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FERMENTATION, CELL CULTURE AND BIOENGINEERING

Kinetics of sugars consumption and ethanol inhibition in carob pulp fermentation by *Saccharomyces cerevisiae* in batch and fed-batch cultures

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Abstract The waste materials from the carob processing industry are a potential resource for second-generation bioethanol production. These by-products are small carob kibbles with a high content of soluble sugars (45–50%). Batch and fed-batch *Saccharomyces cerevisiae* fermentations of high density sugar from carob pods were analyzed in terms of the kinetics of sugars consumption and ethanol inhibition. In all the batch runs, 90–95% of the total sugar was consumed and transformed into ethanol with a yield close to the theoretical maximum (0.47–0.50 g/g), and a final ethanol concentration of 100–110 g/l. In fed-batch runs, fresh carob extract was added when glucose had been consumed. This addition and the subsequent decrease of ethanol concentrations by dilution increased the final ethanol production up to 130 g/l. It seems that invertase activity and yeast tolerance to ethanol are the main factors to be controlled in carob fermentations. The efficiency of highly concentrated carob fermentation makes it a very promising process for use in a second-generation ethanol biorefinery.

Keywords Fermentation · Carob by-products · Bioethanol · Fed-batch culture · Stirred reactor

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Introduction

Research into new carbon sources among industry by-products and agricultural residues for use in bioethanol production is an essential and a sustainable strategy for the successful development of renewable biofuels. Second-generation bioethanol processes that use agro-industrial wastes, have, besides their low cost, the additional advantage of the absence of ethical concerns about the use of potential food resources. In this way, they can compete with products derived from fossil resources in terms of economical and energetic sustainability, resource availability, supply reliability, and environmental friendliness.

The carob tree, *Ceratonia siliqua* L., is a perennial leguminous tree that grows mostly in soft and dry places, in poor and calcareous soils of Mediterranean countries [3]. Usually carob pods are extensively used as a raw material for syrups production, or as a cocoa substitute in the food industry, or even directly in the diet of farm animals [18]. According to recent data, carob pod production worldwide amounts to nearly 400,000 tons/year, of which 50,000 tons is produced each year in the Algarve, south Portugal. Its price has fluctuated and is about 200 €/ton. The carob seeds represent 10% of the weight of the fruit and the pulp represents the other 90% [10]. Several authors have reported that carob pulp composition has a high content of sugars, mainly sucrose (more than 30%), fructose, and glucose with values of 20–50% [15], 45% [2], 48–56% [22], and 28–82% [4]. This gave economical relevance to carob pulp as an inexpensive and available feedstock for various biological products, e.g., dextran and fructose [18], citric acid [17], and ethanol [16]. The by-products from the carob processing industry are small carob kibbles, which contain a high content of sugar (mainly sucrose, glucose, and fructose) which are easily extractable by water, producing sugar-rich syrups [10].

One of the methods that enhances ethanol productivity is the very high gravity technology (VHG), which involves preparation and fermentation of syrups containing a high sugar concentration (>250 g/l), to achieve a high ethanol yield [9, 23]. This process has several advantages for industrial applications such as the increase in both ethanol concentration and fermentation rate, which reduces capital costs, energy costs per liter of alcohol, and the risk of bacterial contamination [9]. However, VHG technology is particularly susceptible to incomplete fermentation, which has been attributed to osmotic and ethanol stresses and would result in the loss of cell viability, growth, and weak fermentation performance of yeasts [23]. On the other hand, it has been claimed that aeration may improve the fermentation performance of yeasts [8].

The purpose of this study was to analyze in detail the performance of an autochthonous strain *S. cerevisiae* (F13A), producing ethanol from highly concentrated carob pulp extracts, in an aerated stirred tank reactor (STR) and to identify the factors that limit the efficiency of this fermentation process. As ethanol toxicity [1, 21] and incomplete fermentation had already been identified as relevant factors affecting productivity, the kinetics of sugars consumption and ethanol toxic effects were specifically addressed. Regulation and control of sugar consumption in *S. cerevisae* is very well known at molecular level [24]. To summarize the abundant knowledge in basic terms relevant for the present research, it can be noted that glucose and fructose use the same facilitated diffusion system. Glucose has greater affinity than fructose for that system, competitively inhibiting fructose transport. On the other hand, glucose also represses invertase synthesis. Thus, when glucose is present at a concentration above the threshold value, invertase is not synthesized and, consequently, sucrose is not hydrolyzed until glucose concentration is consumed to levels below the threshold. Other alternative systems have been described at biochemical and molecular levels. For example, a proton symport, specific for fructose [6, 7] and also a sucrose permease [19] that could transport sucrose to cytoplasm where it would be hydrolyzed by the intracellular invertase were described. In the current work we have assumed that these alternative mechanisms are not relevant from a quantitative point of view, and that the industrial process can be accurately described taking into account only the major components: the facilitated diffusion for monosaccharide transport and the secreted invertase for sucrose hydrolysis.

