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Continuous ethanol production in a nonconventional five-stage system operating with yeast cell recycling at elevated temperatures

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Three ranges of increasing temperatures (35–43, 37–45, 39–47°C) were sequentially applied to a five-stage system continuously operated with cell recycling so that differences of 2°C (between one reactor to the next) and 8°C (between the first reactor at the highest temperature and the fifth at the lowest temperature) were kept among the reactors for each temperature range. The entire system was fed through the first reactor. The lowest values of biomass and viability were obtained for reactor R_3 located in the middle of the system. The highest yield of biomass was obtained in the effluent when the system was operated at 35–43°C. This nonconventional system was set up to simulate the local fluctuations in temperature and nutrient concentrations that occur in different regions of the medium in an industrial bioreactor for fuel ethanol production mainly in tropical climates. Minimized cell death and continuous sugar utilization were observed at temperatures normally considered too high for *Saccharomyces cerevisiae* fermentations.
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Introduction

Fuel ethanol production using the yeast *Saccharomyces cerevisiae* has been carried out using fed-batch and continuous processes for many years [7]. Temperature maintenance in such large-scale fermentors (thousands of liters capacity) has been difficult and expensive in tropical climates. Efficiency of ethanol production is dependent on the type of process used. For example, in a multistage system involving three and five bioreactors, higher values of specific ethanol productivity were obtained than those in a single-stage system for the conversion of cane molasses into ethanol under nonaseptic conditions [2].

S. cerevisiae attains its maximal temperature of growth at around 34–37°C in batch cultures [17]. However, strains of *S. cerevisiae* showing growth at 42°C on solid medium have been reported [6,10]. Cells approaching stationary phase simply become reversibly adapted to the increases in ethanol and temperature or they gradually develop an enhanced thermotolerance [14]. Evidence which suggests that thermotolerance and tolerance to ethanol are interactive properties has been reported [1,13]. The exposure of yeast cells to a near lethal temperature often leads to a certain degree of adaptation so that a previously lethal temperature is tolerated following a mild heat shock [4]. The application of a sublethal heat stress seems to increase longevity of yeast cells [16]. However, little is known about the effects of temperature heterogeneity and/or instability, as found in large industrial reactors employed for fuel ethanol. Great reductions in ethanol production rate (up to 50%) caused by heat shocks have been described in a continuous system [3]. In addition, decreases in ethanol production rate in repeated batch fermentations [12] have been observed when the temperature was gradually raised from 30 to 35°C.

A five-stage bioreactor system was used in the present study with the aim to simulate the fluctuations in temperature (repeated temperature shocks) and nutrients that occur in large industrial reactors (thousand of liters) where control of the temperature is difficult due to high external temperatures. The effects of such stressful and unstable conditions on growth, viability, and sugar metabolism regarding both ethanol formation and sugar consumption were analyzed and discussed in the context of cell physiology in yeasts exploited in industrial fermentations.

Materials and methods

Microorganism and media

A hybrid strain of *S. cerevisiae* (63M) was used as a starter of the fermentation system. This hybrid strain resulted from the crossing of haploids of the thermotolerant strains [8] ET-2 (*MAT a ura*) and OSMO 8 (*MAT* α *lys*). Strains were maintained on yeast extract, peptone, and glucose (YPD) medium at 4°C with subculturing every 4 months. The following media at pH 4.5 were used (sugar concentration expressed as total reducing sugar, wt/vol): (a) 5% sugar cane syrup for growth of the starter culture (25 ml) in a 125-ml Erlenmeyer flask for 16 h in a rotary shaker (250 rpm) at 30°C; (b) 10% sugar cane syrup for the initial propagation of the culture in the fermentation system at 30°C; and (c) 15% sugar cane syrup supplemented with 0.2% ammonium sulfate and 2% K_2HPO_4 [15].

