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Characterization and Properties of an Inulinase from a Thermophilic Bacteria

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SUMMARY

*The inulinase of the thermophilic bacterial strain LCB41 (*Bacillus *sp.) was produced in fermentor using a mineral medium containing inulin as carbon source. The enzyme content was as high as the known inulinase producers and most of the activity was found in the culture medium. The enzyme was stable at high temperature and active at neutral and slightly basic pH. Fructose is liberated as the sole reaction product of inulin hydrolysis, classifying the enzyme as an exoinulinase. Inulin and sucrose were both hydrolyzed at appreciable rates with an (I/S) ratio of 0·40 and* $(V_m/K_m)_I/(V_m/K_m)_S = 9{\cdot}9$. *The enzyme was less inhibited than yeast invertase or* Kluyveromyces fragilis *inulinase at high sucrose concentrations. The inulinase of strain LCB41 is a good candidate for industrial hydrolysis of inulin or sucrose.*

INTRODUCTION

Inulin is a fructan found as a reserve carbohydrate in the tubers and roots of various plants like Jerusalem artichoke, dahlia or chicory. These renewable substrates offer interesting perspectives for the production of fructose syrups. The fructans are formed of a linear chain of fructose units with a terminal glucose, all being linked by β-2,1 osidic bonds. The degree of polymerization varies from 4 to 35.

Since enzymatic hydrolysis of inulin has been shown to give higher yields than acid hydrolysis (Fleming & Grootwassink, 1979; Toran-Diaz *et al.*, 1980), there is a need for an inulinase preparation with high

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hydrolysis performances. Inulinases are 2,1-β-D-fructan-fructanohydrolases (EC 3.2.1.7), most of them split-off fructose units from inulin. The enzymes from several yeasts and fungi have been studied in the laboratory scale and their properties reviewed (Vandamme & Derycke, 1983).

There are few reports on bacterial inulinases (Uchiyama *et al.*, 1973; Vandamme & Derycke, 1983) in the literature. In our laboratory, bacterial strains growing on inulin as the sole carbon and energy source have been isolated from soil samples (Allais *et al.*, 1986, 1987). Thirty-two mesophilic, five thermotolerant and four thermophilic bacterial strains were isolated and studied. All bacteria showed inulinase activity and are candidates for an industrial source of enzyme. Among them, strain LCB41 (*Bacillus* sp.) was selected for the following reasons: (a) it is a thermophilic strain able to grow up to 60°C; (b) it produces an extracellular inulinase; (c) the enzyme level (200 units liter^{-1}) compares favorably to the yeast one.

In this paper the enzymatic properties of this thermostable inulinase are described and its possible use for inulin (or sucrose) hydrolysis is suggested.

MATERIALS AND METHODS

Enzyme production

Strain LCB41 *Bacillus* sp. was isolated in the laboratory on a mineral medium containing inulin (Allais *et al.*, 1987). It was maintained on agar slants containing (per liter of distilled water): inulin (Merck), 2·0 g; $(NH_4)_2SO_4$, 1·0 g; KH_2PO_4, 3 g; $MgSO_4.7H_2O$, 0·5 g; mineral salt solution (Cooney & Levine, 1972), 2 ml; agar, 20 g. The pH was adjusted to 8·0. After incubation at 50°C (24 h) the slants were stored at 4°C and transferred every month.

Liquid batch cultures (1·2 liters) were grown in a fermentor (2 liters, Biolafitte) under the following conditions: impeller speed 200 rpm, aeration rate 1·2 VVM (volume of air per liquid volume per minute), temperature 50°C, pH regulated at 8·0. The medium described for slant preparation was used (agar omitted). Inulin was sterilized separately at 110°C and added to the mineral salts (sterilized at 120°C) before inoculation. The inoculum culture was prepared in erlenmeyer flasks using the same conditions, for 17 h. The optical density (620 nm) of the cell suspension was adjusted to 1·0 by dilution with medium and the fermentor culture was started with an 8% inoculum (v/v). Samples were

taken at regular intervals from the fermentor and analyzed for biomass, total and reducing sugars, and inulinase activity.

At the end of fermentation (11 h) the cells were separated by continuous centrifugation and the clear supernatant was used as the enzyme source.

