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Optimization of the Simultaneous Saccharification and Fermentation Process Using Thermotolerant Yeasts

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ABSTRACT

Different treatments to improve the thermotolerance of fermenting yeasts for simultaneous ethanol saccharification and fermentation process of cellulosic materials have been examined. Yeasts of the genera *Saccharomyces* and *Kluyveromyces* were tested for growth and fermentation at progressively higher temperatures in the range of 42–47°C. The best results were obtained with *K. marxianus* LG, which was then submitted to different treatments in order to achieve thermotolerant clones. A total of 35 new clones were obtained that dramatically improved the SSF of 10% Solka-floc substrate at 45°C when compared to the original strain, some with ethanol concentrations as high as 33 g/L.

Index Entries: Cellulosic materials; ethanol fermentation; simultaneous saccharification and fermentation; thermotolerant yeasts; mutants.

INTRODUCTION

Simultaneous saccharification and fermentation (SSF) involves the enzymatic saccharification of cellulose and the simultaneous yeast fermentation of sugars to ethanol in the same vessel. The primary advantages of this system are: avoidance of the necessity of separate reactors for saccharification and fermentation and enhancement of the rate of cellulose hydrolysis, because catabolite repression is minimized.

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For a successful SSF process, it is necessary that the fermentation and saccharification systems are compatible (i.e., similar pH, temperature, and optima substrate concentration). One main problem associated with the SSF process is the different temperatures for saccharification and fermentation. Since the optimum temperature for the enzymatic activity is usually in the range of 50–60°C, much higher than those tolerable by yeasts, the use of thermotolerant yeast strains (yeasts able to ferment at ≥ 40°C) would be desirable.

Although thermotolerant yeasts could offer many advantages to the fermentation industry, there have been few reports of selection of strains that produce ethanol at elevated temperatures. Hence, the purpose of this article was to increase the thermotolerance of different yeast strains selected in a previous work (*1*). Strategies for enhancing thermotolerance of the yeasts include the following:

1. Adaptation by cultivation at progressively higher temperatures;
2. Selection of survivors from a gentle heating process of the broth; and
3. Exposures of the yeasts cells to mutagenic agents, such as UV light and ethylmethanosulfonate, and selection of the survivors after cultivation of the treated cells at high temperatures.

MATERIALS AND METHODS

Microorganisms

Yeast strains utilized in this work included: *Saccharomyces cerevisiae* NCYC 975 *Saccharomyces cerevisiae-pretoriensis* FDHI (this strain was described and donated by Benítez [2] [Univ. Sevilla, Spain]), *Kluyveromyces fragilis* 2671, kindly provided by Van Uden from Gulbenkian Institute (Lisbon-Portugal), *Kluyveromyces marxianus* 2713, and *Kluyveromyces marxianus* LG. These two last strains were obtained from Santamaria (Univ. Politécnica, Madrid, Spain) and Guiraud (Univ. of Langedoc, Montpellier-France), respectively. All the cited strains have been described as thermotolerant in a previous work (*1*).

Culture Media and Inocula Preparation

The medium employed for growth and fermentation assays consisted of (g/L): yeast extract (Difco), 2.5; peptone (Oxoid), 5.9; NH_4Cl, 2.0; KH_2PO_4, 1.0; $MgSO_4$, 0.3; and glucose, 30. Agar was added to the described broth for plate preparation at a concentration of 20 g/L. In SSF experiments, glucose was replaced with 10% (w/v) Solka-floc (88% cellulose content). All components of the fermentation were autoclaved for 20 min at 121°C, and a sterile filtrated enzyme loading of 15 FPU/g substrate was added.

The yeast inocula cultures were prepared by aseptically transferring a loopful of cells from collection agar slants to 100-mL Erlenmeyer flasks with 25 mL of growth medium. The flasks were incubated on a Braun rotary shaker (model certomat H) at 170 rpm and 42°C for 16 h.

