ISSN 1330-9862
(FTB-3272)

original scientific paper

Modelling of Ethanol Production from Red Beet Juice by *Saccharomyces cerevisiae* under Thermal and Acid Stress Conditions

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Received: December 5, 2012
Accepted: January 16, 2014

Summary

In this work the effects of pH and temperature on ethanol production from red beet juice by the strains *Saccharomyces cerevisiae* ITD00196 and *S. cerevisiae* ATCC 9763 are studied. Logistic, Pirt, and Luedeking-Piret equations were used to describe quantitatively the microbial growth, substrate consumption, and ethanol production, respectively. The two *S. cerevisiae* strains used in this study were able to produce ethanol with high yield and volumetric productivity under acid and thermal stress conditions. The equations used to model the fermentation kinetics fit very well with the experimental data, thus establishing that ethanol production was growth-associated under the evaluated conditions. The yeast *S. cerevisiae* ITD00196 had the best fermentative capacity and could be considered as an interesting option to develop bioprocesses for ethanol production.

Key words: *Beta vulgaris* L.; modelling parameters; logistic, Pirt and Luedeking-Piret equations

Introduction

The consumption of fossil fuels has contributed to environmental pollution. Therefore, ethanol has gained increasing attention in recent years as an alternative fuel. In fact, ethanol is blended with gasoline as an oxygenating additive to reduce the use of fossil fuels and CO_2 emissions (*1*). Ethanol is produced by chemical processes through ethylene hydration, and in biological processes by fermentation of sugars from different sources. Many sugar crops that are suitable for fermentation include sugarcane, fruits, sweet potato, sweet sorghum (*2*), agave must (*3*), and sugar beet juice (*4*). The processing of sugar beets to make bioethanol can be a convenient process, if it has ecological benefits and can be produced on large scales without affecting food provisions (*4,5*).

Red beets in Mexico are used in the colorants industry, but their juice and bagasse can be sources of carbohydrates for ethanol production. The microorganisms that can be used for ethanol production include fungi such as *Mucor indicus* (*6*), bacteria such as *Zymomonas mobilis* (*7*), and yeast such as *Kluyveromyces marxianus* (*8*). However, *Saccharomyces cerevisiae* is the most commonly used yeast in industrial ethanol production (*9*). During fermentation processes, the activities of microorganisms closely respond to changes in the environmental conditions, which are accompanied by variations in the mass transfer and the metabolic behaviour of the microorgan-

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ism (*10*). Some important environmental conditions for the fermentation process are: pH, temperature, sugar concentration, and strain type (*11*). Information regarding the influence of temperature and pH on the kinetic parameters is still necessary to understand and improve the ethanol productivity; as such, mathematical models are valuable tools for this purpose. Several structured and unstructured mathematical models have been developed which attempt to describe the fermentation reaction. These models have been largely developed for the purpose of describing quantitatively the experimental data, as well as to support fermentation reactor design and operation (*8*). Unstructured models have been used to describe the relationship between growth, microbial process, substrate consumption and product synthesis.

The aim of this study is to evaluate the effects of pH, temperature, and strain type on ethanol production from red beet juice using logistic, Pirt and Luedeking-Piret equations. Unstructured models were used to estimate how these factors affect the fermentation kinetics.

Nomenclature

X biomass concentration/(g/L)
X_0 biomass concentration at t=0/(g/L)
t time/h
X_{max} maximum biomass concentration/(g/L)
μ specific growth rate/h^{-1}
μ_{max} maximum specific growth rate/h^{-1}
S sucrose concentration/(g/L)
S_0 sucrose concentration at t=0/(g/L)
$Y_{x/s}$ biomass per sucrose yield/(g of biomass per g of sucrose)
$Y_{p/s}$ ethanol per sucrose yield/(g of ethanol per g of sucrose)
m maintenance coefficient/(g of sucrose per g of biomass per h)
P concentration of the product/(g/L)
P_i concentration of the product at t=0/(g/L)
α growth-associated coefficient for the product/(g of ethanol per g of biomass)
β non-growth-associated coefficient for the product/(g of ethanol per g of biomass per h)
Q_p volumetric ethanol productivity/(g of ethanol per L per h)

Materials and Methods

Yeast strains

The strain *S. cerevisiae* ITD00196 was obtained from the yeast collection of the Technological Institute of Durango, Durango, Mexico. This strain was isolated from the must of *Agave duranguensis* (*12*). *S. cerevisiae* ATCC 9763 isolated from distillery was also used. These strains were chosen because it has been reported that they are good ethanol producers (*12,13*). The strains were maintained on yeast extract peptone dextrose (YPD) agar slants containing the following (in g/L): yeast extract 10, peptone 20, dextrose 20 and agar 16, at 30 °C.