Inulinase assay

Enzyme activities were assayed using inulin as substrate. The incubation mixture (0·5 ml) contained inulin (10 g liter^{-1}) in a 50 mM potassium phosphate buffer, pH 7·0. After enzyme addition the mixture was incubated for 10 min at 30°C and the reaction stopped by addition of 2 ml of Somogyi reagent (Somogyi, 1952) and 1·5 ml of water. The mixture was then boiled for 15 min, cooled to room temperature and 1 ml of Nelson reagent (Nelson, 1944) was added. The optical density at 550 nm was immediately measured. Blanks were run simultaneously with enzyme and substrate solutions. A calibration curve was obtained using a standard solution of fructose. One inulinase unit was the amount of enzyme which liberates one micromole of fructose per minute under the above conditions.

Invertase assay

Sucrose hydrolysis activity was measured in an incubation mixture (0·5 ml) containing 50 mM sucrose dissolved in a 10 mM potassium phosphate buffer pH 7·0. After enzyme addition, the mixture was incubated for 10 min at 30°C and the reducing sugars assayed as described for the inulinase assay. The calibration curve was obtained using an equimolar standard solution of glucose and fructose. One invertase unit was the amount of enzyme which hydrolyzed one micromole of sucrose per minute.

The invertase activity was also assayed in the Sumner conditions: sucrose concentration was 4·5% (w/v) in a 10 mM sodium acetate buffer, pH 4·7. Incubation was at 20°C for 5 min and reducing sugars were assayed as before. One Sumner unit was the amount of enzyme which liberates 1 mg of inverted sugar in 5 min in the above conditions. In some experiments the temperature was 34°C and the pH 7·1.

Analytical methods

Biomass was determined by the dry cell weight method using 5 ml of culture medium. Total sugars were measured by the anthrone method

(Sattler & Zerban, 1948) and reducing sugars by the Somogyi–Nelson method (Nelson, 1944; Somogyi, 1952). Thin layer chromatography was done on Merck silicagel plates (200 × 200 × 2·5 mm). The migration solvent was ethylmethylketone/acetic acid/water in the proportions 3/1/1 (v/v/v). Sugars were revealed by spraying a 0·5% solution of naphtoresorcinol in a mixture of acetone and phosphoric acid 22/1 (v/v).

RESULTS AND DISCUSSION

Inulinase production in the fermentor

The kinetics of growth and inulinase production were first studied in a fermentor with the pH controlled at 8·0, temperature 50°C, aeration rate 1·2 VVM and agitation speed 200 rpm. The results are shown in Fig. 1. The specific growth rate was 0·37 h^{-1} and a cell concentration of 0·22 g $liter^{-1}$ was obtained in 11 h. At this point 62% of the inulin was consumed and the cell yield was 0·18 g g^{-1}. During the whole fermentation the amount of reducing sugars in the culture medium was less than 35 mg $liter^{-1}$. The inulinase activity in the culture medium increased to reach a maximum value of 200 units $liter^{-1}$. Some activity was also detected in the cell fraction but it did not exceed 20% of the total activity. The enzyme production was not proportional to cell growth, as shown by the increase in specific activity (inulinase units divided by biomass concentration) from 30 u g^{-1} ($t = 2{\cdot}3$ h) to 920 u g^{-1} ($t = 6$ h).

These data for cell growth and inulinase production are similar to the ones already reported in shake-flasks (Allais *et al.*, 1987). The same amount of enzyme (200 u $liter^{-1}$) was produced in a very short time (11 h). Inulinase production by yeast is in the same range of enzyme level (Vandamme & Derycke, 1983). Strain LCB41 offers several advantages: (a) fermentation at 50°C reduces the probability of contamination; (b) the enzyme is extracellular; (c) the fermentation time is much shorter than for yeast. However, a further increase in enzyme level will be helpful for industrial production.

