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Production, purification and characterization of an extracellular inulinase from *Kluyveromyces marxianus* var. *bulgaricus*

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The yeast *Kluyveromyces marxianus* var. *bulgaricus* produced large amounts of extracellular inulinase activity when grown on inulin, sucrose, fructose and glucose as carbon source. This protein has been purified to homogeneity by using successive DEAE-Trisacryl Plus and Superose 6HR 10/30 columns. The purified enzyme showed a relative molecular weight of 57 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and 77 kDa by gel filtration in Superose 6 HR 10/30. Analysis by SDS-PAGE showed a unique polypeptide band with Coomassie Blue stain and nondenaturing PAGE of the purified enzyme obtained from media with different carbon sources showed the band, too, when stained for glucose oxidase activity. The optimal hydrolysis temperature for sucrose, raffinose and inulin was 55°C and the optimal pH for sucrose was 4.75. The apparent K_m values for sucrose, raffinose and inulin are 4.58, 7.41 and 86.9 mg/ml, respectively. Thin layer chromatography showed that inulinase from *K. marxianus* var. *bulgaricus* was capable of hydrolyzing different substrates (sucrose, raffinose and inulin), releasing monosaccharides and oligosaccharides. The results obtained suggest the hypothesis that enzyme production was constitutive. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 63–69.

Keywords: inulin; inulinase; *Kluyveromyces marxianus* var. *bulgaricus*; extracellular enzyme

Introduction

The extracellular glycoprotein, inulinase, catalyzes hydrolysis of the polyfructoside inulin outside the cell wall. The enzyme also hydrolyses sucrose, both within and outside the cell wall [23]. For a microorganism to utilize inulin as its carbon and energy source, production of an extracellular inulinase is required to hydrolyse the fructan into fermentable monosaccharides. Fructans are polyfructose molecules that can be subdivided into two categories, the inulin and the levan type. The inulin fructans are found as carbohydrate reserves in several plants, including chicory, dandelion, dahlia, and Jerusalem artichoke. They consist of a linear chain of (2-1)-linked fructose molecules and, depending on the plant source, may have between 5 and 45 fructose monomers [2]. The levan fructans produced by many bacterial species consist of branched molecules with predominantly (2-6)-linkages branched at C1 [10,11]. Inulinases have been purified from yeast [7,21,25,31], molds [9,27] and bacteria [1,3,8,29,30] and have potential uses in the preparation of fructose syrups from inulin and invert sugar in the food industry [19,28]. The results of the study on the production and purification of an extracellular inulinase by *Kluyveromyces marxianus* var. *bulgaricus* are presented.

Materials and methods

Strain

K. marxianus var. *bulgaricus* ATCC 16045 was obtained from the Bioengineering Laboratory, Campinas State University, São Paulo, Brazil. The strain was maintained on solid medium a 4°C. The medium composition (g/l) was comprised of the following: Difco yeast extract 10.0; Difco Bactopeptone 20.0; glucose 20.0 and Difco agar 20.0. Cells were harvested from 18 slants and used to inoculate liquid medium.

Culture media

Liquid media with 1% (w/v) glucose, sucrose or fructose were obtained from Reagen and raffinose, inulin from dahlia tubers and, inulin from Jerusalem artichoke were obtained from Sigma. Media were supplemented with 1% yeast extract and the pH was adjusted to 3.0 with orthophosphoric acid.

Culture conditions

The yeast was inoculated into 250 ml Erlenmeyer flasks containing 50 ml of medium incubated at 30°C for 48 h in a temperature-controlled shaker (New Brunswick Scientific, USA) operated at 200 rpm. The culture was pelleted by centrifugation at 10,000×*g* for 10 min.

Fractionation of cultures for inulinase activity assays

Supernatant enzyme: The organism was grown at 30°C in a continuous laboratory fermenter with a working volume of 600 ml, aeration of 0.2 vvm, pH 3.0 and sucrose as carbon source. One hundred milliliters of culture was harvested by centrifugation at 4°C (10 min, 10,000×*g*). The supernatant was used as a source of extracellular enzyme.