Besides, it was claimed [11] that fed-batch fermentations could have several potential advantages compared with batch fermentation, because they could extend the exponential growth phase and minimize the potential repressive effects of carob medium components. So, this study used batch and fed-batch cultures in aerated STRs. To the best of our knowledge, this is the report on sugar consumption kinetics and ethanol inhibition in high density carob pulp fermentations, performed in batch and fed-batch cultures, using an autochthonous yeast strain for further application at a second-generation biorefinery.

Materials and methods

Saccharomyces cerevisiae growth and pre-inoculum

Three different strains of *S. cerevisiae* were used: F13A, isolated from a local environment, as the target strain; and two reference strains, namely ISA1000, an industrial winery strain, and PYCC 3507, a collection strain widely used in previous works on ethanol tolerance. These strains were maintained on solid YEPD medium (peptone 20 g/l, yeast extract 10 g/l, glucose 20 g/l, agar 15 g/l) and spread on sterile Petri dishes. Pre-inoculums were prepared by growing 4-day cultures on solid YEPD medium. Individual yeast colonies were transferred to 250-ml shake flasks, containing 50 ml of liquid YEP medium (yeast extract 3 g/l, peptone 5 g/l) with carob pod extract containing a total sugar concentration of 200 g/l. They were incubated in an orbital shaker (NeifoPentlab, Portugal), at 150 rpm and 30°C, until cultures reached late exponential phase. These fresh cells were used as inoculums in shake flasks, batch and fed-batch assays.

Aqueous carob pulp extraction

The carob kibbles were dried to constant weight, ground, and the powder was suspended in distilled water at solid/liquid ratio 3:10 (w/v). Up to four leaching tests were conducted, with half initial ratio, to take full advantage of all soluble sugars in the carob pulp. These mixtures were homogenized at 150 rpm, 25°C for 1 h as suggested by Manso et al. [10, 11]. After this period, to clarify the carob pulp extract, the mixtures were centrifuged at 7,500*g*, at 4°C for 15 min (Beckman J2-MC Centrifuge with a JA14 rotor), filtered through a 0.22-μm membrane filter, and stored at −20°C. Each extraction tested was repeated twice with three replicates per experiment.

Fermentation conditions

Batch experiments in shake flasks

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 cells concentration of 1×10^6 cfu/ml. Fermentations were performed at high

initial hydrocarbon density (VHG), with sugar concentrations of 241, 248, and 249 g/l of carob pod extract (CPE), for *S. cerevisiae* PYCC 3507, F13A, and ISA1000, respectively. The initial pH was 6.5 for all assays. Flasks were incubated in an orbital shaker (NeifoPentlab, Portugal) at 150 rpm, 30°C for approximately 72 h. At the beginning of the experiments and every 2 h, samples were collected for analysis. Optical density, 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.

Batch fermentation in an aerated STR

The strain F13A batch fermentation was performed in a 3-l STR (2.4 l working volume, ADI 1010/1025, Apllikon, Holland) operated at 30°C, 250 rpm and aerated at 0.3 l/min (0.13 vvm). It was inoculated to reach an initial cell density of about 1×10^7 cfu/ml. The growth medium was YEP (yeast extract 3 g/l, peptone 5 g/l), with carob extract and antifoam B (Sigma A-5757) at 0.1 ml/l. The initial pH was 6.5. A Rushton-type turbine and an L-shaped sparger were employed, for aeration. The fermentation was monitored online using the BioXpert program, version 2.1. The temperature, pH, and dissolved oxygen were measured with specific probes and the values were constantly registered. Samples were taken immediately after inoculation and at regular intervals to determine the optical density (OD) at 600 nm, dry weight (DW) biomass, and sugar and ethanol concentrations, as described in "Analytical methods". Three independent experiments were performed and the observed differences in the parameters values were not significant.

Fed-batch experiments

The fed-batch fermentation was performed in a 3-l STR (ADI 1010/1025, Apllikon, Holland) containing 1,600 ml YEP (yeast extract 3 g/l, peptone 5 g/l), carob extract, and antifoam B (Sigma A-5757) at 0.1 ml/l, with an initial pH value of 6.5. The F13A strain was used at an initial cell concentration of about 1×10^7cfu/ml. A Rushton-type turbine and an L-shaped sparger were employed. The fermentation was operated at 30°C, 250 rpm, with an aeration of 0.13 vvm. Initial total sugar concentration was 249 g/l. After about 20 h, when glucose had been consumed completely, a volume of 750 ml of YEP medium with carob pulp extract at 290 g/l (total sugar) was added. Thirty hours later, glucose was exhausted again and another 750 ml of fresh carob pod medium was added. The fermentation was also monitored online and samples were collected as described for batch fermentation experiments.

