Intrinsic Activity of Inulinase from *Kluyveromyces marxianus* ATCC 16045 and Carbon and Nitrogen Balances

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Summary

Kluyveromyces marxianus ATCC 16045 was cultivated in a batch on minimal medium to overproduce inulinase. The fermented broth was purified by fast protein liquid chromatography and ultrafiltration, and a pure enzyme fraction was obtained. SDS-PAGE electrophoresis allowed calculating molecular mass of 59 kDa, while nitrogen determination by the micro-Kjeldahl method allowed evaluating intrinsic inulinase activity of 879 IU/mg. These results were then used to perform material balances of the fermentation process, which suggested that no more than 0.1 % either of carbon or nitrogen initially present in the medium were incorporated in the extracellular inulinase released under different cultivation conditions. The information obtained in this study can be used for future proposal of metabolic models describing inulinase overproduction by this yeast.

Key words: carbon source, nitrogen source, inulinase production, *Kluyveromyces marxianus*

Introduction

Stoichiometric models can be proposed to describe bioprocesses making use of simple material balances based on kinetic results of product formation and substrate uptake. Such a simple tool allowed checking the validity of proposed networks of reactions and provided information on the metabolic routes actually involved in several bioprocesses, namely xylitol production by suspended (*1–4*) and immobilized yeast cells (*5,6*), 2,3-butanediol and acetoin production by *Acetobacter hansenii* (*7*), and hydrogen production by *Enterobacter aerogenes* (*8,9*).

In view of possible use of this methodology to describe the overproduction of an extracellular enzyme, it is needed to include in the network a general and simplified stoichiometric equation describing its formation from limiting substrates. This operation requires knowledge of the intrinsic specific activity of the enzyme expressed as activity units per unit of enzyme mass. In the particular case of inulinase overproduction by *Kluyveromyces marxianus*, determination of such an activity is hindered by the difficult purification of enzyme solutions and quantification of its mass.

Inulinases from different microbial cultivations have been characterized up to now mainly in terms of molecular mass and specific activity referred to total protein content. GrootWassink and Fleming (*10*) prepared concentrated solutions of *Kluyveromyces fragilis* inulinase using a three-step purification protocol consisting of autolysis, ultrafiltration and precipitation with acetone, which allowed obtaining specific activity of 1338 IU/mg. The resulting inulinase solution contained only three little fractions of contaminant proteins besides the desired enzyme. Man-

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zoni and Cavazzoni (*11*) obtained concentrated inulinase solutions from *Candida kefyr*, *Candida tropicalis*, *Kluyveromyces cicerosporus* and *K. fragilis* by 10 kDa ultrafiltration and obtained specific activities in the range 72.3–192.7 IU/mg. Azhari *et al.* (*12*) detected two inulinase fractions (81 and 53 kDa) from *Aspergillus* sp. Finally, Pandey *et al.* (*13*), reviewing literature data dealing with inulinase production by yeasts and fungi, reported molecular masses in the range 53–300 kDa and specific activities in the range 21–350 IU/mg.

However, all these studies made reference to specific inulinase activity referred to unit of mass of total protein expressed as albumin. Although very useful for comparison purposes, these relative determinations cannot directly be used for carbon and nitrogen balances to be applied in inulinase synthesis, because its elemental composition is different from that of albumin. This conclusion is suggested by the work of Rouwenhorst *et al.* (*14*), who obtained different values of specific inulinase activity using different methodologies for protein determination, among which those of Lowry *et al.* (*15*) and Bradford (*16*) and that proposed by the same authors for more accurate determination using total organic carbon analysis (*17*).

The aim of the present work was to set up an accurate methodology for determination of the intrinsic activity of inulinase from *K. marxianus* as well as to make possible future formulation of a stoichiometric model of its overproduction using kinetic data of substrate (carbon, nitrogen and oxygen sources) uptake and formation of products, including biomass, inulinase, metabolic by-products and carbon dioxide.

