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Properties of a Thermostable Nonspecific Fructofuranosidase Produced by *Cladosporium Cladosporioides* Cells for Hydrolysis of Jerusalem Artichoke Extract

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ABSTRACT

Thermostable invertase (E.C. 3.2.1.26) and inulinase 2,1-β-D-fructan fructanohydrolase (E.C. 3.2.1.7) activities were produced by *Cladosporium cladosporioides* grown on sucrose, inulin, yam extract, or Jerusalem artichoke. The ratio I (inulinase)/S(invertase) activity was between 0.31 and 0.36. Both activities had high temperature optima (60°C) and were stable during pretreatment for 4.5 h at this temperature. Whole cells of *C. cladosporioides* were used for batch fructose production from Jerusalem artichoke extract at several concentrations. With the highest extract concentration used (260 g total sugars/L), total hydrolysis was achieved in 150 min at 60°C. Thin-layer chromatography of the enzymatic hydrolysis of inulin and Jerusalem artichoke extract showed that from the beginning of the reaction, fructose was the only product released. This suggests an exoaction mechanism, β-D-fructofuranoside fructohydrolase [E.C. 3.2.1.2.6]

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Index Entries: Insulinase; invertase; high fructose syrup; Jerusalem artichoke hydrolysis; *Cladosporium cladosporioides.*

INTRODUCTION

Enzymatic hydrolysis of fructose-containing carbohydrate can produce in one step high fructose syrup, practically free from glucose. Alternatively, this hydrolyzed material can be used for fermentation in alcohol production (*1–6*). In order to make the fructan enzymolysis process economically competitive with the high fructose syrup from corn starch, the degree of polymerization, the source of fructans, the rate of inulinase production, and the action mechanism and thermal operational stability of the enzyme require careful attention. The principle of using a thermostable inulinase is industrially significant because the enzyme can be used at higher temperatures for longer times, resulting in increased productivity.

Inulinase activity has been found in a wide variety of microorganisms (*7–8*) being a typical feature of fungi and yeast in which there is occurrence of both inulinase and invertase activities. There has been a general misunderstanding in the literature that the inulase (EC 3.2.1.7) is an enzyme with exosplitting activity. However, the systematic name fructan fructanohydrolase implies that it liberates fructans from the substrate inulin. In fact, the observed inulase activity is represented by a nonspecific fructofuranosidase that fits better under the EC 3.2.1.26 classification because of its exosplitting activity. In this paper, for the sake of continuity with the literature both activities are referred to, but may of course be the result of one and the same enzyme protein.

Regarding the optimal temperature and thermal stability, the inulinase from *Arthrobacter ureafaciens* was reported as the most thermostable, retaining 90% of the activity after pretreatment at 60°C for 30 min without substrate (*7*). Nevertheless, it was with the enzyme from *Kluyvermoyces fragilis* (*6,9–15*) and *K. marxianus* (*5,16–20*) that the majority of studies has been made. Immobilization of inulase from *K. fragilis* on 2-aminoethylcellulose did not improve the enzyme optimal temperature, which decreased from 55 to 45°C (*10*). Entrapment, of the whole cells of *K. marxianus* has been reported in agar (*16*), open pore gelatin (*17,18*) and calcium alginate (*5,19*). Only with the gelatin entrapped cells was there increase in optimal temperature from 50 to 60°C. This immobilized derivative was stable to pretreatment at 60°C for 2 h without substrate, but the operational stability for reuse in several batch cycles was reported only at 50°C (*18*). The inulinase (Novozyme) purified from *Aspergillus ficuum* displayed both exo- and endoaction and invertase activity (*21,22*). Using the enzyme at optimal temperature (60°C), complete hydrolysis of 13% w/v inulin was achieved in 48 h (*21*). A purified preparation from *Panaeolus papillonaceus*

