Development of a "Stress Model" Fermentation System for Fuel Ethanol Yeast Strains

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ABSTRACT

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During industrial scale fuel ethanol fermentations, yeast encounters a multitude of stress factors that impose constraints on growth and fermentative metabolism. These stresses include high sugar concentration, elevated temperature, high ethanol concentrations, low external pH and the weak organic acids lactic and acetic. Yeast strains which are tolerant to these stresses and able to synthesize high ethanol concentrations in their presence would be most desirable for use in industrial scale fuel ethanol production. In this study, a "stress model" fermentation system was developed as a tool to screen candidate yeast strains for relative stress resistance. The stress model was designed on the basis that the degree of ethanol produced by a particular strain would be indicative of the stress resistance of that particular strain. Eight strains of *Saccharomyces cerevisiae,* each with different backgrounds and fermentative capabilities, were screened for relative stress resistance using the stress model. The results obtained indicate that the sum of the stress factors in the stress model exceeded the tolerance level of most of the strains screened (approximately 40%). Two strains in particular, J006 and A007, displayed superior fermentative performance and produced significantly ($P < 0.01$) higher final ethanol concentrations when compared to the other strains.

Key words: Alcoholic fermentation, corn mash, fuel ethanol production, industrial yeasts, *Saccharomyces cerevisiae,* yeast stress resistance.

INTRODUCTION

Fuel ethanol is produced by fermenting sugars derived from starches or other carbohydrate-containing raw materials. It can be produced from a wide variety of feedstocks including corn, wheat, barley, sorghum, sugar beet, sugar cane, and cellulose, as well as various agricultural wastes. The majority of fuel ethanol currently produced in North America uses field corn as a feedstock, as it is the most abundant and economical source of starch[13]. Very high gravity (VHG) corn mashes containing ≥30% dry solids are typically prepared, and the starch is hydrolyzed to glucose by specific liquefying and saccharifying enzymes. Today, many commercial fuel ethanol production facilities employ simultaneous saccharification and fermentation (SSF), where the saccharifying enzyme and yeast are added to the corn mash at the same time.

During industrial scale corn mash fermentation processes, yeast may be confronted with a variety of environmental stresses that can lead to impairment of yeast growth and fermentative metabolism. For instance, when the yeast is inoculated into VHG corn mashes, a considerable hyperosmotic stress is imposed on the cells due to the high sugar concentration in the medium. As fermentation proceeds, the cells must cope with the progressive accumulation of ethanol in the corn mash. Depending on the ethanol production facility, other stress factors such as increased temperature, low pH and the weak organic acids, lactic and acetic acid, may also be encountered by the yeast during fermentation. Under the appropriate conditions, these stress factors can cause the loss of yeast cell viability, reduced yeast growth, increased fermentation times, decreased fermentation rates and possibly stuck or sluggish fermentations[6,16,22].

Even a slight reduction in ethanol yield can constitute a considerable economic loss to the ethanol producer. As such, yeast strains which are capable of tolerating various forms of stress and are capable of synthesizing large quantities of ethanol in their presence would be most desirable for use in industrial scale fuel ethanol production. Employing strains which have the ability to respond and adapt to stressful conditions and maintain a high stress resistance during industrial alcoholic fermentation would greatly improve the speed and efficiency of the fermentation process. Accordingly, the objective of this study was to develop a "stress model" fermentation system that could be used to screen candidate yeast strains for relative stress resistance, and evaluate the suitability of a specific strain for use in industrial ethanol fermentations.

MATERIALS AND METHODS

Yeast strains screened for relative stress resistance

A total of eight *Saccharomyces cerevisiae* strains were screened for relative stress resistance using the stress model fermentation system: T002, R003, RW004, I005, J006, A007, B008 and W009 (Table I). A control *S. cerevisiae* strain S001, was employed during the screening process. All strains were single colony isolates obtained from the culture collection at Alltech, Inc. that were recoded for the purpose of this study. The yeast strains were identified as *S. cerevisiae* by the bioMerieux ID 32 C

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Table I.

Strain identification	Yeast code (Alltech, Inc.)	Characteristics or comments	Background
S001	362-83-1-S001	Starts fermentation fast, high alcohol producer	Industrial ethanol strain
T002	211-90-T002	Ferments at high temperature, high alcohol producer	Industrial ethanol strain
R003	273-29-1-R003	Fast fermenter; ferments at high temperature	Russian beverage distillery strain
RW004	384-32-3-RW004	Ferments at high temperature, high alcohol producer	Rice wine strain
I005	384-32-4-I005	Ferments at high temperature	Indian beverage distillery strain
J006	384-32-5-J006	High alcohol production, high sugar tolerance	Jamaican cane juice strain
A007	384-32-6-A007	Growth at high temperature	Associated with fermentation
B008	384-32-7-B008	High alcohol producer	Super-attenuated beer strain
W009	384-32-8-W009	High alcohol producer, high sugar tolerance	Wine strain

Analytical Profile Index (API) system in accordance with the manufacturer's instructions. The strains were further classified as ale strains because they were able to grow well at 37°C, but were unable to assimilate and ferment the disaccharide sugar melibiose[2,3]. All yeast strains were maintained on slants, which were stored at 4°C and sub-cultured monthly. Slants were prepared with Yeast Malt (YM) agar according to the manufacturer's instructions. YM agar contains 3 g/L of yeast extract, 3 g/L of malt extract, 5 g/L of peptone, 10 g/L of dextrose and 20 g/L of agar.

