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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*.

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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

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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

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101 efficient alcoholic fermentation. The glucose in carob residue substrate, at a concentration
102 above at threshold value, represses invertase synthesis and sucrose hydrolysis does not occur
103 until the glucose concentration reaches values below the threshold [6].

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

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

111 The establishment of the best technological conditions to achieve the highest ethanol
112 productivities and yields for 2nd generation biofuel production, using carob industrial wastes
113 as raw-material, was a major goal of the present work. In order to identify the factors that
114 limit the fermentation efficiency, Crabtree effect, invertase repression and ethanol toxicity
115 were studied in the present work, using kinetics and energetic approaches.

116

117 Materials and Methods

118 Microorganism

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

121

122 Culture media and preculture conditions

123 The strain was maintained on solid YEPD medium (peptone 20 g/l, yeast extract 10 g/l,
124 glucose 20 g/l, agar 15 g/l). Inocula were made in 250 ml shake flasks, containing 50 ml of
125 liquid YEP medium (yeast extract 5 g/l, peptone 10 g/l) supplemented with carob extract.

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126 The cultures were incubated in an orbital shaker (NeifoPentlab, Portugal), at 150 rpm and
127 30ºC, until it reach late exponential growth phase. These cultures were used as inocula to get
128 a initial cell concentrations of about 1 x 10^7 cells/ml.
129
130 **Aqueous carob extraction**
131 The carob residue extract was prepared as described in Lima-Costa et al. [6]. The
132 carob kibbles were dried to constant weight, ground, and the powder was suspended in
133 distilled water at solid/liquid ratio 30 % (w/v). This mixture was homogenized at 150 rpm, 25
134 ºC for 1 h. After this period, to clarify the carob extract, the mixtures were centrifuged at
135 22000 g, at 4 ºC for 25 min (Beckman Instruments, E.U.A), filtered through a 11 μm
136 membrane. Aqueous carob extract had a concentration of approximately 140 g/l total sugars.
137 For the assays at higher sugar concentrations the carob extract was concentrated using a rotary
138 evaporator (Heidolph 94200, Germany) at a temperature of 70 °C. The carob extract
139 concentrate is stored at a temperature of -20 ° C [7].
140
141
142 **Culture conditions**
143 Batch fermentations were performed at laboratory scale, in 250 ml shake flasks
144 containing 100 ml of YEP medium, supplemented with different concentrations of carob pulp
145 extract at an initial fresh cells concentration of 1 x 10^7 cells/ml. Fermentations were
146 performed at different initial carbon concentrations of 20, 50, 100, 150, 200, 250 and 300 g/l
147 of carob pod extract (CPE). The initial pH was 6.5 for all assays. Flasks were incubated in an
148 orbital shaker (NeifoPentlab, Portugal) at 150 rpm, 30 ºC for 96 h. Samples were collected for
149 analysis at the beginning of the experiments and every 2 hours. Absorbance at 590 nm, dry
150 weight (DW), pH, sugar consumption, and ethanol production were measured in the broth as

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151 described in ‘‘Analytical methods’’. Each assay was conducted in three replicates and
152 repeated twice.
153

154 **Analytical methods**

155 Absorbance was measured spectrophotometrically (Cintra 202 GBC DBUV
156 instrument, Australia) at 590 nm. Nutrient medium was used as a blank. Absorbance values at
157 590nm were converted into biomass concentration (g DW/l), using a standard curve. DW was
158 determined by centrifuging the cultures (Hettrich Zentrifugen Universal 320), as described
159 previously in Lima-Costa et al. [6]. Sugars and ethanol analyses were performed by high
160 performance liquid chromatography (HPLC) using samples previously centrifuged at 13400 g
161 for 10 min. Analyses were performed on a Beckman System Gold HPLC (Beckman, USA)
162 equipped with a Jasco 1530 refractive index detector (Jasco, Japan). To analyze sugar
163 concentrations, a Purospher STAR NH_2 column (Merck KGaA, Germany) was used with an
164 isocratic elution of acetonitrile:water (75:25) at 35ºC. Ethanol determinations were performed
165 on an OH AY column (Merck KGaA, Germany), at room temperature with an isocratic
166 elution of 0.002 N H_2SO_4 at 0.5 ml/min.

