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Kinetic and Energetic Parameters of Carob Wastes Fermentation by *Saccharomyces cerevisiae*: Crabtree Effect, Ethanol Toxicity, and Invertase Repression

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Carob waste is a useful raw material for the second-generation ethanol because 50% of its dry weight is sucrose, glucose, and fructose. To optimize the process, we have studied the influence of the initial concentration of sugars on the fermentation performance of *Saccharomyces cerevisiae*. With initial sugar concentrations (S_0) of 20 g/l, the yeasts were derepressed and the ethanol produced during the exponential phase was consumed in a diauxic phase. The rate of ethanol consumption decreased with increasing S_0 and disappeared at 250 g/l when the Crabtree effect was complete and almost all the sugar consumed was transformed into ethanol with a yield factor of 0.42 g/g. Sucrose hydrolysis was delayed at high S_0 because of glucose repression of invertase synthesis, which was triggered at concentrations above 40 g/l. At S_0 higher than 250 g/l, even when glucose had been exhausted, sucrose was hydrolyzed very slowly, probably due to an inhibition at this low water activity. Although with lower metabolic rates and longer times of fermentation, 250 g/l is considered the optimal initial concentration because it avoids the diauxic consumption of ethanol and maintains enough invertase activity to consume all the sucrose, and also avoids the inhibitions due to lower water activities at higher S_0.

Keywords: Bioethanol, carob pod, fermentation, invertase synthesis, *Saccharomyces*, second-generation biofuels

Introduction

One of the challenges of this century is the progressive shift from fossil energy to renewable fuels. Biofuels are one of the solutions to the continuous rising of oil prices, exhaustion of fossil sources, greenhouse gas emissions reduction, and dependence on the Middle East volatile politics. The requirements of the Kyoto Protocol and Bali Action Plan encouraged the search for renewable feedstock as sources for biofuels. Fermentation processes stand out in bioethanol production since they transform simple raw materials into products with aggregated value [4]. The answer to these problems could be found in second-generation bioethanol produced by agro-industrial residues, since its use does not compete with food resources; it also allows the exploitation of raw materials with low commercial value and arranges an alternative to their disposal. A wide variety of these raw materials are used as carbon sources for bioethanol production; namely, sugarcane molasses, beet molasses, pineapple, orange, and sweet lime residues or carob industrial wastes [4, 6, 8, 12].

Carob tree (*Ceratonia siliqua* L.) grows in the Mediterranean region and southwest Asia. Approximately 50,000 tons of carob pod is produced each year in Algarve, south region of Portugal [6].

The carob pod pulp exhibits a sugar content higher than

sugar cane, and the analysis of some Turkish carob varieties showed that the most abundant sugar is sucrose with 29.9–38.4% (w/w), followed by fructose with 10.2–11.5% (w/w), and the less abundant is glucose with 3.30–3.68% (w/w) [17, 18].

The high level of sugar content combined with low prices makes the carob-based nutrient medium an advantageous alternative to carbon sources for ethanol production.

Many research groups have developed intensive studies to obtain efficient fermentative organisms, low-cost substrates, and optimal conditions for fermentation [6, 17]. The persistent search for different low-cost carbon sources brings as a consequence a large variability of complex polysaccharides and increases the need of understanding the hydrolysis processes and how the resultant sugars are metabolized and converted into ethanol.

To accomplish a high ethanol yield and increased productivity, the optimal fermentation conditions have been subjected to substantial improvements like the integration of very high-gravity (VHG) technology, by using heavily concentrated substrate. However, several problems are associated with VHG technology. One of these is the incomplete fermentation process caused by several stress conditions, in particular and most important, the osmotic effect of the high sugar concentration on the initial stage and the ethanol inhibition during the production stage. These stress conditions would result in loss of cell viability and growth, and weak fermentation performance [19].

Saccharomyces cerevisiae is a microorganism predominantly selected, since it has a good fermentative capacity as well as high tolerance to ethanol and other inhibitors [10]. At high glucose concentration of the medium, catabolite repression occurs [7] as both the expression of the specific genes involved in the tricarboxilic acid cycle, oxidative phosphorylation, glyoxylate cycle, gluconeogenesis, and the metabolism of the other sugars are repressed. Simultaneously, the expression of genes involved in alcoholic fermentation is induced and will result in the preferential consumption of glucose over the other carbon sources [11].