Preparation of yeast inocula for the stress model fermentations

Prior to the start of fermentation (approximately 42 h before), a small quantity of pure yeast culture was used to inoculate 50 mL of fresh sterile YM broth. (YM broth consists of the same ingredients as YM agar without the agar). The yeast was cultivated in an orbital shaker at 30°C (200 rpm) and allowed to grow for 24 h. An aliquot (10 mL) of this 24 h culture was used to inoculate 1 L of sterile YM broth (4 flasks, each with 250 mL of broth) and grown for an additional 18 h. After 18 h of growth, the yeast culture was harvested by centrifugation (4000 × *g* for 10 min) at 4°C. The cell pellets were re-suspended in 40 mL of sterile deionized water. An appropriate volume of the cell suspension was added to 200 g of mash in order to achieve an inoculation rate of 1×10^6 cells/% dry solids/mL mash. A cell count was conducted on the culture to ensure the correct inoculation rate was attained for each fermentation.

Corn mash preparation

Liquefied mash (with approximately 30% [w/v] dry solids) was prepared using corn (US #1) purchased from a local supplier (Thompson & Shearer, Nicholasville, KY). Corn was ground using a hammer mill (Model No. 9506TF, Bliss Industries, Inc., Ponca City, OK) fitted with a #4 screen (1.588 mm mesh openings). To prepare the mash, approximately 1379 g ground corn was slowly added to 2621 mL of tap water pre-heated to 60°C. The slurry was continuously mixed during the cooking phase using a Silverson Homogenizer (Model L4RT). Following the addition of corn, 0.33 mL of α-amylase (High T DS – 145,000 amylase units/mL, Alltech, Inc., Nicholasville, KY) per 100 g of corn was added to reduce viscosity. The slurry was heated to 85°C, and maintained at this temperature for 20 min. To achieve starch gelatinization, the mash was autoclaved at 121°C for 20 min. After cooling to 85°C, 0.67 mL of α-amylase per 100 g corn was added, and the mash was maintained at 85°C with constant stirring (Cole-Parmer Stir Pak Laboratory Mixer Model No. 50002-30) for 1 h. Water lost during autoclaving was made up with sterile water. The antibiotic product Lactoside 247 (Alltech, Inc., Nicholasville, KY) was added (5 μg/mL) to prevent bacterial contamination and maintain consistency between experiments.

Stress model fermentation design

The stress model fermentations were performed in 500 mL Erlenmeyer flasks containing 200 g of corn mash. The parameters of the stress model fermentation system used to screen candidate *S. cerevisiae* strains for relative stress resistance are illustrated in Table II. Lactic acid (85%, Fisher, Cat. No. A162-500) and acetic acid (glacial, Fisher, Cat. No. A491-212) were added to the corn mash to achieve concentrations of 4% (w/v) and 0.3% (w/v), respectively. After weak acid addition, the mash pH was adjusted to pH 4 using 8M KOH. Amyloglucosidase (Allcoholase II L400, Alltech, Inc., Nicholasville, KY) was used for saccharification of the dextrins at an addition rate of 0.08% the weight of grain. Urea, at 0.016% the weight of mash in each flask (0.032 g/200 g mash), was used as the nitrogen source. The temperature was maintained at 37°C throughout fermentation in an incubator shaker (New Brunswick Scientific, Model C25KC). The stress model fermentations were performed in duplicate and repeated twice. Samples were withdrawn for analysis from each flask at 6, 18, 24, 48, 72 and 96 h incubation times. Fermentation rates were calculated from the linear portions of the curves generated as ethanol was synthesized over time during the initial 18 h of fermentation.

HPLC analysis of fermentation samples

Concentrations of ethanol, carbohydrates (dextrin, maltotriose, maltose and glucose), and lactic and acetic acids were determined by high-performance liquid chromatography (HPLC). Fermentation samples were centrifuged (4000 × *g* for 15 min) and the supernatant filtered (0.20 μm filter) prior to analysis. A 20 μL portion of a sample or standard solution was injected onto a Bio-Rad HPX-87H

Table II.

Corn mash solids	30% (w/v) dissolved solids
Lactic acid	4% (w/v)
Acetic acid	0.3% (w/v)
pH	4
Fermentation temperature	37°C

