Title

REVALORISATION OF STRAWBERRY SURPLUSES BY BIO-TRANSFORMING ITS GLUCOSE CONTENT INTO GLUCONIC ACID

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Abstract

Modern societies produce massive surpluses of food, by-products and wastes that increase the interest for their revalorisation. This work examines the use of a culture of *Gluconobacter japonicus* CECT 8843, without pH control, to convert selectively the glucose content of industrially pasteurized strawberry purée into gluconic acid for the development of new beverages. However, depending on the initial concentration of glucose, the microorganism could transform the acid formed into other compounds; for this reason, in this work the effect of initial sugar concentration on the preservation of the acid was investigated. The results show that the gluconic acid formed in strawberry purée containing no added sugars started to disappear after glucose depletion, but the acid concentration remained constant if sugar-enriched purée was used. The use of this industrial substrate resulted in the presence of yeasts and hence in some fructose uptake; however, the fructose consumption was negligible until after 20–30 h. The use of food by-products is an excellent opportunity not only to recover valuable compounds but for the development of new chemical and biotechnological approaches for their revalorisation. This strategy should improve regional economies and contribute to a sustainable management of these underexploited resources.

Key words

Strawberry surpluses, Revalorisation, Gluconic acid, *Gluconobacter japonicus* CECT 8843, Strawberry purée.

1. Introduction

Efficiently producing, conditioning, distributing and using food continues to be one of the crucial problems in both developed and underdeveloped zones. About 1.3 billion tons of food is not used each year owing to farming, storage, transportation, processing, distribution or even consumption losses (Gustavsson et al., 2011).

Food by-products and wastes revalorisation practices for the recovery/production of compounds of high interest are gaining much attention. This strategy should improve regional economies and contribute to a sustainable management of these underexploited resources (Galanakis and Schieber, 2014; Naziri et al., 2014; Perez-Jimenez and Viuda-Martos, 2015).

The problem of food surpluses is worse when the food is easily degradable. Such is the case, for example, with surpluses and residues from fruit and vegetable production processes, which account for about 50% of the global production of these products (Gustavsson et al., 2011). Although the situation varies geographically, developed countries typically generate fruit surpluses that cannot be sold for direct consumption and have a strongly adverse economic impact on their production zones. This is particularly so with strawberry, the first five world producers of which are China, United States, Mexico, Turkey and Spain (Faostat, 2015); the last country generates a surplus of about 20% of this fruit each year that is used mainly to obtain strawberry purée for various food production purposes.

The composition of strawberry purée, with a sugar content ranging from 30 to 200 g/L depending on its concentration grade, makes it a natural substrate with a high potential

for obtaining various added-value bioproducts. One possible use of strawberry purée is for obtaining a gluconic acid based bio-oxidation product by converting the glucose into gluconic acid while preserving the initial content in fructose. Mixing with other fermentation products can be used to obtain new highly aromatic, refreshing non-alcoholic beverages sweetened by the fructose remaining in the medium (Cañete-Rodriguez et al., 2016, 2015, 2012; Sainz et al., 2012).

A search for microorganisms effecting this bioconversion led to acetic acid bacteria (Garcia-Garcia and Gullo, 2013; Raspor and Goranovic, 2008). Previous studies (Cañete-Rodriguez et al., 2015, 2016) showed that *Gluconobacter japonicus* CECT 8443 has a high potential for performing the sought transformation in a selective manner. The problem, however, is not limited to finding an appropriate microorganism; in fact, acetic acid bacteria can behave widely differently and lead to also different products depending on the particular cultivation medium and operating conditions. Also, preserving the sensory and antioxidant properties of the raw material requires avoiding sterilization prior to inoculation. As a result, fulfilment of the primary aim may be compromised by the presence of the natural micro flora of the fruit that includes mainly yeasts (Cañete-Rodriguez et al., 2015).

The result of the action of acetic acid bacteria depends largely on the prevailing metabolic pathway (Deppenmeier and Ehrenreich, 2009; Olijve and Kok, 1979a). The conversion of glucose into gluconic acid involves the nearly stoichiometric oxidation of the substrate on the outer surface of the cytoplasmic membrane via glucono-δ-lactone as intermediate product (King and Cheldelin, 1958). However, the resulting gluconic acid can undergo further conversion into keto-gluconates (Ano et al., 2011; Beschkov and Velizarov, 1995; Buse et al., 1992; Olijve and Kok, 1979a, 1979b; Stubbs et al., 1940;

Träger et al., 1992). In addition, glucose and gluconic acid can be used via the pentose phosphate and the Entner-Doudoroff pathways (Levering et al., 1988; Olijve and Kok, 1979a, 1979b; Prust et al., 2005). The end result is strongly influenced by the pH and available concentration of glucose in the medium (Olijve and Kok, 1979a, 1979b; Velizarov and Beschkov, 1998, 1995).

Thus, in this work, the effect of enriching strawberry purée with sugars on the preservation of the formed gluconic acid in the fermented end-product has been examined.

2. Material and methods

2.1. Raw material

Two different media were used as fermentation broths. One was strawberry purée (SP) industrially produced and pasteurized (92 ºC for 90-120 s) by Hudisa Desarrollo Industrial, S.A. (Lepe, Spain) from fresh strawberries. The purée, containing approximately 34 g/L of sugars (roughly 50% glucose and 50% fructose) was stored at 0–4 ºC prior to use. The other fermentation broth was enriched purée (ESP) obtained by enriching the previous broth with sugars up to a total concentration of 135–140 g/L (again 50% glucose and 50% fructose approximately).

2.2. Microorganism

G. japonicus CECT 8443 was selected for its high selectivity towards glucose relative to fructose (Cañete-Rodriguez et al., 2015, 2016; Navarro, 2011; Navarro et al., 2013; Sainz et al., 2016).

2.3. Preparation of inocula

G. japonicus was stored frozen at –18 ºC in a 50:50 (v/v) water/glycerol mixture as cryoprotective agent. Prior to use, it was reactivated by thawing, and seeding in 125 mL of GYP liquid medium first [50 g/L glucose, 10 g/L yeast extract and 20 g/L bacteriological peptone] and tilted agar GY tubes [50 g/L glucose, 10 g/L yeast extract and 30 g/L agar].

