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Selection of Thermotolerant Yeasts for Simultaneous Saccharification and Fermentation (SSF) of Cellulose to Ethanol

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ABSTRACT

A total of 27 yeast strains belonging to the groups *Candida, Saccharomyces,* and *Kluyveromyces* were screened for their ability to grow and ferment glucose at temperatures ranging 32–45°C.

K. marxianus and *K. fragilis* were found to be the best ethanol producing organisms at the higher temperature tested and, so, were selected for subsequent simultaneous saccharification and fermentation (SSF) studies.

SSF experiments were performed at 42 and 45°C, utilizing Solkafloc (10%) as cellulose substrate and a cellulase loading of 15 FPU/g substrate. Best results were achieved at 42°C with *K. marxianus* L. G. and *K. fragilis* L. G., both of which produced close to 38 g/L ethanol and 0.5 ethanol yield, in 78 h.

Index Entries: Cellulose; simultaneous saccharification and fermentation (SSF); thermotolerant yeasts; ethanol fermentation; enzymatic hydrolysis.

INTRODUCTION

The simultaneous saccharification and fermentation process (SSF) is being studied as a promising method for transforming cellulosic materials

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into ethanol (*1–3*). It has been reported to improve hydrolysis rates and yields when compared to those systems involving a separate hydrolysis and fermentation steps (*4–7*). The SSF process has also the advantage of requiring a lower enzyme loading and less expensive equipment (*2*). Despite these benefits, the potential of the SSF system is limited by the widely different temperature requirements: for optimal activity of the cellulolytic enzymes, about 50°C, and that normally used for growth of the fermenting microorganisms, about 35°C. One way to overcome this problem would be to use thermotolerant yeasts. This would allow operation of SSF at high temperatures, thereby increasing the hydrolysis rate of the cellulose by the enzyme system. Because of the benefits of obtaining ethanol from cellulosic biomass, by conducting SSF at 40°C and above, various groups have attempted the selection of thermotolerant yeast strains (*4,5*). Hacking et al. (*10*) have identified some yeast strains belonging to the groups *Saccharomyces, Candida,* and *Kluyveromyces* as being the most capable of growth and ethanol production from glucose at 40°C.

The present work is an attempt to select yeasts capable of producing ethanol in good yield at temperatures higher than 40°C in order to employ them in further SSF processes. Earlier results on thermotolerance of different yeast groups (*8,9*) have been taken into account, and so, yeast strains belonging to the groups cited above have been chosen for screening.

Additional SSF tests to produce ethanol, utilizing Solka-floc as a substrate at 42°C, have also been developed with the selected yeasts.

MATERIALS AND METHODS

Microorganisms

A total of 27 yeast strains belonging to the groups *Saccharomyces* (12), *Candida* (9), and *Kluyveromyces* (6) were screened. One of the strains, *S. cerevisiae-pretoriensis* FDH-I, was obtained at the University of Sevilla (Spain) by crossing *S. cerevisiae* FSP414/6 and *S. pretoriensis* D517-4B, getting a fusion product showing a high thermotolerance (*10*).

Culture Media

Media employed for yeast growth and for fermentation tests consisted of (g/L): yeast extract (Difco) 2.5, peptone (Oxoid) 5.0, NH_4Cl 2, KH_2PO_4 1, $MgSO_4$ 0.3 and glucose 50. Agar plates were prepared by addition of 20 g/L agar to the above medium.

In SSF assays, glucose was substituted with 10% Solka-floc (potential glucose content in Solka-floc was 94.5% d.w.b.), and cellulase (15 FPU/g of substrate) was also added (*see* next point).

Cellulase Complex

The cellulolytic system used in SSF and hydrolysis assays was kindly provided by J. Pourquie from IFP (Institut Français du Petrole). It was obtained by ultrafiltration of a culture of the mutant *Trichoderma reesei* CL-847 followed by freeze-drying of the liquid fraction containing the enzyme. This fraction exhibited final enzymatic activities of 0.65 and 0.8 IU/mg of protein, for filter paper and β-glucosidase, respectively.

Initial Yeast Selection

To determine their ability to grow at different temperatures, the yeast strains considered in this work were sown on agar plates. The plates were incubated at temperatures of 32, 37, and 40°C for 3 d. After that time, those strains showing poor growth or none at all were discarded for subsequent fermentation purposes.

