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Isolation and Characterization of Thermophilic Bacterial Strains with Inulinase Activity

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Nine bacterial strains growing on inulin as the sole carbon and energy source were isolated from soil samples by enrichment culture on a mineral medium. Four of the strains were thermophilic and belong to the genus *Bacillus*. The thermophilic strains synthesized a β-fructosidase that was active on both inulin and sucrose. The presence of inulin in the culture medium is necessary for enzyme synthesis. Most of the activity on inulin was recovered in the culture medium, and the enzyme was synthesized during cell growth.

Inulin is a linear fructose polymer of plant origin found in the tubers of Jerusalem artichoke in large amounts. After enzymatic hydrolysis, it is potentially useful for the production of high-fructose syrups (17) or as a substrate for alcohol production by fermentation (4, 6, 14; J.-J. Allais, E. Favela Torres, and J. Baratti, Biotechnol. Bioeng., in press).

Inulinases are β-fructosidases used for the hydrolysis of inulin. These enzymes were first isolated from plants. The microbial enzymes from yeasts and fungi have been intensively studied (17), while bacterial inulinases are less well known (13, 15). In a previous study in our laboratory, a total of 32 mesophilic bacterial strains with inulinase activity were isolated and characterized (1).

In this paper, we describe the isolation and characterization of four thermophilic bacterial strains with inulinase activity. These strains synthesize thermostable enzymes which are of potential industrial use for inulin or sucrose hydrolysis.

MATERIALS AND METHODS

Culture media. The isolation medium contained (per liter of distilled water): $(NH_4)_2SO_4$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; KH_2PO_4, 3.0 g; inulin (E. Merck AG), 2.0 g; and mineral salt solution (2), 2 ml. Unless otherwise noted, the pH was adjusted to 7.0. The mineral salts and inulin were sterilized separately at 120 and 110°C, respectively.

Isolation of bacterial strains. Enriched natural sources of bacteria included compost which had been treated daily with Jerusalem artichoke juice for 2 weeks and a soil sample from a Jerusalem artichoke field. Both samples were from Sousse, Tunisia. For the enrichment culture, 1 g of soil was dispersed in 30 ml of medium and incubated at 50°C. After 24 h, 1.5 ml of the culture was transferred into 30 ml of fresh medium, and, after an additional 24 h, 0.5 ml was inoculated into 30 ml of medium. Single colonies were then isolated from inulin agar and purified by five cycles of liquid culture and single-colony isolation.

Batch cultures. Erlenmeyer flasks (100-ml volume) were charged with 30 ml of medium, inoculated (10%) with a culture previously incubated for 18 h, and incubated at 50°C for 24 h. A 2-liter fermentor was also used, under conditions previously described (1).

Analytical methods. Biomass was determined by dry cell weight, total protein was determined by the method of Lowry et al. (5), total sugar was determined by the anthrone method (11), and total reducing sugar was determined by the Nelson method (10) as modified by Somogyi (12). The activity on inulin or sucrose was measured as the rate of liberation of reducing sugars, as previously reported (1). Cells from 5 ml of culture medium were isolated by centrifugation (the supernatant was labeled fraction E), suspended in 10 mM potassium phosphate buffer (pH 7) containing 5 mM cysteine and 10^{-4} M phenylmethylsulfonyl fluoride, and homogenized with a French press (4.9×10^8 Pa, four passages). The suspension was then centrifuged at 12,000 × *g* for 10 min, and the supernatant was labeled fraction I. Jerusalem artichoke juice was prepared as previously reported (Allais et al., in press).

RESULTS

Isolation of bacterial strains. Nine bacterial strains growing on a mineral medium containing inulin as the sole carbon and energy source were isolated by enrichment culture as described in Materials and Methods. Five strains were able to grow at both 50 and 37°C, showing a typical thermotolerant pattern (Table 1). The four remaining strains were determined to be thermophilic since they could grow at 50°C, but not at 37°C. On average, three strains per soil sample were isolated. This average is most probably the result of both sample choice (soil from Tunisia) and treatment in situ with

TABLE 1. Isolation of thermophilic inulin-degrading bacterial strains

Strain	Soil sample[b]	Growth at:		Specific growth rate[c] (per h)
		37°C	50°C	
LCB34	C1	+	+	
LCB35	C2	+	+	
LCB36[a]	C2	−	+	0.65
LCB37	C1	+	+	
LCB38	C1	+	+	
LCB39	C2	+	+	
LCB40[a]	JA	−	+	0.63
LCB41[a]	JA	−	+	0.54
CB42[a]	JA	−	+	0.61

[a] Identified as a *Bacillus* sp.
[b] C, Compost; JA, Jerusalem artichoke field.
[c] Cultivated in the selection medium at pH 7.0 and 50°C.