As it is justified in Cañete-Rodriguez et al. (2015), the inoculum was prepared by seeding in 250 mL Erlenmeyer flasks containing 125 mL of GYP liquid medium that was previously autoclaved at 121 ºC for 15 min. After shaking in an incubator at 29 ºC and 150 rpm for 24 h, the medium was supplied with 125 mL of strawberry purée that was previously sterilized by autoclaving under identical conditions. After 24 h of additional incubation, the inoculum (the 250 mL of the final mixture) was ready for addition to the fermentation tank, which had previously been loaded with 3 L of pasteurized strawberry purée.

2.4. Determination of cell concentrations

Yeast cell concentrations were determined by direct counting under a microscope, using a Neubauer chamber as described elsewhere (Baena-Ruano et al., 2006). Bacterial cell concentrations were not determined since no accurate cell counting was possible owing to the characteristics of the purée (Cañete-Rodriguez et al., 2016).

2.5. Determination of sugars, gluconic acid and total acidity

Sugars and gluconic acid were quantified with the following enzyme kits from Megazyme®: K-GLUC 07/11 for glucose, K-FRUGL 12/12 for fructose and K-GATE 12/12 for gluconic acid. Additional information about the kits can be found at the Megazyme web site (Megazyme, 2016). Total acidity was measured by acid-base titration. Determinations were performed at least in triplicate and the resulting standard deviations are given in the figures.

2.6. Fermenter

Fermentation runs were conducted batchwise in a Biostat® 5 L bioreactor equipped with pH, agitation, dissolved oxygen and temperature controls. An average volume of 3 L, agitation at 500 rpm, a temperature of 29 ºC and a dissolved oxygen at 20% air saturation were used in each run. The pH of the medium was allowed to evolve freely throughout. Since the fermentation substrate was unsterilized, the equipment required thorough cleaning between runs. Tests with SP and ESP media were replicated eight and eleven times respectively.

3. Results and discussion

Figures 1 and 2 show some of the experimental results for both media. Only in ESP the gluconic acid formed remained unaltered (Fig. 1b).

The two fermentation substrates also differed in pH and acidity; thus, SP exhibited an increase in pH and a decrease in acidity at a late stage (Fig. 2a), ESP exhibited no change in either variable after about 15 h of fermentation (Fig. 2b).

In order to explain these results, it should be considered that preserving the sensory properties of the product required using pasteurized rather than sterilized purée. Therefore, the end product was the result of the action not only of the bacterial inoculum, but also of yeasts present in the purée. As can be seen from Fig. 3, the yeasts regained activity and grew substantially after 20 h of fermentation. Confirming authors´previous results (Cañete-Rodriguez et al., 2015, 2016), Fig. 3 testifies to the fact that biological activity in the medium was due mainly to bacteria during the first 20 h, after which yeasts´activity started to be important.

Futhermore, one of the most salient features of *Gluconobacter* spp. is their over-flow metabolism (Deppenmeier and Ehrenreich, 2009; King and Cheldelin, 1958; Kulhanek, 1989; Träger et al., 1992), by which, in a first phase, a substantial fraction of glucose is rapidly oxidized to gluconic acid and scarcely assimilated by these microbes as a result. These bacteria can also use glucose via the pentose phosphate pathway (Kitos et al., 1958; Olijve and Kok, 1979a); however, the activity of this pathway is strongly dependent on pH and on the glucose concentration of the medium. For example, glucose consumption via the penthose phosphate pathway is considerably inhibited below pH 3.5–4 as well as at glucose concentrations over the range 0.9–2.7 g/L (5–15 mM) (Olijve and Kok, 1979a). Therefore, selectively converting glucose into gluconic acid and preserving the latter seemingly requires working either without pH control or using a value below 3.5–4. Also, the process is favoured by glucose concentrations above 5–15 mM. In any case, the behaviour of these microorganisms is rather complex and its

outcome depends to a greater or lesser extent on the particular cultivation medium used. Based on the foregoing, in this work the pH was not controled. Interestingly, the initial pH of the strawberry purée, 3.3, fell below the previous range.

Gluconic acid formed from SP started to decline, once all the glucose was used, and eventually disappeared completely from the medium after about 70 h, see Fig. 1a. This result led us to examine the influence of the initial concentration of sugars by enriching the original medium to obtain ESP. As can be seen from Fig. 1b, the concentration of gluconic acid remained constant after, approximately, 17 hours of cultivation.

Acidity and pH changes during the fermentation process are also worth noting. Thus, as can be seen from Figs. 1 and 2, the increase of gluconic acid concentration in both SP and ESP entails the increase in total acidity and decrease in pH at an early stage (i.e., until glucose was depleted); also, acidity and the gluconic acid concentration peaked at that stage. In addition, the apparent yields of gluconic acid relative to glucose ($Y_{GA/G}$) were identical in both media (Fig. 4) and similar to those previously obtained by other authors when glucose uptake via the pentose phosphate pathway is very low owing to the low pH and relatively high concentration of glucose (Olijve and Kok, 1979b; Velizarov and Beschkov, 1995). This suggests that glucose was exclusively used by the bacteria and mostly for oxidation to gluconic acid.

The two types of substrate evolved markedly differently after the early fermentation stage. Thus, in ESP, the gluconic acid concentration, acidity and pH changed similarly throughout the rest of the fermentation cycle, and the high concentration of gluconic acid obtained seemingly governed pH and acidity. On the other hand, the low concentration of gluconic acid formed in SP, roughly four times lower than in ESP, apparently resulted in additional bio-reactions after about 10 h of fermentation that

transformed the acid and caused important changes in the pH and acidity profiles (see Figs. 1a and 2a).

In fact, whereas pH and acidity evolved in parallel during the central part of the fermentation cycle (Fig. 2a) in SP, gluconic acid clearly decreased with time (Fig. 1a). Explaining these results requires several aspects to be considered; on one side the mechanisms for gluconic acid consumption and on the other side the potential influence of yeasts over the whole system (particularly over the period from 20 to 40 h). A previous study by the authors involving blank tests (Cañete-Rodriguez et al., 2015) revealed that when no bacteria are inoculated, glucose consumption started after about 20 h, the time needed for yeast activity to be triggered, and fructose about 1-2 h later; however, sugar uptake resulted in minimal changes in total acidity and pH. Therefore, the pH and acidity changes observed when the bacteria are present were due to their activity and very likely associated to the presence of gluconic acid and other acids resulting from its metabolism.