Cell wall enzyme: The cell pellet obtained after centrifugation was suspended and incubated for 1 h at 30°C in 10 ml of enzyme

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release buffer (ERB) (50 mM potassium phosphate, pH 7.0, 10 mM 2-mercaptoethanol, 10 mM dithiothreitol and 2 mM $MgSO_4$). The suspension was then spun at 4°C at 10,000×*g* and washed twice, first with 5 ml of ERB and then with 5 ml of sonication buffer (50 mM potassium phosphate, pH 7.0, 10 mM $MgSO_4$). Enzyme activities present in the supernatant phase were referred to as cell wall.

Cell-bound enzyme: After the release of cell wall enzyme and washing, cells were suspended in 5 ml of sonication buffer and sonicated at 4°C using a Vibra Cell 600 W disintegrator (Sonics) for 5 min. Cell debris was removed by centrifugation at 4°C for 15 min at 10,000×*g* and resuspended in 5 ml of sonication buffer. Enzyme activities in the supernatant were referred as cell-bound enzyme.

Chromatographic supplies

Inulin from dahlia tubers, inulin from Jerusalem Artichokes, sucrose, raffinose, fructose, glucose, DEAE-Trisacryl, polyacrylamide gel electrophoresis (PAGE) reagents, and gel filtration molecular weight markers were obtained from Sigma; Superose 6 HR 10/30, Sephacryl S-200 HR were from Pharmacia (Uppsala, Sweden); chromatographic equipment were from Bio-Rad. Silica gel 60 was obtained from Merck. All other reagents were of analytical grade.

Extraction and purification of extracellular inulinase

Culture (48 h old) filtrate were collected by centrifugation (10,000×*g*), dialyzed overnight against 4 l of distilled water, lyophilized, dissolved in a small volume of 5 mM sodium phosphate buffer and applied to DEAE-Trisacryl (13×1.6 cm) column equilibrated with 5 mM sodium phosphate buffer, pH 6.8. The column was washed with the same buffer and eluted with a 100 ml linear gradient of 0–0.3 M sodium chloride in 5 mM sodium phosphate (pH 6.8). The flow rate throughout the experiment was 2 ml/min, and effluent was collected in fractions of 1.34 ml.

HPLC

Further purification was performed using small-scale high-performance gel filtration chromatography on a Superose 6 HR 10/30 (Pharmacia) prepacked column (10×300 mm, 24 ml) and a volume void of 7.22 ml with a Bio-Rad model 2800 chromatography apparatus. The column was equilibrated with 50 mM sodium phosphate buffer. Elution was carried out at room temperature with 150 mM sodium chloride at a flow rate of 0.4 ml/min and proteins were detected at 280 nm.

Determination of molecular mass

To estimate the molecular mass of inulinase, the Superose 6HR column was calibrated with standard proteins: thyroglobulin (669 kDa); β-amylase (200 kDa); alcohol dehydrogenase (150 kDa) and ovalbumin (66 kDa). All standards and samples were passed through the column at least twice, and the mean of their elution volumes was taken. Fractions (0.5 ml) collected were examined using a UV/280 nm monitor and assayed for inulinase activity.

Sodium dodecyl sulfate (SDS)-PAGE

Denaturing electrophoresis was performed by the method of Laemmli [14] with Tris–glycine–SDS buffer (pH 8.3) for 11% and 7% gels. Samples were mixed in a 1:1 ratio (vol/vol) with Tris–glycerol β-ME buffer and boiled for 1 min (100°C) before application. To visualize proteins, gels were stained with Coomassie Brilliant Blue R-250. Apparent molecular weight was determined using a Sigma kit (SDS-6) which contained: soroalbumin bovine (66 kDa), ovoalbumin (45 kDa), pepsin (34.7 kDa), trypsinogen (24 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa). Proteins were also mixed with Tris–glycerol–βME buffer and boiled for 1 min (100°C) before electrophoresis.

Nondenaturing PAGE

PAGE performed on 7% (w/v) polyacrylamide gels was carried out by the method of Davis [5] at a constant current of 4 mA, with Tris–glycine buffer (pH 8.9) as the running buffer.

Inulinase activity

Inulinase activity was determined using inulin from dahlia tubers, raffinose and/or sucrose as substrates by determining the amount of reducing sugar released [18]. Assays consisted of 0.025 ml of enzyme solution, 10 ml of 1% inulin from dahlia or 0.8% sucrose, or 0.8% raffinose in 50 mM citrate phosphate buffer pH 5.0. Reaction mixtures were incubated at 40°C and aliquots of 1.0 ml were withdrawn at intervals (0–30 min) for quantification of reducing sugars. One enzyme unit was defined as the amount of enzyme which yields 1.0 μmol of product in a minute at 40°C, using fructose as the standard [26].