Materials and Methods

Microorganism and fermentations

The microorganism utilized in this study as extracellular inulinase producer, *Kluyveromyces marxianus* ATCC 16045, was maintained in yeast malt agar at 4 °C. Cryogenic pre-inocula (20 % glycerol at –80 °C) were prepared by 24-hour incubation in 500-mL baffled Erlenmeyer flasks containing 100 mL of the complex medium described by Kalil *et al.* (*18*) at 30 °C so as to obtain suspensions with absorbance *A*=0.5.

Batch fermentations were carried out either on minimal (*19*) or complex medium (*18*) at the optimum temperature of 30 °C (*20*), 450 rpm and aeration of 1 vvm in a 3.0-litre fermentor, type Bioflow III (New Brunswich Scientific, Edison, NJ, USA), with 2.0 L of working volume, after inoculation with biomass from the above suspensions and addition of small amounts of antifoam, type Aratrop (Alcamo Química, Indústria e Comércio Ltda, Sertãozinho, SP, Brazil). A 2 M NaOH solution was added to control the pH at its optimum value (3.5) (*20*). Samples were being withdrawn throughout the fermentations to determine the levels of the main metabolites, inulinase, biomass and total proteins. Additional fermentations were also carried out varying the initial sucrose concentration from 10 to 40 g/L, while 5 g/L of ammonium sulfate was always used as a nitrogen source.

The effect of aeration was investigated using 1 vvm of 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 %.

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 absorbance at 600 nm to the dry biomass 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). The resulting biomass elemental composition was used to calculate C-mol of biomass.

Concentrations of acetate, ethanol, sucrose, glucose and fructose during fermentations were determined at 40 °C by a HPLC device consisting of 9010 Solvent Delivery System, RI-4 Refractive Index Detector, 9095 Autosampler (Varian, Walnut Creek, CA, USA), SPH99 Column Thermostat (Spark Holland, Emmen, The Netherlands), and Millennium Chromatography Manager (Waters, Milford, MA, USA), equipped with an anionic exchange HPX87H column (Biorad Laboratories, Hercules, CA, USA) using a sulfuric acid solution (pH=1.0) as a mobile phase at a flow rate of 0.7 mL/min.

The dissolved oxygen (DO) level was monitored online using a polarographic probe (Mettler Toledo, Greifensee, Switzerland).

The amount of carbon dioxide released during fermentation was determined gravimetrically by collecting the exhausted air from the fermentor in a 1.0 M KOH solution. The resulting potassium carbonate was precipitated as $BaCO_3$ by the reaction with a saturated solution of $BaCl_2$. The $BaCO_3$ suspension was then filtered through a preweighed filter with 0.22-µm pore diameter, and the cake was washed with cold distilled water in the filtration system and finally dried at 104 °C up to constant mass (approximately 24 h). The carbon dioxide mass was finally calculated from the resulting $BaCO_3$ dry mass.

The nitrogen content of pure inulinase was determined with the micro-Kjeldahl method, while that of total proteins in the fermented broth according to Lowry *et al.* (*15*).

Determination of inulinase activity

Inulinase activity was assayed according to Santos (*21*). The enzyme solution (0.5 mL) was mixed with 4.5 mL of 2 % sucrose 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, and the rate of appearance of reducing sugars was determined by the colorimetric method of 3,5-dinitrosalicylic acid (*22*). One unit of inulinase activity was defined as the amount of enzyme able to hydrolyze 1 µmol of sucrose per min, under the above conditions.

Inulinase purification and concentration

The fermented broth was produced using 10 g/L of sucrose in minimal medium and was concentrated passing first through a 1-kDa cellulose ultrafilter, model Prep/scale-TFF 1 ft², cat. N. CDUF001LA (Millipore Cor-

poration, Bedford, MA, USA) and then through a 50-mL stirred cell model 8050 employing a 30-kDa YM30 DIA-FLO® utrafiltration membrane (Amicon, Beverly, MA, USA). Inulinase was then purified by a fast protein liquid chromatography (FPLC) system (Pharmacia-Biotech, Uppsala, Sweden), using a Q Sepharose FF anion exchange resin and 0.05 M phosphate buffer at pH=6.0 with 0–0.8 M NaCl gradient, and the resulting fractions with inulinase activity were concentrated with a 10-mL stirred cell model 8010 employing the same ultrafiltration membrane as above.