Molecular transport is a determining factor of cellular metabolism, mainly when the carbon source is not the preferential one, as in the fructose and sucrose case in *Saccharomyces cerevisiae*. Glucose and fructose use the same facilitated diffusion system, but glucose has a prevailing affinity, inhibiting competitively fructose transport. Invertase hydrolysis should balance the monosaccharides' supply of the medium and their yeast consumption, in a way that the medium osmolality remains at a minimum value during the fermentation [16]. It was also shown, in the same work, that regulation of the invertase activity could result in a more efficient alcoholic fermentation. The glucose in carob residue substrate, at a concentration above threshold value, represses invertase synthesis, and sucrose hydrolysis does not occur until the glucose concentration reaches values below the threshold [6].

In general, the lower affinity of hexose transporters for fructose, when comparing with glucose, explains the residual fructose prevalence at the end of fermentation. However, the role of sugar transport systems in efficient fermentation processes remains unsolved [14].

In this work, carob waste fermentations with low and high initial sugar concentrations were performed, and the kinetic and energetic parameters of cell growth, as well as the consumption rates of glucose and fructose, and sucrose hydrolysis were calculated in each of the media with different initial sugar concentrations.

The establishment of the best technological conditions to achieve the highest ethanol productivities and yields for second-generation biofuel production, using carob industrial wastes as raw material, was a major goal of the present work. In order to identify the factors that limit the fermentation efficiency, the Crabtree effect, invertase repression, and ethanol toxicity were studied in the present work, using kinetics and energetic approaches.

Materials and Methods

Microorganism

An industrial winery strain of *S. cerevisiae* F13A was used [13]. This strain has been widely used in our previous works owing to its ethanol tolerance [6].

Culture Media and Preculture Conditions

The strain was maintained on solid YEPD medium (peptone 20 g/l, yeast extract 10 g/l, glucose 20 g/l, and agar 15 g/l). Inocula were made in 250 ml shake flasks, containing 50 ml of liquid YEP medium (yeast extract 5 g/l and peptone 10 g/l) supplemented with carob extract. The cultures were incubated in an orbital shaker (NeifoPentlab, Portugal), at 150 rpm and 30°C, until they reached the late exponential growth phase. These cultures were used as inocula to get initial cell concentrations of about 1×10^7 cells/ml.

Aqueous Carob Extraction

The carob residue extract was prepared as described in Lima-Costa *et al*. [6]. The carob kibbles were dried to constant weight and ground, and the powder was suspended in distilled water at a solid/liquid ratio of 30% (w/v). This mixture was homogenized at 150 rpm, 25°C for 1 h. After this period, to clarify the carob extract, the mixtures were centrifuged at 22,000 ×*g*, at 4°C for 25 min (Beckman Instruments, EUA), and filtered through a 11 μm membrane.

Aqueous carob extract had a concentration of approximately 140 g/l total sugars. For the assays at higher sugar concentrations, the carob extract was concentrated using a rotary evaporator (Heidolph 94200, Germany) at a temperature of 70°C. The carob extract concentrate was stored at a temperature of −20°C [6].

Culture Conditions

Batch fermentations were performed at laboratory scale, in 250 ml shake flasks containing 100 ml of YEP medium, supplemented with different concentrations of carob pulp extract at an initial fresh cell concentration of 1×10^7 cells/ml. Fermentations were performed at different initial carbon concentrations of 20, 50, 100, 150, 200, 250, and 300 g/l of carob pod extract (CPE). The initial pH was 6.5 for all assays. Flasks were incubated in an orbital shaker (NeifoPentlab, Portugal) at 150 rpm, 30ºC for 96 h. Samples were collected for analysis at the beginning of the experiments and every 2 h. Absorbance at 590 nm, dry weight (DW), pH, sugar consumption, and ethanol production were measured in the broth as described in Analytical Methods. Each assay was conducted in three replicates and repeated twice.