167 **Determination of kinetics and energetics parameters of growth**

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

174 The rates of sucrose hydrolysis, measured as grams of sucrose per hour per gram of dry
175 weight, were calculated as the slope, fitted by linear regression, of the values of sucrose

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176 concentration versus time, during the first 3 to 6 hours of fermentation, divided by the cell dry
177 weight corresponding to the middle time of the interval. The specific rates of ethanol
178 production, q_{Eth}, during the exponential phase were calculated as:

179 $$q_{Eth} = \frac{\mu}{Y_{X/E}} \quad (1)$$

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

183 $$q_{Eth} = \frac{dEth/dt}{X_{max}} \quad (2)$$

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199 # Results and Discussion

200 **Influence of the initial carob sugars concentration on biomass growth kinetics**

201 To analyze if the sugars present in carob waste triggered a Crabtree effect in *S.*
202 *cerevisiae,* fermentations runs were performed at eight different initial sugar concentrations
203 from 20 to 350 g/l. The corresponding values of biomass, ethanol production and total sugar
204 consumption, at these different concentrations, performed as described in Methods, are
205 displayed in Fig.1. At any initial sugar concentration, except at the lowest one (20 g/l), cell
206 growth presented a biphasic kinetics with an exponential phase and a stationary phase. In
207 contrast, at 20 g/l a diauxic growth could be observed and, instead of the stationary phase, a
208 second growth phase, consuming ethanol as carbon source could be measured, with a μ of
209 0.0049 h^{-1} (Table 1). Between 20 and 100 g/l, transitions between the exponential and the
210 stationary phases were very abrupt and correspond to the exhaustion of sugar in the culture
211 (Fig. 1, A, B and C). At the highest tested concentrations (250 to 350 g/l), the sugars were no
212 longer the limiting factor when the stationary phase was reached, because at this point there
213 were still sugars available in the medium (Fig. 1, F, G and H). In these cases, we submit that
214 ethanol was the factor limiting growth. At concentrations higher than 250 g/l the cells were
215 not even able to consume all the sugars added and, consequently, the final biomass decreased.
216 In relation to specific growth rates (μ), longer exponential growth phases were found with
217 increased sugar concentrations (Fig. 1), but with decreasing μ values (Table 1). Several
218 physiological mechanisms, underlying this decrease, were identified. The initial sugar
219 concentration affected the biomass yield factor, i.e., less biomass was produced per gram of
220 sugar consumed, due probably to the osmotic stress (Table 1).

221 The energetic efficiency of the sugar catabolism affected also the value of μ. During
222 the first 3 hours of culture, for S_0 below 150 g/l, there was sugar consumption but no ethanol
223 was produced (Fig. 1), which indicates that oxygen was available and the catabolism was

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224 completely oxidative. After that time ethanol begun to be produced and the catabolism was
225 progressively fermentative. This change in the efficiency of energy metabolism determined
226 the decrease in the yield factor biomass/sugar from 0.13 to 0.02 g/g (Table 1). To analyze
227 more deeply the effect of the initial sugar concentration on the yeast fermentation
228 performance, the data on the different sugars consumption and biomass and ethanol
229 production were analyzed quantitatively and the corresponding rates of sugars consumption
230 and ethanol production in the different growth phases were calculated.
231

232 **Kinetics of ethanol production and consumption during the growth and stationary**
233 **phases, depending on the different initial carob sugar concentrations.**

234 Ethanol profiles at the different initial sugar concentrations were determined along the
235 whole growth curve. From these values, the specific rates of ethanol production or
236 consumption (q_E) were calculated, as described in Material and Methods, and the values are
237 included in Table 1. In this table, a positive sign was added to q_E when there was net
238 production of ethanol, and a negative one, when there was net consumption. As mentioned
239 before, ethanol was always produced during the growth phase, at any initial sugar
240 concentration. From 20 to 100 g/l of initial total sugar an increase of q_E, from 0.4 to 1.0 g of
241 ethanol per g of biomass per hour was observed. That was the maximal production rate
242 reached because at higher initial sugar concentrations the rate decreased (Table 1). The
243 increase of q_E can be explained by the Crabtree effect that occurs in these species, which
244 consists in the repression of the synthesis of some components of the respiratory chain
245 (cytochromes) at high external glucose concentrations, enhancing fermentation process [7].
246 This Crabtree effect hypothesis was supported by the yeasts behavior because, when all the
247 glucose had been consumed, cytochromes synthesis was derepressed, and ethanol was in fact
248 oxidized. At 20 g/l of initial sugar, after glucose exhaustion, the yeasts were completely