Fermentation Assays

Fermentation assays at 40, 42, and 45°C were carried out in 100 mL Erlenmeyer flasks, each containing 50 mL of fermentation medium, on a rotary shaker (Braun mod. Certomat H), at 150 rpm. Flasks were coupled with rubber stoppers with fermentative tubes filled with 2.5 mL of 50% sulphuric acid, to prevent ethanol evaporation. The flasks were inoculated with 10% (v/v) of yeasts' cultures that had been grown at 35°C for 16 h on the rotary shaker at 170 rpm.

SSF and Enzymatic Hydrolysis Assays

SSF experiments were carried out utilizing the medium already described and under the same conditions, except temperature, as mentioned for fermentation. SSF tests were carried out at 42 and 45°C.

Flasks were periodically checked during the assays for ethanol, glucose, reducing sugars and residual cellulose contents, as well as for cell viability. Cellobiose concentration was also determined in some of the assays.

In parallel with SSF trials, enzymatic hydrolysis tests of Solka-floc were carried out at 42°C, utilizing the same work conditions (10% substrate, 15 FPU/g enzyme loading). Samples were periodically collected from the reaction media and then analyzed for glucose and cellobiose content.

Taking into account an initial content of a potential glucose of 94.5 g/L in the media, and considering glucose to ethanol conversion ratio of 0.5 (determined as g ethanol formed/g glucose consumed), the saccharification efficiency (SE) in SSF tests was calculated as

$$SE = (E \times 0.5 + G) / 94.5$$

where E = ethanol concentration (g/L) in the media at a determinate time, and G = glucose in the media (g/L) at the same time.

Analytical Procedures

Cell viability of the yeasts was controlled by sowing the samples on agar plates containing growing medium and checking for cell growth after three days of incubation.

Enzymatic activities (filter paper and β-glucosidase) were determined according to the methods described by Mandels et al. (*11*) and Bailey and Nevalainem (*12*), respectively.

Total reducing sugars and glucose were determined by the Nelson-Somogyi (*13*) and hexokinase glucose-6-phosphate dehydrogenase (Glucoquant Kit, Boehringer-Mannheim) methods, respectively. Cellobiose was determined by HPLC, using a Hewlett-Packard apparatus equipped with a RI detector and a column of Aminex HPX87 P at 85°C, and water flow rate of 0.6 mL/min.

Ethanol was determined by GLC, utilizing a Konik 2000C Series apparatus with FI detector and a column of Carbowax 20 *M* (2 m × 1/8 in) at 95°C. Injector and detector temperature: 125°C.

Residual Cellulose

Residual cellulose was estimated as described by Spindler et al. (*4*).

RESULTS

Initial Screening of Yeast Strains

Yeast strains belonging to *Saccharomyces, Candida,* and *Kluyveromyces* genera were screened for growth at 32, 37, and 40°C on agar plates. All yeast strains tested were able to grow at 32 and 37°C, but only 19 grew at 40°C (12 *Saccharomyces,* 2 *Candida,* 5 *Kluyveromyces*). As mentioned before, strains that did not grow (or showed very poor growth) at the last temperature were excluded from further studies.

Fermentation tests with the selected yeasts were then run at 40 and 42°C in fermentation medium containing 50 g/L glucose. Ethanol concentration was measured after 20 and 40 h (*see* Table 1). Further incubation did not result in any significant increase in ethanol production by any of the strains screened.

From these 19 strains, those showing ethanol production greater than 15 g/L after 40 h were considered for assays at 45°C. Results of experiments at this last temperature are given in Table 2. *Kluyveromyces fragilis* and *Kluyveromyces marxianus* were the best strains for fermentation, producing amounts of ethanol between 18–22 g/L 48 h after inoculation.

Table 1
Ethanol Production (g/L) for Different Yeast Strains
at 40°C and 42°C in a Medium Containing 50 g/L Glucose

Yeast strains	40°C		42°C	
	20 h	40 h	20 h	40 h
S. cerevisiae 87	2.4	2.7	3.0	4.3
S. cerevisiae 157	12.7	16.2	7.1	9.5
S. cerevisiae 211	9.9	10.7	1.9	2.1
S. cerevisiae 1701	5.9	6.2	3.6	3.6
S. cerevisiae 20	14.7	16.8	11.5	13.9
S. cerevisiae 34	15.6	12.7	4.7	5.6
S. cerevisiae 178	15.6	12.7	9.4	11.2
S. cerevisiae 1685*	14.7	16.9	16.5	20.9
S. cerevisiae-pretoriensis FDH-I*	21.1	20.5	16.8	20.7
S. pretoriensis FJF-414	3.6	4.0	4.1	5.2
S. fermentati ACA-4	14.9	16.1	12.7	14.4
S. montanum 462	18.9	18.0	11.3	13.6
C. obtusa 1944	9.3	9.1	6.2	5.9
C. lusitaniae 1799*	20.6	21.0	15.4	18.0
K. marxianus 2713*	20.9	20.7	22.2	20.8
K. marxianus L.G.*	20.8	20.3	20.7	20.6
K. fragilis 2671*	20.5	20.1	20.7	20.4
K. fragilis L.G.*	20.8	21.0	21.7	20.6
K. veronae 1853*	21.6	21.1	21.1	20.6

