A Strategy to Compare Yeast Strains and Improve Cell Survival in Ethanol Production Processes above 30°C.

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At industrial scale, sucrose is converted into ethanol in fermentation processes in which high amounts of both yeast cells and sugar are used. When the temperature increases, the cell lethality, caused by the concomitant effects of high temperature and high sugar levels, is aggravated by increasing levels of ethanol within the medium. By decreasing the sugar concentration in the reactor feed, cell death occurring can be diminished or avoided at increased temperatures. The fed-batch strategy proposed here allows the comparing of strains in regard to their tolerance to ethanol and osmotic stresses at increasing temperatures. Thus, a superior yeast strain is expected to present and maintain higher viability, greater ethanol productivity and lesser biomass formation during fermentation under industrial stress conditions.

Keywords *Saccharomyces;* ethanol; high temperatures; high cell density; viability; high sugar concentration

1. Introduction

Classical fermentations (bioethanol, beer, wine, sake, distilled spirits, baker's yeast) are usually carried out using strains of *Saccharomyces cerevisiae* as starter. Fed-batch cultivations have successfully been used in fuel-ethanol production. Cell reuse avoids costs with the inoculum propagation, since additional consumption of medium and a longer fermentation time will not be required. The use of high cell densities reduces the fermentation periods. Brazilian alcohol plants operate at high sugar concentrations (10% to 20% total reducing sugar, w/v) and high cell densities (10-12% of wet cell mass, v/v). High cell densities minimize the effects of substrate and product inhibition [1], making it possible to carry out fermentations in shorter periods. If the biomass formation is significant, decreases in ethanol production will occur. In Brazilian alcohol plants, the biomass surplus obtained at the end of each fermentation cycle is discarded before starting a new cycle and it has been used in the preparation of animal feed [2]. The increase in the inoculum size led to an increase in the cell viability of a thermotolerant mutant of *Saccharomyces cerevisiae* which was fermenting 15% glucose in batch culture at 40°C [3]. Glycerol is the main by-product of fuel ethanol production while the levels of other by-products are less relevant as in the case of beverage production. In a fermentation process, the extracellular proportion of glycerol and bioethanol can be altered in favor of increased ethanol formation by adjusting parameters such as sugar concentration, pH, temperatures and inoculum size [4].

Despite the achievements in the field of ethanol production, halted or slow fermentations still persist as a threat lasting for six to seven months of operation with cell reuse, in ethanol plants located in a tropical country where temperatures above 34°C can be reached within the reactors. A long succession of fermentation cycles with cell reuse is not always possible, depending on drops in viability, intensity of the yeast propagation and bacterial contamination. Process interruption or a sluggish fermentation can be prevented by correcting nutritional deficiencies of the medium or by adjusting the operational conditions. The interruption of the fermentation process in an ethanol plant causes devastating economical losses. Thousands of liters of ethanol production can be lost every day. Optimal temperatures for ethanol

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production using *Saccharomyces cerevisiae* can vary within the range of 20-35°C, depending on strain and process conditions [5, 6].