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249 derepressed and were able to perform a diauxic growth, consuming all the ethanol that has
250 been produced (Fig. 1A). A similar pattern could be observed at 50 up to 200 g/l of initial
251 sugars, but with an apparent lower derepression, as measured by the specific rates of ethanol
252 consumption, that decreased from 26 mg of ethanol per gram of biomass per hour at an initial
253 glucose concentration of 6.1 g/l (Fig. 2A) to 10 mg of ethanol per gram of biomass per hour at
254 an initial glucose concentration of 44.8 g/l. (Fig. 2C, Table1). Apparently, this low
255 consumption did not provide energy enough to synthesize new biomass and, although ethanol
256 consumption could be measured, no increase in biomass could be detected (Fig. 1 B, C, D, E,
257 Table 1). It may be argued that, once glucose was exhausted, derepression should take place
258 completely. An alternative hypothesis to explain the differences observed with increasing S_0
259 values may be based on the action of the ethanol accumulated, that would inhibit both the
260 synthesis of new biomass and the synthesis of the components of the respiratory chain, e.g.,
261 the derepression of respiratory chain [6].

262 At concentrations higher than 200 g/l of initial sugar, none of the accumulated ethanol
263 was consumed during the stationary phase (Fig. 1 and Table 1). On the contrary, at these high
264 concentrations the alcohol continued to be produced by the metabolically uncoupled cells,
265 unable to grow but yet able to ferment (Table 1). However, the specific ethanol production
266 rates were much lower (0.11 to 0.13 g/g.h) than those of the exponential phase (0.67 to
267 0.84g/g.h) (Table 1), indicating that the accumulated ethanol was partially inhibiting the
268 fermentation.

269 From a stoichiometric point of view, it can be concluded that the initial sugar
270 concentration showed a strong effect on the final amount of ethanol accumulated in the
271 culture (see Table 2). Up to 200 g/l total sugar, all the ethanol was produced during the
272 exponential phase. However, at these low sugar concentrations, after glucose exhaustion,
273 ethanol was completely consumed when S_0 was 20 g/l and in significant amounts at 50 and

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274 100 g/l. This behavior changed at S_0 concentrations higher than 250 g/l, at which ethanol was
275 not consumed but produced by the uncoupled stationary cells, in an amount similar or even
276 higher than that produced by the exponential cells (Table 1 and 2). It must be remarked that at
277 these high S_0 values, the stoichiometric relation between sugar consumed and ethanol
278 produced (Eth_{net}), which included the alcohol produced by both exponential and stationary
279 cells, showed values near to the maximal theoretical value that can be obtained if all the sugar
280 was fermented: 0.51 g of ethanol per g of sugar (Table 2). The highest ethanol/sugar yield,
281 0.42 g/g was obtained with an initial sugar concentration of 250 g/l.

282

283 **Regulation of sugars consumption: Repression of invertase synthesis and inhibition of its**
284 **activity.**

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

291 It can be observed that sucrose concentration decreased immediately after inoculation
292 at the lowest initial sugar concentration (20 g/l), indicating that active invertase was present.
293 The invertase activity was so high in this condition that the concentration of glucose in the
294 culture increased, because its production by sucrose hydrolysis was higher that its
295 consumption by the cells (see Fig. 2A). This immediate sucrose hydrolysis was also present at
296 100 g/l fermentation, although at a lower rate, as shown by the rate of sucrose disappearance
297 and by the fact that the concentration of glucose did not increase. However, at fermentations
298 performed at higher S_0 (see the case of 250 g/l in Fig. 2C) sucrose was not immediately

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299 hydrolyzed and only when glucose had been consumed, sucrose hydrolysis showed a high rate.
300 Anyway, at this sugar concentration the invertase activity was enough to hydrolyze all the
301 added sucrose (Fig. 2C). At even higher S_0 concentrations, 350g/l (Fig. 2D), it was observed
302 that invertase activity increased very slowly, even when glucose concentration was very low,
303 indicating that, although derepression may have taken place, either the enzyme was not being
304 synthetized, due to the action of the accumulated ethanol (about 40 g/l) and/or its activity was
305 being inhibited by the high osmolality of the medium (water activity, a_w of 0.964) value [3,6].
306 This dependence of invertase activity on the water activity of the culture has been previously
307 reported [17]. In any case, with S_0 equal to 350 g/l the invertase activity was so low that
308 sucrose was not completely hydrolyzed and 52 % of the initial sucrose concentration
309 remained in the culture, even after 96 hours of fermentation.