also displayed inulinase and invertase activities. The optimal temperature between 60–65°C was the same for both activities but the thermal stability was similar only at 50°C and below. After pretreatment at 60°C for 1h, without substrate, the residual inulinase activity was around 80%, whereas the invertase retained only 50% of the activity (*23*). One of *A. niger* inulinase isoforms had similar thermal stability (*24*) to that of *A. ficuum* inulase. The immobilization of inulinase from *A. ficuum* on chitin did not improve enzyme thermal stability. This derivative retained approx 50% of the initial activity after pretreatment at the optimal temperature of 60°C for 5 h without substrate (*25*). Stability to reuse was at 40°C, which is still a low temperature for industrial use (*25*).

Studies carried out in our laboratory showed that *Cladosporium cladosporioides* produced a thermostable inulinase when grown on yam- or inulin-containing medium (*26*). Further investigation showed that both inulinase and invertase activities were produced by *C. cladosporioides* grown on several carbon sources. All free cells were stable at the optimal temperature of 60°C when used for hydrolysis of various concentrations of Jerusalem artichoke extracts.

MATERIALS AND METHODS

C. cladosporioides, isolated from yam (*26*), was grown at 28–30°C with shaking in 250 mL Erlenmeyer flasks containing 100 mL of liquid medium at pH 5.5–6.0. The following sterilized liquid media were used: inulin 5% (w/v) supplemented with yeast extract 1% (w/v); sucrose 5% (w/v) supplemented with yeast extract 1% (w/v); yam extract 0.8% (w/v, total sugars); Jerusalem artichoke extract 3.2% (w/v, total sugars). Growth started with 10% (v/v) inoculum. At appropriate time intervals, cells were harvested by filtration, washed with cold distilled water, followed by 0.1*M* sodium acetate buffer, pH 5.0. Cell samples were used for inulinase and invertase assays and for biomass determination.

Jerusalem artichoke extract was prepared by soaking the powder of Jerusalem artichoke tubers supplied by J. O. B. Carioca, Federal University of Ceara, Brazil) in hot (80°C) distilled water (32 g/100 mL). The resultant was homogenized in a blender, filtered by squeezing through a gauze cloth, and centrifuged at 8000*g* for 15 min. Yam extract was prepared from peeled yams (*Dioscorea* sp.), which were sliced and cooked (14 g/100 mL) in boiling distilled water. The thick suspension was filtered through a gauze cloth. Samples were withdrawn from extracts of Jerusalem artichoke and yam for assay of total reducing sugars by dinitrosalicylic acid (DNS) method (*27*) after acid hydrolysis. Biomass was determined by dry wt measurements.

Enzyme assay: Inulinase activity was determined by the assay of reducing sugars released during the incubation with shaking of 200 mg cells (wet wt) in 2 mL of 2% (w/v) inulin (Sigma) at 40°C in 0.1*M* sodium acetate buffer, pH 5.0. Invertase activity was determined using the same conditions except inulin was replaced by 2% (w/v) sucrose. The reducing sugars were assayed by the DNS method (*27*). One unit of activity was defined as the amount of enzyme that releases 1 μmol of reducing sugars/min at 40°C.

The optimal temperature for inulinase and invertase activities was investigated under the enzyme assay conditions at temperatures ranging from 40 to 80°C. The maximal activity was taken as 100%. Thermal stability was determined by the preincubation of cell samples (200 mg, wet wt) at pH 5.0 in 0.1*M* sodium acetate buffer over the temperature range of 40–70°C, for 4.5 h. After rapid cooling, the residual inulinase and invertase activities were assayed under the standard conditions using the optimal temperature (60°C). All experiments were done in duplicate.