Enzyme preparation

The cells from the previous experiment were separated by continuous centrifugation and the supernatant concentrated by ultrafiltration with a hollow fiber cartridge (Amicon DC-2). The concentrated solution (13-fold) was used as enzyme preparation. More than 95% of the original activity was recovered in the concentrate. The storage stability of this

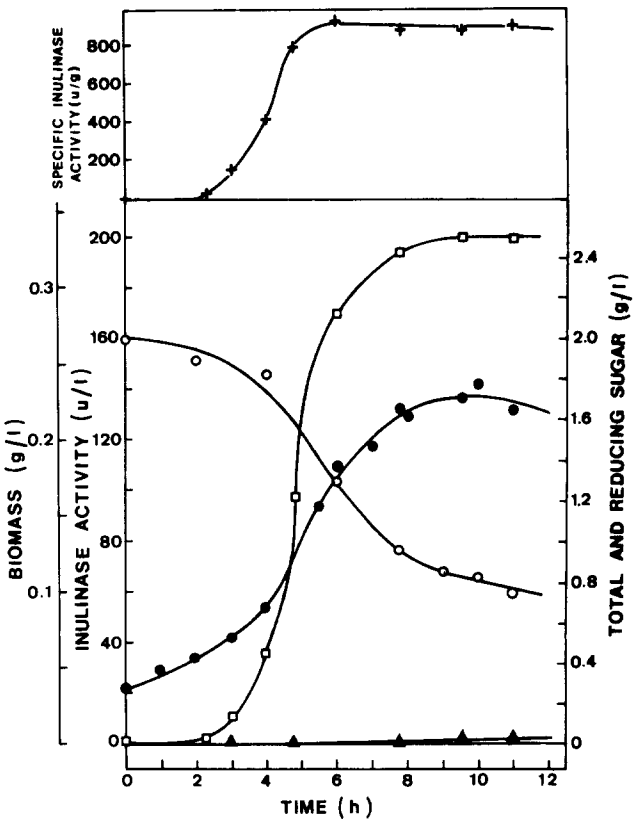

Fig. 1. Kinetics of inulinase production in the fermentor with strain LCB41. Key: ●, biomass; ○, inulin; ▲, reducing sugars; □, inulinase activity in culture medium. Top graph: specific inulinase activity (+) expressed in units per gram of cell.

preparation, at 4°C, was checked during a period of 2 months. As shown in Table 1, there was no loss of activity during this period, indicating a high stability of the inulinase preparation. This one-step preparation method can be easily scaled up to larger volumes if necessary.

Effect of temperature on activity

The effect of temperature on inulinase activity was checked between 10 and 80°C (Fig. 2). The activity increased from 10 to 45°C and then decreased slowly. At 60°C a total of 70% of the maximal activity was measured. In the conditions used, a maximal activity was found at 45°C. This low value is quite surprising since the enzyme is produced by a thermophilic bacteria able to grow up to 60°C. The difference between

TABLE 1
Storage Stability of Inulinase Preparation[a]

Time (*day*)	*Remaining activity* (%)
0	100
26	105
29	107
54	109
60	109

[a] The enzyme preparation was stored at 4°C. The remaining activity was assayed in standard conditions with inulin as substrate.

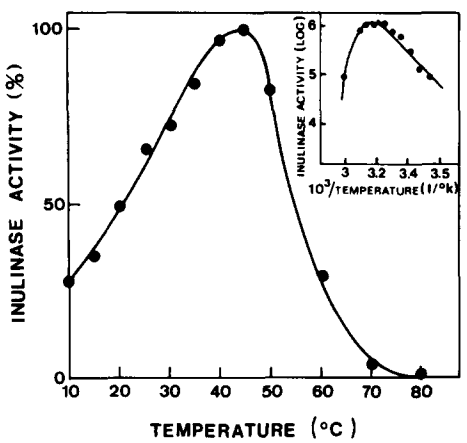

Fig. 2. Effect of temperature on the inulinase activity. Activity was assayed with inulin as substrate in the conditions described in 'Materials and Methods' and the incubation temperature was varied from 10 to 80°C.

these optimal temperatures for enzyme activity and cell growth might result from the different conditions used for growth and enzyme assay (buffer, pH, etc.). The optimal temperature of the inulinase from strain LCB41 is similar to the one reported for *Arthrobacter ureafaciens* (50°C) (Uchiyama *et al.*, 1973), *Candida kefyr* (50°C) (Negoro, 1973; Negoro & Kito, 1973) and *Candida salmenticensis* (46°C) (Guiraud *et al.*, 1980), but lower than the one for *Kluyveromyces fragilis* (55°C) (Nahm & Byun, 1977; Negoro, 1978), *Aspergillus* sp. (60°C) (Zittan, 1981) and *Aspergillus niger* (55°C) (Derycke & Vandamme, 1984). The Arrhenius plot is also shown in Fig. 2. An activation energy of 7·1 kcal mol^{-1} was esti-

mated from this plot. This value is close to the one (8·0 kcal mol^{-1}) reported for the *K. fragilis* enzyme (Nahm & Byun, 1977).