Protein determination

Protein was determined by the method of Lowry *et al.* [16] modified by Hartree [13] using bovine serum albumin as standard.

Influence of temperature, pH and cations on inulinase activities

The optimum temperatures for inulin, raffinose and sucrose hydrolysis by inulinase were determined at temperatures ranging from 30°C to 80°C. Thermal stability data were obtained by incubating the enzyme at 40, 45, 50°C for different time intervals, after which the remaining activity was determined. The effect of pH on inulinase activity was studied using 100 mM citrate phosphate buffer (pH 3.0–6.0). The activity of purified enzyme was investigated in the presence of 50 mM of $CaCl_2$, $BaCl_2$, $MnCl_2$, $FeCl_3$, $ZnCl_2$ and NaCl.

Effect of carbon sources

In order to determine the effect of various C-sources on enzyme in *K. marxianus* var. *bulgaricus*, cells were grown in media containing various carbon sources or in medium without a main carbon source. After 72 h incubation at 30°C, 200 rpm, the activity of secreted inulinase was determined. The enzyme present in each culture filtrate was submitted to the purification process described above. After the last chromatographic step, the specific activity was determined.

Kinetic studies

Comparative studies were performed to determine the K_m and V_{max} values of *K. marxianus* var. *bulgaricus* inulinase in the presence of different substrates: inulin 4.0–20.0 mg/ml; raffinose 0.5–16.0

mg/ml and sucrose 0.5–20.0 mg/ml. The kinetic constant values were calculated from the initial velocity rates as described by Lineweaver and Burk [15].

Hydrolysis products identification

Hydrolysis products from inulin, sucrose and raffinose were analysed by thin layer chromatography on silica gel G-60, using ethyl acetate–acetic acid–formic acid–water (9:3:1:4, by volume) as the mobile phase. Sugar was detected with 0.2% orcinol in sulfuric acid–methanol (10:90).

Results and discussion

Production and distribution of inulinase of *K. marxianus* var. *bulgaricus* in continuous cultures

In carbons and energy-limited continuous cultures of *K. marxianus* var. *bulgaricus*, the highest inulinase yields were obtained with sucrose as the limiting substrate. The levels of inulinase in sucrose-limited chemostat cultures were strongly dependent on the dilution rate. Enzyme levels (sum of cell-bound enzyme, cell wall enzyme and supernatant enzyme) decreased from a maximum of 107 U/ml at $D=0.05\ h^{-1}$ to 0.81 U/ml at $D=0.45\ h^{-1}$. The distribution of inulinase activity among the three fractions was determined at various dilution rates (Table 1). The amount of cell wall enzyme exhibited a slight increase with increasing dilution rates, while the cell-bound enzyme decreased and became nil above a dilution rate of $0.30\ h^{-1}$.

The decrease in inulinase activity with increasing dilution rates is primarily caused by increased residual sugar concentrations at higher dilution rates [23]. The presence of higher concentrations of residual substrate led to lower enzyme activity. These observations indicate that the enzyme is regulated by the residual sugar concentration in the culture [4,6].

Purification of supernatant inulinase

Preliminary experiments indicated that inulinase activity of *K. marxianus* var. *bulgaricus* was lost when it was precipitated by ammonium sulfate. The best precipitation results were reached with acetone (18.80 U/mg protein), ethanol (15.60 U/mg protein) and lyophilization (14.33 U/mg protein). Although the organic solvents showed good results, we opted for lyophilization because of its simplicity. Table 2 summarizes the typical results of the purification protocols for extracellular inulinase. Fractions with activity were pooled, dialyzed against distilled water, lyophilized and dissolved in a small volume of sodium phosphate buffer. After DEAE-Trisacryl chromatography (Figure 1), the fractions with inulinase activity were pooled, dialyzed, lyophilized and the specific activity of inulinase at 40°C was determined to be 84.1 U/mg protein and a purification of 34-fold was achieved.

Preparations were examined using SDS-PAGE (Figure 2, line 2) and exclusion chromatography (Figure 3). No other protein bands were detected on the SDS polyacrylamide gels, either with Coomassie Brilliant Blue or with the more sensitive silver staining (results not shown), indicating that the enzyme preparations contained only one protein.