The yeast's capacity to produce ethanol is dependent on the tolerance of a strain to ethanol, temperature and other properties such as secretion of invertase. Strains isolated from alcohol plants showed different capacities of producing invertase when fermenting sugar-cane juice at 38°C [5]. An improvement of invertase secretion [7] was obtained due to the use of a mutant strain of *Saccharomyces cerevisiae* capable of fermenting molasses containing 15% total sugar at 40°C. Increases in invertase secretion were also reported elsewhere over the time course of an ethanol production process [5, 7]. Thus, invertase secretion is not diminished during the ethanol production at high temperatures and this represents an advantage for high-temperature fermentations. Concerning ethanol yields and viability, strain 19G isolated from reactors during the operation of Brazilian plants showed maximal ethanol yields within the range of 40°C to 45°C when fermenting 15% sugar-cane syrup (total reducing sugar). Nevertheless, decreasing viabilities were observed for strain 19G above 37°C [5]. In addition, the ethanol yield decreased at 30°C when ethanol was added to the medium at concentrations above 6% (v/v) [8]. In an industrial process, the tolerance to ethanol and temperature of a starter strain can be affected by the genetic and physiological stability of the yeast population as indicated by the generation of variants and/or mutants over a long-lasting processes operation with cell reuse [9]. The incidence of wild yeasts is another source of alteration of the yeast population in an industrial process. It has to be remembered that an industrial process operates under non aseptic conditions. In addition, lactic acid bacteria can tolerate higher temperatures (e.g., optimum growth at 55-58°C, [10]) than those tolerated by the yeast cells [11], so that competition of bacterial cells for the carbon source increases when the temperature is raised. The effects of lactic and acetic acid produced by bacteria on ethanol production are much smaller in corn mash cultures than in a laboratory medium [12]. Smaller variations in the pH values around 4.5-5.5 during fermentation of corn mash were due to its buffering capacity [13]. The yeast biomass is also expected to play a relevant role in the buffering capacity of the medium, particularly in fermentations operated at high cell densities. Due to transport phenomena [14], the equilibrium established between the interior of cells and the medium is expected to alter the medium pH immediately after inoculation.

The optimization of an industrial process in respect to ethanol concentration, productivity and yield requires the quantification of the dynamic behavior of yeast population at very high ethanol concentrations and this is not a simple task [15]. When the extreme limits of the yeast's tolerance to ethanol and temperature are previously determined in a fed-batch process, it is easier to define optimal monitoring strategies for a fermentation process operating under strict conditions. In the present work, the determination of extreme limits of the yeast's tolerance to high temperatures, sugar concentrations and high inoculum showed that it is possible to obtain high ethanol productivity in short fermentation periods.

2. The fermentation process

2.1 Yeast strains

A hybrid yeast (strain 63M) constructed by using isolates obtained from industrial fermentation systems [16] and an industrial strain BG1, kindly donated by Açucareira Zilo Lorenzetti S/A Macatuba/SP/Brazil were assayed and compared.

2.2 Inoculum propagation in a sterilized medium

The inoculum propagation was carried out at 30°C in the synthetic medium containing 10% sucrose [17]. Portions of 50 mL of synthetic medium were distributed into 250 mL Erlenmeyer flasks before sterilizing for 20 min at 120°C and one atmosphere pressure. This medium was enriched with 2% yeast extract to guarantee vigorous growth. Each Erlenmeyer was inoculated with fresh pre-culture to obtain an initial cell density of 0.85 g L^{-1} and then left for a 12-hour propagation period in a rotary shaker operating at 125 rpm. Cells were harvested by centrifuging at 5000 *g* for 2 min at 4°C. Harvested cells

were re-suspended in sterilized water followed by a second centrifugation for washing of the cell pellet. In a next step, the washed cells were re-suspended in sterilized water resulting in a concentrated yeast cream (160 g L^{-1}) used as inoculum for high cell density fermentations. Despite being poorer than an industrial medium, a defined medium has the advantage of giving more constant and reliable results at laboratory scale.

2.3 Fermentation system

The system used was the one constructed by Marconi, S.A, Piracicaba, Brazil, model MA5002/4/200. The system is composed of four 200 mL mini-reactor units equipped with independent devices for pH adjustment (base addition), magnetic stirring (around 100 rpm), and sampling which are independently and simultaneously operated. Temperatures are thermostatically controlled by circulating water from a water-bath. A reflux cooler column attached to each mini-reactor was refrigerated by circulating water at 4°C. More time is required and the risks of contamination are greater between fermentation cycles when large volumes of medium are centrifuged. Less contamination is the advantage of fermenting small volumes of medium at laboratory scale, particularly when fed-batch cultures are carried out independently and at the same time.