The preinoculum was prepared by inoculating the slant culture into a 125-mL Erlenmeyer flask with 100 mL of YPD medium and growing it on a rotary shaker at 120 rpm (Thermo Fisher Scientific, Waltham, MA, USA) and 30 °C for 12 h. The cell population was quantified in a Neubauer chamber. Fresh YPD liquid medium (100 mL) was inoculated at an initial concentration of 10^6 cells/mL and was incubated at 120 rpm and 30 °C for 12 h. This inoculum was used in the fermentation of red beet juice at an initial concentration of 10^6 cells/mL.

Substrate

Red beets (*Beta vulgaris* L.) were obtained from a local market in Durango, Mexico. They were washed and cut into slices to ensure a rapid rate of juice extraction. The juice was obtained with a juicer (Turmix, Zurich, Switzerland), and then preserved at 4 °C to prevent any possible degradation during storage. The amounts of total sugars (*14*) and yeast assimilable nitrogen (*15*) were determined in the red beet juice as previously described.

Fermentation conditions

Two pH levels (pH=2.8 and 5.5), two temperatures (30 and 37 °C), and two *S. cerevisiae* strains (ITD00196 and ATCC 9763) were applied to determine the best conditions for ethanol production. First, pH=2.8 was chosen because Páez-Lerma (*12*) found that this pH was the lowest required for the growth of many strains of *S. cerevisiae*. On the other hand, pH=5.5 was chosen because it has been demonstrated that *S. cerevisiae* shows an intracellular pH near 5.5 when the external pH is 3.0 (*16*). The temperatures used were selected because the *S. cerevisiae* ITD00196 strain was isolated from an environment where an average temperature is 30 °C (*17*), while 37 °C is the highest temperature at which many *S. cerevisiae* strains are able to grow (*3*). Experiments were performed in triplicate. The codified variables from the experimental matrix are summarized in Table 1.

Table 1. Experimental conditions used to perform the fermentations

Assay	pH	Temperature / °C	Strain
E_1	2.8	30	ITD
E_2	5.5	30	ITD
E_3	2.8	37	ITD
E_4	5.5	37	ITD
E_5	2.8	30	ATCC
E_6	5.5	30	ATCC
E_7	2.8	37	ATCC
E_8	5.5	37	ATCC

The collected data were analyzed for between-subject effects to determine statistical differences between the different treatments. Multivariate and between-subject effects tests were used to determine the interactions between factors. The Statistical Package for the Social Sciences (SPSS, v. 17, SPSS Inc, Chicago IL, USA) was used for all statistical tests. The significance level was set at p=0.05.

The fermentation of red beet juice was carried out in 500-mL Erlenmeyer flasks without stirring or aeration. Each flask contained 200 mL of juice. The medium was supplemented with $(NH_4)_2SO_4$ up to a C/N ratio of 73, since it has been demonstrated that this nitrogen level stimulates yeast metabolism (*18*). The medium pH was adjusted using 2.5 M H_2SO_4, and it was sterilized for 15 min at 121 °C. All flasks were inoculated and incubated without stirring for 30 h. Temperature, initial pH and strain type were fixed as mentioned above. Samples were collected from the flasks at regular intervals to determine growth, sugar consumption and ethanol production.

Analytical methods

Assimilable nitrogen content of the red beet juice was estimated by the formol titration method (*15*). Biomass concentration was quantified (in cells/mL) with a Neubauer counting chamber and also by gravimetric analysis after drying to a constant mass. Samples were filtered through a 0.2-μm nylon membrane, and the cell-free supernatant was employed to measure sugar content by the phenol sulphuric acid assay (*14*) and ethanol concentration by high-performance liquid chromatography (HPLC series 1200, Agilent Technology, Palo Alto, CA, USA).

Total sugar content was determined by a modified phenol-sulphuric acid method using glucose as the standard (*14*). The sample was filtered, and 1 mL of it was transferred to a glass tube. Then 0.6 mL of 5 % (by mass per volume) phenol and 3.6 mL of 98 % H_2SO_4 were added. The mixture was shaken and incubated at room temperature for 30 min, and the absorbance was read at 490 nm. A calibration curve was established using glucose as the standard.

Ethanol concentration was determined by HPLC using water as the eluent at the flow rate of 0.4 mL/min with a Carbomix column (H-NP10, Sepax Technologies, Inc., Newark, DE, USA) operating at 80 °C. A refractive index detector was employed.

