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A Multipotential Hydrolytic Reactor Using the Yeast Strain *Kluyveromyces marxianus*

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ABSTRACT

In previous publications, we described the continuous production of D-fructose from enzymatic hydrolysis of inulin with immobilized permeabiliized cells of *Kluyveromyces marxianus* and the increase of productivity obtained by using a mutant selected by NTG action on the wild-type strain. By improving reactor performance, it has been possible to reach 2000 g/L/d of liberated sugar from an inulin solution with the mutant strain.

In addition, it has been shown that the KF 28 mutant was an invertase and pectinase hyperproducer. These enzymatic activities are also secreted by the species *Kluyveromyces marxianus*. Therefore, we investigated the possibility of using immobilized cells of this yeast as a multipotential hydrolysis reactor. A sucrose hydrolysis reactor and a pectin hydrolysis reactor were set up. It is shown here that the majority of the optimized parameters from the inulin hydrolysis reactor can be transported directly to the other reactors. However, some parameters have to be adapted, especially for pectin hydrolysis.

Index Entries: *Kluyveromyces marxianus;* mutant; inulinase; invertase; pectinase.

INTRODUCTION

D-fructose production involves the utilization of naturally occurring D-fructans; a commonly considered one is inulin (2, 1 β-D fructan). To hydrolyse inulin, different strategies can be utilized: the acid process

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(*1,2*) and the classical enzymic process or immobilized-cell reactor (*3,4*). D-fructose production by hydrolysis of inulin with immobilized cells requires that two conditions be satisfied. First, the inulinase in the cell must be accessible to the substrate; second, the resulting sugars must not be metabolized by the cells.

There are different ways to achieve these conditions (*5*). In our case, we immobilized *Kluyveromyces marxianus* cells after a thermal treatment that has been shown to kill the cells (so that they could not use the liberated sugars) and that also increases their permeability. Through this approach, the reactor performances have been improved. In a second optimization step, we selected a mutant strain of *Kluyveromyces marxianus* using a mutagenic agent: NTG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine). The selected mutant—KF28—has been shown to possess an increased inulinase activity. The gain of productivity was about threefold. By optimizing another parameter, the quantity of cells immobilized in each alginate bead, we finally reached a productivity of 2000 g/L/d.

The mutant strain itself (*6*) has also been studied. It exhibits hyperproduction of other enzymic activities: invertase and pectinase are produced in larger quantities by KF28 than by its parent strain. These physiological properties have been used with the aim of setting up a multipotential reactor that would be able to efficiently hydrolyze inulin as well as sucrose or pectin. This paper describes the extent to which it has been possible to transpose the optimized parameters of the inulin hydrolysis reactor. It also outlines improvements required to optimize this multipotential reactor so that further gains in productivity can be achieved by new mutant strains.

MATERIALS AND METHODS

Strains

We cultivated the yeast *Kluyveromyces marxianus* ATCC 12424 and its mutant strain KF28 (*7*).

Inulin extraction, cell permeabilization, and immobilization have been described previously (*8,9*), as have the mutation technique and the screening medium (*7*). Production of free cells has been performed as already described (*8*). In case of modification, this is mentioned in the text.

Assays for Enzymatic Activities

1. Inulinase-invertase-pectinase: After centrifugation, cells are resuspended in sodium acetate buffer (0.05*M*, pH 5.0) and permeabilized by a thermal treatment as patented by Thonart et al. (*9*). The cellular activity is then measured. Each enzyme assay is performed during 15 min at 37°C. Reactions are stopped by heating the mixture to 100°C for 5 min. Blanks are made by

denaturing enzyme (5 min at 100°C) before incubation. Four milliliters of the reaction mixture is added to 1 mL of supernatant or resuspended cells. The reaction mixture for the inulinase assay is 5% inuiln in sodium acetate buffer (0.05*M*, pH 5.0); for the invertase assay, 10% sucrose is employed in the same buffer. For the pectinase assay, 0.5% pectin is added to the same buffer.

One unit on inulinase or pectinase is defined as the amount of enzyme necessary to liberate one μmole of reducing sugars/min at 37°C and pH 5.0. The reducing sugars are measured with dinitrosalicylate (*see below*). For inulinase, a reference solution is made with 20m*M* fructose; for the pectinase assay, we use a solution of $15 \times 10^{-3} M$ galacturonic acid. The pectin used for pectinase assay is a citrus pectin of Fluka (AG 76280), containing 58% galacturonic acid.