2.4 Analytical assays

Determinations were: a) cell viability using the methylene blue method [18]; b) residual sugar using the 3,5-dinitrosalicylic acid method [19]; c) ethanol using a gas chromatograph (model CG-37; Instrumentos Científicos, São Paulo, Brazil); d) for biomass assays, cells were washed by vacuum filtration and dried at 105°C until constant weight and expressed as g L^{-1}. Ethanol productivity (g $L^{-1}h^{-1}$) was calculated as the ratio between total ethanol produced and the total fermentation time. The $Y_{P/S}$ was the ratio between ethanol produced (g L^{-1}) and sugar consumed (g L^{-1}).

2.5 Feeding strategy applied to the pulse fed-batch process

In an industrial alcohol plant, a yeast cream (concentrated cell suspension) is added to the empty reactor prior to the continuous feeding by molasses or a mixture of molasses and sugar-cane syrup at pH values adjusted to 4.5-5.5 [2], and this leads to increases in the volume of the medium within the reactor at the end of the feeding phase. After the feeding phase, the fermentation continues up to the total exhaustion of the sugar from the medium. The fermentation time usually varies from 6 to 12 hours in an industrial alcohol plant [2]. High cell densities are a prerequisite for high productivity and shorter fermentation times. When a decreasing feeding strategy was applied to the fermentation of black strap molasses, the concentration of reducing sugar increased within the reactor reaching maximal values at the end of the feeding phase [20]. In fact, there is no kinetic model available able to properly describe a process at an industrial scale [2].

For the present strategy, a sterilized synthetic medium was used and pulses of concentrated sucrose solution (400 g L^{-1} to 800 g L^{-1} solution) were added to the reactor containing all the rest of the components of the synthetic medium except for the carbon source. All materials and the medium were sterilized for 20 min at 120°C and one atmosphere pressure. The mini-reactors were prepared and operated as follows:

a) addition of 25 mL concentrated inoculum (160 g L^{-1} suspension) to the empty mini-reactor, followed by the addition of a 2-fold concentrated synthetic medium at pH 4.5 (50 mL), making up an initial volume of 75 mL, devoid of carbon source and maintained under constant stirring (100 rpm).

b) In the first two hours of the fermentation period (feeding phase), decreasing pulses (10.0, 6.0, 4.0, 3.0 and a 2.0 mL added at 30 min intervals) of concentrated sucrose solution (400 g L^{-1} to 800 g L^{-1}) were added to the reactors using a pipette Model L10000 from HTL, so that a final working volume of 100 mL (100g/L to 200 g/L added sucrose) was reached at the end of the feeding phase.

c) A 2.4 mol L^{-1} NaOH solution (~ 0.5 mL) was added immediately after the addition of the first sucrose pulse, in order to restore the pH to 4.5 and prevent cell death. Further variations in pH were recorded and they varied from 3.4 to 4.9 at the end of the fermentation process as described later on in this text.

d) Temperatures of the media (varying from 30°C to 40°C) were kept constant by pumping water from thermostatically controlled by water-baths. Small samples (~ 0.5 mL) were taken from each reactor for assays. The fermentation was considered finished when the ethanol concentration became constant.