Cellulase Source

The cellulase enzyme for SSF experiments was kindly provided by J. Pourquie from IFP (Institut Français du Petrole). This enzyme was obtained by ultrafiltration of a culture of the mutant *Trichoderma reesei* CL-847 followed by a freeze-drying of the liquid fraction containing the enzyme. This fraction was characterized for activities in a previous paper (*1*).

Culture Temperature Gradient Treatments

Assays to adapt the yeast strains to grow at high temperatures were carried out in 250-mL Erlenmeyer flasks containing 50 mL of growing medium. Flasks were inoculated with 10% (v/v) yeast seed culture prepared as above described, and growth studies were carried out at progressively higher temperatures in the range of 42–47°C. Flasks were checked for cell growth after 3 d postinoculation.

Cell Heat Treatment

The selection of *K. marxianus* LG by heating of yeast cultures was conducted by seeding agar plates with 100 μL of a 500–1000 cell/mL containing medium, which was obtained by appropriate dilution of inoculum cultures. The agar plates were incubated at 48°C for 3 d, and then a number of colonies were chosen at random and used to inoculate Erlenmeyer flasks with growth medium. The flasks were incubated for cell propagation at 42°C. One-mililiter aliquots of the obtained cultures were then used to inoculate Durham tubes containing growth medium. Fermentations in the Durham tubes were carried out for 24 h at 47 and 49°C. The thermotolerant cultures were selected according to the intensity of CO_2 formation after the cited period.

Mutagenic Treatments

Two pen ray lamps (Ultraviolet Products, Inc., San Gabriel, CA) were used for UV light exposure. UV light flux was 600 $\mu W/cm^2$ as measured by a UVX Digital Radiometer (Yellow Springs Instrument Co., Model 65). Petri dishes were spread with 50 μL of a previously diluted inoculation medium to a cell density of 1000–2000 cell/mL. The plates were treated with different UV energy doses by varying time exposure. UV doses employed were 1.2, 3.0, 6.0, 8.4, 10.2, and 12.0 mJ/cm^2. After UV irradiation treatments, the plates were incubated for 3 d at 48°C. The colonies growing in these conditions were submitted to the same protocol as described for the heating process for the selection of further SSF tests.

K. marxianus LG cells from an inoculum culture were centrifuged and then resuspended in 20 m*M* sodium phosphate buffer, pH 7.0, for ethylmethanesulfonate (EMS) treatment. Different ethylmethanesulfonate (Sigma) doses, namely 120, 300, 600, 840, 1020, and 1200 μL/100 mL, were then added to the corresponding flasks, which were incubated on a shaking water bath at 28°C for 2 h. Subsequently, selection of colonies for SSF tests was effected as described for the heating treatment. In all cases, cells of *K. marxianus* LG previously adapted to grow at 47°C were employed. As in the case of UV treatment, three agar plates were seeded for each EMS treatment. The experiment was repeated a minimum of three times per dose.

SSF Experiments

The SSF fermentations were carried out in 100-mL flasks, each containing 50 mL of SSF medium. The flasks were inoculated with 10% (v/v) of inoculum medium. SSF flasks were coupled with rubber stoppers with fermentative tubes filled with 25 mL of 50% sulfuric acid to prevent ethanol evaporation. Flasks were periodically checked during the assays for ethanol, glucose, reducing sugars, and residual cellulose contents, as well as for cell viability.

Analytical Procedures

Ethanol was determined by gas chromatography in a Konik 2000 C chromatograph with a stainless-steel column of Carbowax 20M (2 m × 1/8 in). Total reducing sugars were determined by the Nelson-Somogyi (*3*) method, and glucose was measured by the hexokinase glucose-6-phosphate dehydrogenase assay (Glucoquant kit, Boheringer-Manheim). Residual cellulose was determined as described by Spindler et al. (*4*). Cell density was controlled by sowing the samples on agar plates and checking for colony-forming units (CFU) after 3 d of incubation at 30°C.