Determinations of inulinase molecular mass and purity degree

Inulinase molecular mass and purity degree of the active fractions were determined by SDS-PAGE electrophoresis using a 7.5 % acrylamide/bisacrylamide gel and Tris/glycine/SDS buffer. After 30- to 40-minute runs at 150 V, the protein bands were fixed with 25 % isopropanol and 10 % acetic acid. To determine the molecular mass of inulinase we used a high molecular mass standard mixture, HMW Calibration Kit (Pharmacia-Biotech: 330, 18.5, 60, 36 and 67 kDa). To determine the relative mobility (R_f) of a protein, its migration distance from the top of the separating gel to the centre of the protein band was divided by that of the bromophenol blue tracking dye from the top of the separating gel. The R_f values were plotted against the known molecular masses for calibration curve.

Results and Discussion

Intrinsic specific inulinase activity

The presence of only one band for each fraction tested by SDS-PAGE electrophoresis (Fig. 1) pointed out that purification of the cultivation broth of *K. marxianus* ATCC 16045 by the FPLC system provided a practically purified inulinase preparation from minimal medium. In addition, as shown in Table 1, the molecular mass of inulinase was 59 kDa. Rouwenhorst *et al.* (*14*) determined a subunit molecular mass of 64 kDa of the dimeric inulinase for the corresponding monomer produced by *K. marxianus* CS 6556.
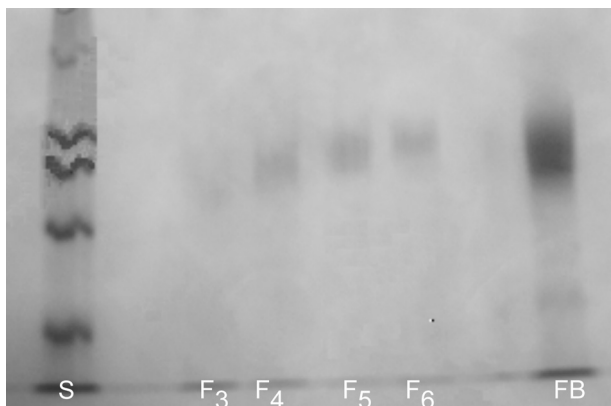

Fig. 1. SDS-PAGE electrophoreses of four fractions of purified inulinase from *K. marxianus* ATCC 16045 (F₃, F₄, F₅ and F₆), fermented broth (FB), and standard enzyme solutions (S)

Table 1. Results of SDS-PAGE electrophoreses performed on inulinase solutions purified by fast protein liquid chromatography and ultrafiltration for determination of inulinase molecular mass

Fraction	First determination			Second determination		
	Run/cm	R_f	M/kDa	Run/cm	R_f	M/kDa
F3	2.210	0.414	62.1	2.114	0.393	63.9
F4	2.298	0.431	59.5	2.244	0.417	60.0
F5	2.472	0.463	54.7	2.366	0.440	56.5

R_f=relative mobility; M=molecular mass

The intrinsic specific inulinase activity was then determined as follows, relating the mass of inulinase present in the purified solution to the initial activity before purification. The primary structure of the enzyme was reported by Laloux *et al.* (*23*) from sequencing the *KmINU1* gene responsible for inulinase expression in *K. marxianus*. The elemental composition of inulinase ($C_{2861}H_{4093}O_{761}N_{694}S_7$) was deduced from this gene sequence, and the ratio of 6.23 g of inulinase per g of nitrogen was calculated from this composition. The starting activity contained in 455 mL of the fermented broth was 1692 IU, and the nitrogen content of the corresponding purified enzyme solution was 0.295 mg of N. Using these data, it was possible to evaluate a protein mass of inulinase present in the sample corresponding to an intrinsic specific activity of 879 IU per mg of inulinase. Although this value is not so far from that obtained by Rouwenhorst *et al.* (*14*) for *K. marxianus* CS 655l cultivation on sucrose (1388 IU/mg), one can recognize as possible causes of variations in such a parameter the different carbon sources used by the above authors (sucrose, inuline or raffinose) as well as the variable degree of glycosylation (*14*) notoriously linked to the protein, which could have appreciably influenced their inulinase mass estimation from experimental data of carbon content.