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

316

317 **Technological consequences of initial sugars concentration on ethanol net production:**
318 **Crabtree effect and invertase repression and inhibition**

319 Taking in consideration the data of Table 2 it can be concluded that, from all the sugar
320 concentrations assayed, 250 g/l is the best concentration to be used in industrial processes for
321 ethanol production from carob wastes sugars. At this sugar concentration the diauxic behavior
322 is not present at all and ethanol is not consumed during the stationary phase (Fig. 1F).
323 Crabtree effect may be at its maximum, glucose respiration is almost completely repressed

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324 and, therefore, catabolism is completely fermentative, with an ethanol/sugar yield of 0.42 g/g,
325 near the maximum (Table 2). Working at this S_0 concentration had another advantage, ethanol
326 is produced by both cells, exponential (82 %) and stationary (18 %). Although invertase is
327 initially repressed, the complete glucose consumption enables its derepression. The
328 determined water activity (a_w) of 0.964 is not low enough to inhibit strongly the hydrolysis of
329 sucrose, as happens at higher concentrations, and sucrose can be completely consumed. At
330 this optimal concentration of 250 g/l, the metabolic rates are slower than at lower S_0 values,
331 due to physiological reasons that have been analyzed above, and the ethanol productivity,
332 although not the highest, is close to the maximal obtained (Table 2). Another remarkable
333 advantage is the high final concentration of ethanol attained, close to 100 g/l in these assayed
334 conditions.

335

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340 the *Saccharomyces cerevisiae* F13A strain by Prof Célia Quintas.

341

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397 **Figure legends**

398 **Figure 1 -** Fermentation profiles of yeast *Saccharomyces cerevisiae* F13A, using aqueous
399 carob residue extract as carbon source at different initial sugar concentrations. A – 20 g/l, B –
400 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.
401 logarithm Ln of DW (filled triangles) and DMFIT modeling predictions (line), ethanol
402 production (filled squares) and total sugar consumption (empty circles). The fermentation was
403 run for 96 h on an orbital shaker at 150 rpm and 30 °C. The experiments were performed
404 three times. Dry cell weight, sugar and ethanol data are average of three replicates.

405 **Figure 2** – Glucose consumption and sucrose hydrolysis time-course in aqueous carob extract
406 fermentations, at different initial sugar concentrations. A – 20 g/l, B – 100 g/l, C – 250 g/l and
407 D – 350 g/l. Glucose consumption (empty squares) and sucrose hydrolysis (filled triangles).
408 The fermentation was run for 96 h on an orbital shaker at 150 rpm and 30 °C. The
409 experiments were performed three times.

410 **Figure 3** – Variation of the initial specific rate of sucrose hydrolysis (moles per gram of DW
411 per hour) with glucose concentration, in the fermentation of aqueous carob extract. Culture
412 conditions were 30°C, 150 rpm during 96 h. Glucose concentrations were 7.5 g/l (empty
413 squares), 17 g/l (filled squares), 22 g/l (empty triangles), 41g/l (filled triangles), 45 g/l (filled
414 diamond), 64 g/l (filled circles) and 82 g/l (empty diamond and circles). An arbitrary linear
415 modeling of the data (line) was also plotted.

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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.

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_{Eth} (g/g h)	Eth (g/l)	t_{interv} (h)	μ (1/h)	T (%)	q_{Eth} (g/g h)	Eth (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	-

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

452 **Table 2**- Final concentrations of biomass, net ethanol accumulated and ethanol/sugar yields,
453 for *Saccharomyces cerevisiae* F13A fermentations, using aqueous carob extract at different
454 sugar concentrations. Culture conditions were 30 °C, 150 rpm during 96 h.

S_0 (g/l)	X_{max} (g/l)	Eth_{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