Analytical Methods

Absorbance was measured spectrophotometrically (Cintra 202 GBC DBUV instrument, Australia) at 590 nm. Nutrient medium was used as a blank. Absorbance values at 590 nm were converted into biomass concentration (g DW/l), using a standard curve. DW was determined by centrifuging the cultures (Hettrich Zentrifugen Universal 320), as described previously in Lima-Costa *et al*. [6]. Sugars and ethanol analyses were performed by a high-performance liquid chromatography (HPLC) using samples previously centrifuged at 13,400 ×*g* for 10 min. Analyses were performed on a Beckman System Gold HPLC (Beckman, USA) equipped with a Jasco 1530 refractive index detector (Jasco, Japan). To analyze sugar concentrations, a Purospher STAR NH_2 column (Merck KGaA, Germany) was used with an isocratic elution of acetonitrile:water (75:25) at 35ºC. Ethanol determinations were performed on an OH AY column (Merck KGaA, Germany), at room temperature, with an isocratic elution of 0.002 N H_2SO_4 at 0.5 ml/min.

Determination of Kinetics and Energetics Parameters of Growth

The specific growth rates (h^{-1}) were calculated using the DMFIT modeling tool (http://modelling.combase.cc) [2]. The biomass yield $Y_{X/S}$ (gram of biomass produced per gram of sugar consumed) and ethanol yield $Y_{E/S}$ (gram of ethanol produced per gram of sugar consumed) were calculated as the slope, fitted by linear regression, of the corresponding values of biomass or ethanol produced versus total sugar consumed at the corresponding time intervals.

The rates of sucrose hydrolysis, measured as grams of sucrose per hour per gram of dry weight, were calculated as the slope, fitted by linear regression, of the values of sucrose concentration versus time, during the first 3 to 6 h of fermentation, divided by the cell dry weight corresponding to the middle time of the interval. The specific rates of ethanol production, q_{Eth}, during the exponential phase were calculated as

$$q_{EtOH} = \frac{\mu}{Y_{X/E}} \quad (1)$$

and the specific rate of production or consumption during the stationary phase were calculated as the slope of ethanol concentration versus time, divided by the constant biomass concentration at that growth phase, X_{max}:

$$q_{EtOH} = \frac{dEth/dt}{x_{max}} \quad (2)$$

Results and Discussion

Influence of the Initial Carob Sugars Concentration on Biomass Growth Kinetics

To analyze if the sugars present in carob waste triggered a Crabtree effect in *S. cerevisiae,* fermentations runs were performed at eight different initial sugar concentrations from 20 to 350 g/l. The corresponding values of biomass, ethanol production, and total sugar consumption at these different concentrations are displayed in Fig. 1. At any initial sugar concentration, except at the lowest one (20 g/l), cell growth presented biphasic kinetics with an exponential phase and a stationary phase. In contrast, at 20 g/l, diauxic growth could be observed and, instead of the stationary phase, a second growth phase consuming ethanol as carbon source could be measured, with a μ of 0.0049 h^{-1} (Table 1). Between 20 and 100 g/l, transitions between the exponential and stationary phases were very abrupt, corresponding to the exhaustion of sugar in the culture (Figs. 1A, 1B, and 1C). At the highest tested concentrations (250 to 350 g/l), the sugars were no longer the limiting factor when the stationary phase was reached, because at this point there were still sugars available in the medium (Figs. 1F, 1G, and 1H). In these cases, we submit that ethanol was the factor limiting growth. At concentrations higher than 250 g/l, the cells were not even able to consume all the sugars added and, consequently, the final biomass decreased.

In relation to specific growth rates (μ), longer exponential growth phases were found with increased sugar concentrations (Fig. 1), but with decreasing μ values (Table 1). Several physiological mechanisms underlying this decrease were identified. The initial sugar concentration affected the biomass yield factor; that is less biomass was produced per gram of sugar consumed, due probably to the osmotic stress (Table 1).