Inulinase purified by DEAE-Trisacryl was active on sucrose as well as on inulin, having a S/I ratio of 8.2. Polydispersity in the inulinase bands apparently is caused by the polysaccharide attached to the inulinase polypeptide (estimated to be 40% with glucose as the standard). The molecular weight of the purified enzyme was estimated to be 57 kDa by SDS-PAGE and 77 kDa by gel filtration. The difference between the methods was probably due to the polydispersity of the protein band in polyacrylamide gel (SDS-PAGE). Rouwenhorst *et al.* [24] compared inulinase treated with Endo-H to remove the carbohydrate chains with non-treated inulinase run on SDS-PAGE and determined the apparent molecular weights to be 72 and 87 kDa, respectively.

Table 1 Total inulinase activities and distribution in carbon-limited continuous cultures of *K. marxianus* var. *bulgaricus* at different dilution rates

Dilution rate (h^{-1})	Total activity[a] (U/ml)	Percent of inulinase in		
		Supernatant	Cell wall	Cell-bound
0.05	107	70	28	2
0.10	105	64.6	34.2	1.2
0.15	67	45.8	55.1	0.1
0.20	65	43.7	57.2	0.1
0.25	51	41.5	58.4	0.1
0.30	9	37.4	62.5	0.1
0.35	1.9	28.7	71.3	–
0.40	0.84	24.4	75.6	–
0.45	0.81	19.6	80.4	–

[a]Enzyme activity was measured with sucrose as substrate.

Table 2 Purification of extracellular inulinase from *K. marxianus* var. *bulgaricus*

Step	Volume (ml)	Total protein (mg)	Total activity (U/ml)[a]	Specific activity (U/mg protein)	Yield (%)	Purification factor
Cell-free medium	575	4217	10,419	2.47	100	1
Dialysis, lyophilization	59	742	18,743	25.2	179.9	10.2
DEAE-Trisacryl, dialysis, lyophilization	32.9	7.3	614	84.1	5.9	34

[a]One unit of inulinase activity is the amount of enzyme which yields 1 μmol of fructose per minute.

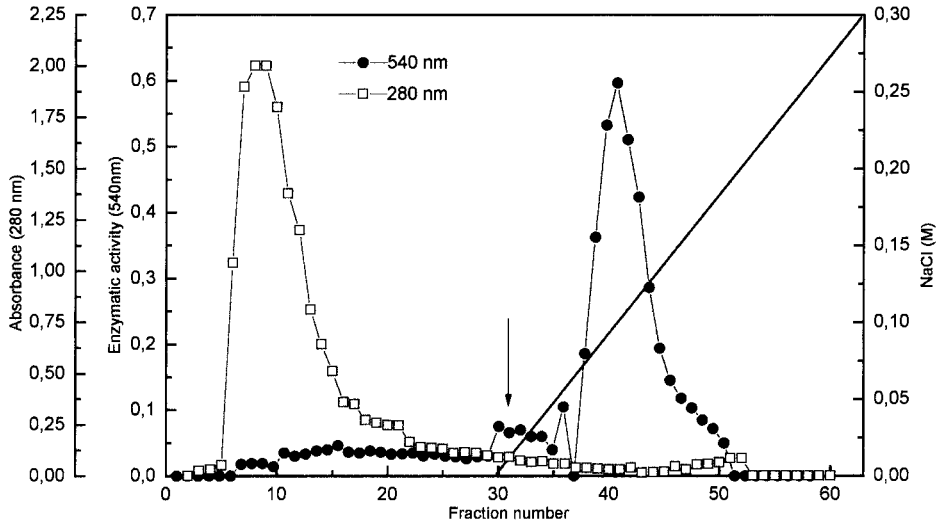

Figure 1 Chromatograph of inulinase preparations on DEAE-Trisacryl. A crude preparation of supernatant inulinase was applied to the column equilibrated with 5 mM sodium phosphate buffer (pH 6.8) and eluted with a 0 to 0.3 M linear gradient of NaCl.