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456 Values are 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
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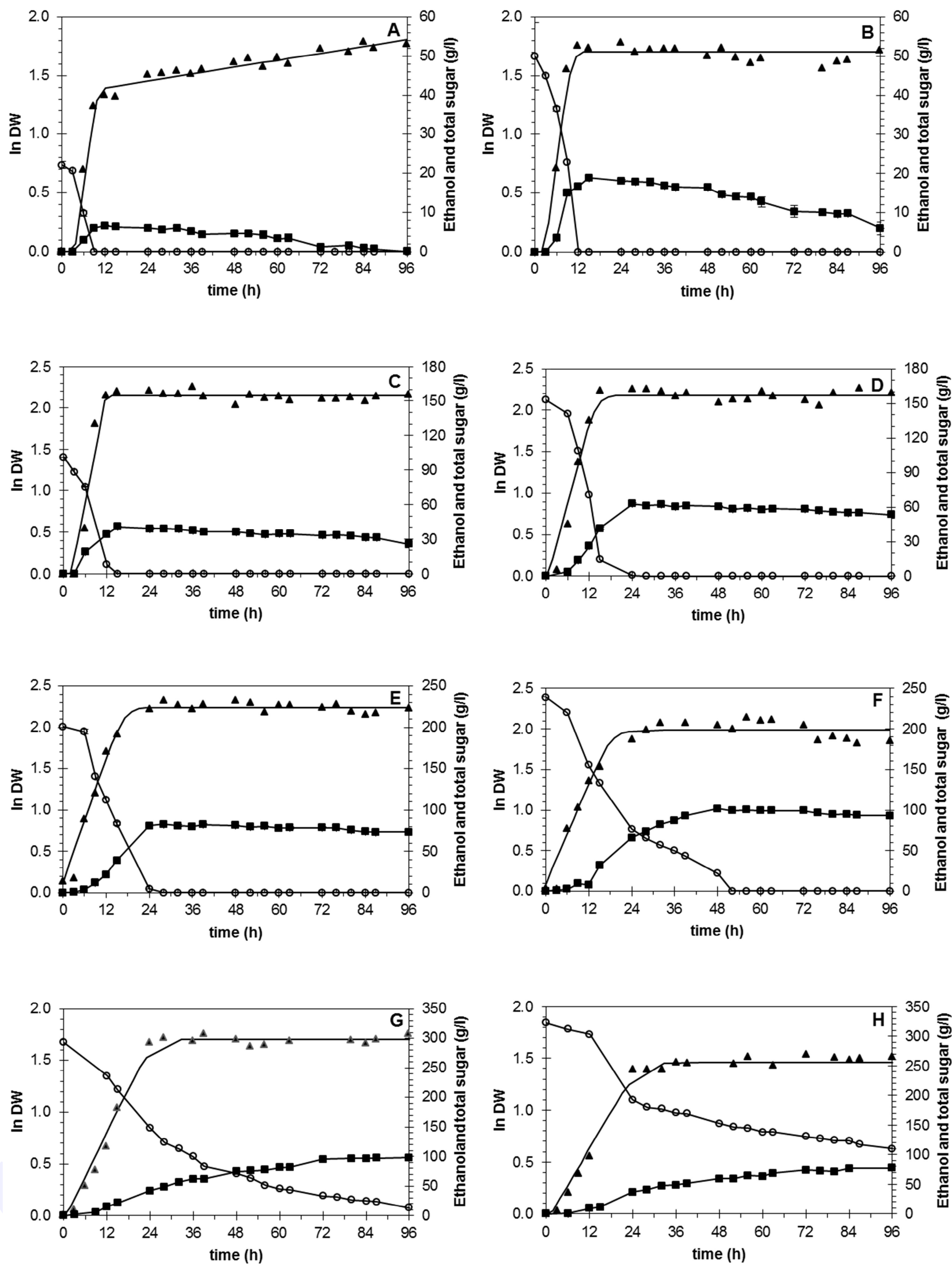
475 **Figures**



476 **Figure 1**

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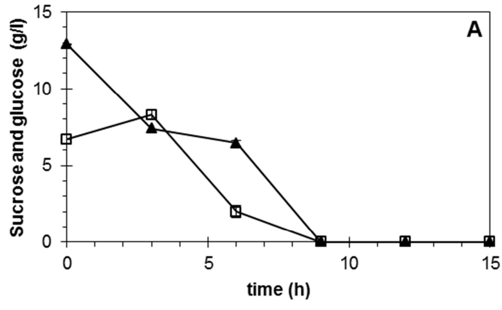