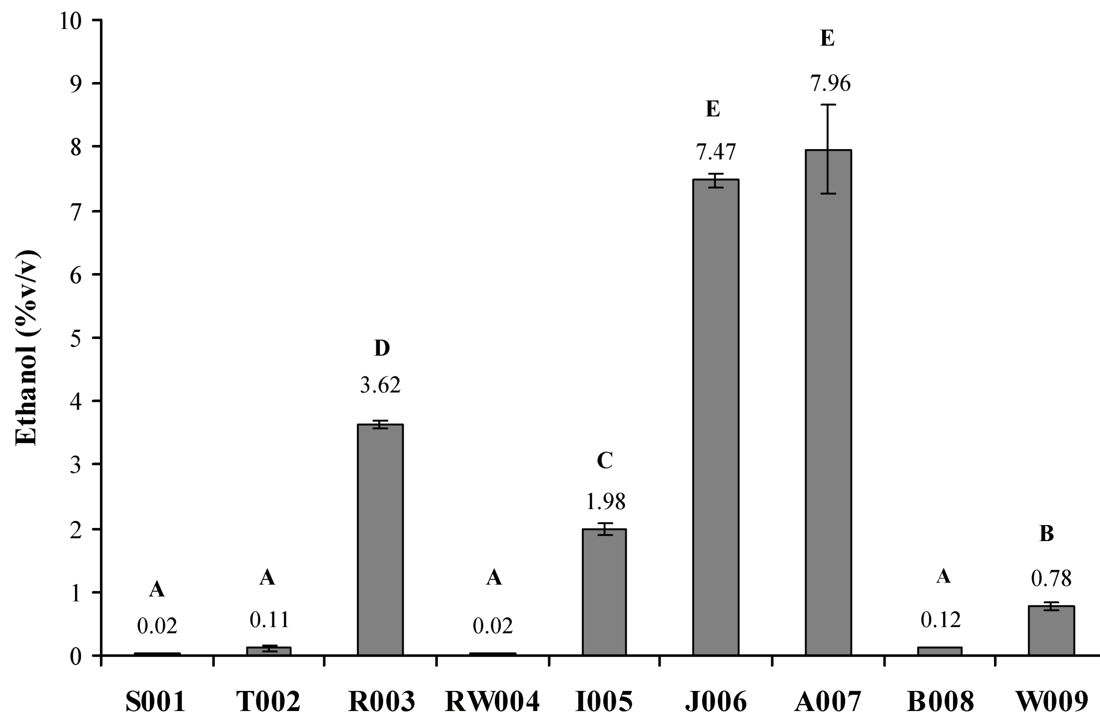

Fig. 1. Final ethanol concentrations (%v/v) produced after 96 h by various *S. cerevisiae* strains in the stress model fermentation. Values are means of duplicate samples from two separate experiments. Error bars indicate ± standard deviation between replicate samples. Means with different letters differed significantly ($P < 0.01$).

Aminex ion exclusion column (Cat. No. 125-0140) coupled to a refractive index detector (Model 2410, Waters Chromatographic Division, Milford, MA). The column was operated at 65°C and sulfuric acid (0.002 M) was used as the mobile phase at a flow rate of 0.6 mL/min. The data were processed by Millennium Software (Waters Chromatographic Division, Milford, MA).

Viable yeast cell numbers during fermentation

Viable yeast cell numbers were determined using the ASBC methylene blue staining method[1]. Cell counts of 24 h fermenting samples were established using a Neubauer Haemocytometer (Fisher, Cat. No. 02-671-5) at 40× magnification with a Nikon Eclipse light microscope (Model No. E400).

Statistical analysis of data

All fermentation rate and final ethanol data were analyzed using the General Linear Model (GLM) procedure of SAS Software (SAS Institute, Cary, NC, USA).

RESULTS

A model fermentation system incorporating a number of stress factors encountered by yeast during industrial fuel ethanol fermentation was developed as a tool for screening candidate yeast strains for relative stress resistance. The model fermentation system, which has been termed the stress model, was designed on the basis that the amount of ethanol produced by a particular yeast strain would be a good indicator of the resistance of the strain to environmental stress. The parameters of the stress model were chosen based on previously published results with the control strain, S001[8,9]. The least stressful combination of the stress factors (corn mash solids level, lactic and acetic acid concentrations and pH) that completely inhibited ethanol production by strain S001 at 37°C was chosen for the stress model (Table II). A total of eight *S. cerevisiae* strains, each with different backgrounds, were screened for relative stress resistance using the stress model fermentation system (Table II). The strains were selected based on previous experiments in this laboratory (Alltech, Inc.) which established that the yeast displayed different growth properties and fermentative behaviours during corn mash fermentation (data not shown). The rate at which ethanol was synthesized during the initial 18 h of fermentation, as well as the final concentration of ethanol (after 96 h), was used to assess the stress resistance of the yeast strains. Yeast strains which were unable to synthesize ethanol in the stress model were considered to be stress-sensitive, while those that could produce relatively large quantities of ethanol were deemed to be stress-resistant.

Effect of yeast strain on the production of ethanol during the stress model fermentation

No significant differences ($P < 0.01$) in the rates at which the various *S. cerevisiae* strains synthesized ethanol in the stress model during the initial 18 h of fermentation were observed. All fermentation rates were <0.01 g/L/h, as only minimal ethanol was produced (<0.10% [v/v]) during that time (data not shown).

However, the quantities of ethanol produced by the various strains after 96 h of fermentation in the stress model differed considerably. The yeast strains were statistically grouped into five categories (A, B, C, D and E)