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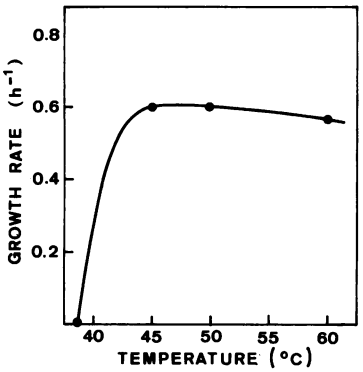

FIG. 1. Effect of temperature on the specific growth rate of strain LCB41. Strain LCB41 was cultivated in shake flasks in the selection medium at pH 7.0 at various temperatures.

Jerusalem artichoke juice. The synthesis of thermostable inulinases by the four thermophilic strains LCB36, LCB40, LCB41, and LCB42 was further studied.

Strain characterization. The four strains were grown in shake flasks in the isolation medium at pH 7.0, and the specific growth rate and inulinase and invertase activities were determined. The growth rate was in the range of 0.54 to 0.65 /h (Table 1). These are high values for bacterial growth on a mineral medium lacking added vitamins or organic nutrients. As shown below, strain LCB41 showed a higher growth rate at pH 8.0. All strains were rod-shaped, aerobic bacteria that form endospores. Hence, they belong to the genus *Bacillus*.

Effect of temperature and pH. The effect of temperature and pH on strain LCB41 was investigated. The strain grew at temperatures from 45 to 60°C at an almost constant growth rate of 0.6/h (Fig. 1). As noted above, no growth was observed at 37°C. Temperatures higher than 60°C were not tested. A temperature of 50°C was selected for further experiments.

The pH of the culture medium showed a slight effect on the specific growth rate, which was around 0.55 to 0.60/h from pH 7.0 to 8.5 (Fig. 2). At pH values of 6.5 and lower, no growth was observed. The activity on inulin in the culture medium as a function of medium pH is also shown in Fig. 2.

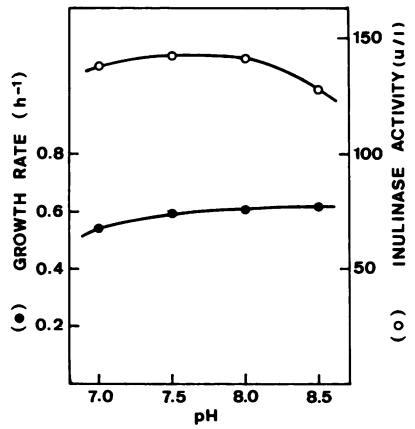

FIG. 2. Effect of pH on the specific growth rate and activity on inulin of strain LCB41. Strain LCB41 was cultivated in shake-flasks in the selection medium at 50°C at various pH levels. Activity on inulin was assayed after 24 h.

TABLE 2. Activity on inulin and sucrose of thermophilic inulin-degrading bacteria[a]

Strain	Bacterial activity of the following fraction[b] (U/liter) on:				Inulin-Sucrose[c]
	Inulin		Sucrose		
	E	I	E	I	
LCB36	73	0	148	10	0.46
LCB40	259	23	473	41	0.55
LCB41	113	35	265	59	0.46
LCB42	93	0	194	5	0.47

[a] Bacterial strains were cultivated in the selection medium at 50°C and pH 7.0 for 24 h.
[b] E, Fraction E; I, fraction I. For details, see Materials and Methods.
[c] Of the total activity (E + I).

The activity was almost constant, with a slight decrease at pH 8.5. From these data, pH 8.0 was selected for further experiments.

Activity on inulin and sucrose. Strains LCB36, LCB40, LCB41, and LCB42 were cultivated on the inulin medium, and enzyme activity toward inulin and sucrose was assayed (Table 2) in the culture medium (fraction E) and in the cell (fraction I). The total activity (fraction E + fraction I) on inulin ranged from 73 to 282 U/ml. The majority (76 to 100%) of this activity was found in the culture medium (fraction E), and only a small amount was present in the cell (fraction I). There were only minor variations among the four strains in both the total activity on inulin and in the localization of this activity. All of the strains also displayed activity on sucrose, in the range of 158 to 514 U/liter. This sucrose-hydrolyzing activity was higher than that on inulin. The inulin-sucrose ratios were very similar for the four strains (0.46 to 0.55). As for inulin, the majority of the activity on sucrose was found in the culture medium (fraction E). The inulin-sucrose ratios for fractions E and I were almost identical for the four strains, which is an indication that these fractions contained only one enzyme active on both inulin and sucrose.