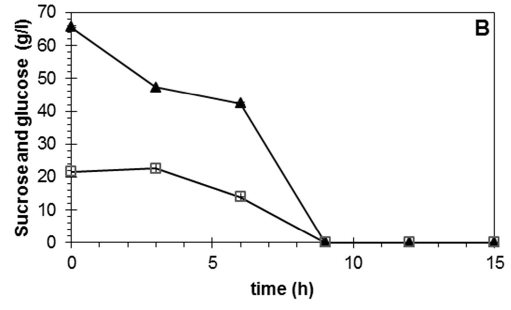

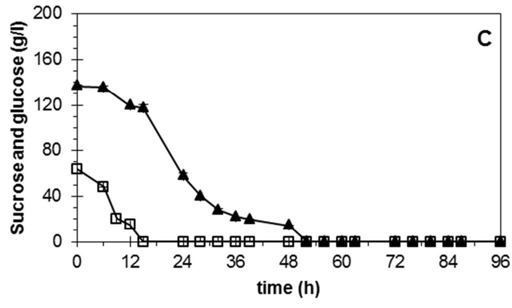

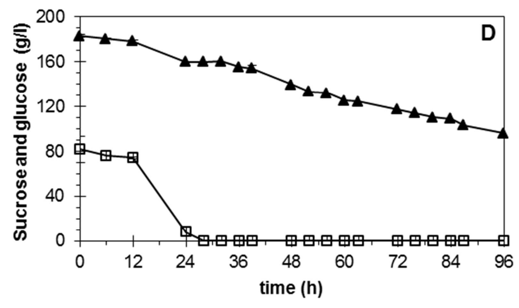

478 **Figure 2**

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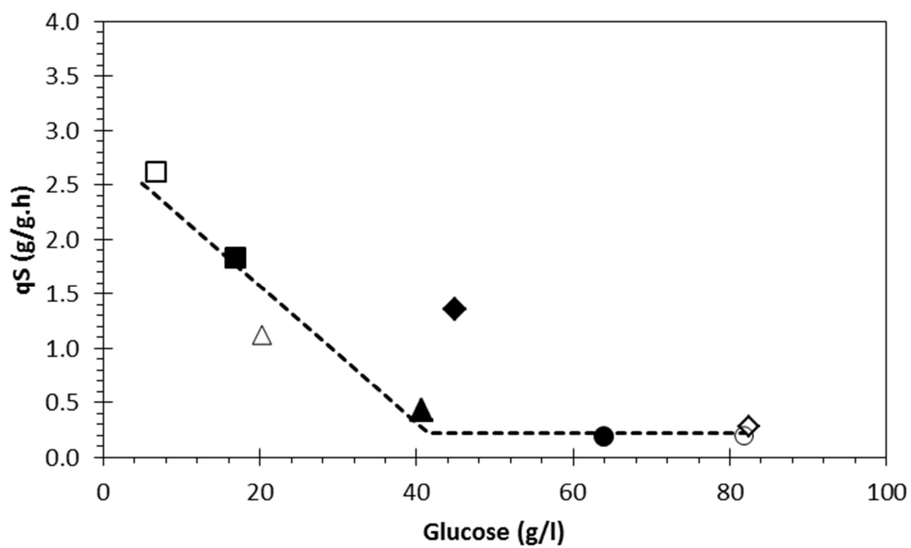

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487 **Figure 3**
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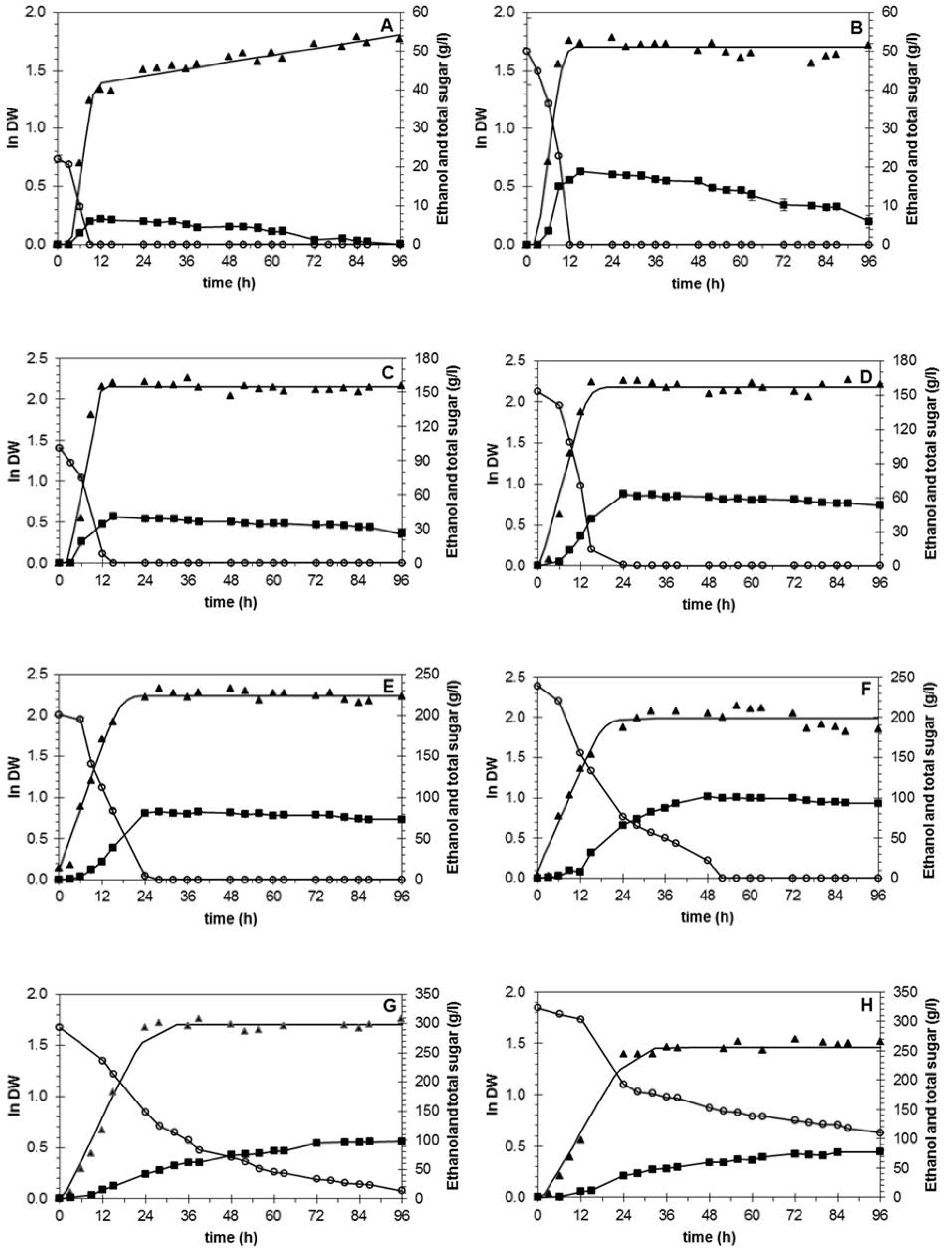

