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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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Brief running title: Carob wastes fermentation by *Saccharomyces cerevisiae*.
Abstract

Carob wastes are useful raw material for 2nd 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, that 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, it 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, 2nd 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 of 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, 12, 8, 6].

Carob tree (*Ceratonia siliqua* L.) grows in the Mediterranean region and southwest Asia. Approximately 50,000 tons of carob pod are 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 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 in 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 to 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, 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 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 the 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 to 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, 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 2nd 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, 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 *Saccharomyces cerevisiae* F13A was used [13]. This strain has been widely used in our previous works due 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, agar 15 g/l). Inocula were made in 250 ml shake flasks, containing 50 ml of liquid YEP medium (yeast extract 5 g/l, 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 it reach late exponential growth phase. These cultures were used as inocula to get a initial cell concentrations of about $1 \times 10^7$ 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, ground, and the powder was suspended in distilled water at solid/liquid ratio 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 22000 g, at 4 °C for 25 min (Beckman Instruments, E.U.A), 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 is stored at a temperature of -20 °C [7].

**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 cells concentration of $1 \times 10^7$ 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, 30 °C for 96 h. Samples were collected for analysis at the beginning of the experiments and every 2 hours. 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 590nm 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 high performance liquid chromatography (HPLC) using samples previously centrifuged at 13400 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\textsubscript{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\textsubscript{2}SO\textsubscript{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](http://modelling.combase.cc)) [2]. The biomass yield Y\textsubscript{X/S} (grams of biomass produced per gram of sugar consumed) and ethanol yield Y\textsubscript{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 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 hours 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:

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

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, $X_{max}$:

$$q_{Eth} = \frac{dEth/dt}{X_{max}} \quad (2)$$
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 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, performed as described in Methods, are displayed in Fig.1. At any initial sugar concentration, except at the lowest one (20 g/l), cell growth presented a biphasic kinetics with an exponential phase and a stationary phase. In contrast, at 20 g/l a 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 $\mu$ of 0.0049 h$^{-1}$ (Table 1). Between 20 and 100 g/l, transitions between the exponential and the stationary phases were very abrupt and correspond to the exhaustion of sugar in the culture (Fig. 1, A, B and C). 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 (Fig. 1, F, G and H). 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 ($\mu$), longer exponential growth phases were found with increased sugar concentrations (Fig. 1), but with decreasing $\mu$ values (Table 1). Several physiological mechanisms, underlying this decrease, were identified. The initial sugar concentration affected the biomass yield factor, i.e., 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 also the value of $\mu$. During the first 3 hours 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. After that time ethanol begun 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 g 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 in the repression of the synthesis of some components of the respiratory chain (cytochromes) at high external glucose concentrations, enhancing fermentation process [7]. This Crabtree effect hypothesis was supported by the yeasts behavior because, when all the glucose had been consumed, cytochromes 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 has 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 energy enough to synthesize new biomass and, although ethanol consumption could be measured, no increase in biomass could be detected (Fig. 1 B, C, D, E, 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, that would inhibit both the synthesis of new biomass and the synthesis of the components of the respiratory chain, e.g., the derepression of 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 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.h) than those of the exponential phase (0.67 to 0.84 g/g.h) (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 (Table 1 and 2). It must be remarked that at these high $S_0$ values, the stoichiometric relation between sugar consumed and ethanol produced (Eth$_{net}$), 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 g 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 *Saccharomyces 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, 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 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, sucrose hydrolysis showed a high rate. Anyway, at this sugar concentration the invertase activity was enough to hydrolyze all the added sucrose (Fig. 2C). At even higher $S_0$ concentrations, 350g/l (Fig. 2D), it was observed that invertase activity increased very slowly, even when glucose concentration was very low, indicating that, although derepression may have taken place, either the enzyme was not being synthetized, due to the action of the accumulated ethanol (about 40 g/l) and/or its activity was being inhibited by the high osmolality of the medium (water activity, $a_w$ of 0.964) value [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 initial sucrose concentration remained in the culture, even after 96 hours of fermentation.

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 is the initial concentration of total sugar in medium, invertase synthesis seems 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 in 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 wastes sugars. At this sugar concentration the diauxic behavior is not present at all and ethanol is not consumed during the stationary phase (Fig. 1F). 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 S₀ concentration had another advantage, ethanol is produced by both cells, exponential (82 %) and stationary (18 %). 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 inhibit strongly 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 S₀ 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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**Figure legends**

**Figure 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 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.

**Figure 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.

**Figure 3** – Variation of the initial specific rate of sucrose hydrolysis (moles per gram of 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.
Tables

Table 1- Kinetics and stoichiometric parameters for biomass, ethanol produced and total sugar consumption for *Saccharomyces cerevisiae* F13A fermentations, with different initial sugars concentration in the aqueous carob extract. Culture conditions were 30 °C, 150 rpm during 96 h.

| S₀ (g/l) | tinterval (h) | µ (1/h) | Yx/s (g/g) | T (%) | qEth (g/l h) | Eth (g/l) | Growth parameters at exponential phase | Growth parameters at stationary phase |
|---------|---------------|---------|------------|-------|-------------|----------|---------------------------------------|--------------------------------------|
|         |               |         |            |       |             |          |                                       |                                       |
|         | µ              |         |            |       |             |          |                                       |                                       |
| 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 | -           |

Values are mean ± SD of three replicates. S₀- Initial total sugar concentration; µ- Specific growth rate; Yx/s- biomass yield, T- % sugar consumed; qEth- Specific ethanol production rate; Eth – Ethanol concentration at the interval.
Table 2- Final concentrations of biomass, net ethanol accumulated and ethanol/sugar yields, for *Saccharomyces cerevisiae* F13A fermentations, using aqueous carob extract at different sugar concentrations. Culture conditions were 30 °C, 150 rpm during 96 h.

| $S_0$ (g/l) | $X_{\text{max}}$ (g/l) | Eth$_{\text{net}}$ (g/l) | $T$ (%) | $Y_{E/S}$ (g/g) | $P_E$ (g/l h) |
|-------------|----------------|----------------|--------|----------------|--------------|
| 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|

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

Figure 1
Figure 2
Figure 3
Fig. 1
Fig. 2
Fig. 3