Gluconic acid is known to be further oxidized to keto-D-gluconic acids (Olijve and Kok, 1979a; Stubbs et al., 1940). However, the process is rather complex and depends on the particular micoorganism and operating conditions. Without pH control, glucose is converted to gluconic acid dropping rapidly the pH to about 3-2.5; under these conditions the acid is not further practically oxidized to keto-acids. Nevertheless, at a controlled pH of 5.5, most of the acid is oxidized to keto-acids; additionally, low initial concentrations of glucose in the medium also favours the formation of keto-acids (Beschkov and Velizarov, 1995, 1998; Seiskari et al., 1985; Weenk et al., 1984). Based on Figs. 1a and 2a and the foregoing, the experimental conditions used in this work (medium SP, with low initial glucose concentration) may have allowed gluconic acid

formed to be partly oxidized to keto-acids, which would be consistent with the observed decrease in the acid concentration without change in total acidity. At a later stage, gluconic acid and the resulting keto-acids may have been used by the bacteria (Gupta et al., 2001; Matsushita et al., 1994) and led to a decreased acidity and increased pH (see Fig. 2a). On the other side, the high initial concentration of glucose in ESP (Fig. 1b) led to the formation of an also high concentration of gluconic acid, and raised total acidity by twice as much as in SP while decreasing the pH to about 2.4. According to Weenk et al. (1984), this low pH would account for the observed preservation of gluconic acid. These results are consistent with those a recent authors´ work in which synthetic and strawberry-based media were used (Sainz et al., 2016).

Additionally, it is well stablished that the presence of $CaCO_3$ in the medium also favours the formation of keto-acids (Beschkov and Velizarov, 1995; Stadlerszoke et al., 1980; Weenk et al., 1984); in fact, the low solubility of calcium keto-gluconates displaces chemical equilibria to their formation. Interestingly, in our case, the low Ca content of strawberry purée —usually about 0.2 g/L (Giampieri et al., 2012; USDA, 2016)— cannot have favoured the formation of keto-acids.

Finally, the concentration of dissolved oxygen also seems to affects the process (Buse et al., 1992). According to these authors, a high concentration of dissolved oxygen substantially boosts activity of gluconic dehydrogenase, which catalyzes the first stage in the formation of keto-acids from gluconic acid; then, an oxygen concentration of about 30% of the saturation level is required to maximize the formation of 2,5-diketo-gluconic acid. Fig. 5 shows the variation of the dissolved oxygen concentration in experiments SP and ESP. In order to minimise volatile losses during fermentation as far as possible, the dissolved oxygen concentration was restricted to 20% of the saturation

level; however, this concentration was impossible to maintain during the exponential growth stages owing to the high oxygen demand of the bacteria to oxidize glucose to gluconic acid; the higher initial glucose concentration in ESP justifies the marked fall of the oxygen curve in ESP. Once all glucose was converted, the dissolved oxygen concentration rapidly returned to the set level; the oxygen depletion concentration observed for experiment SP, between 20 and 40 h, will be discussed later. Although Buse et al. (1992) proved that dissolved oxygen concentrations higher than 25-30% of the saturation level favour the bio-transformation of gluconic acid into keto-gluconic acids, our results show that, in spite of working with a maximum oxygen saturation level of 20%, gluconic acid was transformed in experiment SP. Interestingly, it must be pointed out that Buse et al. (1992) used a medium with a high Ca content (10 g $CaCO_3$/L) and a controlled pH value (3), so these two variables seem to have a stronger influence on the preservation of the gluconic acid than the dissolved oxygen concentration.

Also worth special note was the behaviour of fructose. As can be seen from Figs. 1a and 1b, this sugar started to be used after about 20 h, coinciding with an increase in the concentration of yeast cells (Fig. 3). This coincidence in time suggests a direct relationship between the consumption of fructose and the growth of yeasts; this could be confirmed by the similar mean yields in yeast cells per gram of fructose, Y_{XF}, that can be seen in Fig. 6. Additionally, a recent authors´publication (Sainz et al., 2016), working with a previously sterilized strawberry purée-based medium in which *G. japonicus* CECT 8443 was inoculated, showed that, roughly, no fructose consumption was observed during the first 3 days of fermentation, being necessary 12 days for obtaining a reduction of about 10% in the initial fructose concentration. Therefore, although a small

fraction of fructose may have been used by the bacteria (Cummins et al., 1957; Kitos et al., 1958), the apparent constancy in Y_{XF} of both SP and ESP throughout the fermentation process, as well as the results by Sainz et al. (2016), suggest that this sugar was used mainly by the yeasts. The yeasts shown in Fig. 3 were identified in a previous authors´ work (Cañete-Rodriguez et al., 2015) found to be *Pichia kudriavzevii* and, to a lesser extent, *Saccharomyces cerevisiae*. Although yeasts reached higher concentrations in ESP by effect of its increased availability of fructose, the apparente maximum specific growth rate was relatively low in both substrates, but particularly in ESP. Additionally, from the fructose uptake rate values troughout the fermentations, it can be shown, Fig. 7, that the maximum rate in experiment SP is 45% higher than in ESP; then, from a kinetic point, the environmental conditions prevailing with medium ESP, seem to be detrimental to the yeasts´activity. These results may be explained by the extreme acidity and low pH values obtained in ESP. Although *P.kudriavzevii* has been described as a multi-stress-tolerant strain (Isono et al., 2012), able to growth at high salt concentrations and low pH conditions (pH 2.0), the high gluconic acid concentration and low pH reached in experiment ESP may have had a more negative impact on the yeast´s activity than in the SP case. Additionally, Thomas et al. (2002) studied the effect of weak organic acids on the action of yeasts (*Saccharomyces cerevisiae*) and found it to be the combined function of acidity and pH. Thus, at acetic acid levels of 10 g/L, yeasts grew above pH 2.5 —to an increasing extent with increasing pH— but ceased to grow below that level. Interestingly, the final pH values of our media were 2.8 (SP) and 2.4 (ESP). Therefore, based on the previous comments, the kinetic differences observed for the yeasts in experiments ESP and SP may have been caused by the differences in total acidity and pH.