Effect of carbon source on activity on inulin. Strain LCB41 was cultivated on the mineral medium with different carbon sources to search for inducers or repressors of the enzyme biosynthesis. Growth and activity on inulin were measured (Table 3). Growth on glucose, sucrose, fructose, and inulin was almost identical (optical density in the range of 0.76 to 0.85). Yeast extract sustained a better growth (optical den-

TABLE 3. Effect of carbon source on growth and inulin activity of strain LCB41[a]

Carbon source	Growth (OD at 620 nm)[b]	Activity on inulin (U/liter) of fraction:	
		E	I
Glucose	0.76	51	4
Sucrose	0.85	20	1
Fructose	0.76	7	0
Yeast extract	1.34	15	0
Inulin	0.79	127	68
Inulin + glucose	1.39	106	31
Inulin + sucrose	1.34	91	16
Inulin + fructose	1.35	61	12
Inulin + yeast extract	1.25	176	

[a] Strain LCB41 was cultivated on mineral medium at pH 8.0 with different carbon sources at a concentration of 2 g/liter. Activity was assayed after 24 h.
[b] OD, Optical density.

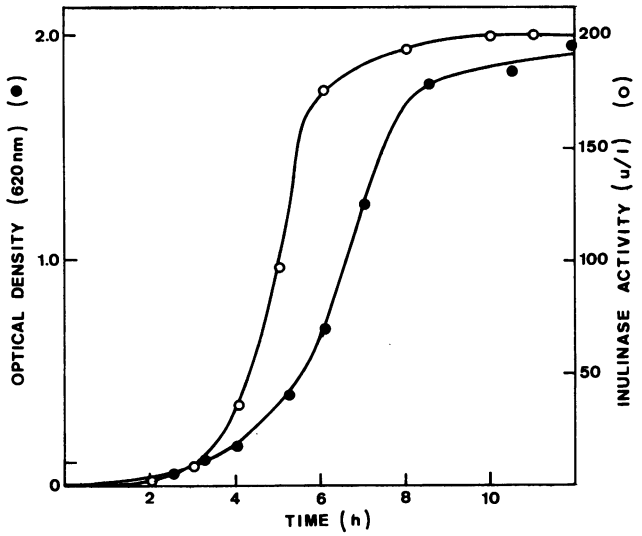

FIG. 3. Kinetics of growth and inulinase activity of strain LCB41 on inulin medium. Strain LCB41 was cultivated in a 2-liter fermentor at pH 8.0 and 50°C.

sity, 1.34). In contrast, the activity on inulin varied widely (13-fold) with the carbon source utilized. The highest activity (195 U/liter) was obtained with inulin in the medium. All of the other sugars showed lower activities: glucose (55 U/liter), sucrose (21 U/liter), fructose (7 U/liter), and also yeast extract (15 U/liter). With fructose as carbon source, the lag phase was greatly increased from 2 to 8 h to 32 h. When the fructose concentration was reduced to 1, 0.5, or 0.1 g/liter, the lag phase was 9 h at all three concentrations, and the inulinase activities were 38, 28, and 2 U/liter, respectively.

Mixed carbon sources, all containing inulin, were also tested (Table 3) to check for catabolite repression of the inulinase biosynthesis. Growth was excellent on all media, and the highest activity on inulin was obtained on a medium containing inulin and yeast extract (192 U/liter). This activity was the same as the one reported for inulin alone. The addition of yeast extract increased the cell growth but not the enzyme activity. The specific growth rate was 1.0/h on this mixed carbon source (doubling time, 41 min). When the culture medium contained inulin and one sugar, the activity decreased 1.4-fold on glucose (137 U/liter), 1.8-fold on sucrose (107 U/liter), and 2.6-fold on fructose (73 U/liter). It is interesting to note that the extent of this inhibitory effect on enzyme level was the same when fructose, sucrose, or glucose was used either as the sole carbon source or as a mixed substrate with inulin. Fructose was the most inhibitory in both cases.

The activity on inulin of strain LCB41 was found mostly in the culture medium (65 to 100%, fraction E; Table 3). The culture medium composition did not show a great influence on enzyme localization.

Inulinase production in a fermentor. The kinetics of growth and inulinase production of strain LCB41 were studied in a fermentor with controlled pH, aeration, and agitation. The temperature was 50°C, the pH was maintained at 8.0, and inulin was the sole carbon source. The results are shown in Fig. 3. Under these conditions, stationary phase was reached in 12 h, with a final optical density of 1.95. This is a much higher value than that obtained in shake flasks (0.79; Table 3). Controlled pH and a higher aeration rate in the fermentor could explain this result. The activity on inulin (culture medium) increased to 200 U/liter. The kinetics of enzyme production paralleled the bacterial growth. More than 80% of the activity on inulin was recovered in the culture medium. There was no variation of the enzyme localization during the fermentation.