The effect of temperature on enzyme stability was checked in a 100 mM sodium acetate buffer, pH 5·0, for an incubation time of 10 min. The results (Fig. 3) show that the activity was stable at 50°C and completely lost at 70°C. The temperature giving 50% inactivation was 63°C in these conditions. The effect of temperature on enzyme stability was almost the same for the following inulinases: *C. kefyr* (Negoro, 1973; Negoro & Kito, 1973), *C. salmentisensis* (Guiraud, 1980), *Debaromyces phaffi* (Demeulle *et al.*, 1981) and *A. niger* (Nakamura, 1978).

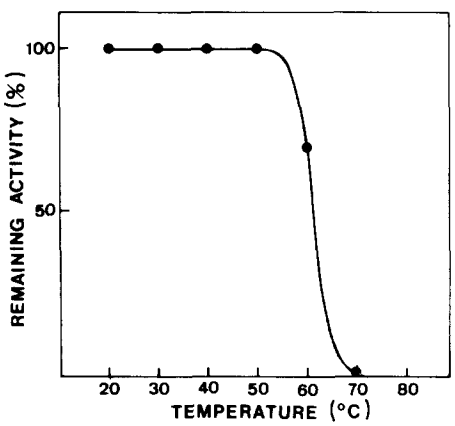

Fig. 3. Effect of temperature on the inulinase stability. The enzyme preparation was incubated for 10 min in a 100 mM sodium acetate buffer at different temperatures from 20 to 80°C. The remaining activity was then assayed at 30°C.

Effect of pH on activity

The activity on inulin was tested at pH values from 3·7 to 9. As shown in Fig. 4(a), the enzyme was not active at pH 4·0 and below, a maximum was attained for pH 6·0 and some activity (45%) remained at pH 9·0. Compared with the yeasts and fungi inulinases the optimum pH is higher (usually 4·5–5·0) and identical to the bacteria *A. ureafaciens* (Uchiyama *et al.*, 1973). The enzyme stability at different pH is also shown in Fig. 4(b). The enzyme was stable from pH 5 to 8 in the conditions used. These results clearly demonstrate that the bacterial inulinase from strain LCB41 showed high activity and stability at neutral or basic pH. This is not the case of the yeasts or fungi enzymes and offers a possibility of new applications for the LCB41 inulinase.

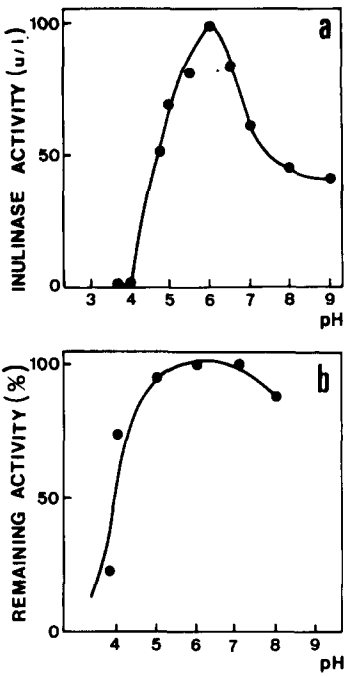

Fig. 4. Effect of pH on the inulinase activity. (a) The activity on inulin was assayed in standard conditions except that the pH was varied from 3·7 to 9·0. (b) The enzyme preparation was incubated for 10 min at 30°C in a 100 mM sodium acetate buffer (from pH 3·7 to 5·0) or in a 100 mM potassium phosphate buffer (from pH 6·0 to 8·0) and the remaining activity assayed in the standard conditions