Finally, it is interesting to note that the dissolved oxygen fall observed in Fig. 5a, between 20 and 40 h, coincided in time with the fructose depletion in experiment SP. In this regard, it should be pointed out that *P.kudriavzevii* (the main yeast present in our media) are decribed as crabtree-negative yeast species (Schnierda et al., 2014; Radecka et al., 2015), so, an important oxygen demand could be expected during this stage; nevertheless, the lower fructose uptake rate found in experiment ESP, allowed the fermenter to keep the dissolved oxygen concentration at the set level for the experiment, see Fig. 5b.

4. Conclusions

Inoculating strawberry purée with *G. japonicus* CECT 8843 without pH control enabled the selective bio-transformation of glucose into gluconic acid. However, the preservation of the acid depended on the initial concentration of glucose in the medium. Thus, gluconic acid formed in strawberry purée containing no added sugars (SP) started to disappear after glucose was depleted; this process comprised two stages according to the evolving of pH and total acidity of the medium. Initially, total acidity remained constant, which suggests that gluconic acid was converted into keto-acids; however, the decreasing acidity during the subsequent stage suggests that the resulting keto-acids were consumed after the gluconic acid concentration fell to low levels. On the other hand, gluconic acid formed in sugar-enriched strawberry purée (ESP) is not further transformed throughout the process.

The need to use an industrially pasteurized substrate inevitably resulted in the presence of yeasts and hence in some fructose uptake. The survived yeast cells are passing

relatively long lag phase of growth after which they enter exponential phase of growth and start to ferment the remained sugar (fructose) in media. However, the amount of fructose used was insubstantial until after 20–30 h.

In any case, obtaining a gluconic acid-based fermentation product useful to formulate other products will require optimizing the fermentation time and such a time depends on the initial concentration of glucose. With a low concentration, the process should be stopped as soon as the glucose has been depleted; with a high concentration, the process can be allowed to develop longer since the resulting gluconic acid will remain unaltered. However, if the end-product must retain the sweetness of fructose in the initial substrate, then one should bear in mind that this sugar starts to be consumed after about 20–30 h —mainly by yeasts.

Therefore, even pasteurized substrate affords the desired biotransformation of glucose into gluconic acid with a high selectivity and a variable content in fructose depending on how long the fermentation process is allowed to develop.

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Figure captions

Fig. 1 - Variation of glucose (○), gluconic acid (▲) and fructose (◊) concentrations in experiments with (a) strawberry purée (SP) and (b) enriched strawberry purée (ESP), with symbols and bars representing experimental values and standard deviations respectively. Solid lines represent best trends. (*FIGURE 1 should be a 2-column fitting image*).

Fig. 2 - Variation of pH (○) and total acidity (•) in experiments with (a) strawberry purée (SP) and (b) enriched strawberry purée (ESP), with dots and bars representing experimental values and standard deviations respectively. Total acidity is expressed as g of gluconic acid per litre. (*FIGURE 2 should be single-column fitting image*).

Fig. 3 - Total yeast cell concentrations in experiments with strawberry purée (SP) (Δ, $\mu_{max} = 0.16 \pm 0.01\ h^{-1}$) and enriched strawberry purée (ESP) (•, $\mu_{max} = 0.11 \pm 0.01\ h^{-1}$). (*FIGURE 3 should be single-column fitting image*).

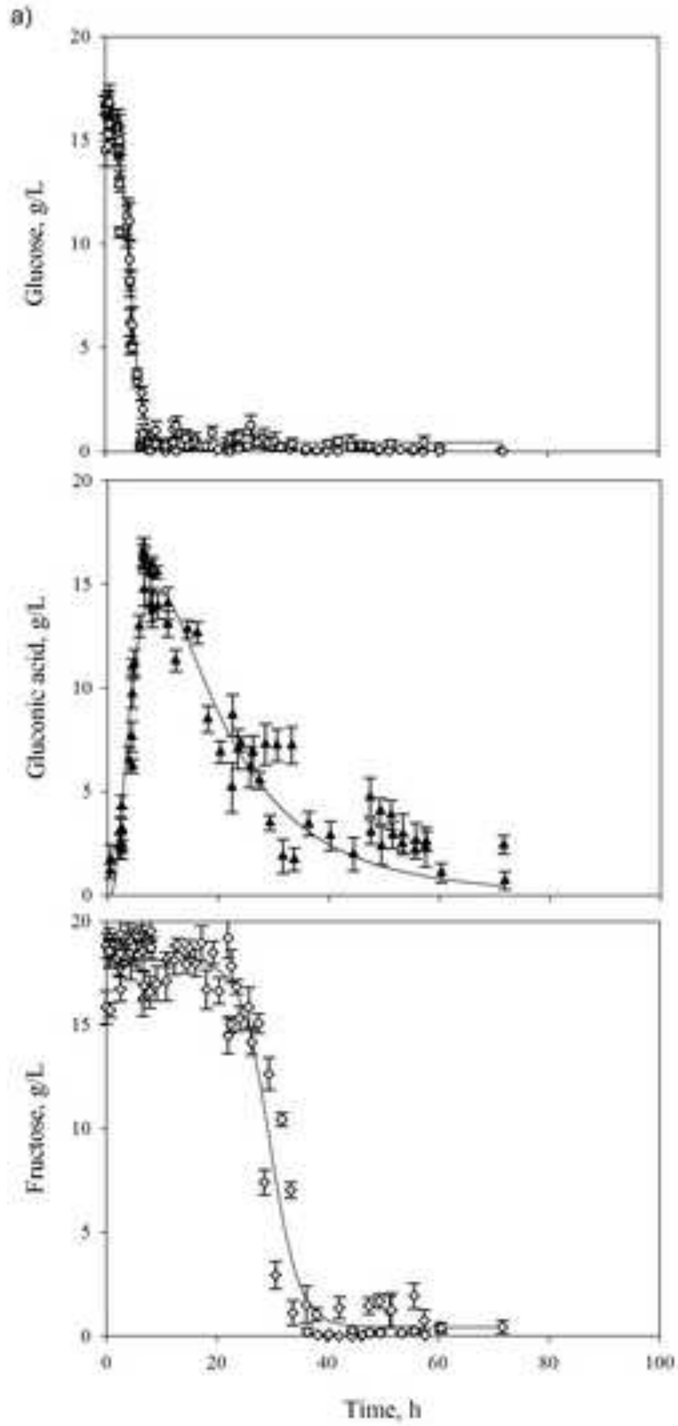
Fig. 4 - Apparent yields of gluconic acid relative to glucose in experiments with strawberry purée (SP) (Δ, Mean value (– – –)= $0.88 \pm 0.11\ g_{gluconic\ acid}/g_{glucose}$) and enriched strawberry purée (ESP) (•, Mean value (–··–) = $0.90 \pm 0.05\ g_{gluconic\ acid}/g_{glucose}$). (*FIGURE 4 should be single-column fitting image*).