One unit of invertase is defined as the amount of enzyme necessary to liberate one μmole of glucose/min at 37°C and pH 5.0. The glucose produced is measured with an automatic analyzer (YSI model 27, Yellow Spring Instruments).

Dinitrosalicylate (DNS) method: 3 mL of DNS (containing 0.05% sodium sulfite) and 1 mL 0.05*M* sodium citrate at pH 5 are added to 0.5 mL of reducing sugars preparation. Coloration is obtained by heating at 100°C for 5 min. The reaction is stopped by adding 1 mL salts of Rochelle (40%) and 15 mL water. Optical density is measured at 500 nm.

2. β-galactosidase activity: After permeabilizing the cells the enzymatic reaction is realized with 0.4% ONPG (*O*-nitrophenyl-β-D-galactoside) as substrate (ONPG is dissolved in phosphate buffer, 0.25*M* pH 7.0). The reaction is performed for 15 min and stopped by adding 2 vol of 1*M* Na_2CO_3. The liberated ONP (*O*-nitrophenol) is a yellow-colored solution and can be measured with a spectrophotometer at 420 nm.

RESULTS

KF28 Hyperproductions

The KF28 mutant has been studied for two other enzymatic activities: invertase and pectinase, known to be secreted by *Kluyveromyces marxianus* (*10*). The mutant has been shown to exhibit significant increases in its inulinase, invertase, and pectinase activities compared to the wild-type strain, and extracellular and cell-bound activities have been investigated (*6*). The pectinase activity of this yeast is only polygalacturonase (*11*).

From the perspective of an immobilized reactor, we are interested only in the cell-bound activities. Figures 1A–C show that inulinase, invertase,

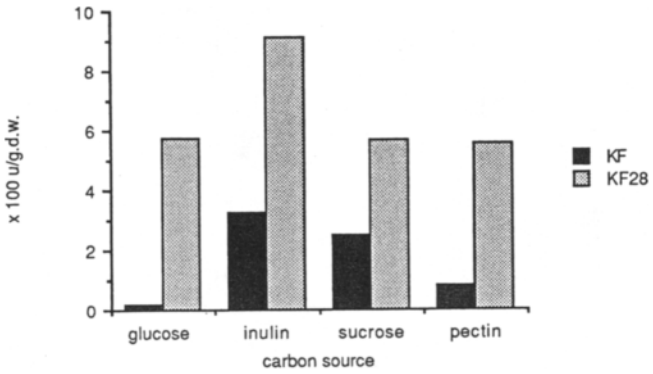

Fig. 1A. Inulinase cell-bound activity exhibited by KF and KF 28 after growth for 72 h on different carbon sources.

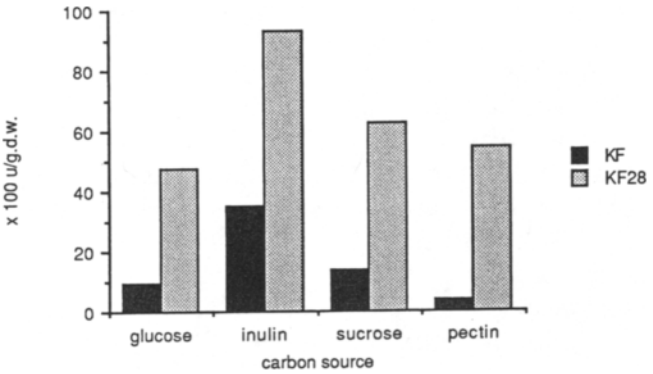

Fig. 1B. Invertase cell-bound activity exhibited by KF and KF 28 after growth for 72 h on different carbon sources.

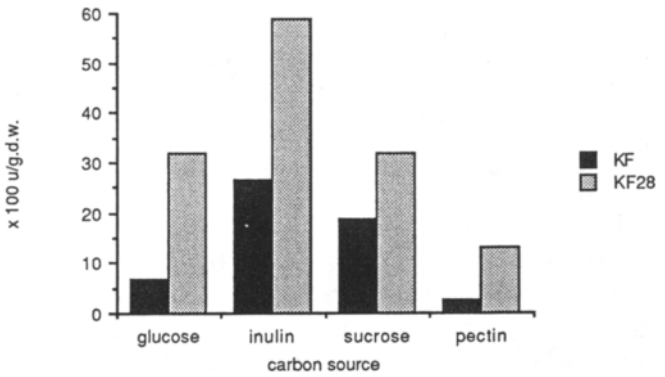

Fig. 1C. Pectinase cell-bound activity exhibited by KF and KF 28 after growth for 72 h on different carbon sources.