Experiments were done with Jerusalem artichoke extracts (2–26%, w/w, total sugars) for determination of time-course hydrolysis by *C. cladosporioides* free cells at 60°C and in 0.1*M* sodium acetate buffer, pH 5.0. The products of the enzymatically hydrolyzed Jerusalem artichoke extract and inulin were identified after 10 times dilution by thin-layer chromatography (TLC) on silica gel 6G (Merck) using solvent systems (*28*) of 1-propanol: ethyl acetate:water 60:20:20 (by vol) and 30:50:20 (by vol). Saccharide spots were revealed with the urea-orthophosphoric acid reagent spray (*29*).

RESULTS AND DISCUSSION

C. cladosporioides cells produce inulinase (I) and invertase (S) activities when grown on sucrose, inulin, and extract of yam or Jerusalem artichoke. The ratio I/S, was between 0.31 and 0.36 (Table 1), although it should be noted that inulin as a substrate experiences greater diffusion problems than sucrose when whole cell enzyme rather than soluble enzyme is being measured. In fact, invertase activity has been reported with inulinases from fungi and yeasts (*7*). Even the highly purified enzyme from *A. ficuum* (*22*) and *A. niger* (*23*) displayed both activities.

Considering the media concentrations used, the best yields of inulinase (110.8 IU/g cells, dry wt) and invertase (350 IU/g cells, dry wt) activities were obtained with 8-d growth of *C. cladosporioides* on yam extract (Table 1). Yam was able to induce the inulinase and invertase activities probably because of the presence of low amounts of sucrose detected in the yam extract, i.e., here, the cultures were C-limited and not N-limited, where excess C represses enzyme production.

Table 1
Effect of Several Carbon Sources at the Production of Inulase and Invertase Activities by *Cladosporium cladosporioides*

Medium	Growth time, d	Activity, IU/g cells dry wt	
		Inulase, (I)	Invertase, (S)
Sucrose (5%)[a]	15	61.23	168.83
Inulin (5%)[a]	15	53.76	147.43
Yam extract (0.8%)[a]	8	110.8	350.0
J. artichoke extract (3 2%)	3	48.36	144.73

[a]Both sucrose and inulin medium were supplemented with 1% w/v yeast extract. Yam extract and Jerusalem artichoke extract concentrations were assayed as total reducing sugars. Activities were assayed under the standard conditions at 40°C, pH 5.0.

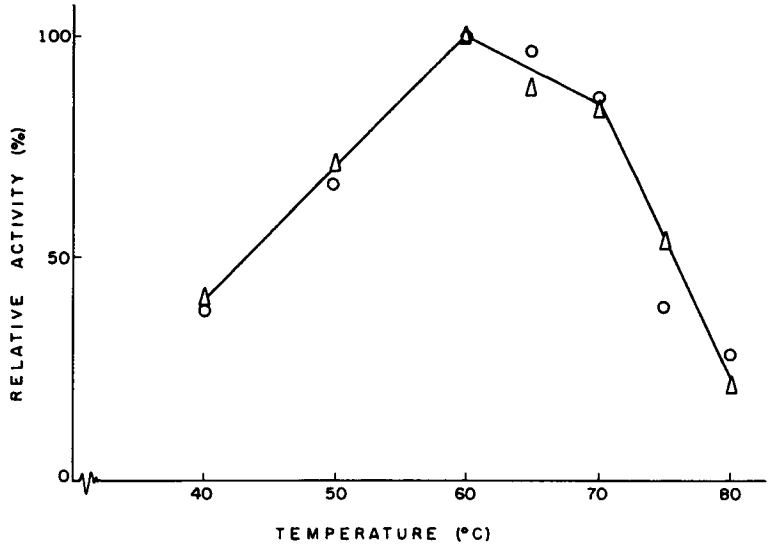

Fig. 1. Optimal temperature of invertase and inulinase from *Clapospórium cladosporioides*. △—△ Invertase; ○—○ inulinase. Incubations of the whole free cells (200 mg wet wt) were performed in 2 mL of 2% sucrose or 2 mL 2% inulin at pH 5.0 at the temperature range of 40–80°C. The maximum activity was taken as 100%.