Fig. 5 - Variation of dissolved oxygen concentration in experiments with (a) strawberry purée (SP) and (b) with enriched strawberry purée (ESP). (*FIGURE 5 should be single-column fitting image*).

Fig. 6 - Apparent yields in yeast cells per gram of fructose in experiments with strawberry purée (SP) (Δ, Mean value (——) = $6.30 \cdot 10^9 \pm 1.60 \cdot 10^9$ yeast cell/$g_{fructose}$) and enriched strawberry purée (ESP) (•, Mean value (− ··−) = $5.73 \cdot 10^9 \pm 1.70 \cdot 10^9$ yeast cell/$g_{fructose}$). (*FIGURE 6 should be single-column fitting image*).

Fig. 7 – Fructose uptake rate in experiments with strawberry purée (SP) (——) and enriched strawberry purée (ESP) (- - -). F represents fructose concentration in $g_{fructose}$/L. Curves were obtained by computing the first derivatives on best trend lines for fructose in Fig. 1. (*FIGURE 7 should be single-column fitting image*).

Figure 1



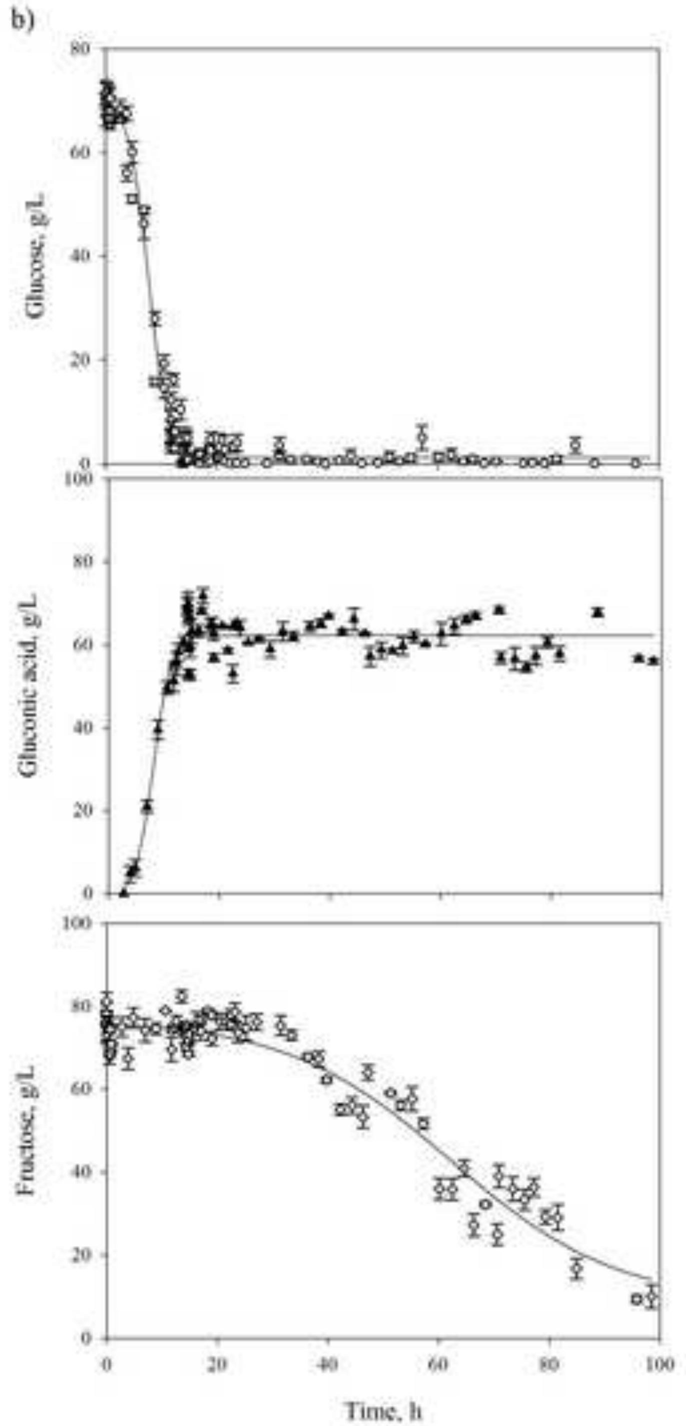
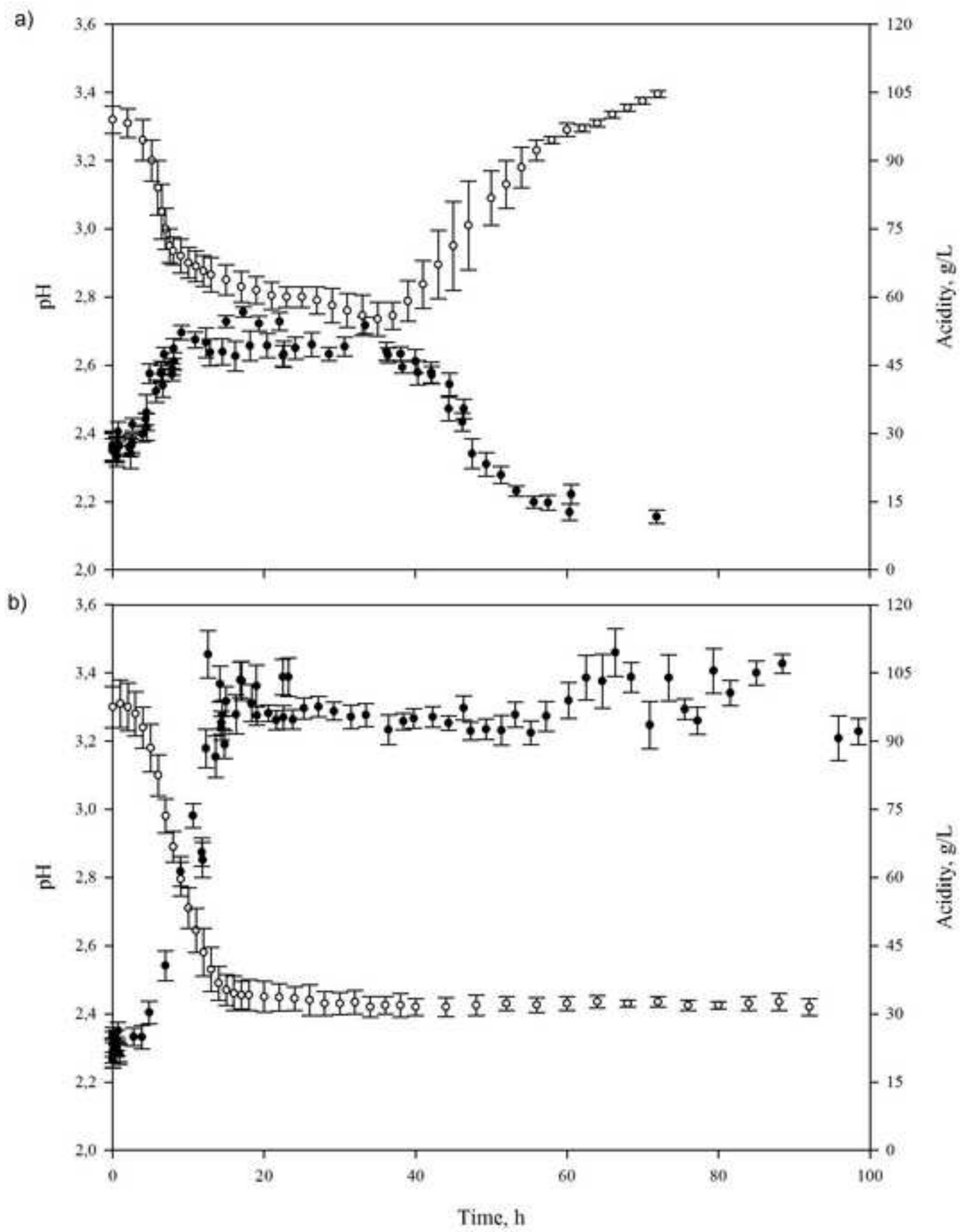