Analytical methods

The OD was measured using a Cintra 202 spectrophotometer (GBC DBUV instrument, software GBC Spectral) at 600 nm. Nutrient medium was used as a blank. OD_{600nm} values were converted into biomass concentration (g DW l^{-1}) by using a standard curve. DW was determined by centrifuging (Hettrich Zentrifugen Universal 320) 7 ml of culture in pre-weighed Falcon tubes at 5,000 rpm for 5 min. The supernatant was removed and the sediment was dried and weighed on a precision balance (XB 12A Precisa), until constant weight.

Sugars and ethanol analyses were performed by 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 NH2 column (Merck KGaA, Germany) was used with an isocratic elution of acetonitrile/water (75:25) at 35°C. 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 fermentation yields and productivities

The specific growth rates (h^{-1}) were calculated using the DMFIT modeling tool (http://modelling.combase.cc). The biomass yield $Y_{X/S}$ (grams of biomass produced per gram of sugar consumed) and ethanol yield $Y_{E/S}$ (grams 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 as a function of time.

Specific glucose and fructose consumption rates were calculated from their concentrations over time, where G_T is the concentration of glucose, G_0 the initial concentration in carob pod, G_H glucose from sucrose hydrolysis, and G_C the glucose consumed by cells. Thus the total mass balance of glucose in the broth is

$$G_T = G_0 + G_H - G_C \quad (1)$$

The evolution with time can be obtained by derivatization as follows, G_0 being constant:

$$dG_T/dt = dG_H/dt - dG_C/dt \quad (2)$$

When rates are expressed in molar terms, the rate of glucose production from sucrose is equal to its rate of hydrolysis, $-dS/dt$, because each mole of sucrose produces 1 mole of glucose and another of fructose. The rate of consumption by cells is equal to the specific rate of glucose consumption, q_G, expressed as moles of glucose consumed

per gram of biomass per hour, multiplied by the present biomass X (g/l). Thus, substituting these parameters in Eq. 2, q_G can be calculated as

$$q_G = (dG_H/dt - dS/dt)/X \quad (3)$$

dG_T/dt and dS/dt were estimated as the slope of the plots of glucose and sucrose concentrations over time at successive time intervals, by linear regression, and X as the biomass concentration corresponding to the middle time of the interval.

Statistical analysis

Kinetic biomass and ethanol parameters determined for *S. cerevisiae* PYCC 3507, ISA1000, and F13A strains were subjected to statistical analysis of variance followed by Student–Newman–Keuls test ($P < 0.05$) with SigmaPlot software (version 12 for Windows).

Results and discussion

Ethanol fermentation of carob pulp extract in shake flasks

Fermentations using the three different *S. cerevisiae* strains, ISA1000, PYCC 3507, and F13A, were performed in shake flasks, using carob pulp extract at high initial sugar concentrations (235–255 g/l). The purpose of this work was to evaluate the fermentation performance and ethanol tolerance of the new isolate strain F13A versus the reference strains ISA1000 and PYCC 3507. They were also useful to prove that the main features of carob pod fermentation by *S. cerevisiae* were common to several strains

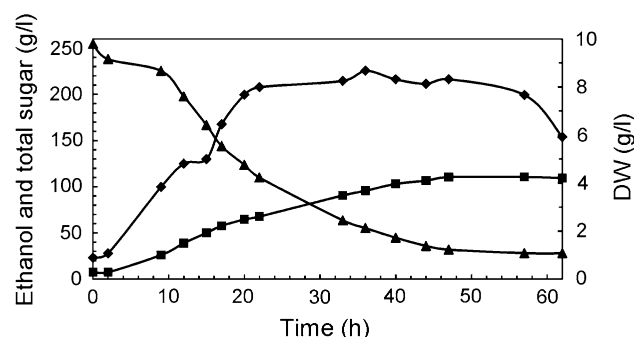

Fig. 1 Time course of ethanol (*filled squares*), biomass (*filled diamonds*) production, and total sugar consumption (*filled triangles*) in batch cultivation performed in a 3-l STR, equipped with a Rushton turbine and an L-shaped sparger, for *S. cerevisiae* F13A, fermentation on CPE (255 g/l as initial sugar concentration). Culture was carried out at 30°C, 250 rpm, and 0.13 vvm. The experiments were performed three times. Dry cell weight, sugar and ethanol data are average of three replicates

