Title

PREPARATION OF A PURE INOCULUM OF ACETIC ACID BACTERIA FOR THE SELECTIVE CONVERSION OF GLUCOSE IN STRAWBERRY PURÉE INTO GLUCONIC ACID

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Abstract

Strawberry surpluses, which may account for about 20 % of the whole production, could be an example of what is happening with other fruits. This surplus is largely transformed into strawberry purée which, although is especially suitable for various bioconversions, is mainly used as an additional ingredient to produce other foods. With a view to using strawberry purée to obtain a new, naturally sweet beverage containing no glucose, the conversion of glucose into gluconic acid, while maintaining the original fructose of the purée, was assessed. Additionally it is important to preserve the sensory and nutritional properties of the fruit so pasteurized rather than sterilized purée must be used.

The use of *Gluconobacter japonicus* strain (CECT 8443) was studied by batch experiments. Different preparation stages were evaluated for ascertaining whether the inoculum required pre-cultivation in strawberry purée and whether using pasteurized rather than sterilized substrate would influence prevalence of the inoculated strain over unwanted microorganisms –yeasts, mainly.

The strain converted glucose into gluconic acid preserving the original fructose content of the substrate. The optimum inoculum preparation conditions involved microbial growth in Glucose Yeast Extract Peptone synthetic medium for 24 h first and in sterilized strawberry purée for 24 h additional then.

Key words

Gluconic acid, *Gluconobacter japonicus* CECT 8443, inoculation, strawberry purée.

1. Introduction

A substantial fraction of strawberry production cannot be sold as whole fruit and results in considerable economic losses in producing regions. Based on the most recently available data, China is the first strawberry producer, with 39.0 % of the world figure, followed by United States, Mexico, Turkey and Spain with 17.7 %, 4.9 %, 4.8 % and 4.1 % respectively (Faostat, 2015).

In Spain, strawberry surpluses, which account for about 20 % of the whole national production, are largely used to obtain purées with or without pips and variable concentration levels that are used to produce other foods. Strawberry purée, with a sugar concentration of 30–200 g/L, is especially suitable for various bioconversions intended to increase its added value.

One potentially viable product from strawberry purée is a new drink resulting from the combination of a strawberry gluconic acid ferment and other strawberry fermentation products such as vinegar (Cañete-Rodríguez et al., 2012; Sainz et al., 2012). The end product should be a non-alcoholic beverage with an appealing aroma and an acid, refreshing taste naturally sweetened by fructose present in the original fruit. By virtue of the decreased glycemic index of fructose relative to glucose, this new product might be especially indicated for individuals on restricted diets.

Research into gluconic acid production has aroused interest for several decades now. In fact, its multiple applications in the food, pharmaceutical and construction industries, among others, have promoted studies on its production and uses (Ramachandran et al., 2006; Singh and Kumar, 2007). The properties of this mild organic acid have propitiated a broad range of applications in the food industry including its use as a pickling, sweetening, acidifying, leavening or flavouring agent. In parallel, the pharmaceutical industry has exploited its properties as a chelating agent for calcium and

iron by using the corresponding salts to treat human deficiencies in these metals (Ramachandran et al., 2006).

Although gluconic acid can be obtained in various ways, fermentation continues being more efficient than chemical procedures. The most widely used microorganism for this purpose is the fungus *Aspergillus niger*; however, the use of fungi for the purposes of producing beverages is not recommended due to two factors: on one side, the low pH and low water activity of the substrate reduce the efficiency of the biotransformation and, on the other side, the risk of producing mycotoxins, which is one of the main concerns for using fungi in the food industry. For these reasons, some acetic acid bacteria (AAB) including *Gluconobacter oxydans* are becoming increasingly important in this respect (Singh and Kumar, 2007).

Based on the foregoing, in this work it has been explored the possibility of biotransforming part of strawberry surpluses into gluconic acid ferment with a view to obtaining a complex product for the production of new beverages. The first challenge met in the process was obtaining gluconic acid from glucose in strawberry purée while retaining the fructose content of the fruit as far as possible. Although AAB cultures produce gluconic acid, their ability to further oxidize it to ketogluconic acids (Beschkov et al., 1995; Stubbs et al., 1940) has led the food industry to favour use of the fungus. However, the need to preserve the fructose content of the fruit and avoiding the health hazards of fungi while minimizing ketogluconic acid formation via controlled fermentation conditions (viz., pH, sugar concentrations) (Beschkov et al., 1995; Levering et al., 1988; Ramachandran et al., 2006) has promoted research into the potential of AAB for obtaining gluconic acid ferments (Gullo and Garcia-Garcia, 2012; Raspor and Goranovic, 2008). It is well-known the mechanism by which many *Gluconobacter* strains use glucose (Ano et al., 2011; Attwood et al., 1991; Beschkov et

al., 1995; Deppenmeier and Ehrenreich, 2009; Olijve and Kok, 1979a, 1979b; Velizarov and Beschkov, 1994; Weenk et al., 1984). Evidences have been presented of a mechanism that determines the partitioning of glucose metabolism through the direct oxidative pathway to gluconic acid (overflow metabolism) and the pentose phosphate pathway; the carbon flux through each pathway is quite dependent on pH and glucose concentration. For instance, the pentose phosphate pathway is importantly repressed at pH values below 3.5 and above a threshold value of 0.9 – 2.7 g glucose/L, resulting, in these cases, in an accumulation of gluconic acid in the medium.