RESULTS

Apaptation of Yeasts by Growing at Progressively Higher Temperatures

Attempts to increase the thermotolerance of the yeasts utilized in this work by cultivating them at progressively higher temperatures were made in a 42–47°C range. All yeast strains tested did not grow at 45°C when cells previously grown at 42°C were directly incubated at that temperature, but growth was observed in all cases when a previous adaptation of the microorganisms was effected by successively cultivating them at 42 and 44°C prior to incubation at 45°C. Nevertheless, *Saccharomyces* species

Table 1
Ethanol Production and Yields
(Referred to as the Ratio g Ethanol/g Potential Glucose in Solka-floc)
in SSF tests at 45°C after 78-h Fermentation Period
with Selected Clones Obtained by the Heating Process

Clone reference	Ethanol max., g/L	Residual glucose, g/L	$Y_{P/S}$
C-7	27.8	10.9	0.29
C-9	29.0	10.5	0.30
C-10	29.6	4.8	0.30
C-13	29.2	6.4	0.30
C-14	29.1	6.4	0.30
C-15	29.4	3.8	0.30
C-22	32.8	9.7	0.34
C-23	32.4	8.6	0.33
C-24	25.0	15.3	0.26
C-25	33.0	4.3	0.34
C-27	32.1	6.3	0.33

showed very poor growth and were excluded for secondary assays. Additionally, cell growth at 47°C was only observed for *K. marxianus* LG, and accordingly, this strain was chosen for subsequent studies.

Selection of Thermotolerant Clones Induced by Heating, UV Irradiation, and EMS Treatments

K. marxianus LG grown at 42°C was incubated at 48°C on agar plates. Thirty colonies of those observed on the plates were chosen at random and transferred into Durham tubes containing growth medium, where further incubation at 47°C was effected. Based on the CO_2 formation after these fermentation assays, 13 clones were chosen and later incubated on agar plates at 49°C. From these 13 clones, those showing the best results (*see* Table 1) were then selected for subsequent 10% Solka-floc SSF assays at 45°C following the same procedure. *K. marxianus* LG C-22, C-23, C-25, and C-27 produced about 33 g ethanol/L from 100 g Solka Floc/L at 45°C in 78 h.

Kluyveromyces marxianus LG was also treated with different UV and EMS doses, and then (as for the heat treatment) a total of 11 clones were selected for further SSF tests by their fermentation ability in Durham tubes for each of the cited treatments at 49°C. In Tables 2 and 3, the results of SSF tests with the selected clones after UV irradiation and EMS treatments, respectively, are shown. When comparing these results to

Table 2
Ethanol Production and Yields
(Referred to as the Ratio g Ethanol/g Potential Glucose in Solka-floc)
in SSF tests at 45°C after 78-h Fermentation Period
with Selected Clones Obtained by the UV Treatments

Clone reference	Ethanol max., g/L	Residual glucose, g/L	$Y_{P/S}$
UV-21	29.1	5.6	0.30
UV-24	25.3	6.3	0.26
UV-25	29.9	5.4	0.31
UV-37	23.7	9.5	0.24
UV-38	25.5	7.7	0.26
UV-39	27.7	8.2	0.28
UV-40	27.9	6.5	0.29
UV-41	28.5	4.7	0.29
UV-42	27.3	9.1	0.28
UV-44	31.2	1.6	0.32
UV-52	24.1	11.2	0.25

Table 3
Ethanol Production and Yields
(Referred to as the Ratio g Ethanol/g Potential Glucose in Solka-floc)
in SSF tests at 45°C after 78-h Fermentation Period
with Selected Clones Obtained by the EMS Treatments