of the species and not a strain-specific particularity. Growth, sugar consumption, and ethanol fermentation displayed a similar time course for the three strains characterized by (1) an initial exponential growth that reached the stationary phase when only 2/3 of sugars had been consumed, (2) 90–95% of the ethanol was produced during growth phase and 5–10% during the stationary phase, and (3) 5% of sugar remained not consumed even after 60 h of culture. Typical growth profiles were obtained for all strains and the corresponding values of kinetics and energetics parameters are shown in Table 1. The specific growth rates (μ) did not show statistically significant differences among strains and the percentage of total sugar consumption is similar for ISA1000 and F13A strains. The latter culture exhibits longer t_{stat}, the time necessary to achieve the stationary phase. Table 1 also shows the ethanol parameters. Maximum ethanol accumulated (E_{max}) and the ethanol productivities (P_E), i.e., E_{max} divided by the time needed to reach that maximum, were also statistically similar for ISA1000 and F13A strains. All the strains evidenced the uncoupling between the biomass growth and the ethanol production at the stationary phase, and the capacity of the non-growing cells to keep a slow ethanol production. The maximum ethanol production around 106 g/l was attained at 55 h on stationary phases, for F13A and ISA100 strains (Table 1). The biomass yields were low (0.067 and 0.064 g/g, respectively) for PYCC 3507 and ISA1000 and a bit lower (0.055 g/g) for strain F13A. This is an advantage in processes in which catabolites and not biomass are the industrial objective. Moreover, the high yield of ethanol/sugar obtained for all tested strains was close to the theoretical maximum (0.51 g/g), which implied that almost all consumed sugar was fermented and not used for biomass synthesis. This high experimental ethanol yield suggested that good conditions for carob pod sugar stoichiometric conversion into ethanol had been established and further improvements had to be made to metabolic rates and productivity.

As the most relevant ethanol-related parameters of tested strains were statistically not different and because of its lower biomass productivity and autochthonous nature, F13A was chosen for further studies in the bioreactor.

Batch fermentation of carob pulp extract in STR

Batch fermentations using *S. cerevisiae* F13A were performed in a 3-l aerated STR, equipped with a Rushton turbine and an L-shaped sparger, at 0.13 vvm. Carob pulp extract with a high sugar concentration (255 g/l) was used. The kinetics of biomass and ethanol production and sugars consumption were characterized in order to understand the physiology of the fermentation process.

Table 1 Kinetic biomass and ethanol parameters determined for *S. cerevisiae* PYCC 3507, ISA1000, and F13A strains in VHG fermentations at initial carbon source concentrations of CPE of 241, 249, and 248 g/l, respectively, in shake flasks at 30°C, 150 rpm and in batch fermentation in a 3-l STR, with 255 g/l of CPE

Strains/production mode	μ (h^{-1})	t_{stat} (h)	$T_{sug\ C}$ (%)	X_{max} (g/l)	$Y_{X/S}$ (g/g)	E_{stat} (g/l)	E_{max} (g/l)	$Y_{E/S}$ (g/g)	P_E ($g\ l^{-1}\ h^{-1}$)
PYCC 3507/flask	$0.168 \pm 0.014^{a,b}$	25.8 ± 0.79^{a}	89.9^{a}	12.9^{a}	0.067 ± 0.013^{a}	95.60^{a}	103.2 ± 4.1^{a}	0.48 ± 0.013^{a}	2.11 ± 0.15^{a}
ISA1000/flask	$0.163 \pm 0.017^{a,b}$	26.0 ± 1.05^{a}	95.3^{b}	13.2^{b}	0.064 ± 0.022^{a}	100.5^{b}	105.7 ± 8.2^{a}	0.44 ± 0.018^{b}	1.94 ± 0.45^{b}
F13A/flask	0.100 ± 0.133^{a}	28.2 ± 1.50^{b}	95.4^{b}	11.5^{c}	0.055 ± 0.014^{b}	103.0^{c}	106.5 ± 8.2^{a}	0.45 ± 0.004^{b}	1.94 ± 0.45^{b}
F13A/batch–STR	0.133 ± 0.010^{a}	23.0 ± 1.11^{c}	89.0^{a}	8.08^{d}	0.052 ± 0.004^{b}	71.20^{d}	110.6 ± 0.65^{b}	0.45 ± 0.006^{c}	2.04 ± 0.15^{a}
F13A/FB1–STR	0.226 ± 0.062^{b}	15.07 ± 0.97^{d}	47.6^{c}	6.08^{e}	0.048 ± 0.010^{b}	50.50^{e}	67.0 ± 1.26^{c}	0.48 ± 0.017^{a}	3.64 ± 0.14^{c}
F13A/FB2–STR	0.079 ± 0.274^{c}	8.57 ± 0.97^{e}	52.0^{d}	7.05^{f}	0.029 ± 0.003^{c}	69.70^{f}	99.6 ± 2.04^{d}	0.47 ± 0.011^{d}	0.65 ± 0.06^{d}
F13A/FB3–STR	0.011 ± 0.081^{c}	20.98 ± 4.67^{f}	61.8^{e}	5.75^{g}	0.007 ± 0.005^{d}	96.30^{g}	126.7 ± 0.86^{e}	0.50 ± 0.005^{e}	0.69 ± 0.07^{d}