3. Fed-Batch Cultures at 30-40°C: Changes in the Sucrose Concentration in the Reactor Feed.

3.1 Thermotolerant strain 63M

Fig. 1 shows that drops in cell viability (Fig. 1B) were practically avoided after a 4-hour fermentation period when the amount of the sucrose added to the reactors was ≤ 150 g L^{-1} in relation to the final working volume. The lower values of viability shown by strain 63M at sucrose concentrations above 150 g L^{-1} seem to be due to a fast accumulation of internal ethanol over short fermentation periods. Short fermentation periods (3h to 6h in Fig. 1C) indicate that strain 63M has a great fermentation activity. Despite the fast sugar uptake shown by strain 63M at low sucrose concentrations (≤ 150 g L^{-1}), high values of final viability were obtained due to the use of a high cell density. In a report from literature, longer fermentation periods were obtained whenever the temperature decreases within the reactor [6]. In the present work, very low amounts of the reducing sugar (Fig. 1C) accumulated within the medium during the feeding of the reactor. Ethanol production was practically over at the end of the feeding phase when sucrose concentrations ≤ 150 g L^{-1} were used (Fig. 1A). At sucrose concentrations ≥ 150 g L^{-1}, dramatic decreases in viability of strain 63M were only observed (Fig. 1B) when the sugar was completely exhausted from the medium (Fig. 1C).

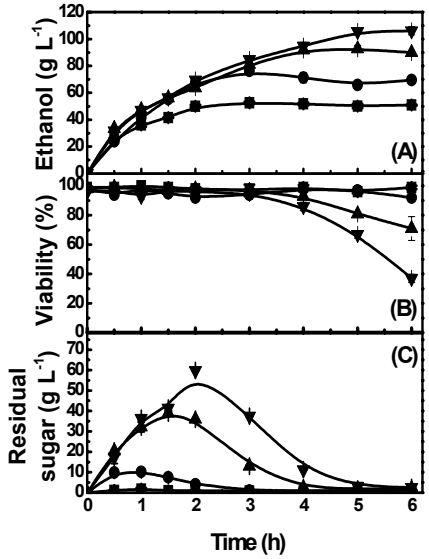

Fig. 1 Effect of the total sugar (■= 100 g L^{-1}, ● =150 g L^{-1}, ▲= 180 g L^{-1}, ▼=200 g L^{-1}) added by pulses of concentrated sucrose solutions to the reactors at 37°C on the ethanol yield (A), viability (B) and residual sugar (C) of strain 63M.

Variations in final pH, biomass and viability are described in Table 1. The initial pH of the medium, re-adjusted after the addition of the first sucrose pulse, varied from 3.4 to 4.7 at the end of the fermentation processes. Thus, the variations in pH were small in the fed-batch processes and less dependent on the changes in sugar concentration. Biomass increments (Table 1) achieved at the end of the fermentation varied from 2.3 g L^{-1} (equivalent to a 5.8% increment when fermenting 100 g L^{-1} sucrose at 40°C) to 4.8 g L^{-1} (equivalent to a 12.0% increment when fermenting 200 g L^{-1} sucrose at 30°C and 37°C). In a succession of fed-batch fermentations, increase in the biomass at the end of each

fermentation cycle is necessary due to the requirement of cell renewal. In the present work, drops in the viability of strains 63M were significant at the end of the fermentations carried out above 37°C particularly at increasing sugar concentrations. The lower values of final viability (37 ± 3% at 37°C and 29 ± 5% at 40°C), given by strain 63M at the end of the fermentation of 200 g L^{-1} sucrose, indicate that it is not possible to carry out successive fermentations with cell reuse at temperatures ≥ 37°C using this sugar concentration. Despite the very low viability obtained for strain 63M fermenting 200 g L^{-1} sucrose at temperatures ≥ 37°C, increments in biomass were obtained due to the contribution of intact but non viable cells at the end of the fermentation. Nevertheless, literature describes that the maintenance of viability during fermentation is favoured by high cell densities [3]. The limiting effect of increasing temperatures on the fermentation capacity of strain 63M is due to losses in viability. Thus, sugar concentrations below 180 g L^{-1} (in relation to the final working volume) greatly improved the values of final viability at 37°C. At 40°C, the drops in viability of strain 63M were also greatly minimized by reducing the amount of sucrose added (100 g L^{-1} to 150 g $L^{-}1$) to the reactors as shown in Table 1.

Table 1 Effect of sucrose concentration added to the reactors containing synthetic medium on final pH, biomass and viability of strain 63M at temperatures ranging from 30°C to 40°C.