Fig. 1

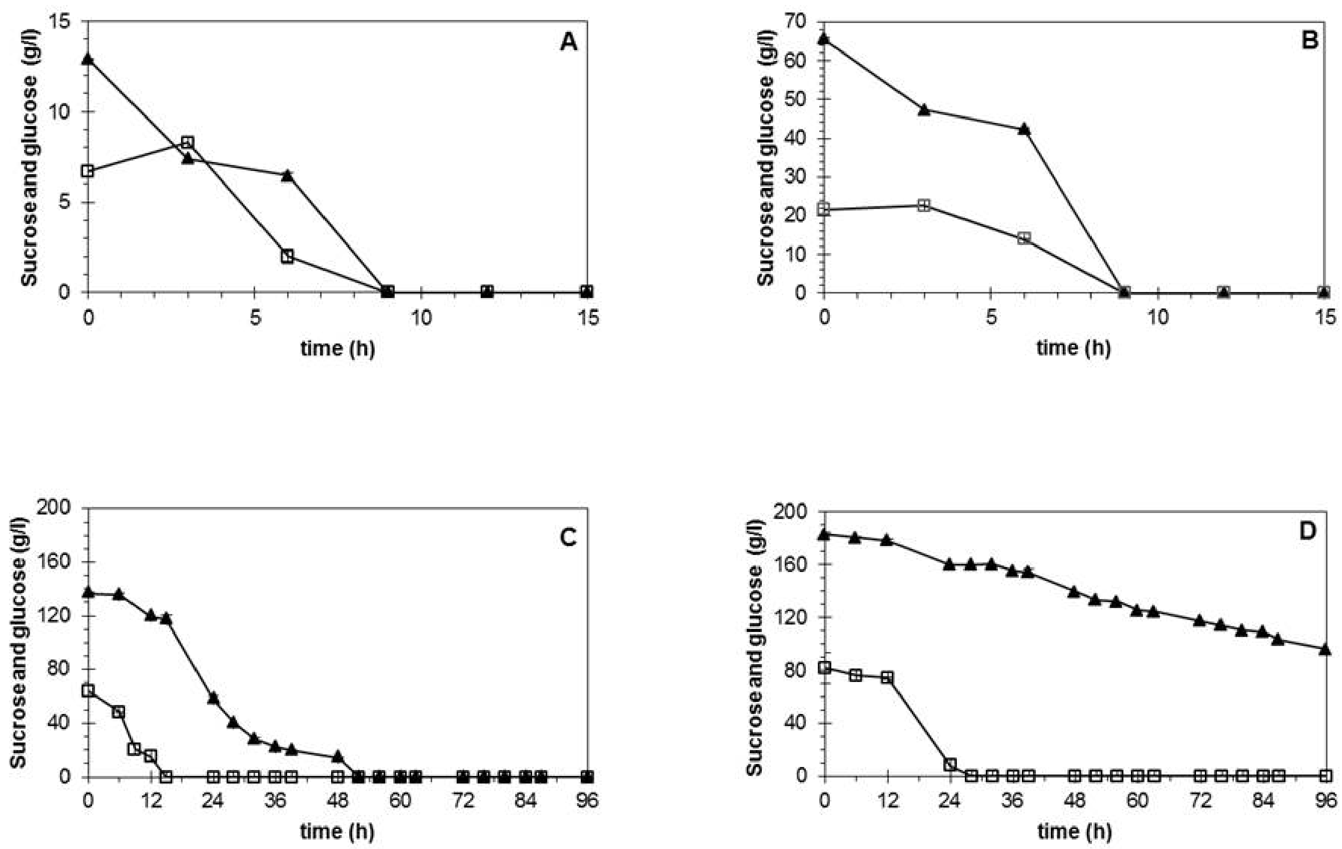

Fig. 2

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