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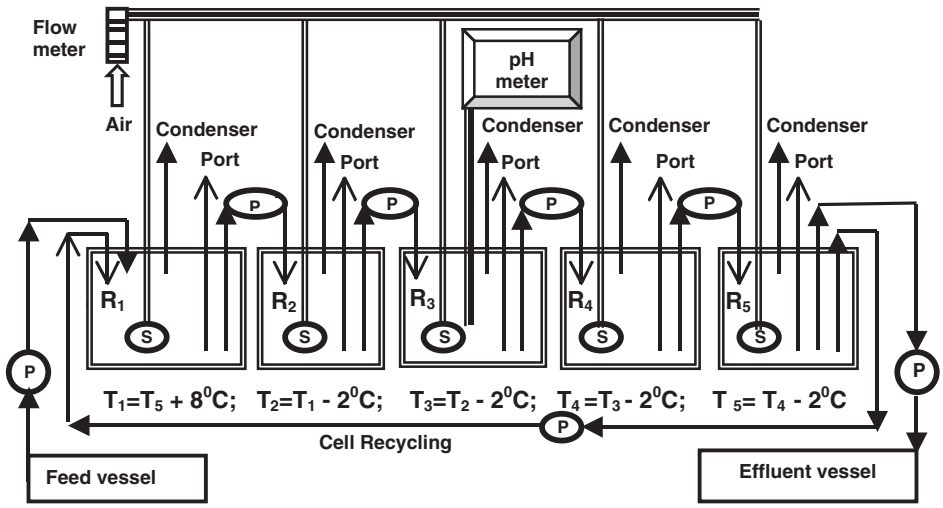

Figure 1 Schematic diagram of the five-stage cascade system set up for continuous operation with cell recycling at increasing temperatures: S=spargers; P=pump drivers. Sampling ports and condensers were adapted to each reactor. When the equilibrium was reached in each reactor operating at 30°C, three ranges of increasing temperatures (in direction opposite to the feed flow) were sequentially applied to the system: 35°C (reactor R_5) to 43°C (reactor R_1); 37°C (reactor R5) to 45°C (reactor R_1); and 39°C (reactor R_5) to 47°C (reactor R_1). The difference in temperature from one reactor to the next was 2°C while an 8°C difference was kept between the last reactor R_5 and first R_1.

Analytical methods

Residual sugar was assayed using the 3,5-dinitrosalicylic acid method [8,11]. The samples containing sucrose were acid-hydrolyzed as described previously [11] and reducing sugar was determined. Ethanol was assayed in a gas chromatograph (model CG-37; Instrumentos Científicos, Sao Paulo, Brazil) and the biomass was evaluated by turbidimetric measurement at 570 nm (Micronal spectrophotometer, model B-345; Micronal, Sao Paulo, Brazil) using a standard curve correlating absorbancy to dry weight. Viability was determined by the methylene blue method [9]. Data are the averages of the measurements obtained daily for each reactor when equilibrium was attained.

Five-stage cascade system

The system shown in Figure 1 was made up of five reactors (24.5 cm high × 8.5 cm id) linked in series. Variable-speed peristaltic pumps (Superohme pump driver; Piracicaba, Sao Paulo, Brazil) were used for feeding of the system and transfer of the culture from one bioreactor to the next. The bioreactors were placed in waterbaths and heated to different temperatures (±0.5°C). The aeration rate of the entire system was controlled by a flow meter (model 0365; Cole-Parmer, IL) located between the air compressor line and the branched (five branches) stainless steel tubes (0.5 cm id) so that air was injected at the bottom of each bioreactor through Teflon porous spargers (10-mm-diameter sparger containing pores of 1 mm id) fitted to each branched tube as shown in Figure 1. An autoclavable pH electrode (model 365; Cole-Parmer) was inserted into vessel R_3 to control the pH of the entire system (pH controller model 5652-00; Cole-Parmer) by automatic addition of 1.5 M NaOH using a variable-speed peristaltic pump. The whole system was sterilized for 1 h at 120°C (1 atm). Refrigerated condensers (tap water at 25°C) were attached to each reactor to minimize the ethanol evaporation during the operation of the system.