Mode of action on inulin

The inulinase preparation was incubated with inulin and samples were taken at 1, 5, 10, 20 and 60 min from the reaction mixture. They were analyzed by thin layer chromatography in conditions where inulin, oligofructosides, glucose and fructose were separated. The results (Fig. 5) clearly demonstrate that fructose is the first product of inulin hydrolysis. No glucose or oligofructosides appeared in the samples tested at early stages (5 and 10 min) of hydrolysis. This is a typical mode of action of an exoinulinase attacking the inulin molecule (n sugar residues) from the fructose side and liberating one fructose unit and an oligofructoside containing $n-1$ sugar residues. Most of the described inulinases showed an exoinulinase mode of action (Vandamme & Derycke, 1983). However, endoinulinases have been isolated and studied from *Aspergillus* sp. (Nakamura *et al.*, 1978; Zittan, 1981; Ettabili & Baratti, 1987). The bacterial inulinase from *A. ureafaciens* degrades inulin giving a difructose anhydrid as product (Tanaka *et al.*, 1972).

Effect of inulin and sucrose concentrations on activity

The effect of inulin concentration (in the range 10–100 g liter^{-1}) and sucrose concentration (in the range 20–180 g liter^{-1}) on the enzyme

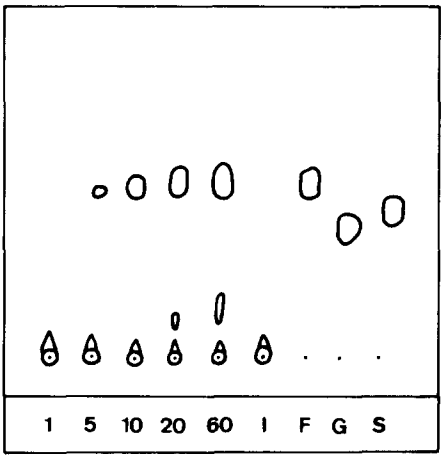

Fig. 5. Thin layer chromatography of the reaction products from inulin hydrolysis. Samples 1, 5, 10, 20 and 60 were taken from the reaction mixture at the corresponding time (min) of incubation. Samples I, F, G and S were standards for inulin, fructose, glucose and sucrose.

activity was measured in the optimal conditions for temperature (45°C) and pH (8·0). The results in Fig. 6 show that Michaelis–Menten kinetics were obtained in both cases. Several authors (Nahm & Byun, 1977; Grootwassink & Fleming, 1980; Vandamme & Derycke, 1983; Derycke & Vandamme, 1984) have reported non-Michaelian kinetics on inulin. They explain this behavior by the low solubility of this substrate and by the presence of polymers of different molecular weight in the commercial preparation used. This was not the case in our experiments; the inulin preparation (Merck) contained less than 0·6% reducing sugars and was homogeneous in terms of molecular weight. Using a molecular weight of 5400 for inulin a K_m of 15·4 mM and a V_m of 3·8 μmol min^{-1} liter^{-1} were calculated. In the same conditions of temperature and pH the kinetic parameters for sucrose hydrolysis were $K_m = 255$ mM and $V_m = 6{\cdot}4$ μmol min^{-1} liter^{-1}. These kinetic parameters are compared in Table 2 with other inulinases. The K_m value for inulin of strain LCB41 is close to the one observed for the yeast enzymes. In contrast, the K_m value for sucrose is the highest one reported. As a result, the bacterial enzyme has a stronger inulinase specificity than the yeast enzymes. This is partially reflected in the I/S ratio: strain LCB41 showed the highest one (with the exception of fungal enzymes). A better understanding of the enzyme specificity is given by the ratio of the V_m/K_m for inulin and sucrose. This ratio had a value of 9·9 for strain LCB41. According to the definition of Ettabili (1985), a ratio higher than 1 corresponds to a true inulinase and a ratio lower than 1 to an invertase.