Table 1
β-Galactosidase Activity of KF and KF 28
after Growth on Different Carbon Sources (%)

Carbon source	Strain	
	KF	KF 28
Inulin	8.9	12.5
Sucrose	7.7	5.9
Glucose	18.4	8.3
Lactose	100[a]	43.0

[a]100 = activity of the wild-type on lactose medium.

and pectinase are hyperproduced by KF28. A derepression occurs, at least partially, for each enzyme on each carbon source. Nevertheless, inulin remains the best inducer, particularly for pectinase activity.

The β-galactosidase activity of both strains have been assayed on different carbon sources; it appears that the mutant does not hyperproduce β-galactosidase activity (*see* Table 1). This suggests that the mutation could possess the secretion mechanism of *Kluyveromyces marxianus*. To test the potentialities of the mutant sucrose and pectin hydrolysis reactors were set up and operated.

Sucrose Hydrolysis Reactor

Optimized Parameters

All parameters previously determined for optimum immobilization in the case of inulin hydrolysis were applied to this new reactor, especially the aeration rate of the culture in combination with the carbon source (*7*). Both strains, the wild-type and the KF28 mutant, are conditioned in the following way to favor their cellular invertase activity: growth of the cells on inulin and glucose for KF and KF28, respectively, while feeding the reactors with 20 or 40% sucrose for KF and KF28, respectively. These parameters have been determined to be the best in the case of invertase production (unpublished data).

By comparing the two reactors and assuming that each strain is at its optimal operating condition, we should obtain the approximate difference of productivity that results from the effct of the mutation. Results are given in Fig. 2.

Influence of the Bead Size

The performance of the sucrose hydrolysis reactors can be improved further, by decreasing the bead size by a factor of three: the volume of each bead was reduced from 30 μL to 10 μL by blowing out the alginate drop during its formation. Therefore, we obtained better productivity of our sucrose hydrolysis reactor (*see* Fig. 2).

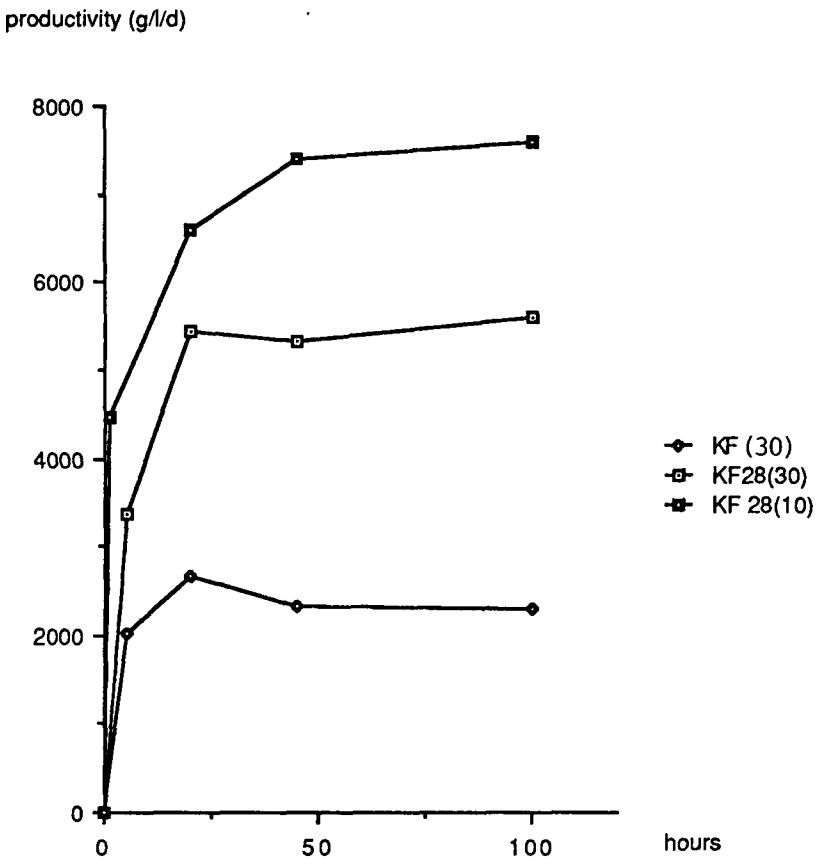

Fig. 2. Productivity (g of liberated sugars/L/d) of three sucrose hydrolysis reactors with KF and KF 28.(10) and (30) indicate bead sizes (μL).