Values are mean ± SD of three replicates. Fed-batch fermentation of *S. cerevisiae* F13A, with 249 g/l of initial CPE sugar, was divided in three phases: FB1, 0–19 h; FB2, 20–49 h; and FB3, 50–160 h. Batch and fed-batch fermentations were performed in a 3-l aerated STR, equipped with a Rushton turbine and an L-shaped sparger at 30°C, 250 rpm and 0.13 vvm

μ specific growth rate, t_{stat} time to attain stationary phase, $Y_{x/s}$ biomass yield, $T_{sug\ C}$ % sugar consumed, X_{max} maximum DW, E_{stat} ethanol concentration when the stationary phase was reached, E_{max} maximum ethanol, $Y_{E/S}$ ethanol yield, P_E ethanol productivity, *FB* fed-batch

Within a column, values followed by the same superscript letter are not statistically different according to Student–Newman–Keuls ($P < 0.05$)

Figure 1 displays biomass formation (DW), ethanol production, and total sugar consumption during the fermentation of carob pulp extract. The kinetics growth parameters of STR fermentation showed no statistically differences (Table 1) and the general growth pattern previously observed in Erlenmeyer experiments was maintained; that is, the stationary phase begun when only 60% of the initial sugar had been consumed, ethanol continued to be produced during the stationary phase, and about 5–10% (p/v) of the sugar remained at the end of fermentation, apparently not being able to be metabolized in those conditions. However, the maximum ethanol production and the stationary ethanol were higher in the bioreactor than in Erlenmeyer cultures (Table 1).

To better analyze the growth process using a very complex carbon source, carob extract, the specific growth rate was determined using DMFIT modeling (http://modelling.combase.cc). Thus, it was possible to define three standardized phases in the growth curve: an exponential growth phase with a constant μ of 0.133 h^{-1}, during the first 14 h; an abnormally long decline phase characterized by growth with a decreasing value of μ, proceeded for the next 8 h; and finally the stationary growth phase ($\mu = 0$), from 23 h to the end of the experiment (60 h). The slow decrease in growth rate could be due to a decrease in the growth yield ($Y_{X/S}$), a decrease in the specific consumption rate of sugar, or both.

This decreasing yield could be attributed to a change in the fermentation/oxidation catabolism balance, because oxygen consumption (oxygen could not be detected after a few hours of culture) and ethanol production had been detected during the experiment.

However it was observed that biomass production and total sugar consumption were tightly coupled, showing a low and constant yield of 0.052 ± 0.004 g of biomass produced per gram of consumed sugar during both growth phases (Table 1). Either with constant or decreasing μ values it was also shown that the ethanol yield (grams of ethanol per gram of sugar consumed) was almost constant and close to the theoretical maximum value of 0.51 g/g. Moreover, during growth phases a lower value of 0.47 g/g (ethanol/sugar) could be calculated, in contrast with a higher value of 0.50 g/g in the stationary phase. As mentioned before, this almost complete conversion of the consumed sugar into fermentation products, which is maintained up to the end of the experiment, is very promising for the use of carob pulp as a raw material for bioethanol production.

Kinetics of sugars consumption

If the decrease in the growth rate could not be explained by a decrease in the biomass yield, it must be due to changes

in the specific rate of sugar consumption. To test this hypothesis, the time course of the different sugars present in the carob extract was measured. In the aqueous carob extract, sucrose was present at high concentration (156 g/l). Initial glucose and fructose concentrations were 38 and 54 g/l, respectively. During the first phase of exponential growth (16 h), only the two aforementioned monosaccharides were consumed (Fig. 2). It was also patent that glucose was the first one to be consumed. During the second growth phase, characterized by a decreasing μ, sucrose was hydrolyzed and the other two sugars maintained at almost constant low concentrations, implying that the monosaccharides produced by sucrose hydrolysis were readily consumed. The third growth phase, stationary phase with μ equal to zero, was similar, in terms of sugar evolution, to the second phase: sucrose hydrolysis, low constant values of glucose, and fructose and sucrose depletion.