DISCUSSION

Five thermotolerant and four thermophilic bacteria utilizing inulin as the sole carbon and energy source were isolated from soil samples. Four of the isolated strains belonged to the genus *Bacillus* and were determined to be thermophilic, since they grew at 45 to 60°C but not at 37°C. The other strains were thermotolerant and were not studied further. The specific growth rate of the thermophilic bacteria was high at 50°C on a mineral medium, indicating that inulin is a good substrate for growing these organisms. Strain LCB41 showed the same specific growth rate at temperatures between 45 and 60°C, and pH did not have a great influence on growth above 7.0, although below this value no growth was observed.

All four of the thermophilic strains showed inulin hydrolysis activity, which was found mostly in the culture medium. Strains LCB40 and LCB41 had the highest activity. In contrast, the activity on inulin was found mostly in the cells of mesophilic bacteria that degrade inulin (1). One explanation for this difference might be that *Bacillus* spp. are gram-positive bacteria that naturally excrete proteins and enzymes, such as proteases, while the majority of mesophilic bacteria belong to the gram-negative genus *Flavobacterium*. Gram-negative bacteria usually excrete fewer proteins than the gram-positive ones. In both kinds of organisms, inulin hydrolysis was rapid, since this step was not growth limiting. Thus, fructan inulin can be properly and effectively hydrolyzed to fructose, not only by a soluble enzyme (in thermophilic *Bacillus* spp.), but also by a cell-bound enzyme (in mesophilic *Flavobacterium multivorum*). Yeast inulinase has also been detected in both culture medium and cell fraction, depending on the nature of the yeast, culture conditions, medium pH, etc. (17). Enzyme secretion is important in industrial applications since enzyme purification is generally much easier.

Sucrose-hydrolyzing activity was also found in the four thermophilic strains. The localization of the sucrose- and inulin-hydrolyzing activities were closely related, which favors the idea that the same enzyme exists with both activities. This is a usual situation for microbial inulinases, which are all active on both inulin and sucrose (17). The insulin-sucrose ratio of the inulinases from the thermophilic bacteria was double the one reported for the inulinases from the mesophilic strains (1), showing a higher specificity of the inulinases from the thermophilic strains for inulin.

When the mineral medium was used, the presence of inulin as the carbon source was necessary for the production of the inulinase by strain LCB41. None of the other tested carbon sources were efficient in promoting enzyme synthesis. This finding favors the idea that enzyme synthesis is induced by inulin. However, these results could also be explained by repression of inulinase synthesis when a carbon source better than inulin (for instance, fructose) is used. This second explanation was further supported by the observation of repression with mixed substrates, for instance, with inulin plus fructose. Since the extent of this repression was low, it could be concluded that the inulinase was most probably an inducible enzyme, with the physiological in-

ducer being inulin, and that some repression of the biosynthesis might occur in the presence of fructose. It should be remembered that inulin hydrolysis produces fructose; thus, the bacterial strain might be able to regulate the inulinase level by a dual mechanism of substrate (inulin) induction and product (fructose) repression.

The kinetics of inulinase production was studied in a fermentor to explore the possibility of industrial production. The enzyme appeared in the culture medium during the exponential growth phase. The inulinase production reached 200 U/liter on inulin. Similar inulinase levels in culture medium have been reported for yeasts. For instance, 390 U/liter was reported for *Kluyveromyces fragilis* (8), and 222 U/liter was reported for *Candida kefyr* (9), but usually these values were obtained after a longer culture time of 48 to 96 h, compared with 11 h in our study. The enzyme level was 10 times higher when *Aspergillus* sp. with a fermentation time of 100 h (3, 7) was used. In contrast, the bacteria *Arthrobacter ureafaciens* showed a low enzyme level of 38 U/liter (16).

Compared with the other inulinase-producing strains, *Bacillus* sp. strain LCB41 showed several advantages for enzyme production. The enzyme productivity of LCB41 (18 U/liter per h) is higher than the productivities of *K. fragilis* (4.0 U/liter per h) and *C. kefyr* (1.9 U/liter per h) and in the same range as that of the *Aspergillus* sp. mentioned above. The possibility of maintaining the culture at 50°C would be a great industrial advantage, since both the sterilization and the fermentor cooling are easier at high temperatures. However, the enzyme concentration produced by LCB41 is still low compared with that for the *Aspergillus* sp. and must be enhanced before such new thermophilic bacterial strains could be considered attractive for use in industrial inulinase production.

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