Data modelling

The logistic, Pirt, and Luedeking-Piret equations can be used to obtain simpler ones in order to establish relationships between the growth and substrate consumption, and growth and product synthesis. Eq. 1 is obtained by integration of the logistic equation, while Eqs. 2 and 3 are obtained by dividing the Pirt and Luedeking-Piret equations, respectively, by the logistic equation followed by integration, as demonstrated previously (*19*):

$$X(t)=\frac{X_{max}}{1+\left(\frac{X_{max}}{X_0}-1\right)e^{-\mu t}} \qquad /1/$$

$$S(X)=S_0-\frac{1}{Y_{X/S}}(X-X_0)-\frac{mX_{max}}{\mu}\ln\left(\frac{X_{max}-X_0}{X_{max}-X}\right) \qquad /2/$$

$$P(X)=P+\alpha(X-X_0)+\frac{bX_{max}}{\mu}\ln\left(\frac{X_{max}-X_0}{X_{max}-X}\right) \qquad /3/$$

The kinetic data were used to fit all kinetic parameters using the Solver function of Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). The simulation program was designed to achieve the minimal normalized error using the Solver function. Eqs. 1–3 were fitted to the experimental data to determine the kinetic parameters for microbial growth (μ_{max} and X_{max}), sugar consumption ($Y_{x/s}$ and m) and ethanol production (α and β). The experimental data along with those generated by the model were analyzed using regression curve fitting (Microsoft Excel) with statistical significance set at p= 0.05.

Results and Discussion

The rates of biomass growth, sugar consumption and ethanol production during batch fermentation of red beet juice by *S. cerevisiae* ITD00196 and ATCC 9763 are shown in Figs. 1–3, respectively. Fig. 1 presents the growth until the maximum concentration of biomass was obtained.

The results presented in Figs. 1a and e show that the highest cell concentrations were reached by *S. cerevisiae* ITD00196 and ATCC 9763 after 16 and 26 h, respectively. These biomass concentrations were 9.6 and 7.9 g/L, respectively, which were obtained when both strains grew at 30 °C and pH=2.8. *S. cerevisiae* ITD00196 had a lag phase of approx. 4 h, and *S. cerevisiae* ATCC 9763 had a longer lag phase of approx. 8 h. This increase in the lag phase is a result of the adaptation of the strain to the medium, which had a more acidic pH than is optimum (pH=5) (*13*).

When the experiment was performed at 30 °C and pH=5.5, the biomass production of both yeasts was reduced in comparison with the conditions of 30 °C and pH=2.8 (Figs. 1b and f). The lag phase of *S. cerevisiae* ATCC 9763 was reduced to 4 h with the increase of pH to 5.5 (Fig. 1f). This shorter lag phase can be explained by the fact that the pH was near the optimum pH of *S. cerevisiae* ATCC 9763. In comparison, Wang *et al*. (*20*) reported a lag phase of 17.3 h using sucrose.

At 37 °C, the growth of both strains at pH=2.8 and 5.5 was different. With the temperature increase from 30 to 37 °C, the biomass production was reduced in all experiments (Figs. 1c, d, g and h). The effects of temperature and pH on both strains induced physiological changes to resist the stress conditions. For example, Hsp30p of *S. cerevisiae* is a plasma membrane-bound heat shock protein with a role in tolerance to environmental stress. It has been reported that Hsp30p inhibits Pma1p, an H^+-ATPase of the plasma membrane, to conserve intracellular ATP reserves under stress conditions. *S. cerevisiae* also activates the expression of the *HSP30* gene in order to resist severe thermal conditions (*21*).

S. cerevisiae ITD00196 showed better adaptation to acid and thermal stress than that shown by *S. cerevisiae* ATCC 9763. These results can be explained by the origin of strain ITD00196, which was isolated from an acidic environment on the musts of *Agave duranguensis*. Yeasts that show thermal and/or acid tolerance are desirable for bioethanol production because this can minimize the risk of contamination as well as the cost of maintaining an optimum temperature.