Figure 2



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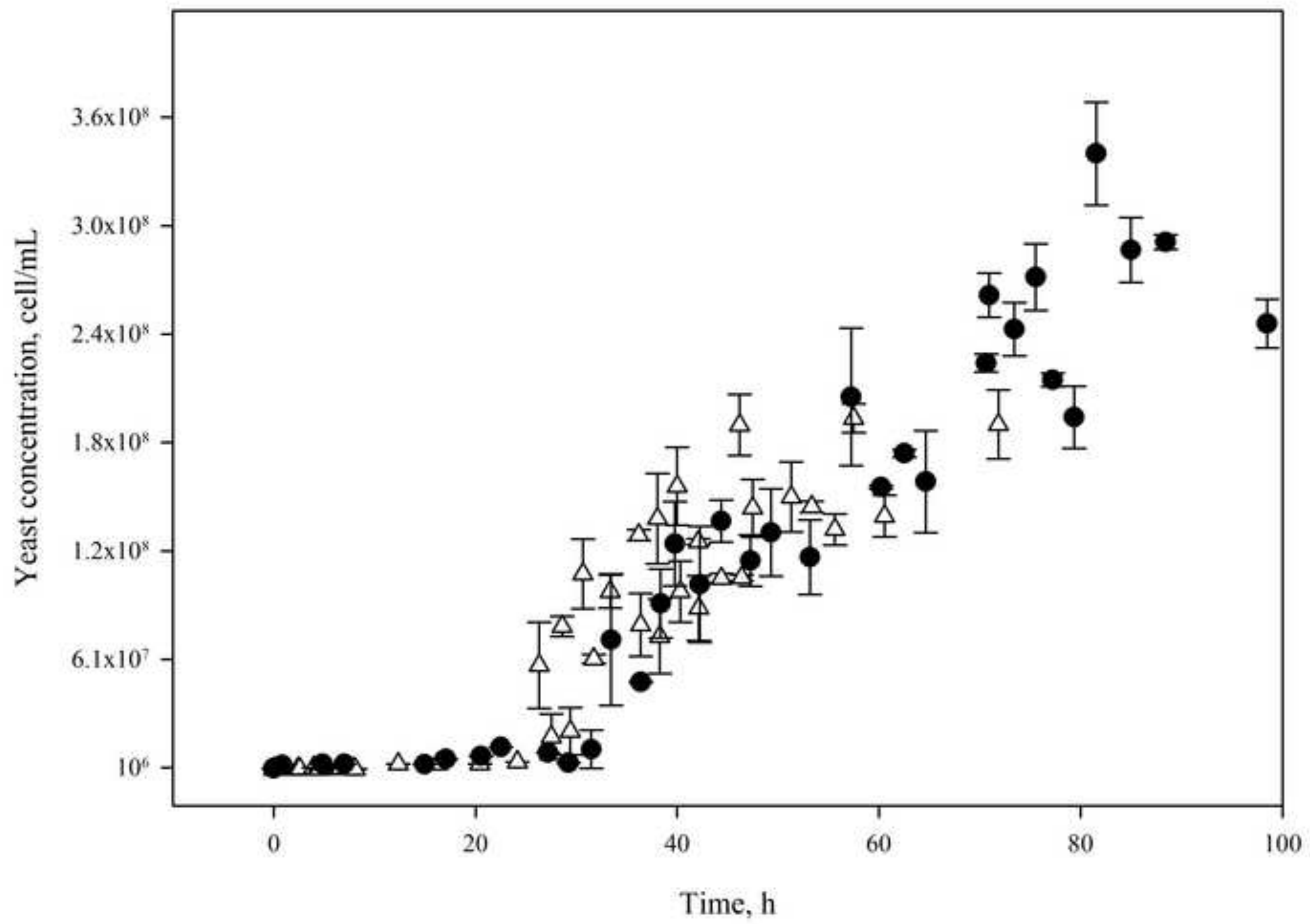