The energetic efficiency of the sugar catabolism affected

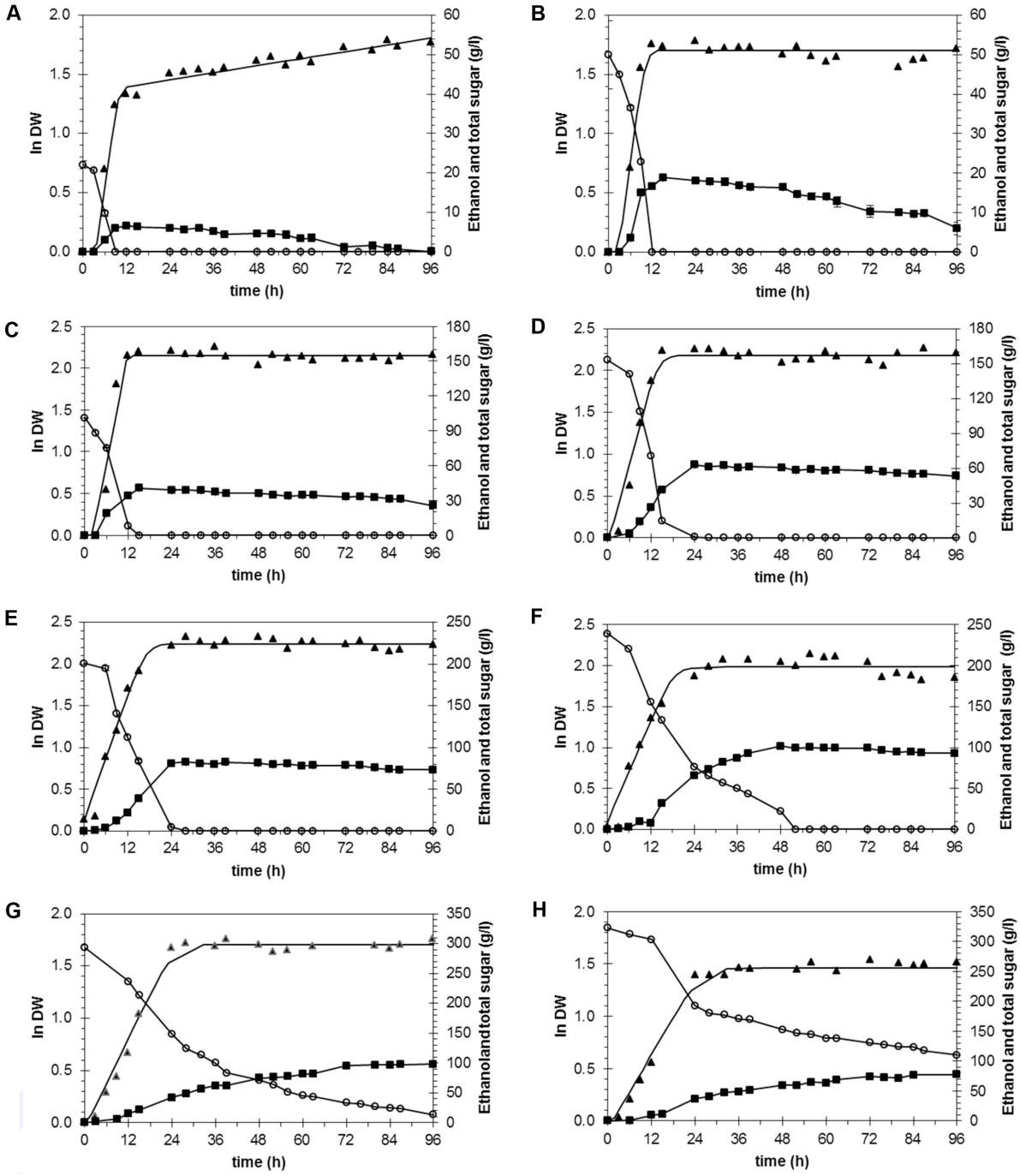

Fig. 1. Fermentation profiles of yeast *Saccharomyces cerevisiae* F13A, using aqueous carob residue extract as carbon source at different initial sugar concentrations.

(**A**) 20 g/l, (**B**) 50 g/l, (**C**) 100 g/l, (**D**) 150 g/l, (**E**) 200 g/l, (**F**) 250 g/l, (**G**) 300 g/l, and (**H**) 350 g/l. Logarithm (Ln) of dry cell weight (DW) (filled triangles) and DMFIT modeling predictions (line), ethanol production (filled squares), and total sugar consumption (empty circles). The fermentation was run for 96 h on an orbital shaker at 150 rpm and 30°C. The experiments were performed three times. Dry cell weight, sugar, and ethanol data are average of three replicates.

also the value of μ. During the first 3 h of culture, for S_0 below 150 g/l, there was sugar consumption but no ethanol was produced (Fig. 1), which indicates that oxygen was available and the catabolism was completely oxidative.