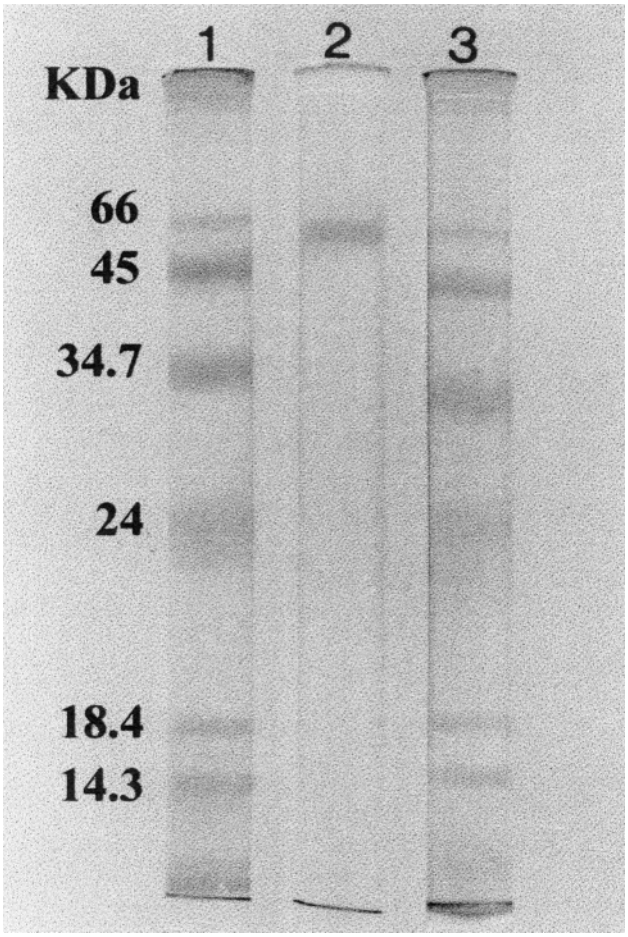

Figure 2 SDS-PAGE of purified extracellular inulinase. Purified protein (25 μg) was applied and the protein was stained with Coomassie Brilliant Blue. The position of the molecular weight markers is given on lines 1 and 3. Line 2: extracellular inulinase.

Characterization of purified inulinase

The characterization of the inulinase produced by the strain *K. marxianus* var. *bulgaricus* was performed in order to verify the optimum temperature for inulin, raffinose and sucrose as substrates. Figure 4 shows the result for these assays, where the optimum temperature was 55°C. The enzyme showed good stability at 40°C over 3.5 h and its half-life at 50°C was 40 min (Figure 5). The energy of activation (E_A) values for these substrates were 17.9, 13.57 and 8.21 kcal/mol, respectively, calculated from the linear portion of the Arrhenius plot. Passador-Gurgel *et al.* [22] also observed the loss of activity after 1 h at 50°C. Guerreiro *et al.* [12] observed that inulinase incubated at 50°C lost activity after 2 h. Furthermore, when incubated at 60°C or 70°C, the half-lives were 20 and 10 min, respectively. Rouwenhorst *et al.* [24] reported a half-life of 30 min at 60°C, while Negoro and Kito [20] reported a fast activity loss at 60°C since at 55°C, the enzyme was stable.

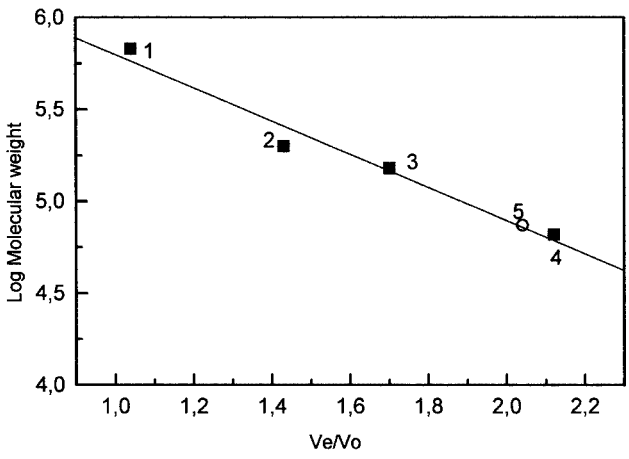

Figure 3 Estimation of molecular weight of purified supernatant inulinase by HPLC (Bio Rad 2800) on a Superose 6HR 10/30 filtration column. The open circle represents the position of purified supernatant inulinase. Standard proteins: (1) thyroglobulin (669 kDa), (2) β-amylase (200 kDa), (3) alcohol dehydrogenase (150 kDa), (4) bovine albumin (66 kDa) and (5) inulinase.