Clone reference	Ethanol max., g/L	Residual glucose, g/L	$Y_{P/S}$
EMS-5	25.0	4.8	0.26
EMS-7	19.6	4.3	0.20
EMS-8	29.4	3.5	0.30
EMS-9	29.2	2.9	0.30
EMS-12	24.9	10.0	0.26
EMS-22	30.3	3.6	0.31
EMS-26	30.8	3.0	0.32
EMS-29	23.6	8.5	0.24
EMS-31	29.5	5.0	0.30
EMS-33	26.0	9.8	0.27
EMS-35	28.1	7.7	0.29

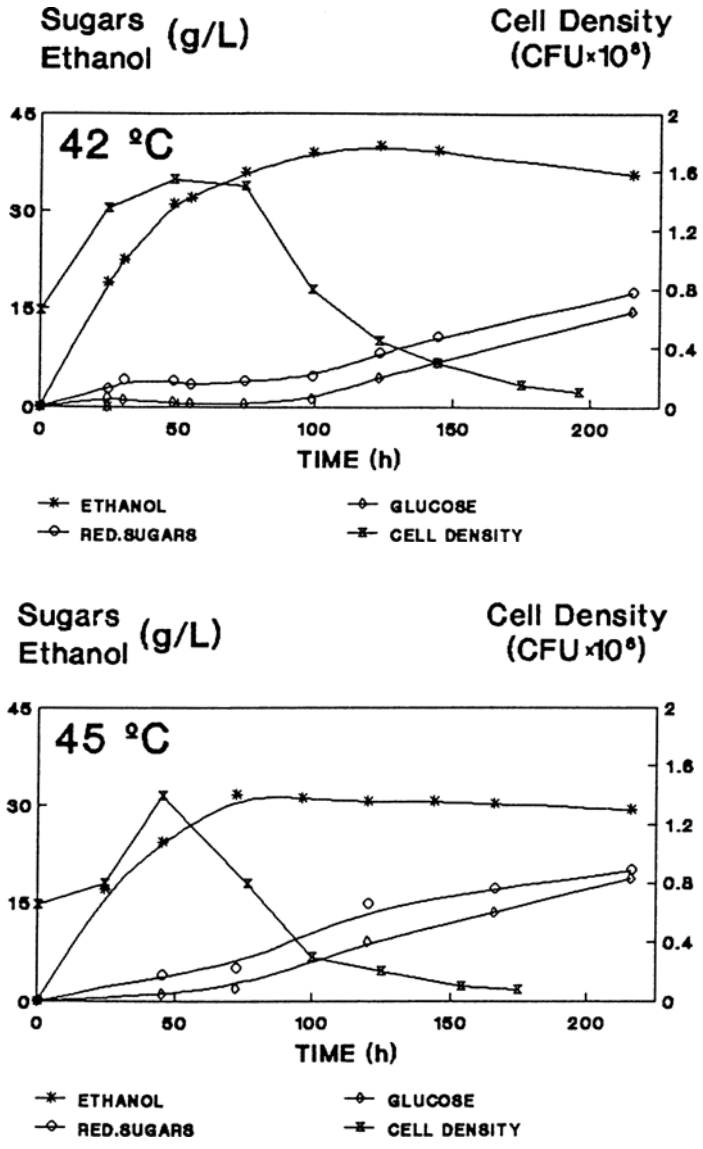

Fig. 1. SSF performance of *K. marxianus* LG C-22 at different temperatures.

those obtained with the thermotolerant clones induced by heating, it can been seen that ethanol yields in the SSF tests were not improved with the mutagenic treatments.