Figure 4

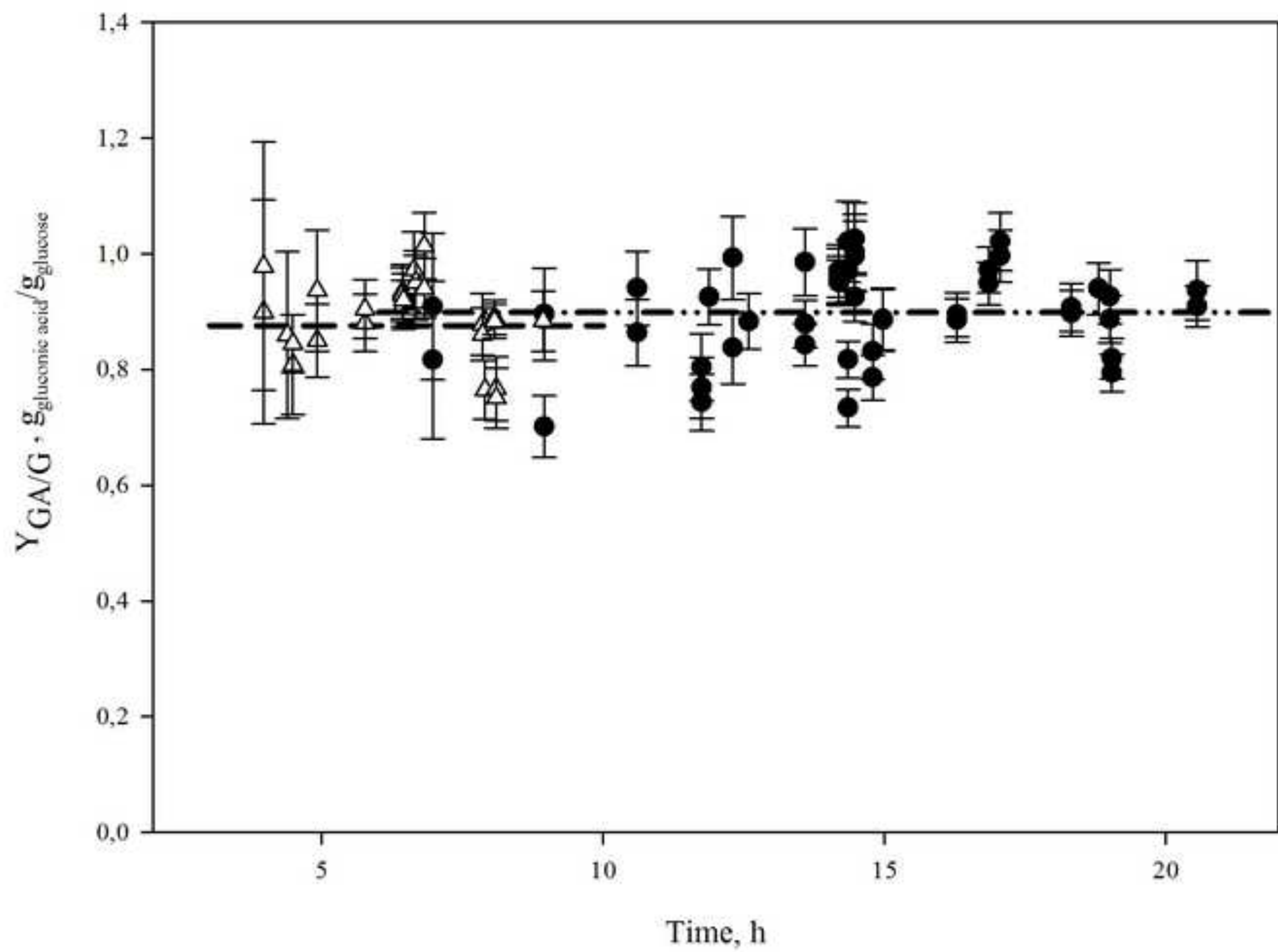

Figure 5