The kinetics of sugars consumption was analyzed quantitatively and specific consumption rates (q) for each sugar were calculated from the data depicted in Fig. 2, as described in Eq. 3. The evolution of q values over time are shown in Fig. 2. As described above, it is assumed that in *S. cerevisiae* cultures sucrose is hydrolyzed by an invertase that is repressed by glucose [12] and its intracellular transport, if present, is not relevant. The only sugars entering into the cells are the monosaccharides, competing for the same permease, with glucose showing much higher affinity. This behavior is also supported by the analysis of the sugar consumption rates (Fig. 2).

At the beginning of fermentation, when glucose is present in high concentration (more than 10 g/l in this case), invertase synthesis is repressed, no sucrose is hydrolyzed, and fructose transport is also inhibited. For these reasons, during the first growth phase, only the monosaccharides are consumed, firstly glucose at the highest rate, and then simultaneously with fructose with an

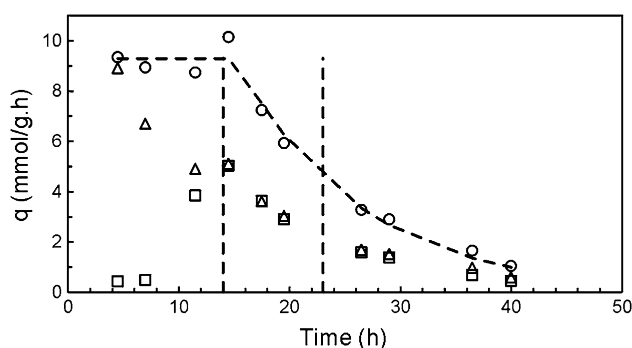

Fig. 2 Total sugar (*circles*), glucose (*triangles*), and fructose (*squares*) specific consumption rates of *S. cerevisiae* F13A in batch cultivation performed in a 3-l aerated STR, equipped with a Rushton turbine and an L-shaped sparger, at aeration rate of 0.13 vvm, using 255 g/l CPE as initial carbon source. Culture conditions were 30°C, 250 rpm, and 0.13 vvm

increased rate for fructose as the glucose concentration is decreasing. So, the hexose permease is working during all this phase at a constant and maximum rate, changing only the proportion of glucose/fructose transported. As glucose concentration decreases with time, its consumption rate decreases, but the fructose consumption rate increases until both sugars are transported at about the same rate at the end of this phase (Fig. 2). Then it is clear that the initial glucose concentration is responsible for the fast cell growth during the exponential phase, highlighting the controlling role of this sugar during the first phase of carob extract fermentation. When the glucose concentration decreases below the threshold value, invertase synthesis is derepressed. Cell growth proceeds with the consumption of the hydrolysis products, glucose and fructose, as soon as they are available, and for this reason their concentrations in the medium are maintained very low. Their concentrations increase a little at 20–30 h, which implies that the sugar hydrolysis rate is higher than the monosaccharide transport rates.

In the second growth phase (14–23 h), in contrast with the first phase in which growth is unrestricted (constant q, constant μ), growth is limited by the availability of monosaccharides provided by hydrolysis. The rate of sucrose hydrolysis limits the growth rate and, as the hydrolysis decreases, the growth rate decreases. At the stationary phase biomass growth stops, but sucrose hydrolysis, monosaccharide transport, and ethanol production continue at a low rate, highlighting the uncoupling between biomass production and fermentation metabolism which happens at this stationary phase.

Kinetics of ethanol production and its inhibitory effects on sugar consumption and growth

As mentioned before, ethanol is produced during the entire fermentation, including the stationary phase. It must be also noted that 90–95% of all consumed sugar is transformed into fermentation products (ethanol and CO_2), because the ethanol/glucose yield is very high (0.45 g/g).

When the stationary growth phase starts (Fig. 1; Table 1), a significant amount of sugar concentration is present in the broth (about 109 g/l), meaning that neither the carbon source nor the pH (whose values varied between 5.08 at the fermentation onset and 4.87 at the end of stationary phase) was the limiting factor. A clear reduction in yeast growth rate when ethanol is present at concentrations between 4 and 10% (w/v) has been previously reported [5, 8, 13, 14]. In this case, the accumulation of ethanol in the broth at the end of the exponential phase (E_{stat}) attained concentrations in the broth of about 7% (w/v) (Table 1). This suggested that ethanol present in the broth was the etiological factor of the inhibition of sugar consumption

and the consequent inhibition of growth. However it was also clear that both processes were not affected in the same way, due to the uncoupling observed at the stationary phase.