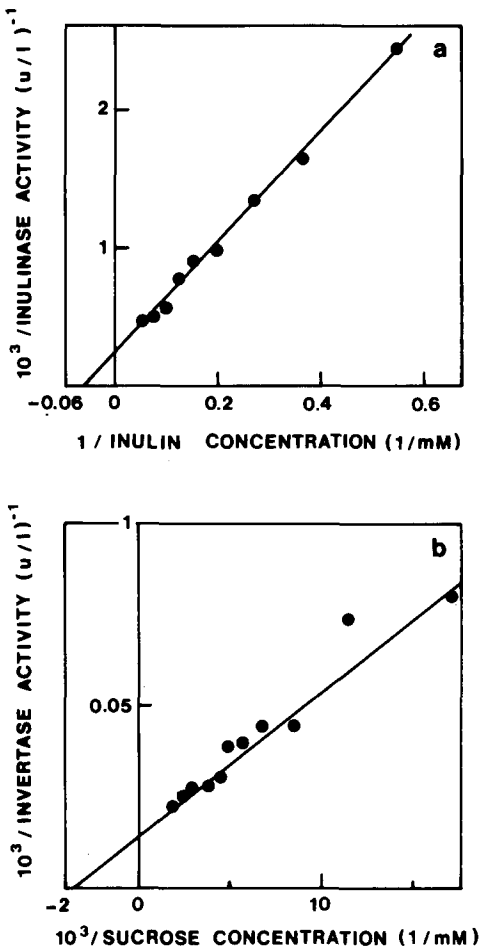

Fig. 6. Effect of inulin and sucrose concentrations on enzyme activity. The standard assays were used except that the substrate concentration was varied from 10 to 100 g litre^{-1} for inulin and from 20 to 180 g liter^{-1} for sucrose.

Comparison of activity on inulin and sucrose

The activities on inulin and sucrose of the enzyme preparation are shown in Table 3. The I/S ratio was 0·40 in the standard conditions for assays. The activity on sucrose was also expressed in Sumner units (20°C, pH 4·7 and sucrose concentration 4·5%). It was 21-fold lower compared with the standard conditions. However, it was 30-fold higher when the Sumner conditions were adapted to the LCB41 enzyme, i.e. temperature was increased to 34°C and pH to 7·1. The preparation was

TABLE 2
Comparison of Kinetic Parameters of Microbial Inulinases

Microorganism	K_m *on inulin* (*mM*)	K_m *on sucrose* (*mM*)	*I/S ratio*[a]	*Reference*
Penicillium sp.				
T.I	0·17	—	1·96	Nakamura & Nakatsu (1977)
T.II	0·23	—	0·65	Nakamura & Nakatsu (1977)
T.III	0·16	—	1·06	Nakamura & Nakatsu (1977)
A. niger				
T.I	1·87	—	0·80	Nakamura et al. (1978)
K. fragilis[b]	8·0	6·7	0·29	Byun & Nahm (1977)
C. salmenticensis[b]	17·0	43·0	0·05	Guiraud *et al.* (1980)
D. phaffii[b]	12·0	31·0	0·13	Demeulle *et al.* (1981)
D. cantarelli[b]	15·0	55·0	0·11	Guiraud *et al.* (1982)
LCB41	15·4	255	0·40	This work

[a] Ratio of activity on inulin to activity on sucrose measured in the standard assay conditions.
[b] These authors have used for inulin a molecular weight of 5000 instead of 5400.

TABLE 3
Comparison of the Activities on Inulin and Sucrose of the Inulinase Preparation from Strain LCB41

Assay conditions[a]	*Substrate*	*Activity* (*unit* ml^{-1})
30°C, pH 7·0, standard conditions	Inulin	2·6
30°C, pH 7·0, standard conditions	Sucrose	6·3
20°C, pH 4·7, Sumner conditions	Sucrose	0·3
34°C, pH 7·1, Sumner conditions	Sucrose	186·5

[a] see 'Materials and Methods' for the definition of enzyme units.

very active in these conditions (186·5 u ml^{-1}), confirming the results observed on inulin as function of pH (Fig. 4).

Effect of high sucrose concentrations

Since the enzyme from strain LCB41 was more active on sucrose than on inulin (I/S = 0·40), it can also be used for sucrose hydrolysis in replacement of yeast invertase. To compare these two enzymes the

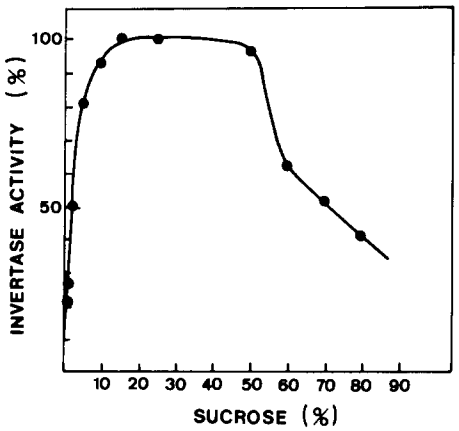

Fig. 7. Effect of sucrose concentration on the sucrose hydrolysis activity of LCB41 inulinase. The initial rate of sucrose hydrolysis was measured at pH 7·0 and 50°C at different sucrose concentrations (weight/volume).

