483.
37. Castrillo, J. I., & Ugalde, U. O. (1993). *Applied Microbiology and Biotechnology*, *40*, 386–393.
38. Pronk, J., Steensma, H. Y., & van Dijken, J. P. (1996). *Yeast*, *12*, 1607–1633.