Fig. 2 shows the substrate consumption by *S. cerevisiae* ITD00196 and ATCC 9763 under acid and thermal

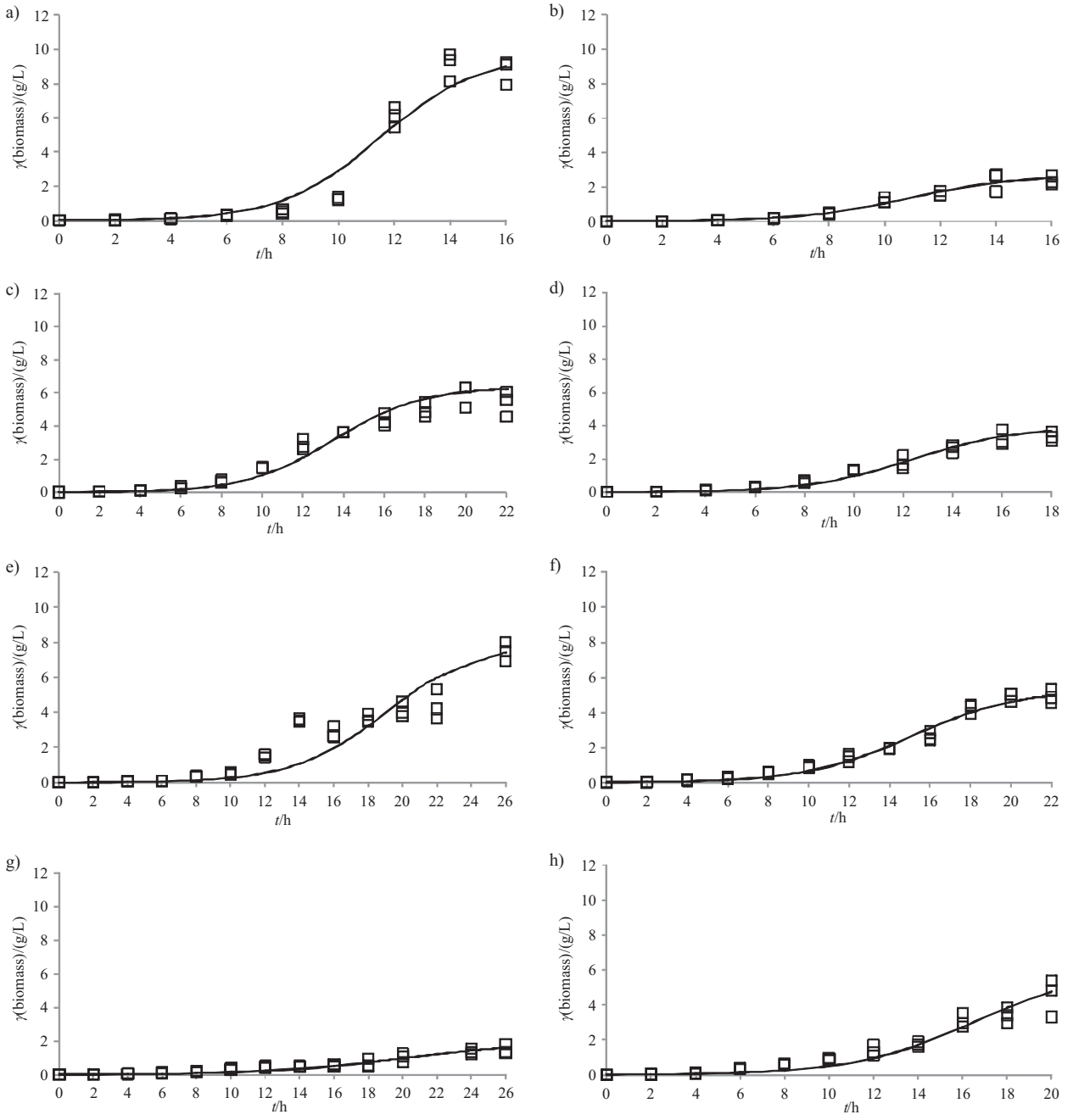

Fig. 1. Biomass growth of *Saccharomyces cerevisiae* ITD00196 (a–d) and *Saccharomyces cerevisiae* ATCC 9763 (e–h) in batch fermentation on red beet juice: a) and e) pH=2.8 and 30 °C, b) and f) pH=5.5 and 30 °C, c) and g) pH=2.8 and 37 °C, and d) and h) pH=5.5 and 37 °C. Experimental data (squares) and model data (line)

stress. At pH=5.5, there were some differences in the sugar consumption rate. All the experiments had better assimilation profile with respect to the experiments carried out at pH=2.8. The results show that the sucrose consumption by *S. cerevisiae* ITD00196 (Figs. 2a–d) was faster in all cases than that shown by *S. cerevisiae* ATCC 9763 (Figs. 2e–h). Thus, the best substrate consumption profiles were at pH=5.5 and 37 °C for both strains.

The ethanol production profiles by *S. cerevisiae* ITD-00196 and ATCC 9763 are shown in Fig. 3. At 30 °C and both pH values, the maximum ethanol production by *S. cerevisiae* ITD00196 was obtained between 18 and 20 h. When the temperature was increased to 37 °C (at both pH values), the maximum ethanol production was obtained between 16 and 18 h. Thus, the start time of ethanol production was similar to the lag phase time for each strain, which suggests that ethanol is a growth-associated product.