Carbon and nitrogen balances

The intrinsic specific inulinase activity was then utilized to calculate the inulinase mass produced in fermentations performed under different conditions (Table 2). As the data of Fig. 2 show, inulinase represented only up to 0.1 % of total carbon balance; therefore, its mass could be considered to be negligible in the overall stoichiometric balances. Moreover, the specific enzyme production ranged between the orders of magnitude of nmol

Table 2. Main experimental results of *K. marxianus* ATCC 16045 cultivations performed under different conditions

Run	1	2	3	4
Sucrose/(g/L)	10	10	40	40
Aeration[a]	Air	Air+O₂	Air	Air+O₂
X_{max}/(g/L)[b]	3.7	3.5	9.1	11
a_{max}/(IU/mL)[c]	6.4	5.3	7.2	6.1
Total proteins/(g/L)	0.89	–	1.22	–
Ethanol/(g/L)	0.49	0.50	7.26	2.46

[a]Aeration performed at 1 vvm; [b]X_{max}=maximum biomass concentration; [c]a_{max}=maximum inulinase activity
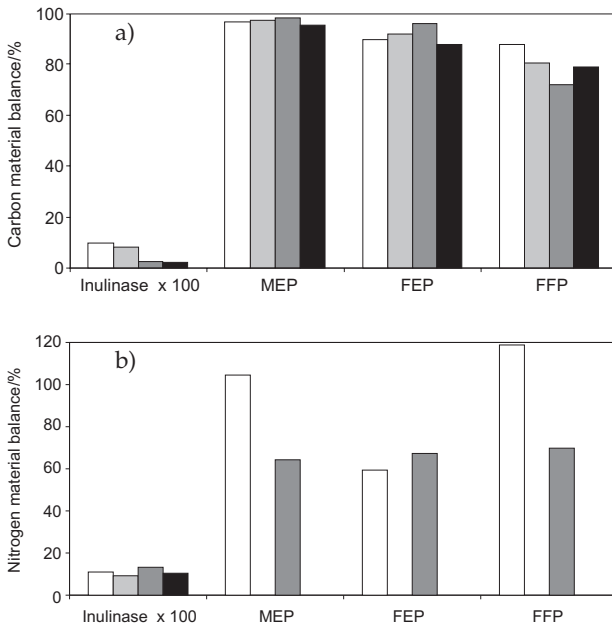
Fig. 2. Inulinase localization and (a) carbon and (b) nitrogen material balances performed during the middle exponential phase (MEP), the final exponential phase (FEP) and the final fermentation phase (FFP) of *K. marxianus* ATCC 16045 cultivations performed under different conditions. All percentages are related to initial carbon and nitrogen contents of the medium. (□) Initial sucrose concentration 10 g/L, air supply 1 vvm; (▨) initial sucrose concentration 10 g/L, air+O_2 supply 1 vvm; (▩) initial sucrose concentration 40 g/L, air supply 1 vvm; (■) initial sucrose concentration 40 g/L, air+O_2 supply 1 vvm

and μmol of inulinase per C-mol of biomass and between μg and mg of inulinase per g of biomass, or sucrose or ammonium sulfate (Table 3).

The carbon material balance was close to 100 % up to the middle exponential phase, and then a progressively increasing fraction was lost (Fig. 2a). These losses (reached 20–30 % of the starting carbon at the end of fermentations) were likely due to either sampling or evaporation of volatile or gaseous products, such as ethanol and carbon dioxide. On the contrary, large variations were observed for nitrogen material balances (from –36 to 19 %) (Fig. 2b), which suggests that the method of Lowry *et al.* (*15*) was inaccurate for determination of the level of extracellular proteins in the specific case under investigation.

