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Improvement of Inulin Hydrolysis Yeast Cell Reactor by Mutants Selection

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ABSTRACT

In a previous publication, we described a continuous production of D-fructose from enzymatic hydrolysis of inulin with immobilized permeabilized cells (*1*). *Kluyveromyces fragilis* ATCC 12424 have been shown to possess inulase activity. The half life of the reactor was at least 1300 h, but productivity was relatively low (around 40 g/L/day).

A selection of 50 mutants was tested on liquid medium for a possible increase of productivity.

In relation to the improvement of the reactor, the most important factor is intracellular inulase activity, and this activity was increased with the KF 28 mutant.

Productivity reached 2000 g/L/day with the increase (of the productivity), proportional to the increase of intracellular inulase activity.

Index Entries: Inulin; inulase; *Kluyveromyces fragilis*; mutants; cell reactor.

INTRODUCTION

A method for D-fructose production involves the utilization of naturally occurring D-fructans. The D-fructan most commonly considered is inulin (2,1-β-D-fructan). Two plants well known for the production of inulin are the Jerusalem artichoke and chicory (*1,2*). The carbohydrate reserves of the Jerusalem artichoke and chicory are considerable (16–20% of wet matter) and these plants have high crop yields (40 t/hectare for chicory and up to 60 t/hectare for Jerusalem artichoke). To hydrolyze inu-

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lin, different strategies can be elaborated, the acid process (*3*), and the classical enzymic process or immobilized cells reactor (*5,6*).

In this publication, we describe the improvement of an immobilized (nonviable) cell reactor. The production of D-fructose syrups from inulin by immobilized whole cells requires that two conditions be satisfied. The inulase in the cell must be accessible to the substrate and the resulting D-fructose (and D-glucose) must not be metabolized by the cells. A single bioconversion is required. Guiraud and coworkers achieved that by using strains with weak fermentation metabolism in a reactor under anaerobic conditions (especially Pichia polymorpha) (*7*). In our case, we used *Kluyveromyces fragilis* (*1*).

Whole cells of *Kluyveromyces fragilis* ATCC 12424 have been shown to possess inulase activity but these yeasts rapidly ferment glucose and fructose. The ability to ferment inulin could be destroyed by thermal treatment; this treatment could kill the cells and increase cell permeability for inulin (*8,9*). Between 60 and 75°C for 5 min., permeabilization was effective, cells were killed, and inulase activity was at its highest. At temperatures higher than 75°C, inulase activity was destroyed. After this thermal treatment, cells were entrapped in alginate beads and incubated in a reactor at 55°C with 10% inulin solution. Productivity varied between 30 and 40 g/L/d with a half life of at least 1300 h. For an industrial application, although the value of the half life is acceptable, productivity must be increased by at least a factor of ten.

In this paper, we describe the selection of mutants effective in the inulase production and the utilization of the mutant cells in an immobilized cells reactor.

MATERIALS AND METHODS

Strain

The wild-type strain used was *Kluyveromyces fragilis* ATCC 12424.

Media

Different carbon sources were used with the same base medium containing 1% yeast extract and 1% peptone. The carbon source concentration was 2%.

Screening Medium

The solid medium had the same composition as the liquid one, with addition of 1.5% agar and 2% inulin as carbon source. After cooling the autoclaved agar nutrient solution to 48°C, cooled inulin powder (from dallhia, Sigma) was added slowly and stirred just prior to plate pouring.

With the inulin concentration at 2%, 0.05% chloramphenicol was added. Undissolved inulin gave the agar plate a creamy opaque appearance.

Isolation of Inulase Mutants

Shaken flask cultures of the wild-type strain were grown to exponential phase in medium (as above) with 2% glucose. Cell suspensions in 0.2*M* sodium phosphate buffer pH 7, were treated with a mutagenesis agent (*N*-methyl-*N'*-nitro-*N* nitrosoguanidine). The final concentration of NTG was .03% and the treatment was carried out at 28°C for 30 min.

After this treatment cells were spread on the medium described above. After growth of the mutagenized clones on these plates for 4 d at 28°C, incubation was shifted to 50°C for one day to increase the formation of clearing zones of inulin hydrolysis around colonies with high inulase content. Colonies with distinctly larger clearing zones than the parent were tested for inulase production in shaken flasks.

Assay for Enzyme Activity

Inulase activity was measured with inulin as the substrate. The assay mixture contained both 5% inulin (final concentration) in 0.05*M* sodium acetate at pH 5.0 (4 mL), and a suitably diluted enzyme suspension (1 mL). After 15 min incubation at 37°C, the reaction was stopped by heating at 100°C for 5 min.