The results presented in Figs. 1–3 show that the used equations fit closely to the experimental data under all tested conditions, which was confirmed by the determination of the degree of fit to the experimental data using the multiple determination coefficients (R^2). Table 2 summarizes the important kinetic parameters of the fermen-

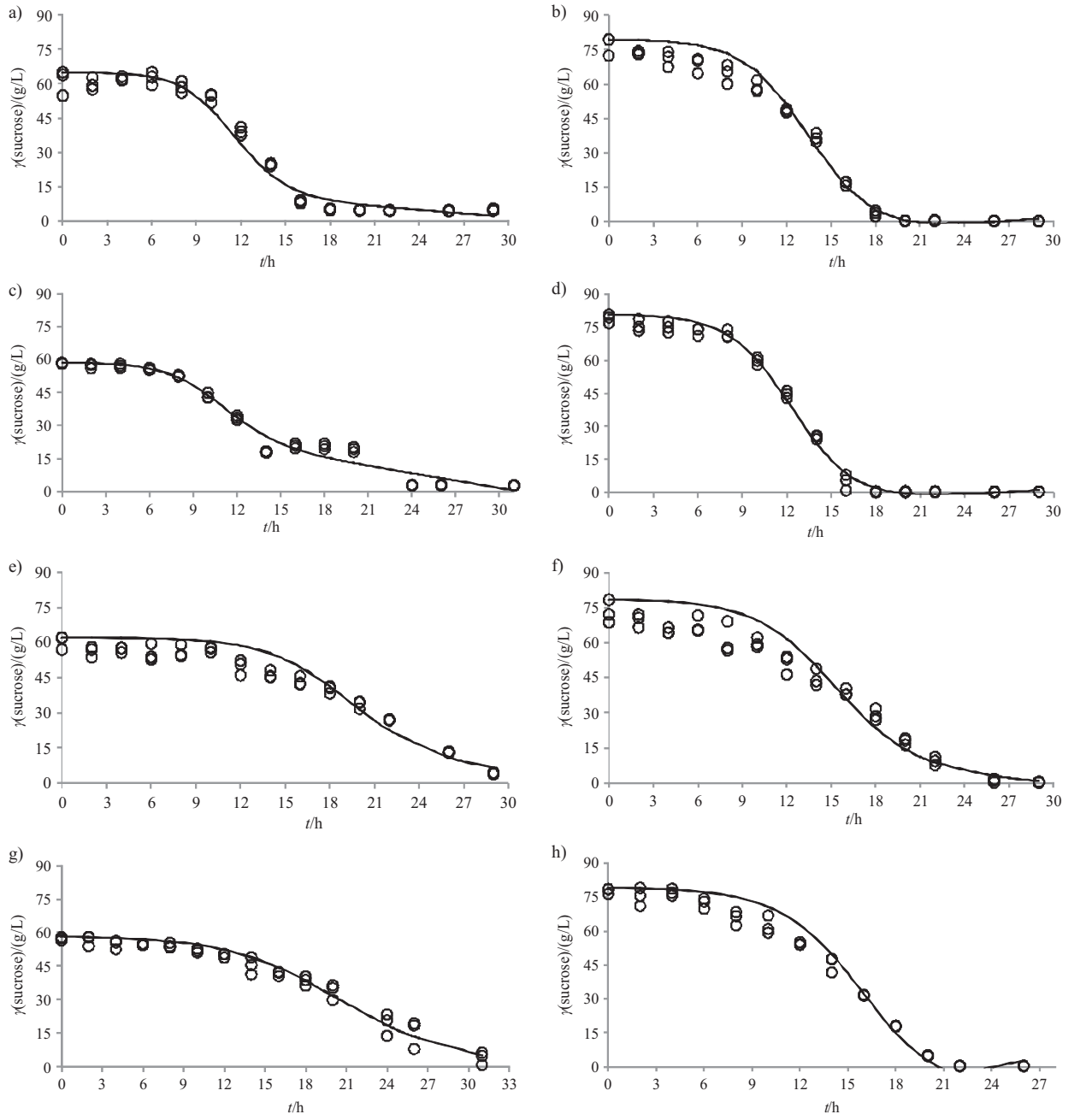

Fig. 2. Sugar consumption by *Saccharomyces cerevisiae* ITD00196 (a–d) and *Saccharomyces cerevisiae* ATCC 9763 (e–h) in batch fermentation on red beet juice: a) and e) pH=2.8 and 30 °C, b) and f) pH=5.5 and 30 °C, c) and g) pH=2.8 and 37 °C, and d) and h) pH=5.5 and 37 °C. Experimental data (circles) and model data (line)