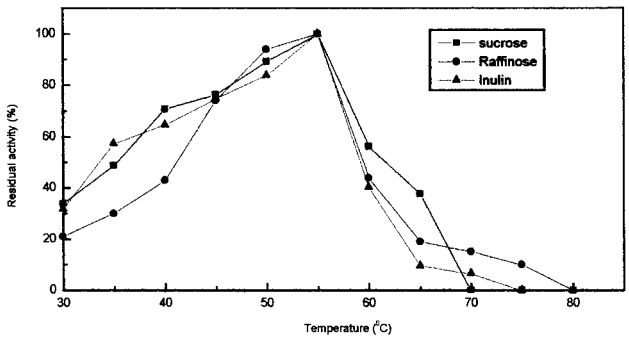

Figure 4 Effect of temperature on inulinase activity. Reactions used 0.8% (w/v) of substrates in 50 mM citrate phosphate buffer (pH 5.0) under constant agitation.

Maximal inulinase activity was obtained at pH 4.75 (Figure 6). Guerreiro *et al.* [12] reported a pH of 5.0, while Michaud *et al.* [17] reported a pH of 4.5. The activity of purified enzyme was investigated in the presence of several cations. Manganese did not affect the activity, calcium inhibited the enzyme by approximately 27%, barium, zinc and sodium inhibited 50%, while ferric chloride completely inhibited the enzyme.

Effect of carbon source

In order to establish the effect of C-source on production of the enzyme, *K. marxianus* var. *bulgaricus* was grown in media containing various carbon sources and in medium without a main carbon source. The specific activities obtained for inulin, sucrose,

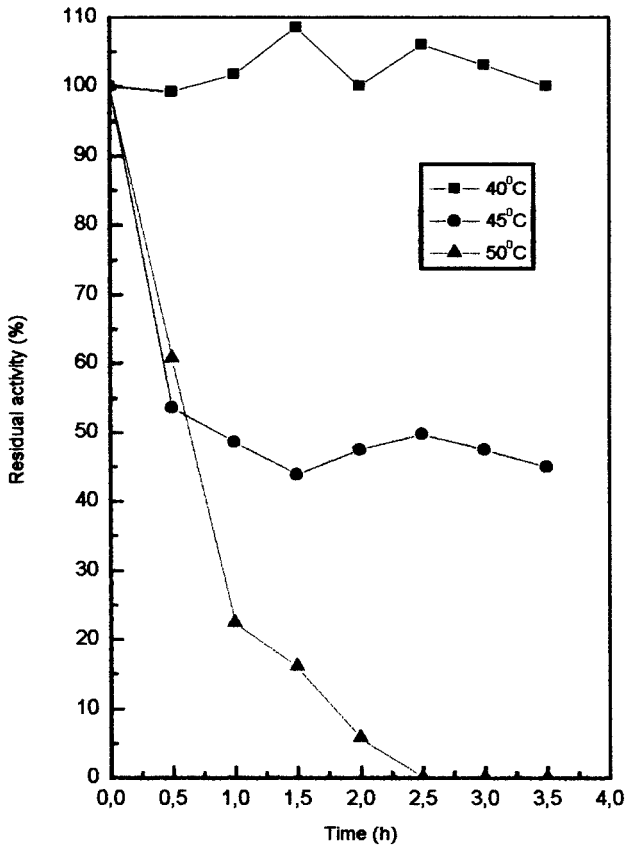

Figure 5 Thermal inactivation of *K. marxianus* var. *bulgaricus* inulinase. Symbols: (■) 40°C; (○) 45°C; (▼) 50°C.

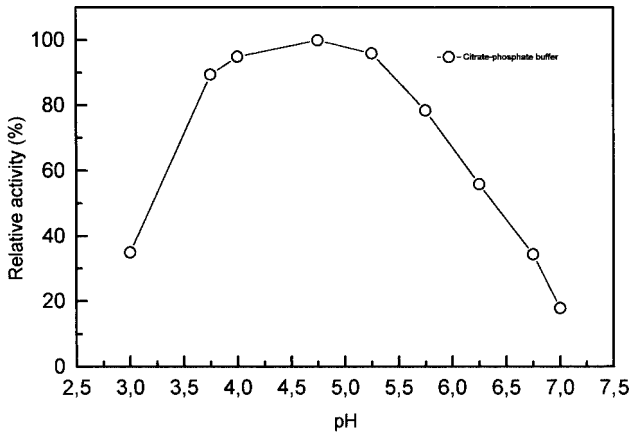

Figure 6 The pH profile of *K. marxianus* var. *bulgaricus* inulinase (40°C, citrate phosphate buffer).