Pectin Hydrolysis Reactor

Keeping in mind the fact that our system of immobilized whole cells could be used as "enzyme bags," we intended to hydrolyze a pectin solution to liberate galacturonic acid. Indeed, the pectinase activity of *Kluyveromyces marxianus* is not an enzyme complex, as in other microorganisms, but a single activity (polygalacturonase) (*11*). The kinetic parameters of pectin hydrolysis for both strains were studied in batch reactors using different pectin concentrations. The results obtained for both strains were that, as expected, as the pectin concentration was increased, more galacturonic acid was liberated (Figs. 3A, B). However, we also noted that the hydrolysis percentage decreased when the pectin concentration was raised. Moreover, the difference of productivity between the wild-type and the mutant was higher for increased pectin concentration (Fig. 4A, B). This phenomenon is not directly related to the mutation, as described below.

Pectin gelation occurs around the beads during hydrolysis. The gel coating produced in this way therefore reduces mass transfer and further

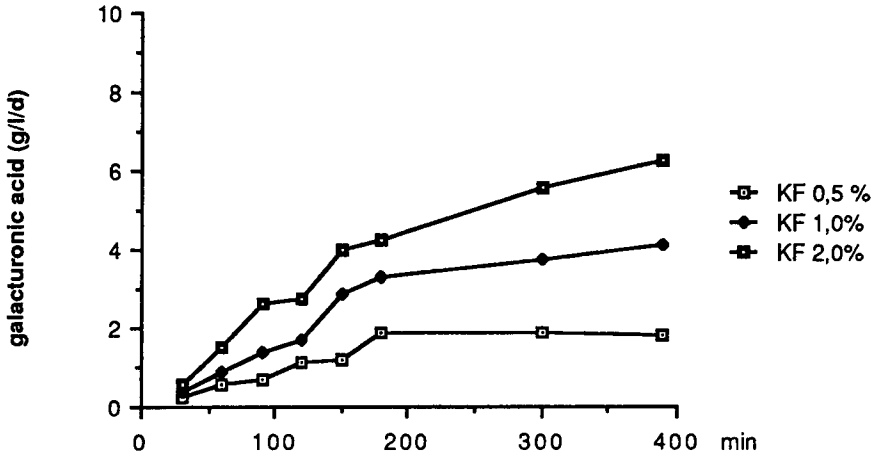

Fig. 3A. Pectin hydrolysis by KF. Galacturonic acid produced with different pectin concentrations.

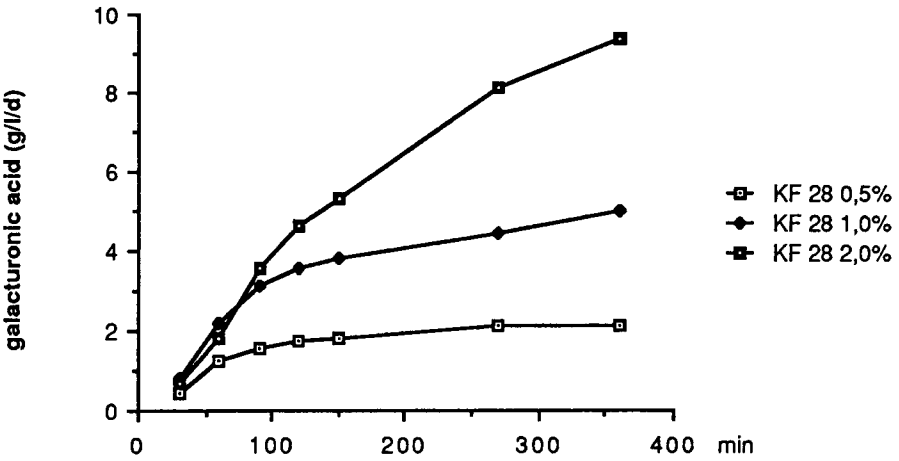

Fig. 3B. Pectin hydrolysis by KF28. Galacturonic acid produced with different pectin concentrations.

enzymic reaction. In the case of the mutant, pectinase activity is sufficiently high to ensure a partial hydrolysis of pectin, so that gelation is postponed. This is not the case for the wild-type strain, for which an increase in pectin concentration more directly affects the hydrolysis rate.

DISCUSSION

The aim of this work was to explore the potential of the KF 28 mutant derived from the yeast *Kluyveromyces marxianus* ATCC 12424. Some of these potentialities include the significant increase in invertase and pectinase cell-bound activities of the KF 28 compared to the wild-type strain (KF).