Typical SSF performance of *K. marxianus* LG C-22 on 10% Solka-floc media at 42 and 45°C is shown in Fig. 1. During the first stage of the SSF experiments (about 100 h at 42°C and 78 h at 45°C), there was a continuous increase in the ethanol content, whereas the glucose content remained

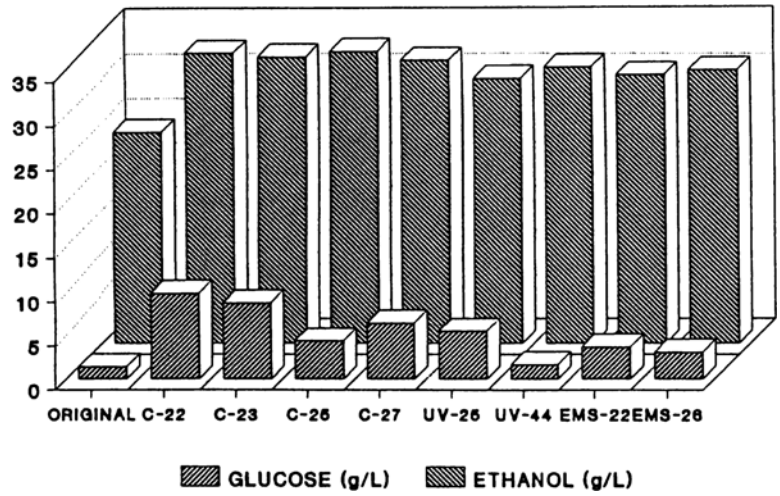

Fig. 2. Comparative SSF results obtained from 78 h fermentation with the best selected *K. marxianus* LG clones and the original adapted strain.

very low. The end of this stage was characterized by cessation of ethanol production and a strong decrease in viable cell density as the fermentation progressed. From here on, ethanol concentration remained more or less constant, but sugar content started to increase, showing continuance of cellulosic activity.

To facilitate comparison between different selected clones and the initial *K. marxianus* LG strain adapted to grow at 47°C, Fig. 2 shows the average amounts of residual glucose and ethanol present in SSF cultures after 78 h fermentation (when maximum ethanol concentration was achieved). In Fig. 3, the remaining cellulose in SSF tests at the end of runs (9 d postinoculation) for the best selected clones is shown. As will be discussed, these results represent significant achievements concerning the thermotolerance and the ability to develop the SSF process at 45°C of the original *K. marxianus* LG strain. The results from 78-h ethanol SSF tests of eight different selected clones of those assayed for the SSF tests also reveal significant improvements in ethanol production in relation to those achieved with the previously adapted *K. marxianus* LG strain. The remaining cellulose present in SSF media after 9 d postinoculation is also significantly lower in the case of the selected clones, thus revealing a higher efficiency to obtain a more complete hydrolysis of the cellulose when the selected *K. marxianus* LG clones are utilized.

DISCUSSION

According the results, the heating, UV irradiation, and EMS treatments have been revealed as suitable alternatives to induce thermotolerant clones of *K. marxianus* LG. UV irradiation and ethylmetanesulfonate, at the

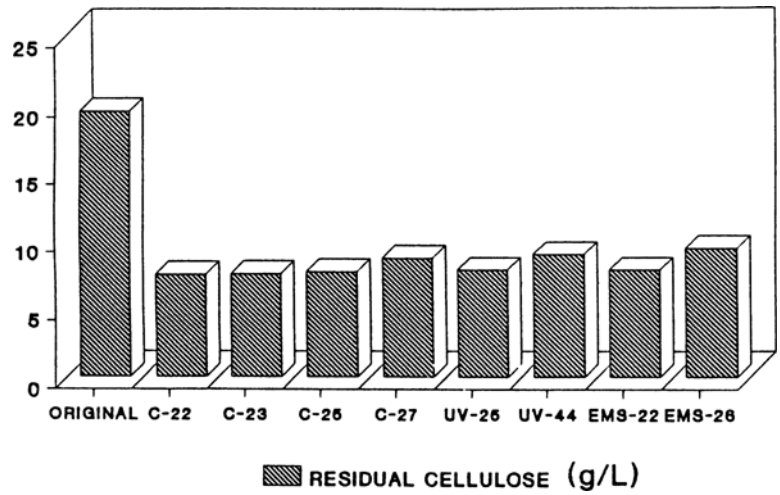

Fig. 3. Cellulase remaining in the SSF medium at end of runs (9 d fermentation) with the best selected *K. marxianus* LG clones and the original adapted strain.

doses employed in this work, were not found to be more effective than heat shock.