Inoculation and operation of the cascade system

The first reactor (R_1) was inoculated with 25 ml of starter culture grown in a rotary shaker. Then, the entire system was aerated with an airflow rate of 120 l h^{-1}, so that cells were maintained in suspension in each reactor (0.6 l medium in each bioreactor) with less cell death at higher temperatures. The culture effluent was continuously pumped back ($F_{recycling}$=0.10 l h^{-1}) from the last reactor (R_5) to the first reactor (R_1) without feeding for 3 days in order to allow an equivalent propagation level of the cells into the five reactors. Continuous feeding of the entire system ($F_{feeding}$=0.12 l h^{-1}=F_{out}) with fresh medium was started through reactor R_1 and the culture was pumped from one reactor to the next at a flow rate of 0.22 l h^{-1} (feeding plus recycling rates). When no significant changes in biomass, viability, residual sugar, and ethanol were observed in each reactor after four consecutive daily samplings, the system was considered to be at equilibrium at 30°C. Then, three ranges of increasing temperatures were

Table 1 Biomass, viability, ethanol, and residual sugar in each reactor for the system operating at 35–43°C[a]

Reactors	Temperature (°C)	Biomass (g l^{-1})	Viability (%)	Ethanol (g l^{-1})	Residual sugar (g l^{-1})
R_1	43	7.4±0.9	89±6	25.2±2.2	45.0±6.8
R_2	41	6.7±0.6	86±9	32.4±2.7	40.2±4.5
R_3	39	5.9±0.5	87±5	39.5±3.0	25.1±2.5
R_4	37	11.2±1.5	88±7	41.9±3.3	12.2±0.9
R_5	35	12.6±1.1	87±8	41.2±2.8	2.2±0.5

[a]Average data (±SD) of daily measurements obtained at steady state during operation of the system for 15 days at 35–43°C.

Table 2 Biomass, viability, ethanol, and residual sugar in each reactor for the system operating at 37–45°C[a]

Reactors	Temperature (°C)	Biomass ($g\ l^{-1}$)	Viability (%)	Ethanol ($g\ l^{-1}$)	Residual sugar ($g\ l^{-1}$)
R_1	45	6.1±1.0	69±16	22.7±2.0	62.1±7.2
R_2	43	5.9±0.8	67±9	23.5±1.5	59.3±4.8
R_3	41	4.8±0.6	56±15	25.2±2.0	48.2±3.1
R_4	39	6.4±0.9	69±13	32.3±3.0	28.5±1.3
R_5	37	8.1±1.1	72±12	38.5±2.8	13.2±3.2

[a]Average data (±SD) of daily measurements obtained at steady state during operation of the system for 25 days at 37–45°C.

sequentially applied to the system, so that the increases in temperature occurred in the opposite direction to the feed flow. In this way, more nutrients were available in reactor R_1 operating at the highest temperature in order to minimize cell death. A difference of 2°C was maintained from one reactor to the next (e.g., 43°C in reactor R_1 and 35°C in reactor R_5) and a second equilibrium was maintained for 15 days. Then, the temperature range of 37–45°C was applied to the system for an additional 25 days. Lastly, the temperature was raised to 39–47°C and the system was operated for 20 days. The pH was kept at 4.5 and samplings were carried out every day through the sampling ports fitted to each reactor. For calculations of the pseudostoichiometric parameters in the final effluent, the entire system was considered as a single system (total working volume of 3 l and a feeding rate of $0.12\ l\ h^{-1}$) operating at a $0.04\ h^{-1}$ dilution rate. The volumetric retention time in the entire system was expressed as the total liquid fermentor volume (3 l) divided by the dilution rate. The fermentation parameters were expressed as the averages (±SD) of the data obtained every day when the entire system was operating at steady state.