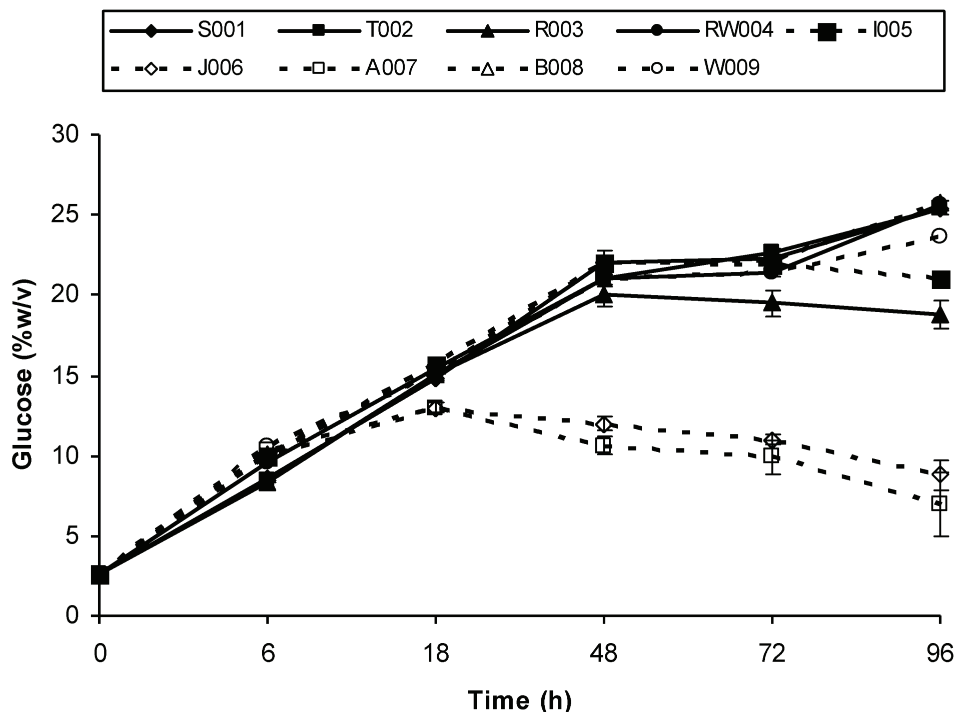

Fig. 2. The concentrations of glucose (%w/v) in the corn mash at various time points during the stress model fermentation with various *S. cerevisiae* strains. Values are means of duplicate samples from two separate experiments. Error bars indicate ± standard deviation between replicate samples.

based on their final ethanol concentrations (Fig. 1). Yeast strains in Group A, T002, RW004 and B008 were susceptible to the imposed stresses and produced only small concentrations of ethanol (≤0.12% [(v/v)]) after 96 h. These strains were grouped with the control strain, S001. Strains W009, I005 and R003, which were grouped independently as Group B, C and D, respectively, were reasonably tolerant of the stress model and produced more ethanol when compared to the stress-sensitive strains. Of these strains, R003 generated the most ethanol (3.62% [v/v]) in the stress model. The most stress-resistant yeast strains screened were J006 and A007 (Group E). The concentrations of ethanol produced by these strains, 7.47% and 7.96% (v/v) respectively, were significantly higher than the ethanol produced by the other yeast strains.

Effect of yeast strain on the utilization/fermentation of mash sugars during the stress model fermentation

The most abundant fermentable sugar available to the yeast during fermentation of the stress model was glucose. However, maltose and maltotriose were also present in the corn mash, particularly within the initial 18 h of fermentation. Yeast inocula and glucoamylase were added to the stress model fermentation concurrently, and as a result, lower saccharides (mainly glucose, maltose and maltotriose) were released and consumed continuously throughout the course of the 96 h fermentation. Due to the continuous activity of the glucoamylase and the subsequent fluctuation of the maltose and maltotriose concentrations in the mash, it was difficult to determine which of the yeast strains were capable of utilizing particular sugars at different stages during the fermentation.

No major differences in the disappearance of glucose were observed among the strains during the initial 18 h of fermentation (Fig. 2). During this time, the glucose concentrations in the mash increased steadily from about 3% (w/v) to approximately 15% (w/v) for all strains. However, after 18 h, the levels of glucose in the mash with the more stress-resistant strains, A007 and J006, were decreased when compared to the remaining yeast. With these strains, the levels of glucose in the stress model mash decreased steadily between 18 and 96 h. The concentrations of glucose remaining in the mash with A007 and J006 at 96 h were approximately 8% and 9% (w/v), respectively. With strains R003 and I005, the glucose in the mash increased until 48 h, at which time it began to decrease. Approximately 19% and 21% (w/v) glucose remained in the mash with these strains, respectively, at the end of fermentation. For the remaining strains, S001, T002, RW004, B008 and W009, the glucose in the mash remained similar from 48 to 72 h, but increased slightly between 72 and 96 h. This increase in glucose concentration towards the end of fermentation probably occurred because fermentation by these strains had slowed, and the glucoamylase activity continued to hydrolyze the higher saccharides into glucose. With S001, T002, RW004, B008 and W009, between 23% and 25% (w/v) glucose was left behind at 96 h of the stress model fermentation.

With the exception of strain J006, no major differences in the pattern of maltose removal from the mash were observed among the yeasts. In the case of J006, the concentration of maltose in the mash was between 0.5% and 2% (w/v) lower at both 6 and 18 h when compared to the other strains (Fig. 3). After 48 h of fermentation, the concentration of maltose in the mash was similar for all