Table 1. Kinetics and stoichiometric parameters for biomass, ethanol produced, and total sugar consumption for *S. cerevisiae* F13A fermentations, with different initial sugars concentration in the aqueous carob extract.

S_0 (g/l)	Growth parameters at exponential phase						Growth parameters at stationary phase				
	t_{interv} (h)	μ (1/h)	Yx/s (g/g)	T (%)	q_{EtOH} (g/g·h)	EtOH (g/l)	t_{interv} (h)	μ (1/h)	T (%)	q_{EtOH} (g/g·h)	EtOH (g/l)
20	0–12	0.224 ± 0.022	0.131 ± 0.005	100.0	0.454	6.70 ± 0.03	12–96	0.0049 ± 0.0005	0.0	-0.016	-6.53 ± 0.80
50	0–12	0.226 ± 0.010	0.113 ± 0.017	100.0	0.816	18.84 ± 0.10	12–96	-	0.0	-0.026	-12.85 ± 1.60
100	0–12	0.207 ± 0.012	0.089 ± 0.006	92.0	1.068	40.55 ± 0.39	12–96	-	8.0	-0.014	-15.01 ± 3.28
150	0–15	0.164 ± 0.012	0.057 ± 0.003	90.5	0.812	41.56 ± 0.74	15–96	-	9.5	-0.013	-9.39 ± 1.42
200	0–15	0.125 ± 0.005	0.044 ± 0.002	58.3	0.881	81.30 ± 0.42	15–96	-	41.8	-0.010	-7.88 ± 0.54
250	0–20	0.076 ± 0.011	0.032 ± 0.002	56.2	0.670	82.56 ± 0.19	20–96	-	43.8	0.110	17.51 ± 0.19
300	0–24	0.070 ± 0.004	0.017 ± 0.001	50.5	0.685	42.10 ± 0.28	24–96	-	44.9	0.173	55.93 ± 1.84
350	0–32	0.054 ± 0.004	0.016 ± 0.001	44.9	-	47.30 ± 1.67	32–96	-	20.8	0.133	-

Culture conditions were 30°C, 150 rpm during 96 h.

Values are the mean ± SD of three replicates. S_0, Initial total sugar concentration; μ, Specific growth rate; Yx/s, biomass yield, T, % sugar consumed; q_{EtOH}, Specific ethanol production rate; EtOH, Ethanol concentration at the interval.

After that time ethanol began to be produced and the catabolism was progressively fermentative. This change in the efficiency of energy metabolism determined the decrease in the yield factor biomass/sugar from 0.13 to 0.02 g/g (Table 1). To analyze more deeply the effect of the initial sugar concentration on the yeast fermentation performance, the data on the different sugars consumption and biomass and ethanol production were analyzed quantitatively and the corresponding rates of sugars consumption and ethanol production in the different growth phases were calculated.

Kinetics of Ethanol Production and Consumption During the Growth and Stationary Phases, Depending on the Different Initial Carob Sugar Concentrations

Ethanol profiles at the different initial sugar concentrations were determined along the whole growth curve. From these values, the specific rates of ethanol production or consumption (q_E) were calculated, as described in Material and Methods, and the values are included in Table 1. In this table, a positive sign was added to q_E when there was net production of ethanol, and a negative one when there was net consumption. As mentioned before, ethanol was always produced during the growth phase, at any initial sugar concentration. From 20 to 100 g/l of initial total sugar, an increase of q_E from 0.4 to 1.0 g of ethanol per gram of biomass per hour was observed. That was the maximal production rate reached, because at higher initial sugar concentrations the rate decreased (Table 1). The increase of q_E can be explained by the Crabtree effect that occurs in these species, which consists of the repression of the synthesis of some components of the respiratory chain (cytochromes) at high external glucose concentrations, enhancing the fermentation process [7]. This Crabtree effect hypothesis was supported by the yeast behavior, because when all the glucose had been consumed, cytochrome synthesis was derepressed and ethanol was in fact oxidized. At 20 g/l of initial sugar, after glucose exhaustion, the yeasts were completely derepressed and were able to perform a diauxic growth, consuming all the ethanol that had been produced (Fig. 1A). A similar pattern could be observed at 50 up to 200 g/l of initial sugars, but with an apparent lower derepression, as measured by the specific rates of ethanol consumption that decreased from 26 mg of ethanol per gram of biomass per hour at an initial glucose concentration of 6.1 g/l (Fig. 2A) to 10 mg of ethanol per gram of biomass per hour at an initial glucose concentration of 44.8 g/l (Fig. 2C, Table 1). Apparently, this low consumption did not provide enough energy to synthesize new biomass and, although ethanol consumption could be measured, no increase in biomass could be detected (Figs. 1B, 1C, 1D, 1E, Table 1). It may be argued that once glucose was exhausted, derepression should take place completely. An alternative hypothesis to explain the differences observed with increasing S_0 values may be based on the action of the ethanol accumulated, which would inhibit both the synthesis of new biomass and the synthesis of the components of the respiratory chain, such as the derepression of the respiratory chain [6].