Results

Continuous fermentation was carried out for a total of 64 days with a volumetric retention time of 75 h. The effects of increasing temperatures on the accumulation of biomass, ethanol, sugar consumption, and viability in each reactor of the system operating in three ranges of temperatures (35–43, 37–45, 39–47°C) are shown in Tables 1–3.

Table 1 shows the data obtained for each reactor when the system was operating at 35°C (in the last reactor R_5)–43°C (first reactor R_1). The lowest biomass ($5.9\pm0.5\ g\ l^{-1}$) was found in reactor R_3 operating at 39°C (located in the middle of the system) and the highest in reactor R_5 ($12.6\pm1.1\ g\ l^{-1}$) operating at 35°C. Viability was maintained at high and quite stable values in all five reactors (86–89%). Ethanol in the medium showed the highest values in reactors R_4 ($41.9\pm3.3\ g\ l^{-1}$) at 37°C and R_5 ($41.2\pm2.8\ g\ l^{-1}$) at 35°C, while the lowest value was obtained in reactor R_1 ($25.2\pm2.2\ g\ l^{-1}$) at 43°C. The residual sugar showed its lowest value ($2.2\pm0.5\ g\ l^{-1}$) in reactor R_5 at 35°C and the highest in reactor R_1 ($45.0\pm6.8\ g\ l^{-1}$) at 43°C.

Table 2 shows data obtained when the system was operated at 37°C (in the last reactor R_5)–45°C (first reactor R_1). Decreases were observed for values of biomass, ethanol, and viability when compared with data obtained in the range of 35–43°C (Table 1). However, increases were observed for the levels of residual sugar in all reactors (Table 2). The lowest value of biomass ($4.8\pm0.6\ g\ l^{-1}$) was obtained in reactor R_3 at 41°C, and the highest in reactor R_5 ($8.1\pm1.1 g\ l^{-1}$) at 37°C. Viability varied from 69% in reactor R_1 at 45°C to 72% in reactor R_5 at 37°C. However, the lowest value of viability (56±15%) was found in reactor R_3 at 41°C located in the middle of the system. The ethanol level showed the highest value in reactor R_5 ($38.5\pm2.8\ g\ l^{-1}$) at 37°C and the lowest in reactor R_1 ($22.7\pm2.0\ g\ l^{-1}$) at 45°C. Similar variations were observed for the residual sugar that showed the highest value ($62.1\pm7.2\ g\ l^{-1}$) in reactor R_1 at 45°C and the lowest ($13.2\pm3.2\ g\ l-^{1}$) in reactor R_5 at 37°C.

Table 3 shows data obtained when the system operated at 39°C (in the last reactor R_5)–47°C (first reactor R_1). Remarkable increases in residual sugar were observed for the residual sugar in all five reactors (values of $84.3\pm5.7\ g\ l^{-1}$ in reactor R_1 at 47°C and $42.2\pm4.5\ g\ l^{-1}$ in reactor R_5 at 39°C) when compared with the levels of residual sugar shown in Tables 1 and 2. Viability decreased particularly in reactor R_3 (36±8% viability) operated at 43°C. The amounts of ethanol in the five reactors did not show great variations ($20.7\pm3.0\ g\ l^{-1}$ in reactor R_1, $19.8\pm2.8\ g\ l^{-1}$ in reactor R_2, and $36.3\pm3.0\ g\ l^{-1}$ in reactor R_5) with the changes in temperature certainly due the evaporation caused by the high temperatures and strong aeration.