tation of red beet juice by *S. cerevisiae* ITD00196 and ATCC 9763. The kinetic parameters show that the strains have a similar growth behaviour. Both strains have the best values of μ_{max} and X_{max} at 30 °C and pH=2.8, and the worst values at 37 °C and pH=2.8. On the other hand, it can be seen that, in general, the kinetic parameter values are greater for the strain *S. cerevisiae* ITD00196. It should be pointed out that the logistic model fits the data very well, as determined by the high R^2 values in all cases. As previously reported (*21*), the maximum specific growth rate depends on the temperature and pH of the medium. In this work, only the temperature and the nature of strains had an effect; we could also see that the effect of pH on the μ_{max} was not significant ($p<0.05$). These results are different from those previously reported, where at pH=5.5 and 2.8 the μ_{max} changed from 0.386 to 0.222 h^{-1} using fructose and *S. cerevisiae* (*22*).

Table 2 shows the kinetic parameter values for ethanol production (Eq. 3). Under all conditions, the growth-associated parameter (α) was significantly large (from 2.3734 to 10.70 g of ethanol per g of biomass), while β was close to zero (from 0.3359 to 0.3109 g of ethanol per g of biomass per h). *S. cerevisiae* ITD00196 showed larger values of μ_{max}, which was also manifested in the kinetics of ethanol production (Figs. 3a–d), since the ethanol con-