The optimal temperature was high (60°C) for both activities (Fig. 1). The full inulinase and invertase activities were retained after pretreatment of the cells at the optimal temperature of 60°C for 4.5 h without substrate (Fig. 2). In fact, it was found that both activities were stable at 60°C, without substrate, for a maximum of 20 h. This heat stability places C. *cladosporioides* as the source of the most thermostable inulinase, consider-

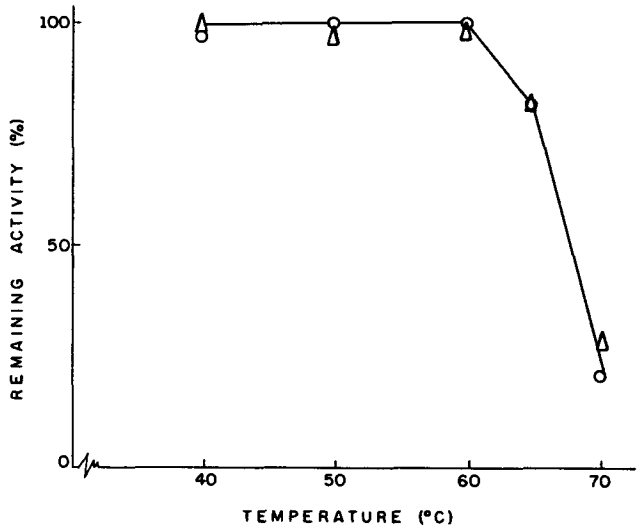

Fig. 2. Thermal stability of invertase and inulinase from *C. cladosporioides*. △—△ Invertase; ○—○ inulinase. Cells were suspended on 0.1*M* sodium acetate buffer, pH 5.0, and were submitted at the temperature range of 40–70°C during 4.5 h. After rapid cooling, the pretreated cells were incubated in 2% sucrose or 2% inulin at 60°C.

ing the best values reported for inulinases from *K. marxianus* (*18*). *A. ficuum* (*21*), and *A. niger* (*24*). Increase of temperature above 60°C sharply decreased the remaining inulinase and invertase activities. Only 80% of these activities remained after pretreatment at 65°C, without substrate.

The time-course hydrolysis at 60°C of Jerusalem artichoke extracts (at 2–26% w/v based on total sugar) showed that, with the most concentrated extract (26%), complete hydrolysis was achieved in 150 min (Fig. 3). A plot of the relative hydrolysis rate vs the substrate (total sugar concentration), where 4.24 mg/mL/min equals the 100% relative hydrolysis rate, showed that the hydrolysis rate was the highest at the 26% w/v substrate concentration (Fig. 4).

Batch hydrolysis at 40°C, using the chitin immobilized inulase purified from *A. ficuum*, achieved a maximum of 80% hydrolysis after 4 h with a dosage of 3840 IU/100 g (total sugar)/L of Jerusalem artichoke extract (*25*). Comparing with this, our results show that the whole (free) cells of *C. cladosporioides* achieved complete hydrolysis in 2.5 h at 60°C, using a dosage of 2100 IU/260 g (total sugar)/L of Jerusalem artichoke extract.

The repeated batch hydrolysis at 60°C of Jerusalem artichoke extract (26% w/v total sugar) was investigated using the same cells for 12 batch cycles of 4 h each. At the end of each batch cycle, the spent extract was replaced by fresh Jerusalem artichoke extract. In all batch cycles with 2.5 h reactions, the hydrolysis was 100%. This shows the high stability of *C. cladosporioides* cells, which at 60°C in several batch cycles displayed the