Considering each reactor separately, the fermentation parameters changed when the temperature of the entire system was raised from 30°C (same temperature in all five reactors) to ranges of increasing temperature. The maximal values of biomass (Figure 2) were found in reactor R_5 at 35°C and reactor R_4 at 37°C while decreases were observed for the other reactors with increases in temperature above 30°C. However, the lowest biomass was found in the reactor R_3 in the three ranges of temperature applied to the

Table 3 Biomass, viability, ethanol, and residual sugar in each reactor for the system operating at 39–47°C[a]

Reactors	Temperature (°C)	Biomass ($g\ l^{-1}$)	Viability (%)	Ethanol ($g\ l^{-1}$)	Residual sugar ($g\ l^{-1}$)
R_1	47	4.8±1.3	68±14	20.7±3.0	84.3±5.7
R_2	45	4.6±1.2	61±6	19.8±2.8	82.1±6.9
R_3	43	4.0±0.8	36±8	21.7±1.9	66.4±4.6
R_4	41	5.5±0.9	48±11	30.6±2.0	59.1±4.9
R_5	39	7.5±1.0	53±12	36.3±3.0	42.2±4.5

[a]Average data (±SD) of daily measurements at steady state during operation of the system for 20 days at 39–47°C.

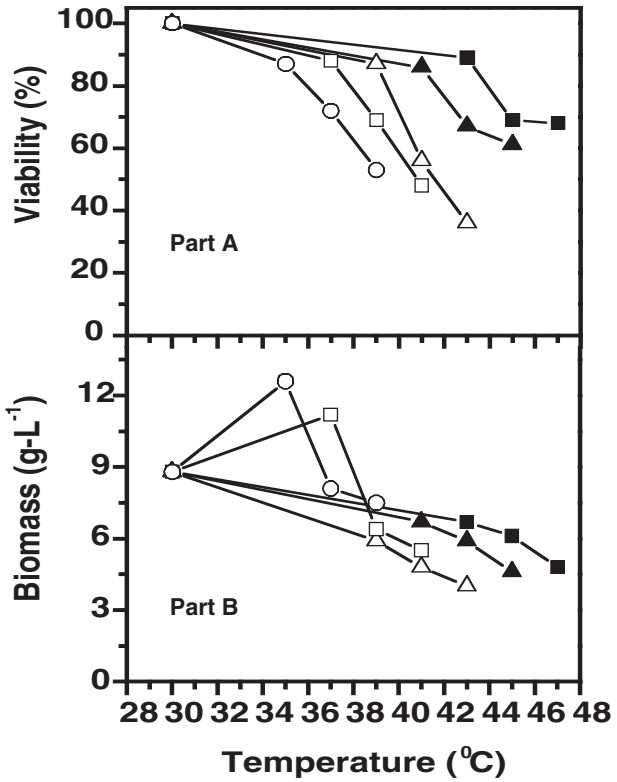

Figure 2 Variations in viability (Part A) and biomass (Part B) occurred in each reactor (R_1 = ■; R_2 = ▲; R_3 = △; R_4 = □; R_5 = ○) during application of the three ranges of increasing temperatures to the fermentation system described in Figure 1.

system. Viability (Figure 2) decreased in each reactor with increases in temperature, but the variations were greater above the following temperatures: above 35°C in reactor R_5, 37°C in reactor R_4, 39°C in reactor R_3, 41°C in reactor R_2, and 43°C in reactor R_1. The lowest viability occurred in reactor R_3 at 43°C (36±8% viability as shown in Table 3). Ethanol levels also decreased in each reactor with increases in temperature, but small differences were observed when the levels of ethanol in each reactor were compared in the three ranges of temperatures applied to the system (Tables 1–3) due to evaporation. The lowest levels of ethanol occurred in reactor R_2 at 45°C and R_1 at 47°C (Table 3) and the highest in reactors R_5 at 35°C and R_4 at 37°C (Table 1). However, variations in levels of residual sugar were greater among the reactors (Tables 1–3) than those observed for the ethanol levels. The lowest values of residual sugar were found in reactors R_5 at 35°C and R_4 at 37°C (Table 1) while the highest values were found in reactors R_1 at 47°C and R_2 at 45°C (Table 3).