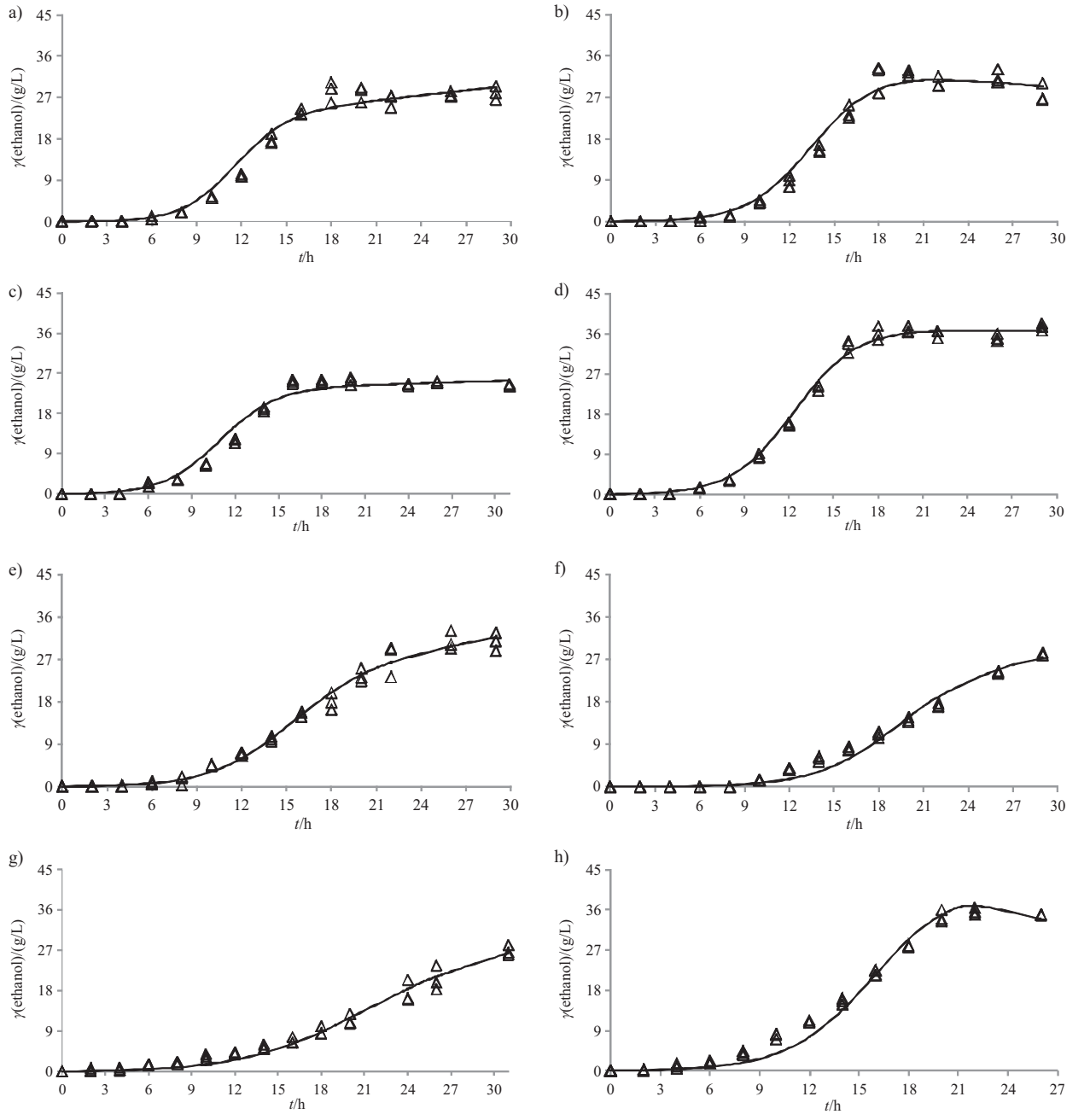

Fig. 3. Ethanol production by *Saccharomyces cerevisiae* ITD00196 (a–d) and *Saccharomyces cerevisiae* ATCC 9763 (e–h) in batch fermentation on red beet juice: a) and e) pH=2.8 and 30 °C, b) and f) pH=5.5 and 30 °C, c) and g) pH=2.8 and 37 °C, and d) and h) pH=5.5 and 37 °C. Experimental data (triangles) and model data (line)

centration reached its highest values in 18 h. On the other hand, *S. cerevisiae* ATCC 9763 showed a slower growth than the strain ITD00196 (evidenced by lower values of μ_{max}), which was also manifested in slower ethanol production, reaching its highest concentrations in 30 h of cultivation. These data suggest that the production of ethanol is growth-associated. It should be noted that the adopted kinetic model (Eq. 3) was able to replicate the production of ethanol with a high level of correlation (R^2=0.97–0.99). In contrast, Ahmad *et al.* (*23*) performed a series of experiments to show that ethanol batch fermentation is a non-growth-associated process that uses glucose. However, these authors used an aeration of 0.075 vvm and an agitation speed of 75 rpm. This discrepancy can be explained by the fact that when oxygen is absent, *S. cerevisiae* produces ethanol in order to reoxidize $NADH^+$ to NAD^+; however, when oxygen is present, it acts as a final electron acceptor (*23*).

The maintenance coefficient (m) of almost all fermentation conditions was low or null (from 0.66 to 0.41), indicating that *S. cerevisiae* ITD00196 and ATCC 9763 mainly utilize sucrose for ethanol and biomass production (Table 2). The sucrose concentration is related to μ_{max} in a similar way to what occurred with the ethanol concentration. The consumption of sugar is faster when μ_{max} is higher, reaching almost total consumption of sucrose in

Table 2. Kinetic parameters estimated by the mathematical model using experimental data

	S. cerevisiae ITD00196				*S. cerevisiae* ATCC 9763			
	Temperature							
	30	30	37	37	30	30	37	37
	pH							
	2.8	5.5	2.8	5.5	2.8	5.5	2.8	5.5
μ_{max}	0.5717 ±0.0199	0.4669 ±0.0285	0.5160 ±0.0191	0.4804 ±0.0154	0.3699 ±0.0211	0.3794 ±0.0031	0.2335 ±0.007	0.374 ±0.034
X_{max}	9.6869 ±0.231	6.3537 ±0.7118	2.7081 ±0.1916	3.9407 ±0.3126	7.9856 ±0.5389	5.3121 ±0.1403	1.9582 ±0.3202	6.0412 ±0.3226
R^2	0.9568	0.9604	0.9553	0.9667	0.8636	0.9803	0.9192	0.9356
α	2.3734 ±0.0487	5.3184 ±0.2913	8.7106 ±0.0879	9.5035 ±0.0972	2.8998 ±0.05326	4.5326 ±0.1047	10.70 ±2.47	9.111 ±0.1508
β	0.037 ±0.0063	–0.044 ±0.0259	0.0341 ±0.0051	–0.0109 ±0.008	0.06021 ±0.0078	0.1047 ±0.053	0.3109 ±0.143	–0.3359 ±0.017
R^2	0.9767	0.9842	0.9816	0.9948	0.9840	0.9859	0.9805	0.9891
$Y_{x/s}$	0.1799 ±0.0044	0.0732 ±0.003	0.07526 ±0.0022	0.0455 ±0.0017	0.1635 ±0.011	0.074 ±0.0065	0.038 ±0.007	0.0511 ±0.0008
m	0.05387 ±0.0056	–0.0848 ±0.0118	0.4089 ±0.0266	–0.100 ±0.035	0.0995 ±0.0279	0.095 ±0.0274	0.2358 ±0.0288	–0.6572 ±0.039
R^2	0.9772	0.9899	0.9738	0.9939	0.9622	0.9706	0.9733	0.9863
Q_p*	1.5733 ±0.1261	1.7418 ±0.1707	1.5677 ±0.028	2.09 ±0.087	0.9706 ±0.009	1.059 ±0.066	0.8691 ±0.036	1.7223 ±0.069