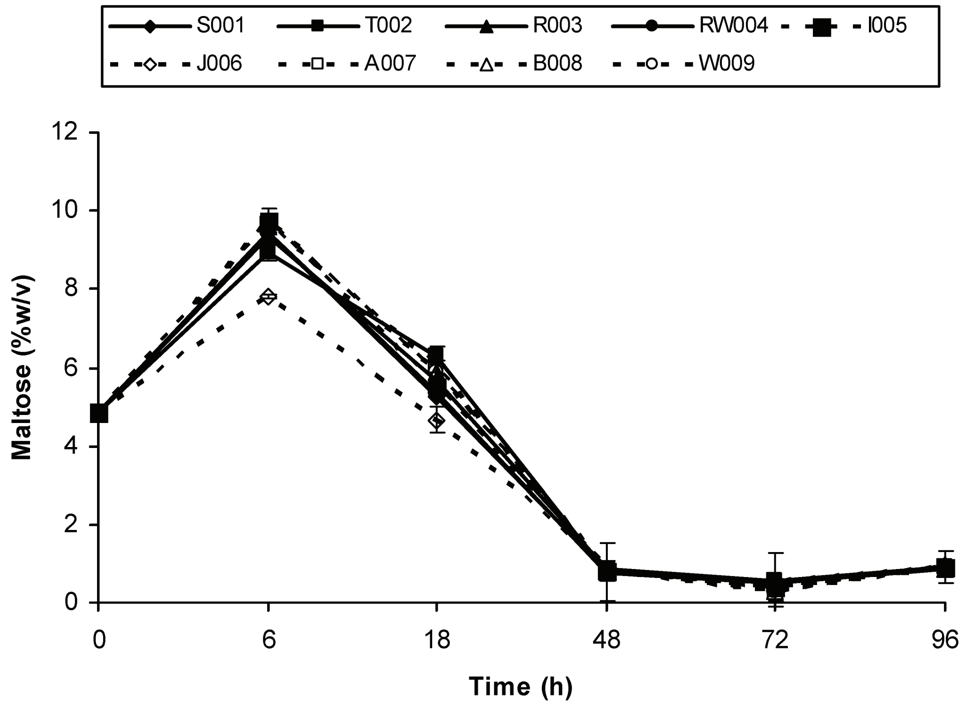

Fig. 3. The concentrations of maltose (%w/v) in the corn mash at various time points during the stress model fermentation with various *S. cerevisiae* strains. Values are means of duplicate samples from two separate experiments. Error bars indicate ± standard deviation between replicate samples.

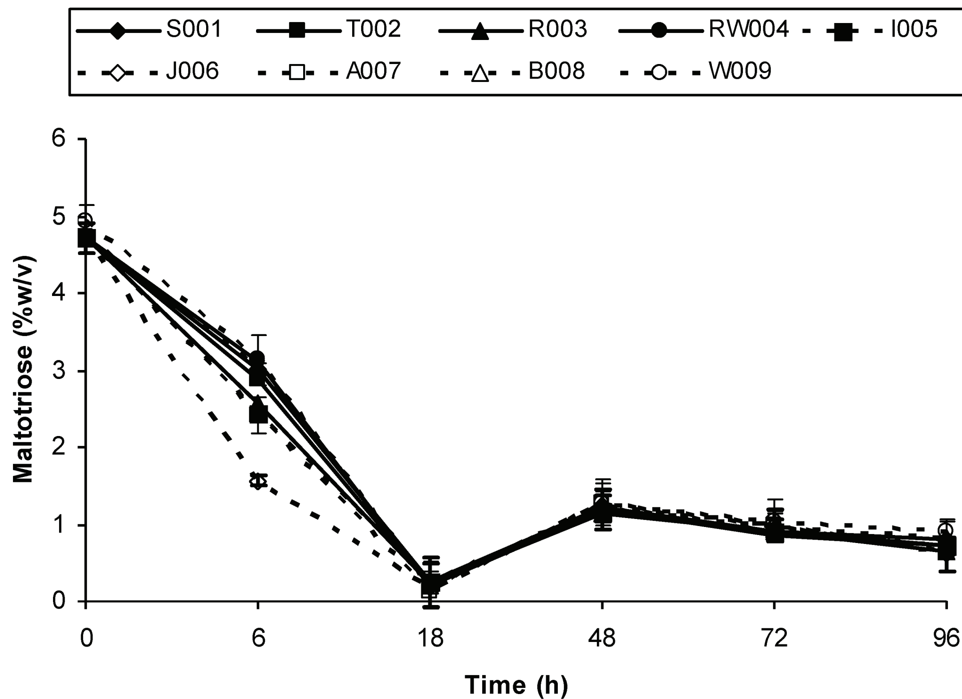

Fig. 4. The concentrations of maltotriose (%w/v) in the corn mash at various time points during the stress model fermentation with various *S. cerevisiae* strains. Values are means of duplicate samples from two separate experiments. Error bars indicate ± standard deviation between replicate samples.

strains. The maltose remaining in the mash after 96 h was approximately 0.9% (w/v).

Differences in the maltotriose profile during the stress model fermentation were also observed with strain J006, but only at 6 h. At this time, the maltotriose concentration in the mash with J006 was approximately 50% less than what was observed with the remaining strains (Fig. 4). Maltotriose concentrations increased to about 1.2% (w/v) at 48 h, and then decreased steadily to about 0.70% (w/v) after 96 h, for all yeast strains screened.

Effect of yeast strain on viable cell number during the stress model fermentation

The number of viable cells of each yeast strain in the mash at 24 h (as determined by methylene blue stain) correlated with the level of ethanol produced by the respective strain in the stress model, as would be expected. The strains that produced low levels of ethanol (S001, T002, RW004 and B008) in the stress model (Fig. 1) possessed low viable cell counts ($\leq 14 \times 10^6$ cells/mL mash) at 24 h (Table III). Strains W009, I005 and R003, which produced appreciable levels of ethanol in the stress model, displayed moderate growth when compared to the other strains. The viable cell counts obtained for these strains were 18, 28 and 34×10^6 cells/mL mash, respectively. The highest viable cell counts were observed for the strains, which produced large quantities of ethanol in the stress model and were considered to be the most stress-resistant of the strains screened. The viable cell counts obtained for these strains, J006 and A007, were 48 and 45×10^6 cells/mL mash, respectively.