Increases in the temperature of the entire system from 30°C to ranges of increasing temperatures (Figure 1) led to variations in the effluent of the entire system (biomass, biomass yield, volumetric productivity, residual sugar, ethanol, volumetric ethanol productivity, volumetric biomass productivity, specific ethanol productivity, specific ethanol production rate) when operating at steady state (Table 4). The lowest levels of residual sugar were obtained in the final effluent when the entire system operated at 30°C (3.0±0.1 g l^{-1}) and at the range of 35–43°C (2.2±0.5 g l^{-1}) while the highest levels of ethanol (47.2±0.7 g l^{-1}) were obtained at 30 and 35–43°C (41.9±4.0 g l^{-1}). However, the highest values of volumetric biomass productivity coinciding with the lowest specific ethanol production rate occurred when the system operated at 35–43°C. Quite high levels of viability were obtained when the system operated at 35–43°C and at 37–45°C (72–87%), but a significant decrease was observed at 39–47°C (52.7±10%). Temperature fluctuations above 37–45°C associated with ethanol formation led to decreases in biomass and increases in residual sugar in the effluent. However, the levels of residual sugar in the effluent (Table 4) were small (13.2±3.2 g l^{-1} at 37–45°C and 42.0±4.5 g l^{-1} at 39–47°C) compared to the much higher levels of residual sugar found in reactor R_1 (62.1±7.2 g l^{-1} at 45°C to 84.3±5.7 g l^{-1} at 47°C) and R_3 (48.2±3.1 g l^{-1} at 41°C to 66.4±4.6 g l^{-1} at 43°C) when the system was operated at 37–45°C (Table 2) to 39–47°C (Table 3). Thus, sugar consumption was improved in the effluent of the system operated at 37–45 and 39–47°C (Table 4) when the values of residual sugar were compared to those obtained for the two last reactors R_4 and R_5 (Tables 2 and 3).

Discussion

The apparatus described here was set up to subject the yeast population to heterogeneous environments. The feeding of the entire system occurred in the direction opposite to the cell recycling, plus the application of gradients of increasing temperatures generated gradients of nutrients, substrates, and product

Table 4 Changes of biomass, ethanol, viability, and residual sugar in the final effluent during application of the ranges of increasing temperatures through out the entire system[a]

Operational conditions		Biomass			Sugar in the effluent	Ethanol				Viability
Ranges of increasing temperatures	Steady state (h)	X (g l^{-1})	$Y_{X/S}$ (10^2)	Q_X (g l^{-1} h^{-1})	(S_{out}, g l^{-1})	P (g l^{-1})	$Y_{P/S}$	Q_P (g l^{-1} h^{-1})	q_P ($g_{ethanol}$ g_{cell}^{-1} h^{-1})	(%)
30°C (all reactors)	96	8.8±1.5	5.99±1.02	0.35±0.06	3.0±0.1	47.2±0.7	0.32±0.01	1.89±0.03	0.21±0.04	93.4±5
35°C (in reactor R_5) –43°C (reactor R_1)	360	12.6±1.4	8.5±0.95	0.50±0.06	2.2±0.5	41.9±4.0	0.28±0.03	1.68±0.16	0.13±0.02	87±8
37°C (in reactor R_5) –45°C (reactor R_1)	600	8.1±0.6	5.91±0.4	0.32±0.02	13.2±3.2	36.3±5.5	0.27±0.04	1.45±0.22	0.18±0.03	72±12
39°C (in reactor R_5) –47°C (reactor R_1)	480	7.6±0.4	7.04±0.4	0.30±0.02	42.0±4.5	30.8±4.1	0.29±0.04	1.23±0.16	0.16±0.02	52.7±10