At concentrations higher than 200 g/l of initial sugar, none of the accumulated ethanol was consumed during the stationary phase (Fig. 1 and Table 1). On the contrary, at these high concentrations, the alcohol continued to be

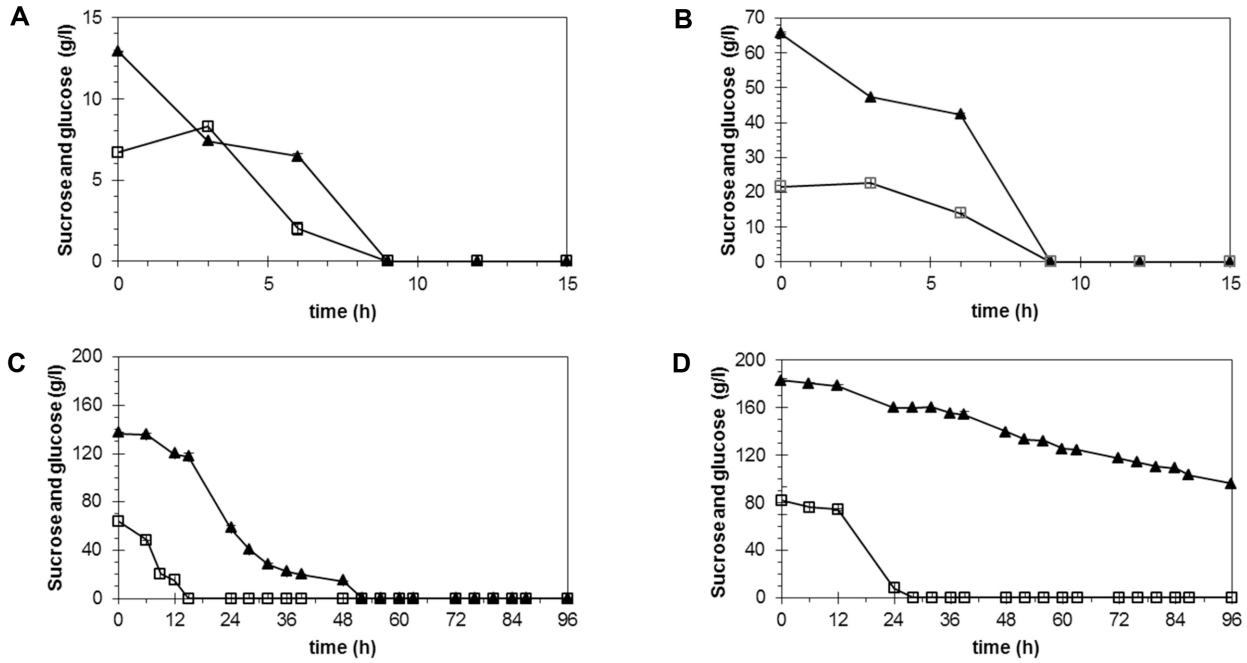

Fig. 2. Glucose consumption and sucrose hydrolysis time-course in aqueous carob extract fermentations, at different initial sugar concentrations.