Temperature (process)	Sucrose [(a)] (g L^{-1})	pH	Biomass [(b)] (g L^{-1})	Viability (%)
30°C (batch)	200	4.0	43.6 ± 0.8	99 ± 1
30°C (fed-batch)	200	3.4	44.8 ± 0.9	99 ± 1
34°C (fed-batch)	150	3.9	43.2 ± 0.2	93 ± 4
	180	4.1	43.9 ± 0.3	99 ± 1
37°C (fed-batch)	100	3.7	42.3 ± 0.2	97 ± 4
	150	4.0	43.2 ± 0.2	94 ± 2
	180	4.4	44.1 ± 0.4	81 ± 1
	200	4.7	44.8 ± 0.4	37 ± 3
40°C (fed-batch)	100	4.4	42.3 ± 1.0	94 ± 2
	150	4.5	43.2 ± 0.4	82 ± 7
	200	4.6	43 .0 ± 0.6	29 ± 5

[(a)] Total sucrose added during the feeding of the reactors and expressed as the sucrose concentration in the final working volume; [(b)] Initial cell density was equivalent to 40 g/L.

Table 2 Ethanol production by strain 63M in synthetic medium containing different amounts of added sucrose and high cell densities at temperatures ranging from 30°C to 40°C.

Temperature (process)	Sucrose[(a)] (g L^{-1})	Time[(b)] (h)	Ethanol Production		
			Final ethanol (g L^{-1})	$Y_{P/S}$ ($g_{ethanol}$ $g^{-1}_{sucrose}$)	Productivity (g $L^{-1}h^{-1}$)
30°C (batch)	200	5	92.2 ± 0.1	0.463 ± 0.002	18.4 ± 0.1
30°C (fed-batch)	200	6	106.5 ± 0.4	0.539 ± 0.002	18.6 ± 0.1
34°C (fed-batch)	150	4	75.3 ± 0 .2	0.506 ± 0.001	18.8 ± 0.1
	180	5	91.2 ± 0.1	0.512 ± 0.006	18.2 ± 0.1
37°C (fed-batch)	100	3	52.6 ± 0.8	0.531 ± 0.008	17.5 ± 0.3
	150	3	76.3 ± 0.5	0.512 ± 0.003	25.4 ± 0.2
	180	5	93.1 ± 0.5	0.523 ± 0.003	18.6 ± 0.1
	200	6	106.2 ± 0.3	0.538 ± 0.002	17.7 ± 0.1
40°C (fed-batch)	100	3	52.6 ± 0.9	0.530 ± 0.009	17.5 ± 0.3
	150	3	75.4 ± 0.3	0.507 ± 0.002	25.1 ± 0.1
	200	6	79.6 ± 0.2	0.452 ± 0.004	13.3 ± 0.1

[(a)] Total sucrose added during the feeding of the reactors and expressed as the sucrose concentration in the final working volume; [(b)] fermentation periods.

Table 2 describes the concomitant effects of increasing temperatures and sugar concentrations on ethanol yields and productivity of strain 63M. The maximal values of final ethanol varied from 52.6 ± 0.8 to 106.5 ± 0.4 g L^{-1}, depending on the sugar concentration and temperature while the ethanol yields ($Y_{P/S}$) varied from 0.452 ± 0.004 g g^{-1} (fermenting 200 g L^{-1} sugar at 40°C) to 0.539 ± 0.002 g g^{-1} (200 g L^{-1} sucrose at 30°C). However, the highest values of productivity (25.4 ± 0.2 at 37°C to 25.1 ± 0.1 g $L^{-1}h^{-1}$ at 40°C) were obtained for strain 63M when the concentration of sucrose added to the reactors was 150 g L^{-1}. High values of productivity were also obtained for other sugar concentrations, except when 200 g/L sucrose was fermented at 40°C due to losses in viability.