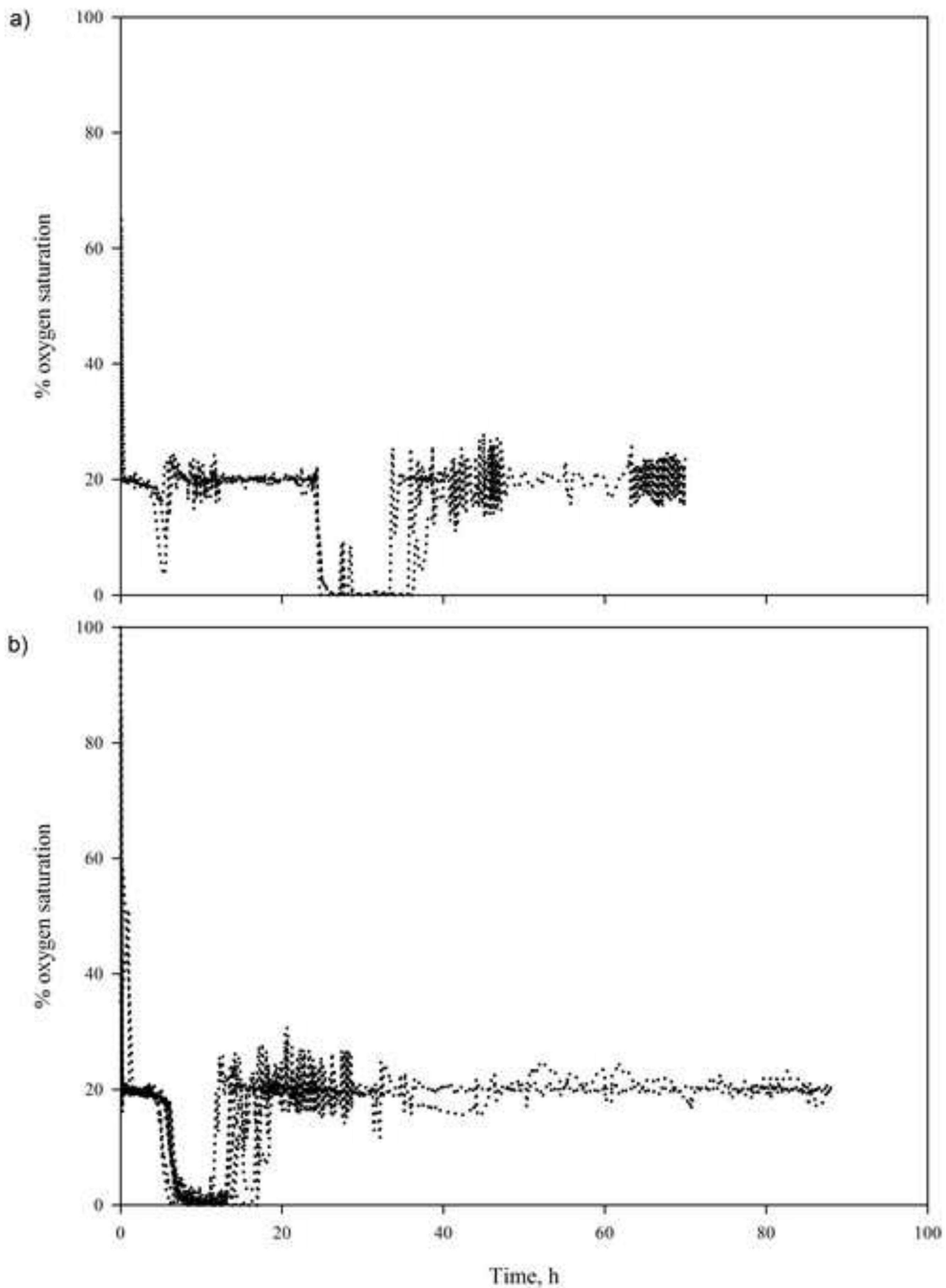

Figure 6

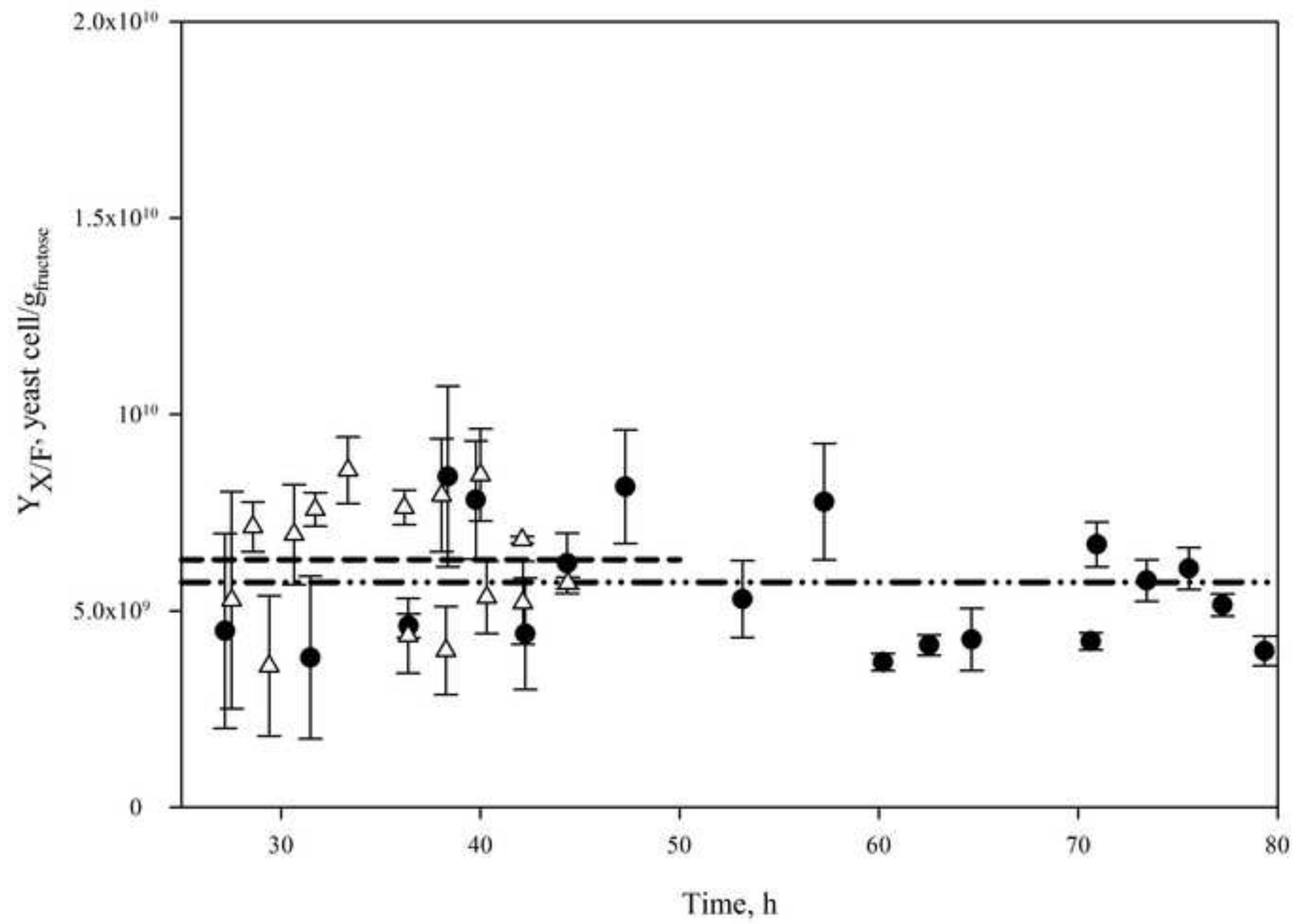

Figure 7