(**A**) 20 g/l, (**B**) 100 g/l, (**C**) 250 g/l, and (**D**) 350 g/l. Glucose consumption (empty squares) and sucrose hydrolysis (filled triangles). The fermentation was run for 96 h on an orbital shaker at 150 rpm and 30°C. The experiments were performed three times.

produced by the metabolically uncoupled cells, unable to grow but yet able to ferment (Table 1). However, the specific ethanol production rates were much lower (0.11 to 0.13 g g^{-1} h^{-1}) than those of the exponential phase (0.67 to 0.84 g g^{-1} h^{-1}) (Table 1), indicating that the accumulated ethanol was partially inhibiting the fermentation.

From a stoichiometric point of view, it can be concluded that the initial sugar concentration showed a strong effect on the final amount of ethanol accumulated in the culture (see Table 2). Up to 200 g/l total sugar, all the ethanol was produced during the exponential phase. However, at these low sugar concentrations, after glucose exhaustion, ethanol was completely consumed when S_0 was 20 g/l and in significant amounts at 50 and 100 g/l. This behavior changed at S_0 concentrations higher than 250 g/l, at which ethanol was not consumed but produced by the uncoupled stationary cells, in an amount similar or even higher than that produced by the exponential cells (Tables 1 and 2). It

Table 2. Final concentrations of biomass, net ethanol accumulated, and ethanol/sugar yields, for *S. cerevisiae* F13A fermentations, using aqueous carob extract at different sugar concentrations.

S_0 (g/l)	X_{max} (g/l)	$EtOH_{net}$ (g/l)	T (%)	$Y_{E/S}$ (g/g)	P_E (g l^{-1} h^{-1})
20	5.96 ± 0.06	0.17 ± 0.01	100.0	0.009 ± 0.001	0.557 ± 0.003
50	5.80 ± 0.24	5.99 ± 1.60	100.0	0.120 ± 0.020	1.256 ± 0.007
100	9.54 ± 0.92	25.54 ± 3.30	100.0	0.255 ± 0.129	2.703 ± 0.026
150	9.60 ± 0.40	32.17 ± 1.60	100.0	0.215 ± 0.051	2.620 ± 0.035
200	10.23 ± 0.24	73.42 ± 0.69	100.0	0.367 ± 0.016	2.962 ± 0.001
250	8.52 ± 0.17	100.07 ± 0.27	100.0	0.419 ± 0.005	2.117 ± 0.024
300	6.30 ± 0.35	98.03 ± 1.86	95.2	0.343 ± 0.026	1.021 ± 0.019
350	4.56 ± 0.17	77.86 ± 1.67	65.8	0.338 ± 0.035	0.811 ± 0.003

Culture conditions were 30°C, 150 rpm during 96 h.

Values are the mean ± SD of three replicates. S_0, Initial total sugar concentration; X_{max}, maximum biomass dry weight; Eth_{net}, Final ethanol, balance between the production and consumption of ethanol; T, % sugar consumed; $Y_{E/S}$, ethanol yield; P_E, ethanol productivity

must be remarked upon that at these high S_0 values, the stoichiometric relation between sugar consumed and ethanol produced ($EtOH_{net}$), which included the alcohol produced by both exponential and stationary cells, showed values near to the maximal theoretical value that can be obtained if all the sugar was fermented: 0.51 g of ethanol per gram of sugar (Table 2). The highest ethanol/sugar yield, 0.42 g/g, was obtained with an initial sugar concentration of 250 g/l.

Regulation of Sugars Consumption: Repression of Invertase Synthesis and Inhibition of Its Activity

As the most abundant sugar in carob pulp is sucrose and, in *S. cerevisiae*, sucrose is not transported inside the cell but hydrolyzed extracellularly, the observed sucrose concentration decrease is a direct measurement of invertase activity [9]. Although the consumption of the three sugars present in the carob pulp (sucrose, glucose, and fructose) was measured along time, in the eight S_0 values assayed in this work, only four of them, those corresponding to 20, 100, 250, and 350 g/l, are displayed in Fig. 2.