3.2 Industrial strain BG1

Industrial strain BG1 was assayed under the same conditions used for strain 63M. The total sugar consumption occurred at three hours of fermentation after adding 15 g sucrose to the mini-reactor (equivalent to 150 g L^{-1} in the final working volume), when strain 63M was used (Fig. 1C). A longer fermentation period (four hours) was required by strain BG1 (Fig. 2C). Fast fermentation is one of the causes of the drops in cell viability, due to a high internal ethanol concentration reached inside the cells at increasing sugar concentrations. As strain 63M fermented sucrose faster, it died more quickly at high temperatures when the sugar concentration was raised within the medium (Fig. 1B). On the other hand, strain BG1 was able to maintain higher viability values than strain 63M when sucrose concentrations greater than 150 g L^{-1} were fermented (Fig. 2B). This seems to be caused by the slower fermentation capacity of strain BG1, as indicated by the longer fermentation times (Fig 2C) required by this strain to reach total sugar consumption.

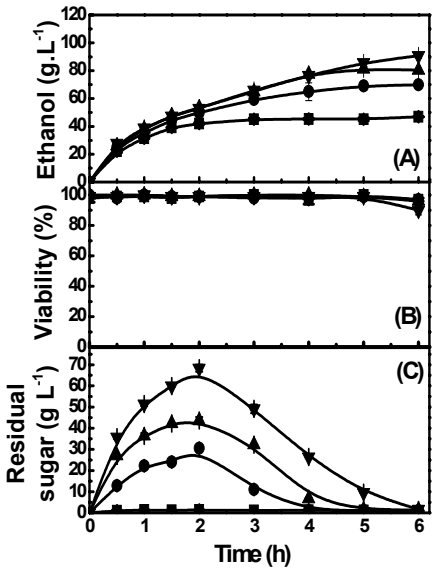

Fig. 2 Effect of the total sugar (■= 100 g L^{-1}, ● =150 g L^{-1}, ▲= 180 g L^{-1}, ▼=200 g L^{-1}) added by pulses of concentrated sucrose solutions to the reactors at 37°C on the ethanol yield (A), viability (B) and residual sugar (C) of industrial strain BG1.

Table 3 describes the effects of both increasing temperatures and sugar concentrations on final values of pH, viability and biomass produced by strain BG1. The final values of viability obtained for strain 63M (Fig. 1B) were lower than those obtained for strain BG1 due to the longer fermentation periods required by strain BG1 to reach total sugar uptake (Fig. 2C). Lower values of ethanol productivity were also shown by the strain BG1 (Table 4) when compared to strain 63M (Table 2). Consequently, strain BG1 showed a low value of final viability only when it was fermenting 200 g L^{-1} sucrose at 40°C (Table 3). As in the case of strain 63M (Table 1), the pH re-adjusted to 4.5 after the addition of the first sucrose pulse to the medium containing cells of strain BG1, varied from 3.6 to 4.9 at the end of the fermentation

(Table 3). Concerning increments in biomass at the end of the fermentation, greater increments in the final biomass values were obtained for strain BG1 (Table 3) than for strain 63M (Table 1). Thus, strain BG1 utilized more nutrients for growth than strain 63M (Table 1) at the expense of losses in ethanol yields as indicated by the final biomasses shown in Table 4.

Table 3 Effect of sucrose concentration added to the reactors containing synthetic medium on final pH, biomass and viability of strain BG1 at temperatures ranging from 37°C to 40°C.

Temperature (process)	Sucrose [(a)] ($g\ L^{-1}$)	pH	Viability (%)	Biomass [(b)] ($g\ L^{-1}$)
37°C (fed-batch)	100	3.6	99 ± 1	44.5 ± 0.5
	150	3.8	98 ± 2	47.1 ± 0.3
	180	3.8	99 ± 1	48.0 ± 0.2
	200	3.8	90 ± 3	49.0 ± 0.3
40°C (fed-batch)	100	4.3	98 ± 1	45.0 ± 0.5
	150	4.5	98 ± 1	46.9 ± 0.2
	180	4.5	93 ± 1	46.4 ± 0.2
	200	4.9	32 ± 9	48.4 ± 0.9

[(a)] Total sucrose added during the feeding of the reactors and expressed as the sucrose concentration in the final working volume; [(b)] Initial cell density was equivalent to 40 g/L.