On the basis of the literature knowledge on the metabolism of this yeast (*24*), hypotheses were formulated about the main metabolic routes (glycolytic pathway, pentose phosphate pathway, fermentation, pyruvate bypass, tricarboxylic acid cycle and oxidative phosphorylation) involved in the formation of the main products. The experimental stoichiometric coefficients listed in Table 3, referring to all the C-containing species, could be used to estimate the corresponding yields of conversion multiplying by their respective molecular masses. These results point out that, under all the conditions tested, the main metabolic products of the overall metabolism of *K. marxianus* were carbon dioxide and biomass, which reached at the end of fermentations $Y_{CO_2/S}$=0.84–0.85 g/g and $Y_{X/S}$=0.16–0.33 g/g, respectively. Both results were

Table 3. Stoichiometric coefficients referring to the production of 1 C-mol of biomass (C-mol$_x$) obtained for *K. marxianus* ATCC 16045 cultivations performed under different conditions*

Run	Substrate		Product								
	Sucrose	(NH$_4$)$_2$SO$_4$	Inulinase	Glucose	Fructose	Pyruvate	Glycerol	Acetate	Acetaldehyde	Ethanol	CO$_2$
	mol/C-mol$_X$	mol/C-mol$_X$	mol·10^{-9}/ C-mol$_X$	mol/C-mol$_X$	mol/C-mol$_X$	mol/C-mol$_X$	mol/C-mol$_X$	mol/C-mol$_X$	mol/C-mol$_X$	mol/C-mol$_X$	mol/C-mol$_X$
MEP[a]											
1	0.690	0.109	107.45	0.185	0.639	0	0.1851	0	0	0.0685	1.84
2	0.735	0.158	183.86	0.100	0.496	0	0.0221	0.0134	0.0196	0.5441	2.88
3	0.690	–	162.50	0.166	0.543	0	0.0327	0.0392	0	0.2115	2.21
4	0.719	0.233	185.05	0.260	0.583	0	0.0249	0.0115	0.0022	0.0464	1.97
FEP[b]											
1	0.250	0.154	363.53	0	0	0.0000	0.004	0	0	0.011	1.64
2	0.463	0.133	91.65	0	0	0.0039	0.010	0.003	0.087	0.529	3.05
3	0.285	–	1.27	0	0	0.0065	0.000	0.021	0	0.102	1.88
4	0.376	0.122	0	0	0	0.0127	0.025	0.003	0.006	0.172	2.48
FFP[c]											
1	0.254	0.095	1063.04	0	0	0	0	0	0	0.011	1.68
2	0.515	0.166	558.93	0	0	0.0048	0.0034	0	0.006	0	3.38
3	0.336	–	1152.59	0	0	0.0108	0	0	0	0	2.22
4	0.356	0.115	347.30	0	0	0.0023	0.0007	0	0	0	2.35

*For conditions of runs 1–4 see Table 2. CH$_{1.94}$O$_{0.76}$N$_{0.17}$ was the elemental dry cell composition used to calculate C-mol of biomass; [a]MEP=middle exponential phase; [b]FEP=final exponential phase; [c]FFP=final fermentation phase

the consequence of the aerobic conditions needed to stimulate inulinase production. In addition, the fermentations with high initial sucrose level (S_o=40 g/L) exhibited the highest ethanol concentrations (2–7 g/L). Strict aerobic conditions ensured by air enrichment with O_2 did not suppress ethanol formation but only reduced it, hence indicating that the synthesis of this metabolite was mainly the result of an overflow of the carbon source through the glycolysis rather than the product of anaerobic fermentation.

These results also suggest that nitrogen was preferably used for biomass synthesis and only to a less extent for the production of extracellular proteins.

Conclusions

The overproduction of inulinase by *K. marxianus* ATCC 16145 cultivation was investigated under different conditions. The intrinsic specific activity of this enzyme was 879 IU/mg. Extracellular inulinase production ranged between the orders of magnitude of nmol and μmol of enzyme per mol of biomass, sucrose or ammonium sulfate, thus representing no more than 0.1 % either of consumed carbon or nitrogen. Therefore, notwithstanding the very high enzyme activity, its inclusion in a stoichiometric model based on material balances could be considered to be negligible.

Material balances, made under different cultivation conditions to better understand the metabolism of *K. marxianu*s ATCC 16045, were satisfactory up to the final exponential phase, whereas significant C loss occurred during the final fermentation phase. Carbon dioxide and biomass syntheses were the main activities responsible for carbon consumption under all the conditions tested, while biomass synthesis and production of extracellular proteins were responsible for most of nitrogen uptake.

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