DISCUSSION

The results obtained indicate that the sum of the stress factors in the stress model exceeded the ability of most of the strains screened to effectively handle these conditions. Approximately 40% of the strains screened (T002, RW004 and B008) were able to synthesize only minimal ethanol (<0.12% [v/v]) from the fermentable sugars in the mash (Fig. 1). These strains which were statistically grouped with the control S001 (based on their final ethanol concentrations), were considered to be stress-sensitive. Strain W009 synthesized significantly more ethanol than S001, T002, RW004 and B008, but the yeast still appeared to be stress-sensitive, since only 0.78% (v/v) ethanol was generated at the end of the stress model fermentation. Strain R003, which synthesized 3.62% (v/v) ethanol in the stress model, was moderately stress-sensitive. This strain produced approximately 80% more ethanol than W009 and 50% more than strain I005. However, the most stress-resistant of the yeast strains screened were J006 and A007. These particular strains synthesized relatively high quantities of ethanol (7.47% and 7.96% [v/v], respectively) in the stress model when compared to the other strains screened.

The differences in the levels of stress-resistance that were observed between the various strains during the stress model fermentation can be partially attributed to the rate and degree of utilization of the glucose in the mash. The strains which displayed poor stress-resistance (T002, RW004, B008 and the control S001) utilized only minor amounts of glucose as high concentrations of this sugar (approximately 25% [w/v]) remained in the mash at the end of the stress model fermentation (Fig. 2). Strains W009, I005 and R003, which were all considered to be more stress-resistant, were able to utilize the glucose in the mash to a greater extent. After 96 h, the glucose concentrations remaining in the corn mash with strains W009, I005 and R003 were approximately 23%, 21% and 19% (w/v), respectively. The glucose in the mash decreased even further for the most stress-resistant strains, A007 and J006, with only 8% and 9% (w/v) respectively, remaining at the end of the stress model fermentation.

One of the yeast strains screened via the stress model, strain A007, was unable to metabolize the disaccharide maltose (as determined by API ID 32C identification system, data not shown). Therefore, the slow disappearance of maltose from the mash observed with A007 was not surprising since it is unlikely that this strain would be capable of utilizing or taking up maltose from a mixed sugar medium. Strains S001, T002, R003, RW004, I005, J006, B008 and W009, on the other hand, are capable of metabolizing maltose (API, data not shown) at least when the sugar is present in the medium by itself. However, all but one of these strains (J006) appeared to utilize maltose very slowly, if at all, during the stress model fermentation, as the maltose concentrations in the mash with these strains were similar to the levels observed with the non-maltose utilizing strain A007 (Fig. 3). Strain J006 was the only yeast which appeared to utilize the maltose in the mash during the stress model fermentation. With this strain, the maltose in the mash disappeared more rapidly, and to a greater extent, when compared to the other strains screened. Hazell and Attfield[10] established that yeast strains which displayed rapid maltose utilization possessed considerably higher levels of maltose utilizing enzymes when compared to strains which utilized maltose slowly. More recently, it was established that the capacity for maltose transport, rather than the hydrolysis of maltose, is the main factor which limits the rate of maltose fermentation[15,17]. However, the apparent increased consumption of maltose by J006 cannot be explained solely by increased maltose permease (or other maltose utilizing enzymes) since the expression of the *MAL* genes required for maltose fermentation is repressed in the presence of glucose[11,14,24]. In this study, the yeast was exposed to glucose under all conditions (i.e. during preparation of yeast inocula [YM broth] and during fermentation of corn mash). Therefore, the possibility arises that the difference in maltose fermentation exhibited by J006 in the stress model fermentation resulted from decreased sensitivity of this strain to glucose repression, or inactivation of maltose permease by the glucose in the YM broth or corn mash. Furthermore, since ethanol may irreversibly inactivate maltose transport during the final stages of VHG fermentation[17], probably by inhibiting maltose permease activity[18], it is plausible that the slower disappearance of maltose (when compared to J006) exhibited by the strains which synthesized ethanol in the stress model (R003,

Table III.

Yeast strain	Viable cell count[a]
S001	1×10^6
T002	12×10^6
R003	34×10^6
RW004	11×10^6
I005	28×10^6
J006	48×10^6
A007	45×10^6
B008	14×10^6
W009	18×10^6

[a] Yeast cell counts are means of duplicate samples from two separate experiments.

I005, and to a lesser extent W009) occurred as a result of the ethanol in the corn mash. Larger quantities of ethanol were synthesized by J006 and the maltose permease of this strain may have been more tolerant to the high ethanol concentration in the mash than those of the other strains.