μ_{max}=maximum specific growth rate (h^{-1}), X_{max}=maximum biomass concentration (g/L), α=growth-associated coefficient for the product (g of ethanol per g of biomass), β=non-growth-associated coefficient for the product (g of ethanol per g of biomass per h), $Y_{x/s}$=biomass per sucrose yield (g of biomass per g of sucrose), m=maintenance coefficient (g of sucrose per g of biomass per h), Q_p=volumetric ethanol productivity (g of ethanol per L per h)

*not estimated by the model

similar times to those observed for the highest concentrations of ethanol produced. This reinforces the hypothesis that the studied yeasts utilize sucrose mainly for growth and ethanol production. If m has a zero or negative value, the term m can be removed from Eq. 2. On the other hand, the maintenance coefficients obtained during fermentation at pH=2.8 and 37 °C were high with respect to the other conditions. These results indicate that a significant portion of the carbon source was used for maintenance in both strains, which agrees with the fact that the lowest values of X_{max} were obtained under these fermentation conditions.

The profiles of biomass, product, and substrate by the two strains were different. The test of between-subject effects showed significant ($p>0.05$) differences between pH, temperature and strain type on the values of $Y_{x/s}$ and Q_p. The evaluation of factor interactions (pH--temperature-strain type) on X_{max}, β, $Y_{x/s}$, m, Q_p and $Y_{p/x}$ showed significant ($p<0.05$) differences. Hence, the growth rate of biomass changed and was modified as a function of pH, temperature and strain type during the fermentation of red beets (Fig. 1). The concentration of biomass decreased with an increase of temperature due to thermal stress.

The volumetric ethanol productivity (Q_p) was also determined. The Q_p values of *S. cerevisiae* ITD00196 were greater than those of *S. cerevisiae* ATCC 9763 under all fermentation conditions tested. The maximum Q_p values for *S. cerevisiae* ITD00196 and ATCC 9763 were 2.09 and 1.74 g/(L·h), respectively. In comparison, Raposo *et al.* (*24*) reported a Q_p value from 0.24 to 0.57 g/(L·h), while Çaylak and Sukan (*25*) reported a value of 1.1 g/(L·h), using sucrose in both cases. In addition, Atiyeh and Duvnjak (*26*) determined a Q_p value of 2.97 g/(L·h) using sugar beet molasses. The variability of these results can be explained by the different fermentation conditions and by the origin of the strain.

On the other hand, Araque *et al.* (*27*) reported that the strains can grow at 35–45 °C on glucose, providing ethanol yields of 50–80 %, with respect to the theoretical yield. In this work, we observed ethanol yields of 80–92 %, with respect to the theoretical yield. We also found that the theoretical ethanol yield was decreased due to by-product formation, such as acetic acid (data not shown).

Benjaphokee *et al.* (*9*) reported a strain of *S. cerevisiae* that shows multiple stress tolerance. This strain was generated by a spore-to-cell hybridization technique (without recombinant DNA technology), which utilizes glucose and produces ethanol under acid and thermal stress conditions (pH=3.5, 41 °C). In this work, we reported two strains that produce ethanol even at pH=2.8 and 37 °C, without recombinant DNA technologies to obtain ethanol overproduction. *S. cerevisiae* ATCC 9763 was able to grow under thermal and acid stress conditions; however, the fermentative capacity was low in comparison with that of *S. cerevisiae* ITD00196.

Conclusion

The two strains of *S. cerevisiae* utilized in this study were able to produce ethanol with a high yield and volumetric productivity under acid and thermal stress conditions. The equations used to model the fermentation kinetics fit very well with the experimental data and establish that the ethanol production was growth-associated under the evaluated conditions. It is important to point out that this mathematical model and the fitted values of its parameters are valid for the conditions used in this study. The yeast strain *S. cerevisiae* ITD00196 had the best fermentative capacity and could be considered an interesting option to develop bioprocesses for ethanol production.

Acknowledgements

We thank UPIDET of the Technological Institute of Durango, Mexico, for valuable help in this work. This study was supported by scholarships from Movilidad ECEST 2011–2012 and FOMIX-HGO 2008-98068. We would also like to thank Arthi Rathinasabapathy for her assistance in preparing this manuscript.

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