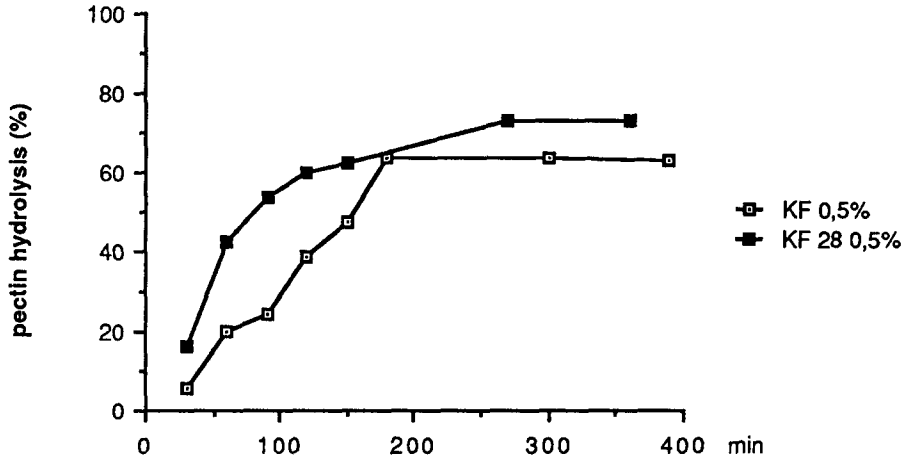

Fig. 4A. Pectin hydrolysis by KF. Hydrolysis rates obtained with different pectin concentrations.

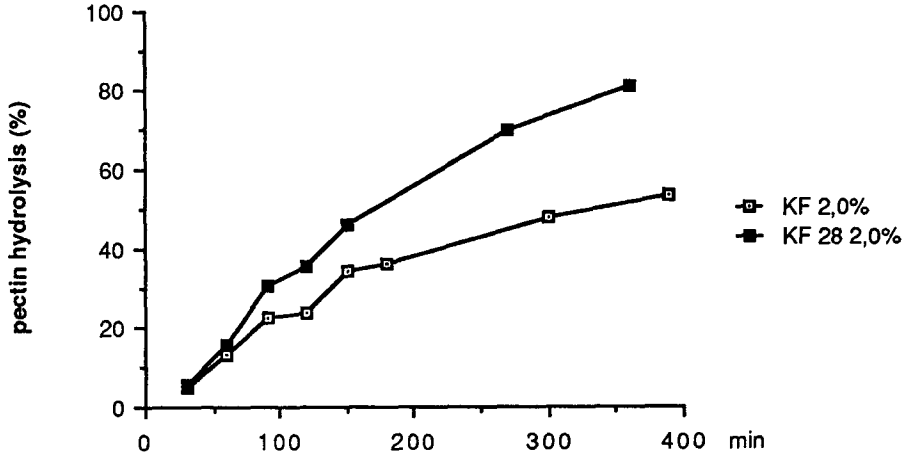

Fig. 4B. Pectin hydrolysis by KF28. Hydrolysis rates obtained with different pectin concentrations.

Since inulin hydrolysis reactors using KF 28 achieved high performances, sucrose and pectic hydrolysis reactors was assembled accordingly.

For sucrose hydrolysis, improved hydrolysis rates were reached with the KF 28 strain using optimized operating conditions transposed from the inulin reactor: 5600 g of liberated sugars/L/day were obtained compared to 2600g/L/day with the wild-type. Productivity was improved further by varying a new parameter: the bead size. By reducing the bead volume from 30 to 10 μL, it has been possible to reach 7600 g/L/day.

This may be the optimum obtainable under these conditions. This kind of reactor could be further enhanced by selecting other improved strains, such as a second-generation mutant from KF 28 or a recombinant strain, using genetic engineering. In the case of pectin hydrolysis, we cannot

draw the same conclusions: though the mutant is actually a pectinase hyperproducer, as shown by cell-bound activities measurements, the batch process using this strain did not perform as well as expected. The pectin gelatin phenomenon causing this lower level rate is probably the result of Ca^{2+} free ions: indeed, it is known that low methylated pectins form gels only in the presence of free metallic ions (*12,13*). The multipotentiality of the reactor is jeopardized by this chemical interaction, even if we can imagine a second-generation mutant that could overcome this limited mass transfer by a very high pectinase activity. As the reactor has to work with any kind of pectic substrate, the problem can be solved only by modifying the cell-immobilization method.

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