The ethanol inhibition was analyzed quantitatively in terms of the variation of relative specific rate of total sugar consumption (qS/qS_{max}) compared with relative specific growth rate (μ/μ_{max}) during the time course of fermentation (Fig. 3). The values for the instantaneous μ were calculated as the derivative of the growth curve, as described in "Materials and methods". The values of total sugar consumption rates were plotted against the corresponding values of the ethanol concentration (Fig. 3) and significant differences between the inhibition kinetics were observed. Ethanol accumulation below 50 g/l did not inhibit monosaccharide transport and consumption. Above this concentration, the relative rate decreased exponentially with the accumulation of ethanol. The inhibition showed by the relative growth rate was more complex because, apparently, inhibition began at lower concentrations of ethanol. However we have already analyzed this early inhibition and have showed that can be explained by the transition between the unrestricted growth on free and abundant monosaccharides and the growth limited by sucrose hydrolysis during the decline phase (Fig. 3). At concentrations above 40 g/l an exponential inhibition could be observed and the relative growth rate drastically declined until complete inhibition. In contrast, the sugar consumption at ethanol concentrations of 80–90 g/l, at which growth was completely inhibited, did maintain a rate equivalent to 25% of the maximum. It is generally admitted that sugar consumption and fermentation are more tolerant to ethanol inhibition than biomass biosynthesis and this could easily be the case in these experiments. However, an alternative explanation could be that the inhibition of invertase activity is stronger than inhibition of monosaccharide transport.

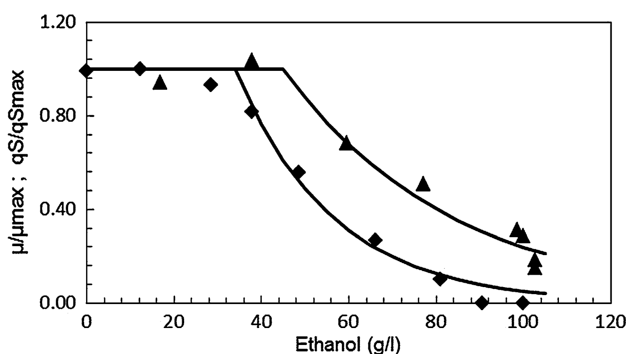

Fig. 3 Correlation of relative specific growth rate (*filled diamonds*) and relative specific substrate consumption (*filled triangles*) with accumulated ethanol concentration in batch cultivation performed in a 3-l aerated STR, equipped with a Rushton turbine and an L-shaped sparger, for *S. cerevisiae* F13A fermentation on CPE (255 g/l as initial sugar concentration). Culture conditions were 30°C, 250 rpm, and 0.13 vvm

To summarize, two different processes seem to affect the efficiency of ethanol production from CPE. First, the rate of sucrose hydrolysis, once free monosaccharides have reached a low concentration after being consumed during exponential growth phase. A similar enzymatic effect was reported by Takeshige and Ouchi [20] with molasses as substrate: a weak invertase activity in the broth gave an insufficient supply of hydrolysates for fermentation that was corrected by adding external invertase. The second factor is the inhibition by the accumulated ethanol in the broth, particularly at ethanol concentrations higher than 40 g/l.

It was thought that a fed-batch culture could alleviate this ethanol fermentation. Addition of fresh medium with CPE, once glucose had been exhausted, should allow more free glucose to be fermented without invertase activity and also the subsequent dilution of ethanol by the addition of new medium would decrease the ethanol concentration in the medium.

Fed-batch fermentation of carob pulp extract in STR

Fed-batch fermentation was performed (Fig. 4) with *S. cerevisiae* F13A under the same operational STR conditions as described for the batch system. Sugar content was monitored in the broth, and carob extract containing 295 g/l of total sugar plus peptone (5 g/l) and yeast extract (3 g/l) was added periodically at 20 and 50 h, times at which glucose had been exhausted. Biomass formation (DW),

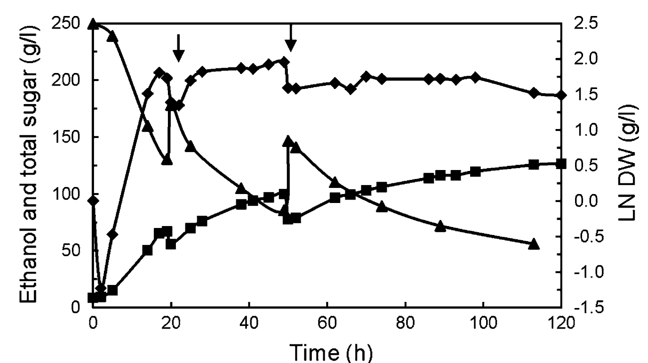

Fig. 4 Time course of ethanol production (*filled squares*), total sugar consumption (*filled triangles*), and DW (*filled diamonds*) of fed-batch fermentation of *S. cerevisiae* in a 3-l aerated STR, equipped with a Rushton turbine and L-shaped sparger, using 249 g/l CPE as initial concentration. Carob extract (290 g/l) was added at 20 and 50 h. Cultures were cultivated at 30°C at 250 rpm, at an aeration rate of 0.13 vvm. The experiments were performed twice. Dry cell weight, sugar and ethanol data are average of three replicates. *Arrows* represent CPE addition

ethanol production, and total sugar consumption were also measured during this fed-batch fermentation.