*Yeast strains selected for further studies

Table 2
Ethanol Production (g/L) for Different Yeast Strains
at 45°C in a Medium Containing 50 g/L Glucose

Yeast strains	45°C		
	8h	24 h	48 h
S. cerevisiae-pretoriensis FDH-I	1.9	2.8	3.6
S. cerevisiae 1685	4.6	6.6	7.0
C. lusitaniae 1799	2.7	5.1	7.4
K. veronae 1853	7.3	7.8	9.2
K. marxianus 2713	9.0	12.9	19.5
K. marxianus L.G.	8.1	16.5	21.9
K. fragilis 2671	8.6	13.8	20.8
K. fragilis L.G.	0.4	15.6	18.2

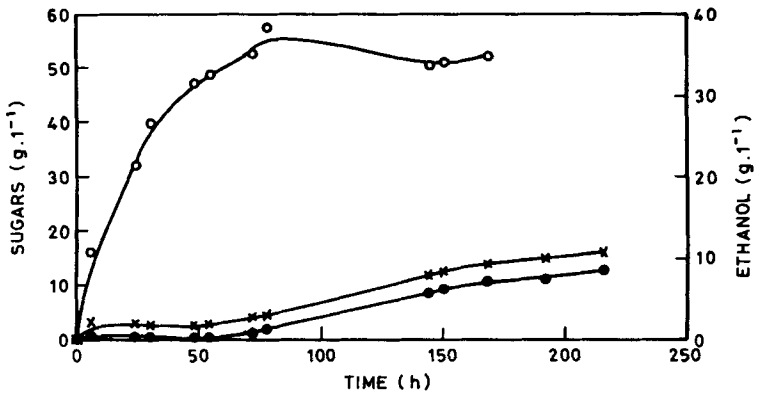

Fig. 1. SSF with *K. marxianus* L. G. at 42°C. Cellulase loading of 15 FPU/g. Substrate Solka-floc 10%. ○, ethanol. ×, reduc. sugars. ●, glucose.

SSF Assays

In order to establish the SSF performance of the four selected strains, SSF experiments at 42°C were carried out using a medium containing Solka-floc (10%) as cellulose source. Yeasts were acclimatized to SSF temperatures by growing them at 35°C, i.e., the temperature employed for inocula preparation.

Reducing sugars, glucose, and ethanol content in the flasks, during the SSF assays with *K. marxianus* strain, are shown in Fig. 1. Results for the experiments with the other *Kluyveromyces* strains followed a similar pattern. Tests of cell viability, carried out on the samples collected at different times, revealed a total loss of cell viability in *Kluyveromyces fragilis* 2671 cultures after 50 h of incubation. The same occurred in *K. fragilis* L. G. and *K. marxianus* L. G. and ETSIAM 2713 flasks, after 78 h. Maxima ethanol concentrations were observed at the same corresponding times. In Table 3, the main fermentation results obtained at the time of cell death with the different strains, are shown. Determinations of the residual cellulose after 216 h postinoculation gave values of (g/L): 7.3; 5.2; 3.5; and 3.7 for *K. fragilis* 2671, *K. marxianus* 2713, *K. fragilis* L. G., and *K. marxianus* L. G., respectively. Significant variations were not detected after that time.

Additional assays at 45°C were not satisfactory. Total loss of cell viability and maximum ethanol concentration in the media not higher than 20 g/L, after 24 h postinoculation, was the case for all *Kluyveromyces* strains tested (data not shown).

Comparative Sugars Release

In Fig. 2, comparative results of glucose and cellobiose release during the SSF assays with *K. marxianus* L. G., and the enzyme hydrolysis tests

Table 3
Fermentation Results for the K. Fragilis and K. Marxianus Strains

Yeast	Ethanol max (g/l)	Time (h)	Residual cellulose (g/l)	Y E/s
K.marxianus L.G.	37.6	78	9.8	0.50
K.marxianus 2713	33.5	78	12.7	0.46
K.fragilis L.G.	37.2	78	10.2	0.50
K.fragilis 2671	25.0	50	35.1	0.50