The amount of reducing sugars produced was assayed with dinitrosalicylate:3 mL DNS (containing .05% sodium sulfite), 1 mL .05*M* sodium citrate, pH 5; and .5 mL reducing sugars preparation. Coloration was obtained by heating at 100°C for 5 min. The reaction was stopped by adding 1 mL salts of Rochelle (40%) and 15 mL water. Optical density was measured at 550 nm (*10*). The reference solution was 20 m*M* fructose, whereas for the blank the enzymic solution was denatured by heating at 100°C for 5 min before hydrolysis.

One unit of activity is defined as the amount of enzyme that can hydrolyze 1 μmol reducing sugar per min at 37°C and pH 5.0. Inulase activity is measured in the medium after centrifugation of the cells; this activity will be referred to as extracellular activity. Inulase activity is also measured on cells permeabilized by a thermal treatment at 65°C for 5 min (cell-bound activity).

Extracellular activity is expressed in unit/mL of culture, whereas cell-bound activity is expressed in unit/mg of cell dry matter.

Cells Immobilization in Alginate Beads

Cells (60 or 90 mg/mL) in .05*M* sodium acetate buffer pH 5 were mixed with an equal volume of 4% sodium alginate. This mixture was

dropped into a 1% $CaCl_2$ solution. The so-formed beads were left for 1 h in this solution and were then filtered before use.

The amount of reducing sugars produced was assayed with the dinitrosalicylate reaction as described above.

Continuous Reactor and Inulin Extraction

They have been described previously (*1*).

RESULTS

Selection of Kluyveromyces Mutants Effective in the Inulase Production

After addition of NTG as described in Materials and Methods, the cells were spread on solid medium containing insoluble inulin. After 3 d incubation at 30°C and a night at 50°C, a clear zone around the colonies appeared. The size of this clear zone is proportional to the amount of inulase activity.

A total of 10,000 colonies were screened and 44 presumptive mutants were picked for testing in liquid culture. The medium contained 2% inulin as carbon source. Table 1 shows the extracellular inulase activities of 10 mutants after 24, 48, and 72 h of growth. Only two mutants were substantial producers: twice as much for KF 15 mutant and three times as much for KF 28 mutant as compared to the wild-type strain.

Table 2 shows the influence of the carbon source (2%) on inulase activity for both the KF 28 mutant and the wild-type strain after 72 h of culture at 29°C.

For the KF 28 mutant, cell-bound inulase activity is induced by galac-

Table 1
Inulase Activity of Presumptive Mutants at 29°C with Inulin as Carbon Source after 24, 48, and 72 H of Culture Expressed in Percent of Wild-Type Strain Activity

	Extracellular activity		
KF, wild-type	24 h, 100%	48 h, 100%	72 h, 100%
KF 8	142	120	101
KF 9	174	178	109
KF 13	179	179	112
KF 15	305	355	203
KF 17	336	333	193
KF 19	152	169	144
KF 25	33	112	117
KF 28	182	287	300
KF 37	179	170	136

Table 2
Influence of Carbon Sources, 2%, on Inulase Activity for Mutant KF 28 and Wild-Type Strain KF Expressed in Percent of the Activity Obtained with 2% Inulin for the Wild-Type Strain

	Extracellular inulase activity	
Carbon source	KF	KF 28
Fructose	37	259
Galactose	0	242
Glycerol	0	206
Inulin	100	337
Lactose	0	356
Sucrose	0	266
Ethanol	0	259
Glucose	0	366
	Cell-bound inulase activity	
Fructose	12	162
Galactose	0	121
Glycerol	0	219
Inulin	100	369
Lactose	0	135
Sucrose	37	188
Ethanol	0	85
Glucose	0	154

tose, glucose, glycerol, lactose, sucrose, and ethanol at different levels. This is not the case for the wild-type strain for which inulin and, to a lesser extent, fructose are inducers. The same results are obtained for extracellular activities.

For technical and economic reasons, it is difficult to use inulin as carbon source for producing inulase. That is why glucose was used at different concentrations with the KF 28 mutant. The best results were obtained at 1% glucose (Table 3). Moreover, the proportion between extracellular and cell bound activities is related to the degree of aeration of the medium (Table 4).

Continuous Reactor for Inulin Hydrolysis by Immobilized Nonviable Kluyveromyces Fragilis Cells

A continuous reactor for inulin hydrolysis was set up in order to test productivity improvements with the mutant KF 28 as compared to the wild-type strain. The characteristics of the reactor with alginate beads were the following: Cells of Kluyveromyces fragilis were permeabilized by treatment at 65°C for 5 min, as described previously (*1*). The incubation temperature was 55°C. The substrate was juice from chicory tubers at pH 5 containing 10% of inulin.