Figure 4 displays the fed-batch (FB) biomass growth, sugar consumption, and ethanol production profiles obtained. DW evolution was modeled using DMFIT software. During the time course of fermentation three growth stages were established: the initial growth, 0–19 h (FB1); growth after the first addition of fresh medium, 20–49 h (FB2); and after the second addition, 50–120 h (FB3). Biomass and ethanol kinetic parameters were determined for each FB stage (Table 1).

The results obtained were those that could be predicted from the performance of the strain in batch fermentations, in the same bioreactor. Growth and sugar consumption rates decreased as the ethanol accumulated increased in successive stages, as predicted by the curve in Fig. 4. At the first stage FB1, the specific growth rate was 0.226 h^{-1}, which decreased to 0.079 h^{-1} in the second stage and to an almost an undetectable 0.011 h^{-1} at the third stage, with an ethanol concentration above 120 g/l. The ethanol inhibition also affected the lag phase that lasted 2 h after the first addition and 9.8 h after the second one.

The DW produced attained the expected value of 5.7 g/l which corresponds to a biomass yield factor of 0.048 g/g, previously obtained in the batch experiment. However, at the second and third stages much lower amounts of biomass were produced (2.7 and 0.8 g/l) corresponding to decreasing yields values of 0.029 and 0.007 g/g (Table 1; Fig. 4). Albeit with low and decreasing rates, it must be remarked that the glucose present in the carob extract was completely metabolized after each addition and ethanol was continuously produced during the three stages, with yields close to the maximum (Table 1), reaching a final concentration of 130 g/l, i.e., 18% more than the value obtained in batch experiments. The continuous presence of glucose, caused by the addition of fresh medium when glucose was exhausted, inhibited fructose consumption and partially repressed invertase synthesis. For these reasons fructose and sucrose concentration were much less consumed and the final medium was strongly enriched in these two sugars. If the initial medium had 250 g/l of total sugar comprising 53% sucrose, 26% fructose, and 22% glucose, the 45.5 g/l sugar content of the final medium inverted these proportions because it was composed of 70% fructose, 30% sucrose, and no glucose at all. Thus, in relation to sugars consumption, the FB did not improve the batch results, because the proportion of residual sugar was slightly higher (13% in FB vs. 5–10% in batch cultures). This can be explained because the addition of fresh media provides not only glucose, but also fructose and sucrose that are not readily consumed either when glucose is present, because of the reasons explained above, or when glucose is exhausted, because then the accumulated ethanol is so high that it strongly inhibits any consumption. Summarizing the results with respect to ethanol production, it must be noted that FB culture improved the batch culture results. At the end of the first growth stage (19 h), before the first nutrient addition, about 65 g/l of ethanol was produced. Production increased up to 100 g/l at 49 h, after the first addition of fresh carob pod and the cells were able to produce more ethanol even during the third stage, albeit at a very slow rate. In this way it was possible to obtain the highest ethanol accumulation, 131.8 g/l, at the third growth stage with the best ethanol yield, 0.50 g/g (Table 1). This value, which is close to the theoretical maximum (0.51 g/g), indicated that all the consumed sugar in the broth was fermented and almost no biomass was formed ($Y_{X/S} = 0.007$) as shown in Fig. 4 and Table 1.

Conclusions

Carob pod aqueous extract is a source of second-generation bioethanol with great potential. It can be fermented at high sugar concentration (250 g/l) with an excellent ethanol/glucose conversion yield (0.47–0.50 g/g) when supplemented with small amounts of C and N sources. The main factors hindering ethanol productivity that were identified in this work are sucrose hydrolysis rate and *S. cerevisiae* ethanol tolerance. It has also been proved in this work that FB culture is a good approach to decrease, at least partially, the ethanol inhibitory effects. External invertase can be added to overcome the limitation of sucrose hydrolysis. Further improvements to increase productivity and decrease costs can be obtained by optimizing (1) the ratio carob pod/additional C and N sources in the fermentation medium; (2) the activity of endogenous and external invertase and, eventually, hexose isomerase, to achieve a complete fermentation of sucrose and fructose; (3) the sequence of fresh medium addition in FB cultures to overcome ethanol toxicity.

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