Table 4 Ethanol production by strain BG1 in synthetic medium containing different amounts of added sucrose and high cell densities at temperatures ranging from 37°C to 40°C.

Temperatures (process)	Sucrose [(a)] ($g\ L^{-1}$)	Time[(b)] (h)	Ethanol Production		
			Final ethanol ($g\ L^{-1}$)	$Y_{P/S}$ ($g_{ethanol}\ g^{-1}_{sucrose}$)	Productivity ($g\ L^{-1}h^{-1}$)
37°C (fed-batch)	100	3	45.2 ±0.7	0.458 ± 0.007	15.1 ± 0.2
	150	4	69.5 ± 0.6	0.467 ± 0.004	17.4 ± 0.2
	180	5	81.2 ± 0.8	0.455 ± 0.005	16.2 ± 0.2
	200	6	90.7 ± 0.9	0.457 ± 0.005	15.1 ± 0.2
40°C (fed-batch)	100	3	47.5 ± 0.1	0.480 ± 0.001	15.8 ± 0.1
	150	3	68.6 ±0.2	0.463 ± 0.001	22.9 ± 0.1
	180	4	72.3 ± 0.7	0.405 ± 0.004	18.1 ± 0.2
	200	5	87.9 ± 0.4	0.442 ± 0.002	17.6 ± 0.1

[(a)] Total sucrose added during the feeding of the reactors and expressed as the sucrose concentration in the final working volume; [(b)] fermentation periods

The maximal amount of ethanol produced by strain BG1 varied from 45.2 ± 0.7 to 90.7 ± 0.9 $g\ L^{-1}$, depending on the sugar concentration and temperature (Table 4). Despite the equally high viability displayed by both strains fermenting 150 $g\ L^{-1}$ sucrose at 37°C (94 ± 2% viability shown in Table 1 for strain 63M and 98 ± 2% in Table 3 for strain BG1), the productivity of strain 63M was superior at 37°C (25.4 $g\ L^{-1}h^{-1}$ using 150 $g\ L^{-1}$ sucrose) to that shown by strain BG1 (17.4 $g\ L^{-1}h^{-1}$) after a three hour fermentation period under the same conditions.

4. Summary

A strategy based on minimizing the toxic effects of ethanol at increasing temperatures is being proposed in order to reduce the cell death when temperatures below 37°C can not be maintained within a reactor due to a limited water supply or deficiencies in the refrigeration system. Above 37°C, the process of fuel ethanol production loses some of its fermentation activity, mainly due to organic acidity, and cell viability to an extent dependent on the fermentation rate, degree of tolerance of the yeast strain to ethanol [21] in the industrial medium. A strain showing a reduced fermentation capacity and a greater biomass

formation is to be highly recommended for use in a biomass accumulation process (e.g., baker's yeast) rather than for fuel ethanol production. The possibility of fermenting a high sugar concentration in the media is of great economical interest to the fuel ethanol industry. But under industrial conditions, the yeast cells are subject to greater impacts from sugar (osmotic) and ethanol stresses. High ethanol yields were obtained in a batch process using a thermotolerant strain and sucrose concentrations varying from 200 g L^{-1} to 250 g L^{-1} [5]. Great drops in viability of strain 63M were observed when sucrose concentrations were above 150 g L^{-1}, possibly due to the accumulation of high amounts of ethanol within yeast cells at high temperatures. Nevertheless, the superior values of ethanol productivity makes strain 63M a superior strain.

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