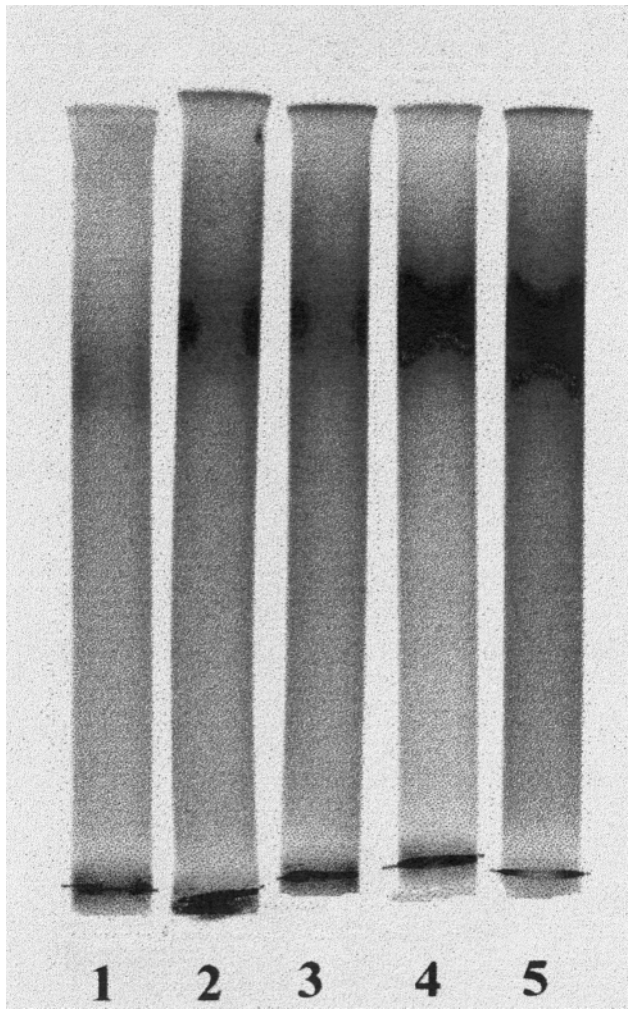

Figure 7 Nondenaturing PAGE of purified inulinase from media with different carbon sources. Activities were determined with glucose oxidase. (1) Sucrose medium, (2) inulin medium, (3) glucose medium, (4) fructose medium and (5) mixture of all media.

glucose and fructose were: 170.0, 35.5, 59.5 and 94.2 U/mg protein, respectively. Inulinase activity in media formulated with inulin from dahlia tubers was significantly higher than that observed in other carbon sources. After enzyme production on different carbon sources, the purified enzyme was submitted to nondenaturing PAGE (Figure 7). The results suggest that the enzyme is produced constitutively.

Kinetic studies

The K_m and V_{max} values were determined in the presence of inulin, raffinose and sucrose. The kinetic constant values for apparent K_m and V_{max} of purified enzyme were 86.9 mg/ml and 53.7 U/mg protein, 7.41 mg/ml and 240.0 U/mg protein; 4.58 mg/ml and 441.0 U/mg protein (Figure 8), respectively. K_m and activation energy showed a highest affinity for sucrose than inulin. Moreover, similar experiments with invertase obtained from Sigma did not hydrolyse inulin, suggesting that the enzyme studied in this research was one inulinase. Although the molecular weight of the polymer varies, Vandame *et al.* [28] reported a K_m value of 7.4 mM for inulin and Guerreiro *et al.* [12] reported K_m 3.04 mM.