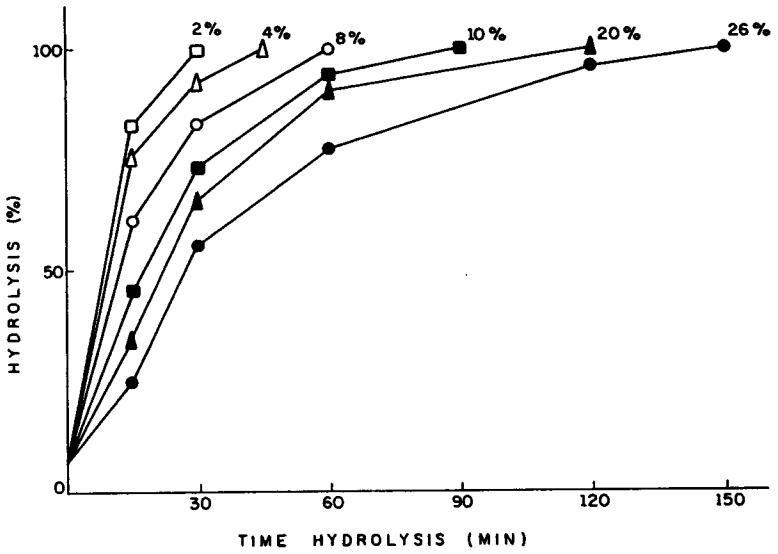

Fig. 3. Time-course hydrolysis of Jerusalem artichoke extracts by *C. cladosporioides,* as a function of total reducing sugar concentration. Incubations of whole cells (200 mg wet wt) were performed in 2 mL of Jerusalem artichoke extracts at 2–26% w/v (total reducing sugars) in 0.1*M* sodium acetate buffer, pH. 5.0, at 60°C.

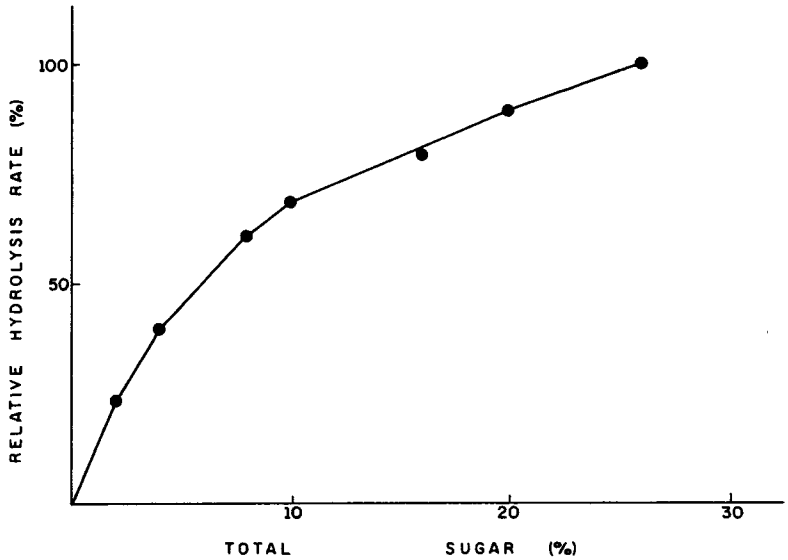

Fig. 4. Effect of total sugar concentration on the hydrolysis rate of Jerusalem artichoke extracts by *C. cladosporioides.* (4.24. mg/mL/min = 100% hydrolysis (taken from results of Fig. 3.))

same operational stability as *K. Marxianus* gelatin-entrapped cells, previously reported as the best but working only at 40°C (*18*). No difference was found when hydrolysis was carried out with Jerusalem artichoke extract prepared in water or 0.1*M* sodium acetate buffer, pH 5.0.

The TLC of the products of the enzymatic hydrolysis of Jerusalem artichoke extract and inulin show that, right from the start of the reaction fructose was released. Since fructooligosaccharides were not detected, it is assumed that the inulinase from C. *cladosporioides* has an exoaction mechanism. Endo- or exoaction, but not both, mechanisms have been reported for inulinase from several microbial sources (*7*). Only the inulinase from *A. ficuum* displayed both endo and exo mechanisms (*21–22*).

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