Differences were also observed with strain J006 in the disappearance of maltotriose from the mash during fermentation of the stress model (Fig. 4). Previous studies have established that maltose uptake and maltotriose uptake are competitive, with maltose being the preferred substrate[7], and that maltose is a potential repressor of maltotriose utilization, allowing consumption to begin only after the majority of the maltose has been depleted[15]. The more rapid disappearance of the maltotriose from the mash with J006 could signify that the maltotriose utilizing enzymes of this strain were not completely repressed by maltose in the stress model fermentation. However, since maltotriose and maltose may possess independent, but closely linked permease systems[25], it is a possibility that S001, T002, R003, RW004, I005, B008, and W009 were unable to utilize the maltotriose and that these strains lack a maltotriose transport system and the enzymes required for its utilization. The inability of these strains to metabolize maltotriose cannot be excluded since other factors such as low external pH, high initial osmotic pressure and high ethanol levels can negatively affect maltotriose utilization during fermentation[25]. The concomitant decrease in both maltose and maltotriose that was observed with J006 (Figs. 3 and 4) could indicate that glucose repression in this strain was at least partially alleviated, or that this strain has diastatic activity. Strain J006 did not appear to possess diastatic ability, as the dextrin concentrations in the mash with this strain were similar to those of the other strains (data not shown). However, since glucoamylase activity was present in the mash and maltose and maltotriose were continuously produced, it cannot be excluded that the faster disappearance of maltose and maltotriose with J006 was partially due to this enzyme activity.

For some of the strains screened, their fermentative performances (or degree of stress resistance determined by the extent of ethanol production in the stress model) can be partially explained by their physiological characteristics and/or the source from which they were isolated (Table I). Strain J006, which was one of the most stress resistant strains screened, was isolated from cane juice and was noted for its tolerance to high sugar concentrations. Cane juice is frequently used for the production of beverage alcohol and fuel ethanol in countries such as Jamaica and Brazil. Crude sugar cane must naturally contains both *Saccharomyces* and non-*Saccharomyces* yeast strains, thus making wild yeast contamination a common problem in industrial alcohol fermentations where this substrate is used[19]. Many of the strains introduced into the alcoholic fermentation process by means of crude sugar cane must have proven themselves to be stress tolerant and capable of high ethanol productivity. As a result, selection of these contaminating yeast strains for industrial alcoholic fermentation purposes has recently become increasingly popular among distilleries utilizing sugar cane juice as a substrate. Six strains have been reported to have been selected from Brazilian distilleries and commercialized as starter strains for fuel ethanol production. The improved fermentative capacities of these selected strains are thought to be due to their genetic and physiological adaptation to conditions which mimic industrial processes[19,20].

Considering that strain J006 was isolated from cane juice, it is plausible that the fermentative performance of this strain in the stress model was due to its ability to survive certain stress conditions such as osmotic pressure, acetic acid and low external pH. Cane juice is a sugar rich medium containing between 13% to 16% (w/v) sugar[21]. Although cane juice is comprised mainly of sucrose, yeast isolated from a high sucrose environment should be more resistant to stress conditions in general, since high-sucrose stress induces the expression of genes involved heat stress and osmotic stress[4]. Furthermore, crude cane juice is commonly contaminated with various yeast species, and acetic acid can be produced by many *Saccharomyces* and non-*Saccharomyces* yeasts. If J006 was isolated from such an environment, it could explain why this strain produced high ethanol concentrations in the stress model fermentation system where the cells were exposed to increased osmotic pressure and acetic acid at low pH.

Strain A007 appeared to be the most stress resistant of the strains screened, as this yeast was capable of producing increased ethanol concentrations in the stress model when compared to the other strains. The exact origin of strain A007 is unknown, but the strain has been noted for its growth at high temperatures in our laboratory (Alltech, Inc.). The apparent thermotolerant characteristics of strain A007 could explain why this strain produced high ethanol concentrations in the stress model.

Strains R003 and I005 were found to be reasonably stress-resistant as they were able to synthesize moderate quantities of ethanol in the stress model when compared to the other strains. These strains have been utilized extensively in beverage distilleries where the major substrates used for alcohol production are wheat and molasses, respectively. In beverage alcohol plants where wheat is used, glucoamylase is usually added to the fermentation to hydrolyse the dextrin material into fermentable carbohydrates. As a result, the most abundant sugar available to the yeast during fermentation of a wheat mash is glucose. The ability of R003 to withstand a high glucose concentration, together with its thermotolerant characteristics, could explain why this strain produced a good ethanol yield in the stress model fermentation. Strain I005 on the other hand, is noted for its ability to produce high quantities of ethanol from molasses, particularly in geographical areas which experience extremely high ambient temperatures. The main sugar available to the yeast during molasses fermentation is sucrose and the ability of I005 to tolerate an increased sucrose concentration may indicate that this strain is resistant to osmotic stress[4]. The capability of I005 to synthesize ethanol from a high sugar concentration at elevated temperature may explain why this strain displayed improved fermentative performance in the stress model when compared to B008 and W009. Research on the isolation of yeast strains with high fermentation efficiencies and superior alcohol producing capabilities from sugar-rich sources such as molasses, has recently been reported[5].