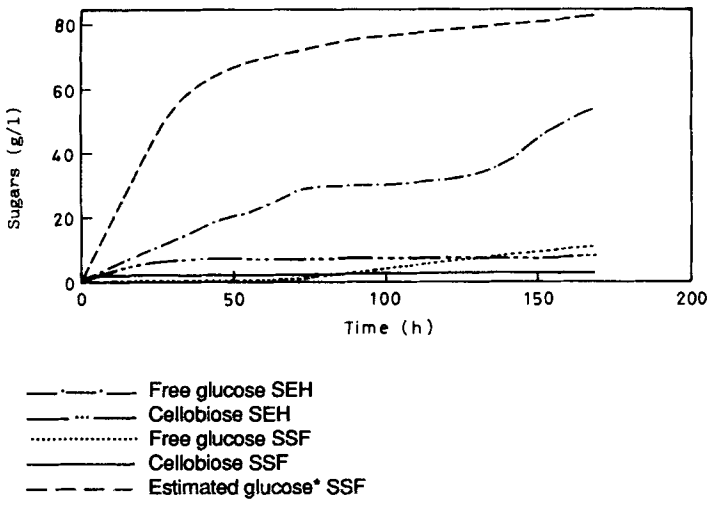

Fig. 2. Comparative glucose and cellobiose release (g/L) to the media during separate enzymatic hydrolysis (SEH) and SSF with *K. marxianus* L. G. of Solka-floc (10%) under the same temperature (42°C) and enzyme loading (15 FPU/g) conditions.

under the same temperature (42°C) and enzyme loading (15 FPU/g) conditions, are shown.

Glucose released in SSF assays was estimated, at a determinate time, from data of free glucose and ethanol concentrations detected at that time, considering an average ethanol yield of 0.5.

DISCUSSION

Initial screening results show that the *Candida* strains tested had the poorest thermotolerance among those of the three genera considered. With the exception of *C. lusitaniae* CBS 1799, they were not able to grow at 40°C, and even the *C. lusitaniae* strain showed decreased ethanol production activity at 42°C (*see* Table 1). *Saccharomyces cerevisiae-pretoriensis* FDH-I, previously cited as thermotolerant strain, achieved a comparative good ethanol production at temperatures of 40 and 42°C, but its activity strongly decreased at 45°C. This may be because of its low ethanol tolerance at that temperature (*10*). On the other hand, *K. marxianus* and *K. fragilis* strains were able to produce the highest ethanol concentrations at 45°C, being the only strains capable of producing similar amounts of ethanol over the temperature interval 40–45°C (*see* tables 1 and 2). This justifies the selection of the cited strains for the SSF trials.

During the first stage of the SSF experiments, at 42°C (Fig. 1), there was a continuous increase in the ethanol content while the glucose content remained very low (lower than 1 g/L). At the end of this stage (*see* Table 3), ethanol concentrations between 33–37 g/L were obtained when utilizing *K. marxianus* L. G. and ETSIAM 2713 and *K. fragilis* L. G. The ethanol yields were high, and varied between 0.46 for *K. marxianus* ETSIAM 2713 and 0.5 for *K. marxianus* L. G. and *K. fragilis* (*see* Table 3). Similar results with respect to ethanol yield were obtained with *K. fragilis* CBS 2671, though ethanol production was significantly lower, because of the earlier cells death.

The end of the first stage was characterized by cessation of ethanol production. This was coincident in time with yeast cell death. From here on, ethanol concentration remained more or less constant, but sugar content started to increase, indicating continuance of cellulasic activity. The decrease in the amount of residual cellulose between 78 h (*see* Table 3) and those at 216 h also corroborate this fact.

On the other hand, (*see* Fig. 2), the amount of glucose released in SSF experiments with *K. marxianus* L. G. was greater than that during a simple hydrolysis process under the same incubation conditions. The SE in SSF after 78 h of incubation at 42°C, when cell death occurred, was much higher (79.6%) than that achieved in enzyme hydrolysis tests (31.7%). Nevertheless, from this time on, the rate of release of glucose was significantly decreased. This indicated a greater inhibition of the enzyme activity, possibly because of the absence of glucose fermentative activity.

Accordingly, one may conclude that if cell viability in the SSF cultures had been longer, higher quantities of cellulose could have been hydrolyzed and fermented. An estimated production of up to 42.5 g/L ethanol might have been achieved.

The results presented here confirm the importance of using thermotolerant yeasts in SSF processes in order to improve hydrolysis rates and achieve higher ethanol production.

Further studies will be carried out in our laboratory in order to increase the thermotolerance of the *Kluyveromyces* strains, either by adequate supplementation of the fermentation media or by natural adaptation of the yeast to high temperature conditions.

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