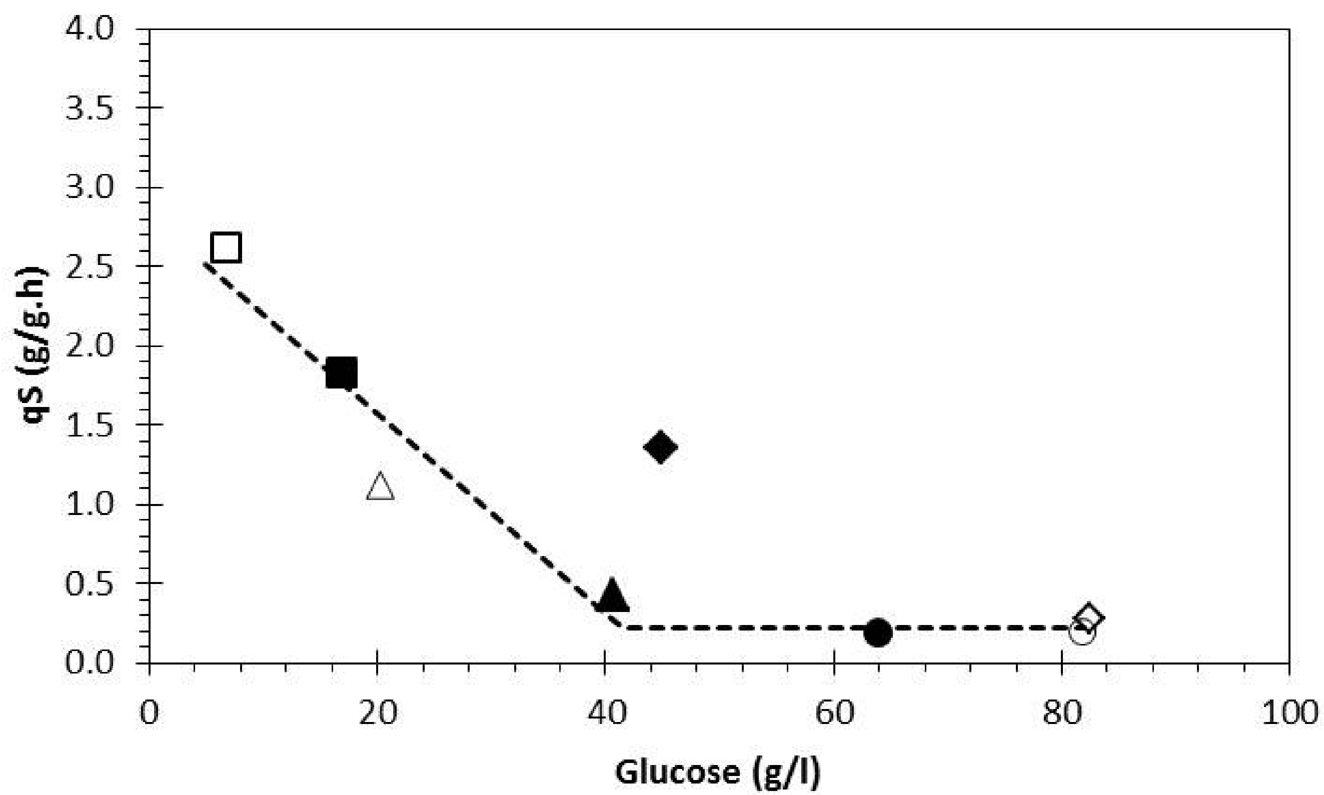

Fig. 3

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