sucrose hydrolysing activity was measured at sucrose concentrations from 1 to 80% (w/v). The results are shown in Fig. 7. The activity on sucrose increased to attain a plateau between 15 and 50% and then decreased. In the same conditions yeast invertase (Combes & Monsan, 1983) and inulinase from *K. fragilis* (Grootwassink & Fleming, 1980) showed a maximal activity at 10% sucrose and then a sharp decrease was observed. For instance, at a sucrose concentration of 50% the relative activities were 97% for LCB41, 76% for yeast invertase and 62% for the *K. fragilis* inulinase. It appeared that the inulinase from the thermophilic bacteria was more active at high sucrose concentrations than the two other enzymes. As a result, it could be useful for the hydrolysis of sucrose when high concentrations of this substrate are used.

Inulin and sucrose hydrolysis

The LCB41 inulinase was then used in a batch reactor for inulin hydrolysis. A temperature of 40°C was selected to improve enzyme stability. High concentrations of 20 and 100 g liter^{-1} of commercial inulin were used. The data (Fig. 8) showed a high rate of hydrolysis for 3 h yielding fructose concentrations of 11·4 (inulin 20 g liter^{-1}) and 25 g liter^{-1} (inulin 100 g liter^{-1}). The initial rates of hydrolysis calculated during 1 h were, respectively, 7 g liter^{-1} h^{-1} (1·4 g unit^{-1} h^{-1}) and 13 g liter^{-1} h^{-1} (2.6 g unit^{-1} h^{-1}). Then the hydrolysis rates decreased and maximal hydrolysis of 70 and 40% were obtained. Product inhibition or enzyme inactivation might explain these results.

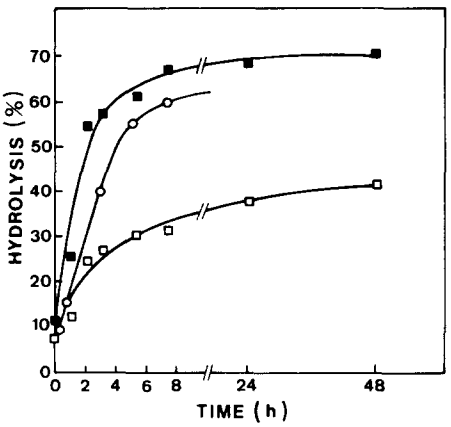

Fig. 8. Kinetics of inulin and sucrose hydrolysis. Inulin (20 g liter^{-1} (■) and 100 g litre^{-1} (□) was incubated at 40°C, pH 8·0, with 5 u liter^{-1} of LCB41 inulinase. Sucrose 100 g liter^{-1} (○) was hydrolyzed in the same conditions.

The hydrolysis of a sucrose solution (100 g liter^{-1}) is also shown in Fig. 8. In 5 h, a reducing sugar concentration of 55 g liter^{-1} was obtained. The initial rate of hydrolysis was 13·3 g liter^{-1} h^{-1} (2·7 g unit^{-1} h^{-1}).

These data demonstrate the high potential of the LCB41 inulinase for inulin or sucrose hydrolysis.

CONCLUSIONS

The properties of the inulinase from the thermophilic bacteria LCB41 were tested to evaluate its potential use for industrial hydrolysis of inulin or sucrose.

The enzyme could be easily produced by growing the strain on a mineral medium containing inulin as sole carbon source. An enzyme preparation was obtained by concentration of the culture medium by ultrafiltration and further used. The effect of pH and temperature on enzyme activity and stability showed two important advantages of the LCB41 inulinase: thermostability (although not as high as expected) and activity at neutral or slightly basic pH.

The LCB41 inulinase was also active on sucrose and less inhibited at high sucrose concentrations than other known enzymes. These properties might be very useful for sucrose hydrolysis in reactors, since less substrate inhibition is expected and thus better productivities in terms of sucrose hydrolysis per unit weight of enzyme can be obtained.

Finally, LCB41 inulinase was demonstrated to be an exoenzyme which hydrolyzed inulin from its fructose end and liberated fructose as a main reaction product.

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