Table 3
Influence of Glucose Concentration for Inulase Activity Expressed in Percent of the Activity Obtained with 2% Inulin as Carbon Source

Carbon source		Extracellular inulase, %	Cell-bound inulase, %
Glucose	2%	124	60
	1%	157	88
	0.5%	77	64
	0.05%	14	25
Inuline	2%	100	100

Table 5 shows the amount of reducing sugars produced (g/L/d) with the mutant KF 28 and the wild type strain. Sucrose (2%) or inulin (2%) were used as carbon source in the culture medium for the production of cells and enzyme.

Productivity is increased more than 10-fold with the KF 28 mutant using sucrose as carbon source. With inulin as the carbon source for producing cells, reactor productivity reaches 1500 g/L/d. Increase of productivity is related to the enzymic activity inside the alginate beads. (Mass transfer limitations are not important.) (Table 6)

If the quantity of cells immobilized in the beads is increased by 1.5, productivity reaches 2000 g/L/d for these productivities. Figure 1 shows the evolution of these productivities as a function of time. The half-life of these reactors was found to be in excess of 250 h.

DISCUSSION

The aim of this work was to continuously produce D-fructose from enzymatic hydrolysis of inulin using immobilized permeabilized cells.

In a previous work, we developed a thermal treatment (5 min at 65°C) that kills cells in order to prevent fermentation of fructose without affecting inulase activity. Moreover, this treatment increases cell permeability for inulin. Unfortunately, while the productivity of the immobilized cell reactor was not acceptable, (around 40 g/L/d), the stability was very good (around 500 h).

The selection of mutants was found to be an effective approach for

Table 4
Influence of Oxygenation on Inulase Activity

Culture volumes,[a] mL	Extra, %	Cell Bound, %	Extra/Cell Bound
25	138	38	3.63
50	124	60	2.07
100	73	54	1.35

[a]Different volumes of medium are used in culture flasks of 250 mL. The activities are expressed in percent of the activity obtained with 2% inulin with KF 28 mutant (*see* Table 3).

Table 5
Influence of Improved Conditions of Strains on Inulin Reactor Productivity

Strain	Carbon used for the enzyme production	Productivity, g/L/d	Cell bound activity, U/g dried cells
KF	sucrose 2 %	40	>70
KF 28	sucrose 2 %	550	864
KF 28	inulin 2 %	1500	1695

increasing intracellular inulase activity and, hence, the productivity of the continuous reactor.

The nonspecific β-fructofuranosidase inulase is an external glycoprotein produced by a number of yeasts and filamentous fungi including *Kluyveromyces fragilis* (*2,11,12*), *Pichia polymorpha* (*7,13*), *Aspergillus* (*14*), among others.

Since yeast cell membranes are impermeable to fructosides, including sucrose, the primary physiological function of inulase suggests an external location. The ratio of periplasmic inulase to soluble inulase depends on culturing conditions. The proportion of endocellular inulase is not significant. High enzyme levels in wild-type strains in batch cultures have been observed only if the media contained oligofructosides such as inulin (*11*). Moreover, experimental limitation of carbon catabolite repression by using the continuous culture technique has shown that the rapidly metabolized substrate sucrose can also support high yields.

For increasing productivity of the reactor, the selection of mutants must be directed towards increased periplasmic and endocellular activity. Moreover, this activity must not be released to the medium by thermal treatment at 65°C for 5 min. This explains why cell bound activity was measured here after thermal treatment of the cells.

The screening method using opaque inulin plates was developed as described in Materials and Methods, and distinct clearing zones of inulin hydrolysis around colonies could be generated. With this technique, 44 mutants were selected. However, in liquid medium, inulase yields varied widely and only 2 were found to have produced 2 to 3 times more than the parent strain.

Productivity of inulase is derepressed on different carbon sources with the KF 28 mutant (*see* Table 2). Cell bound activity of KF 28 mutant reaches 1695 U/g dry wt cells with inulin as the carbon source and 864 U/g

Table 6
Influence of Cells Concentration in Alginate Beads on Reactor Productivity, g of Reducing Sugars Obtained/L/d

Strain	Quantity of cells/100 mL of alginate beads	Productivity, g/L/d
KF 28	6	1500
KF 28	9	2000