It can also be observed that thermoadaptation by successive yeast transfers to higher cultivation temperatures has been successful in improving the thermotolerance of *Kluyveromyces marxianus* LG strain. Yeasts were rapidly killed when cells growing at 42°C or lower temperatures were directly incubated at 45°C; however, cell death was avoided if yeasts were previously allowed to adapt at intermediate temperatures for a limited period of time before the cultivation to the highest temperature. Nevertheless, capacity to grow at 45°C was loss after long-term cultivations which could be explained by different factors that have been reported to influence the thermoresistance of the yeasts under prolonged cultivation cycles. It is known, for example, that at high temperatures, oxygen solubility decreases and the cell's nutritional requirements, i.e., for unsatured fatty acids, increases (*5*). In addition, ethanol intolerance is accentuated at higher temperatures because of increasing membrane rigidity as a result of an increase in the content of saturated fatty acids (*6,7*). These issues could be partly responsible for the reduced cell viability observed in the long-term cultures.

Cell heat process results showed that clones capable of growing at 49°C were the best fermentative clones for SSF tests at 45°C. It could be inferred that the increased resistance of yeasts to thermal inactivation is directly related to their capacity to ferment at higher temperatures. Some authors (*8*) have suggested that heat shock (exposure of cells to high temperature) induces a set of proteins that can be responsible for the acquisition of thermotolerance in heat-stressed cells. In addition, it has

been reported (*9*) that heat shock of *S. cerevisiae* not only induced heat-shock proteins and thermotolerance, but also imparted some degree of ethanol tolerance. UV light (*10*) can also induce synthesis of heat-shock protein. These issues can explain the results showed in Tables 1, 2, and 3 with the selected clones. These results are dramatically better than those obtained in Solka-floc SSF tests at 45°C with the original *K. marxianus* LG strain, which was not able to produce more than 20 g/L ethanol during the fermentation of 10% Solka-floc SSF medium at this temperature (*1*).

The remaining cellulose present in SSF media after 9 d postinoculation (Fig. 3) is also significantly lower, revealing a higher efficiency to obtain a more complete hydrolysis of the cellulose when the selected clones are used. This observation could be explained as a lower inhibition of the cellulolytic enzyme activity by end products that, as glucose, are present in SSF media in lower concentrations in the case of selected clones when compared to an initial adapted strain. This condition is in relation to the capacity of the yeasts to consume and ferment the glucose at higher temperatures.

The results obtained with the selected clone at 42 and 45°C showed that the maximum ethanol concentration in the media was achieved more rapidly as the SSF temperature was increased. However, despite the improvements achieved in this work to carry out the SSF process at temperatures closer to those for optimal activity of the enzyme utilized, the ethanol production is even lower at the higher temperature employed. Spindler et al. (*4*), using mixed cultures of *Brettanomyces clausenii* and *Saccharomyces cerevisiae* at 37°C, obtained 95% equivalent cellulose conversion and 45 g ethanol/L with a residence time of 7 d. These results suggest that the higher SSF temperatures increased the hydrolysis reaction rate and the ethanol production rate, thereby decreasing the residence time. Since sugars are released more rapidly as the SSF temperature increases and all the selected clones are able to grow at 49°C, the ethanol concentrations found in SSF tests at higher temperature are unexpectedly low. This leads the authors to believe that the ethanol intolerance of yeasts, which is more severe with increasing temperature, is responsible for the fact that the yeast does not ferment all of the sugars produced at this temperature. This will be studied in further work, and any method to improve the ethanol tolerance of the selected yeast should be suitable to increase its ability to ferment and conduct the SSF process at higher temperatures with shorter residence times and better productivities.

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