Hydrolysis products

The products of the time course hydrolysis (pH 4.75) of inulin, raffinose and sucrose by the purified inulinase were analyzed by thin layer chromatography. Sucrose was totally hydrolyzed within 2 h. The presence of monosaccharides and disaccharides was observed after 4 h of reaction with raffinose. The inulin hydrolysis products, monosaccharides and oligosaccharides, were detected after 2 h (Figure 9). Yun *et al.* [32] showed that pH has a significant effect on mono- and oligosaccharide production. They also observed, at a more acidic value (pH 4.0), that the contents of fructose and inulobiose increased, in contrast to pH 6.0 where the rate was markedly retarded. Yun *et al.* [32] obtained a high yield of oligosaccharides using a purified endoinulinase from a commercial inulinase preparation. The maximum yield of oligosaccharide ranged from 50 to 150 g inulin/l and was around 96% irrespective of substrate concentrations.

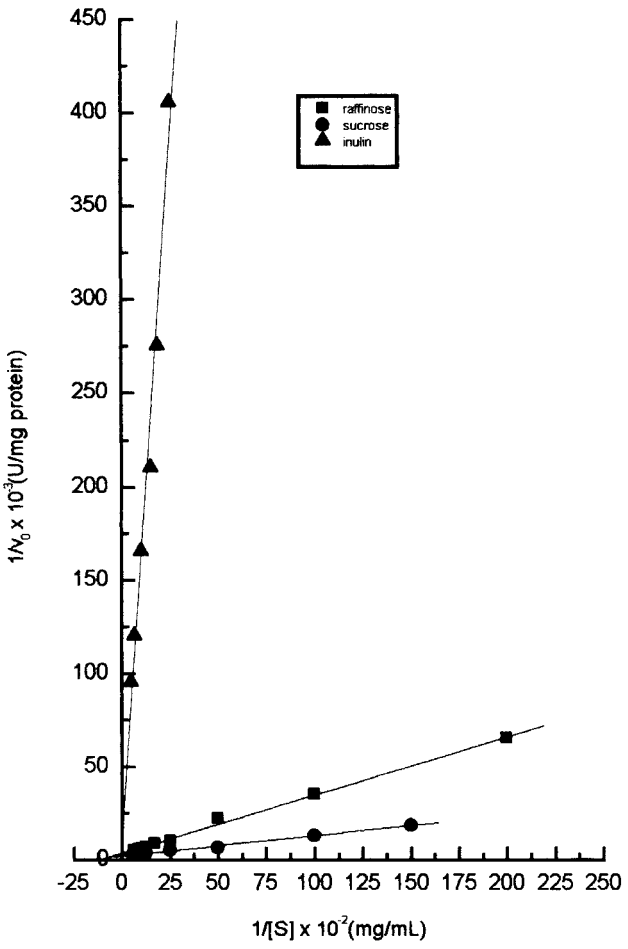

Figure 8 Lineweaver–Burk plots obtained with inulin, raffinose and sucrose. Reactions was performed at pH 5.0 and 40°C.

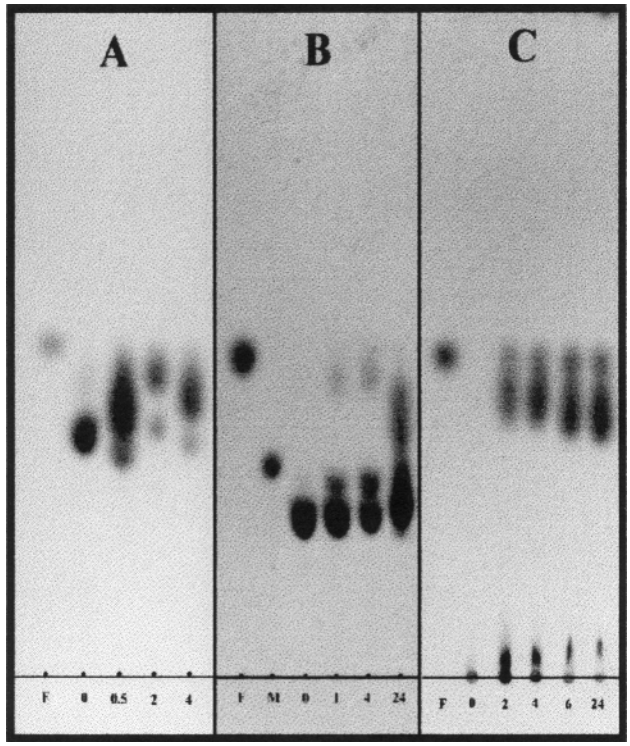

Figure 9 Thin layer chromatography of the products of sucrose (A), raffinose (B) and inulin (C) hydrolysis by purified inulinase, F, Fructose, M, Melibiose.

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