Strains W009 and B008 both displayed poor fermentative performance in the stress model fermentation system. These strains were isolated from wine and super-attenuated beer, respectively. During the production of wine, yeast cells are often exposed simultaneously and sequentially to a variety of stress conditions. Depending on the wine-making process these stresses can include osmotic stress due to high sugar concentration, heat stress, cold stress, nutrient limitation and depletion, weak organic acid stress, low pH and high ethanol concentrations. Since many of the stress factors encountered during wine production were incorporated into the stress model, albeit to a greater degree, the inability of strain W009 to produce a good ethanol yield during fermentation was unexpected. However, since temperatures as high as 37°C (the temperature maintained during the stress model fermentation) are probably not encountered during commercially controlled wine fermentations, it is plausible that strain W009 was unable to tolerate the elevated temperature of the stress model fermentation. Strain B008, on the other hand, was isolated from an environment which is very different from the stress model. Yeasts are typically exposed to moderately high sugar concentrations during commercially controlled brewing fermentations. It is unlikely that the cells would encounter the elevated temperatures and/or weak organic acids to the extent seen in the stress model. Organic acid production by contaminating bacteria is not seen in most controlled brewing fermentations. The fact that B008 was isolated from super-attenuated beer could indicate that this strain is capable of utilizing dextrin compounds, sugars which are not normally metabolized by yeast during a beer fermentation. However, it appears that B008 was unable to utilize dextrin during the stress model fermentation, as the dextrin levels in the mash were similar to those for the remaining strains (data not shown).

The other strains that exhibited poor fermentative performance in the stress model fermentation were T002 and the control S001. These strains were both isolated from commercial yeast strains utilized in the fuel ethanol industry. The inability of these strains to effectively synthesize ethanol in the stress model fermentation was not anticipated, since yeasts are generally exposed to a variety of stress conditions during industrial ethanol fermentations. Strains S001 and T002 are used extensively in the fuel ethanol industry in North America where the primary cereals used for alcohol production are maize, wheat and milo. The dextrin material in mashes prepared with these grains is generally broken down into fermentable sugars through the action of enzymes such as glucoamylase and as a result, the most abundant sugar available to the yeast during fermentation of a fuel alcohol mash is glucose. Since most fuel ethanol plants employ VHG mashes (≥30% dry solids) in an effort to maximise ethanol production, the glucose concentration available to the yeast during fermentation can be very high at certain times. Furthermore, elevated fermentation temperatures are often encountered and lactic and acetic acids are almost always present in industrial mashes due to the presence of contaminating bacteria. Therefore, yeast strains selected for industrial ethanol production should be able to tolerate high glucose concentration, heat stress and the presence weak organic acids without compromising ethanol production. The observation that T002 and S001 were sensitive to the stress model may indicate the need for yeast with improved stress tolerance in the fuel ethanol industry.

It should be mentioned that the concentrations of the weak acids, particularly lactic acid, that were chosen for the stress model are not typical industrial levels. The levels of the acids utilized, 4% (w/v) lactic acid and 0.3% (w/v) acetic acid, were higher than what would be found in a heavily contaminated industrial VHG fermentation. The deliberate contamination of corn mash (approximately 25% [w/v] solids) fermentations with various lactobacilli (added at industrial relevant numbers) has been demonstrated, and these experiments established that contaminating bacteria can generate around 1.7% (w/v) lactic acid and 0.2% (w/v) acetic acid in the presence of yeast[23]. However, these values were established in a laboratory setting, the levels of the acids produced under industrial conditions could be even higher, where the ethanol production conditions are less-controlled. It should also be noted that lactic and acetic acids would not normally be present, at the levels studied, at the start of fermentation (i.e. at 0 h) in an industrial setting. However, water streams such as "backset" (or thin stillage) and process condensate are typically recycled as make up water in fuel ethanol plants. This is done in order to maintain the water balance in the ethanol production facility. These streams often contain high concentrations of bacterial end products, particularly lactic acid in the backset and acetic acid in the process condensate. The levels of lactic acid in backset can range anywhere between 0.6 to 1.7% (w/v) lactic acid, depending on the specific production conditions of the fuel ethanol plant[12]. Although the lactic acid concentrations encountered by yeast at the start of fermentation would rarely reach 4% (w/v) when backset is diluted in mash, significant amounts of lactic acid will be present in the fermenter. High concentrations of lactic and acetic acids were chosen for the stress model, and added at the beginning of fermentation, so that superior yeast strains, capable of growth and ethanol production under extremely stressful conditions, could be isolated.

According to the results obtained, the stress model fermentation system developed in this study could potentially be used as a tool in the fuel ethanol industry, or other fermentation industries where yeast encounter similar stresses, for screening candidate yeast strains for stress resistance. Establishing the stress resistance of a candidate yeast strain in the presence of severe stress would help ethanol producers to select the best strain for efficient fermentation in their particular plant. The screening process is simple and rapid to perform, requiring only a few days. Furthermore, the method does not require any special technology or equipment and hence could be performed easily in any ethanol plant. However, screening candidate yeast strains for stress resistance via the stress model would be only the initial step in selecting the correct yeast strain. The next step would require analysing the fermentative capacity of the strain on a larger scale and under the specific production conditions of the ethanol plant.

Current methods of assessing yeast stress resistance and predicting fermentative performance on an industrial

scale involve analyzing gene expression during alcoholic fermentation, or under various stress conditions that are important during fermentation. These methods give insight into the understanding of the overall stress response during alcoholic fermentation and may suggest mechanisms that yeast utilize to survive the stressful environment. We have confirmed, by analyzing gene expression in yeast strains with different levels of stress resistance, that the stress model fermentation system is an effective method to assess yeast stress resistance for corn mash fermentations. Using quantitative real-time PCR, we found that key stress-related genes were consistently induced and repressed in a stress-resistant and stress-sensitive strain, respectively, during the stress model fermentation. The inability of the stress-sensitive yeast to elicit a stress response appeared to be due to glucose repression, as genes involved in the main glucose repression pathway were significantly up-regulated in this strain.

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