It can be observed that the sucrose concentration decreased immediately after inoculation at the lowest initial sugar concentration (20 g/l), indicating that active invertase was present. The invertase activity was so high in this condition that the concentration of glucose in the culture increased, because its production by sucrose hydrolysis was higher that its consumption by the cells (see Fig. 2A). This immediate sucrose hydrolysis was also present at 100 g/l fermentation, although at a lower rate, as shown by the rate of sucrose disappearance and by the fact that the concentration of glucose did not increase. However, at fermentations performed at higher S_0 (see the case of 250 g/l in Fig. 2C) sucrose was not immediately hydrolyzed, and only when glucose had been consumed did sucrose hydrolysis show a high rate. In fact, at this sugar concentration, the invertase activity was enough to hydrolyze all the added sucrose (Fig. 2C). At even higher S_0 concentrations, 350 g/l (Fig. 2D), it was observed that invertase activity increased very slowly, even when the glucose concentration was very low, indicating that although derepression may have taken place, the enzyme was not being synthetized owing to the action of the accumulated ethanol (about 40 g/l) and/or its activity being inhibited by the high osmolality of the medium (water activity, a_w of 0.964) [3, 6]. This dependence of invertase activity on the water activity of the culture has been previously reported [17]. In any case, with S_0 equal to 350 g/l, the invertase activity was so low that sucrose was not completely hydrolyzed and 52% of the the initial sucrose concentration remained in the culture, even after 96 h of fermentation.

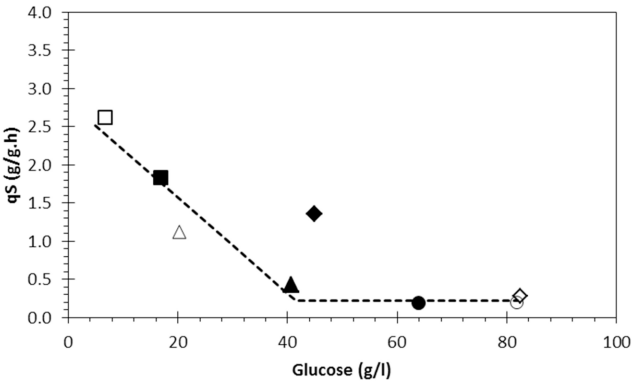

Fig. 3. Variation of the initial specific rate of sucrose hydrolysis (moles per gram of dry cell weight (DW) per hour) with glucose concentration, in the fermentation of aqueous carob extract.

Culture conditions were 30°C, 150 rpm during 96 h. Glucose concentrations were 7.5 g/l (empty squares), 17 g/l (filled squares), 22 g/l (empty triangles), 41 g/l (filled triangles), 45 g/l (filled diamond), 64 g/l (filled circles) and 82 g/l (empty diamond and circles). An arbitrary linear modeling of the data (line) was also plotted.

As mentioned, invertase activity could be quantified as the specific rate of sucrose hydrolysis, calculated as described in Material and Methods, and this rate was taken as a indirect measure of the amount of enzyme synthesized. When these rates were related with the corresponding glucose concentrations in the culture, as depicted in Fig. 3, it could be observed that whatever the initial concentration of total sugar in the medium was, invertase synthesis seemed to be repressed at glucose concentrations higher than 40 g/l (Fig. 3).

Technological Consequences of Initial Sugars Concentration on Ethanol Net Production: Crabtree Effect and Invertase Repression and Inhibition

Taking into consideration the data of Table 2, it can be concluded that from all the sugar concentrations assayed, 250 g/l is the best concentration to be used in industrial processes for ethanol production from carob waste sugars. At this sugar concentration, the diauxic behavior is not present at all and ethanol is not consumed during the stationary phase (Fig. 1F). The Crabtree effect may be at its maximum, glucose respiration is almost completely repressed, and, therefore, catabolism is completely fermentative, with an ethanol/sugar yield of 0.42 g/g near the maximum (Table 2). Working at this S_0 concentration had another advantage, as ethanol is produced by cells in both the

exponential (82%) and stationary (18%) phase. Although invertase is initially repressed, the complete glucose consumption enables its derepression. The determined water activity (a_w) of 0.964 is not low enough to strongly inhibit the hydrolysis of sucrose, as happens at higher concentrations, and sucrose can be completely consumed. At this optimal concentration of 250 g/l, the metabolic rates are slower than at lower S_0 values, due to physiological reasons that have been analyzed above, and the ethanol productivity, although not the highest, is close to the maximal obtained (Table 2). Another remarkable advantage is the high final concentration of ethanol attained, close to 100 g/l in these assayed conditions.

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