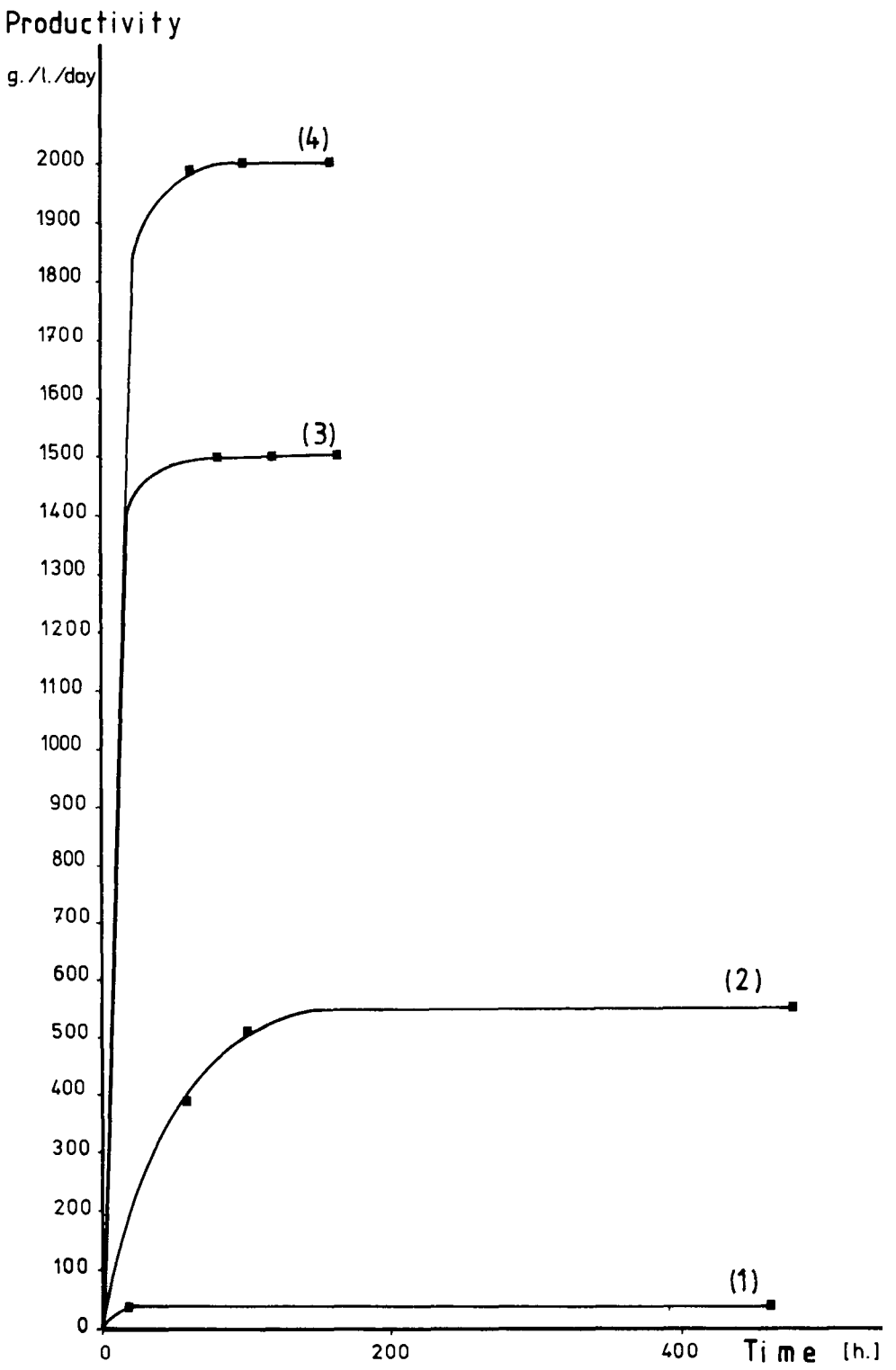

Fig. 1. Evolution of productivity (g reducing sugar/L/D) of *Kluyveromyces fragilis* immobilized in alginate beads.

dry wt cells with sucrose. The limiting factor of the immobilized cell reactor productivity could be the transfer of inulin through alginate beads and/or inulase activity inside the beads. Results of molecular transfer through alginate show that inulin with a molecular weight between 500–10,000 could easily pass through alginate (*15*). For productivity of 40 g/L/d, it is more likely that the limiting factor would be inulase activity. Table 5 and Fig. 1 show that productivity is proportional to the activity up to 1500 g/L/d. Moreover, if the quantity of cells is increased inside the beads, it is possible to reach 2000 g/L/d with the KF 28 mutant and with 9 g of cells/100 mL of alginate; this represents an increase of more than 50%. Fifty-five percent of the maximal theoretical activity is expressed in the reactor with 6 g of cells/100 mL and 48% with 9 g of cells/100 mL.

The sugars produced have been assayed by HPLC and only glucose and fructose have been identified in significant quantities (results not presented).

With these results, it is necessary to increase productivity to study the mechanism of transfer in more detail. This study has begun in our laboratory.

With 1200 g/L/d, a 10 m^3 reactor would produce 20 t/day, and for the period of chicory production (100 d), this reactor could produce 2000 t. This production corresponds to half the Belgian production of fructose from inulin.

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