[a]Average values (±SD) obtained for the final effluent of the entire system are as follows: sugar in the feed flow=150 g l^{-1}; biomass in the effluent=X (g l^{-1}); biomass yield factor=$Y_{X/S}$; volumetric biomass productivity=Q_X (g l^{-1} h^{-1}); ethanol in the effluent=P (g l^{-1}); ethanol yield factor=$Y_{P/S}$; volumetric ethanol productivity=Q_P (g l^{-1} h^{-1}); specific ethanol production rate=q_P ($g_{ethanol}$ g_{cell}^{-1} h^{-1}).

concentrations throughout the entire cascade system. Thus, it was presumed that under such conditions (increasing temperatures plus repeated heat shocks), yeast cells became adapted to the increasing temperatures. Repeated temperature shocks greater than 3°C cause reductions in ethanol production rates up to 50% [3]. However, fluctuations in temperature and ethanol evaporation cannot be avoided in large industrial fermenters due to difficulties related to agitation and cooling when the external temperature reaches high values.

Minimal values of biomass were found in the middle of the cascade system in three ranges of temperatures used in the present study. Both a region of growth deceleration (biomass decreasing from reactor R_1 at the highest temperature to reactor R_3) and acceleration region (biomass increasing from reactor R_3 to R_5 operating at the lowest temperature) were observed (Tables 1–3). A similar behavior was observed for yeast cell viability in the range of 39–47°C (Table 3). Establishment of both acceleration and deceleration growth rates at the center of the cascade system may thus provide a useful insight into aspects of yeast growth and physiology, so that cells at different stages of the cell cycle can be isolated from the same culture. In addition, samples containing cells in different physiological states can be obtained from the same culture for a variety of studies.

It seemed that the residential time of the broth in reactors R_1 and R_2 was not long enough to kill all the cells returning from reactor R_5 at lower temperature. The small differences in viability observed among the reactors in each range of temperature suggested that new cells resulting from budding in the reactors at lower temperatures were compensating for any limitations concerning cell division, which occurred in reactors operating at higher temperatures. In addition, the rate of the cell division process was probably reduced and/or arrested during adaptation at higher temperatures so that adapted cells started dividing at faster rates when the broth returned to reactors at lower temperatures during recycling. The lowest biomass found in reactor R_3 coincided with the equally low viability found in the same reactor when the system was operated in the range of 39–47°C. Thus, this significant cell death/cell division arrest occurred in reactor R_3. A similar decrease in biomass was observed in reactor R_3 when this system was operated using minimal medium containing 2% glucose (Greenhalf and Laluce, unpublished data). The decreases in biomass and viability observed in reactors R_2 and R_3 can also be attributed to negative effects of the ethanol on biomass and viability at higher temperatures.

Optimal temperatures for ethanol production by brewing yeast are higher than those required for growth [5]. Ethanol evaporation and the repeated heat shocks certainly contributed to the decreases in ethanol production rate as shown in a previous report [3]. In the present work, the use of a multistage system made up of five reactors linked in series and showing temperatures decreasing in the direction opposite to the feed flow possibly minimized negative effects of the heat shocks on ethanol formation as shown in a previous work [2]. Considering each range of temperature separately, cell recovery occurred in reactors operated at lower temperatures. Residual sugar in the effluent of the system showed a small increase when the temperature was raised from 35–43 to 37–45°C, suggesting that only a small loss in fermentation capacity occurred. However, the highest value of residual sugar (42.0 ± 4.5 g l^{-1}) was reached in the effluent when the system was operated at 39–47°C (Table 4) while the viability was $52.7 \pm 10\%$. However, a lower value of viability was determined for reactor R_3 ($36 \pm 8\%$ viability; Table 3) situated in the middle of the system when operated in this same range of temperature. Thus, fluctuations in the temperature of the system between 39 and 47°C led to significant sugar consumption followed by maintenance of high viability for extended periods of time at temperatures above 40°C. Gradients of temperature in large reactors may minimize cell death and allow sugar utilization at temperatures usually not permissible for the fermentation activity of *S. cerevisiae* cells.

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