Preserving the properties of the original fruit requires using very mild conditions in the thermal pretreatment of strawberry purée (Gossinger et al., 2009; Hartmann et al., 2008; Klopotek et al., 2005; Patras et al., 2010) that are more akin to pasteurization than to sterilization. Then, a pasteurization treatment allows the efficient reduction of the population size of microorganisms present in the purée but it does not eliminate them completely. Therefore, if no acetic acid bacteria are inoculated and glucose remains in the medium, the low population of yeasts present in the purée after pasteurization is able to grow and ferment the glucose. However, they need a period of time to reach the appropriate population for the alcoholic fermentation of glucose and fructose into ethanol as it will be evidenced in this work.

The primary aim of this work was to optimize the preparation conditions of an AAB inoculum for transforming glucose in pasteurized strawberry purée into gluconic acid while retaining bioactive compounds in the substrate in order to maintain the healthy properties of the fresh fruit and introduce new sensory characteristics. This entailed solving two major problems, namely: the well-known difficulty of culturing AAB (De Vero and Giudici, 2013; De Vero, 2012) and the potentially deleterious effects of other microorganisms present in the raw material.

2. Material and methods

2.1. Raw material

The raw material used was commercial strawberry purée obtained from HUDISA S.A. (Lepe, Spain). The purée was industrially pasteurized at 92 ºC for 90–120 s and stored at 0–4 ºC. Alternatively, the purée can be concentrated by evaporation at low pressure. The purée used contained approximately 50 g sugars/L in a glucose:fructose ratio of ca. 1:1.

2.2. Microorganism

The *Gluconobacter japonicus* strain used (CECT 8443) was isolated from grape must collected in the Mas dels Frares Experimental Cellar (Constantí, Tarragona, Spain) (Navarro et al., 2013). Additionally, in a study using strains from different species of *Gluconobacter* and *Acetobacter* genera, this strain was selected on the grounds of its high rate of transformation of glucose into gluconic acid and the no utilization of fructose in strawberry purée (Navarro, 2011).

2.3. Fermenter

Fermentation runs were conducted batchwise in a Biostat® 5 L fermentation tank equipped with pH, agitation, dissolved oxygen and temperature controls. An average loading volume of 3 L was used. Based on mentioned bibliography references, cultures were kept at 29 ºC, an agitation rate of 500 rpm was used and the pH of the medium was allowed to evolve freely during the cycle, decreasing from a value of 3.35 at the beginning up to a final value of 2.9. Additionally, an initial dissolved oxygen concentration of 20 % the saturation level was used in order to minimize volatile losses

in each run. On the other hand, because the fermentation medium used in most of the experiments was not sterilized, the equipment required thorough cleaning between batches. For experiments under sterile conditions, the fermenter was loaded with medium and sterilized at 121 ºC for 15 min. All tests were performed at least in triplicate and most in quintuplicate.

2.4. Preparation of inocula

CECT 8443 strain was stored frozen at –80 ºC in a 50:50 (v/v) water/glycerol mixture as cryoprotectant. Prior to use, the microorganism was reactivated by thawing, seeded in 125 mL of GYP liquid medium [50 g/L glucose, 10 g/L yeast extract and 20 g/L bacteriological peptone] and then in tilted tubes containing GY agar solid medium [50 g/L glucose, 10 g/L yeast extract and 30 g/L agar], from which the experimental culture was obtained.

Subsequently, 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, each flask was supplied with 125 mL of either sterilized strawberry purée or GYP liquid medium. After 24 h of additional incubation, the inoculum was ready for addition to the fermentation tank; the fermenter was previously loaded with 3 L of strawberry purée that was either industrially pasteurized or additionally sterilized by autoclaving at 121 ºC for 15 min in our laboratory.

2.5. Determination of cell concentrations

Total concentrations of cells were determined by direct counting under a microscope, using a Neubauer chamber of special depth (0.02 mm) as described elsewhere (Baena-Ruano et al., 2006).

2.6. Determination of sugars and gluconic acid

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 manufacturer's website (Megazyme, 2014). All determinations were performed at least in triplicate. The ensuing standard deviations are shown in Figs. 1 and 2.

2.7. Fitting of experimental data

Comparison of the results was facilitated by using regression procedures to identify the equations most closely fitting the experimental data. Significant differences between parameters and whole curves were determined by analysis of variance (ANOVA). Computations were done with the advanced scientific graphing and statistical analysis platform SigmaPlot® for Windows ver. 11.0 (Sigmaplot, 2014).

3. Results

This work was aimed at ascertaining whether the inoculum required pre-cultivation in strawberry purée and whether using pasteurized rather than sterilized substrate would influence prevalence of the inoculated AAB strain. It should be noted that using pasteurized purée was intended to preserve the sensory properties of the fruit as far as possible —this would have been impossible with a more drastic thermal treatment such

as sterilization, which would have denatured the fruit to a variable extent (Gossinger et al., 2009; Klopotek et al., 2005; Patras et al., 2010).

The inoculum used in the first experiment, designated E1, was prepared in two stages, namely: (1) microbial growth in 125 mL of GYP medium for 24 h and (2) further growth for 24 h after addition of an identical volume of GYP. The resulting inoculum was added to the fermentation tank, which had previously been loaded with 3 L of pasteurized strawberry purée.

The second experiment, E2, only differed from the first one in that the second stage used 125 mL of sterilized strawberry purée instead of GYP medium.

In the third experiment, E3, the inoculum was prepared as in E2 but it was added to a fermenter containing 3 L of sterilized strawberry purée.

Experiments E1–E3 were stopped once the glucose had been depleted.

Finally, a negative control experiment, designated WI, using not inoculated pasteurized strawberry purée, was allowed to develop in the fermenter until all sugar was depleted.

By way of example, Fig. 1 shows the variation of glucose, gluconic acid and fructose concentrations in E1, with dots representing experimental values.

For further comparison, the glucose and gluconic acid concentrations were fitted to the following general function in order to obtain the solid lines in the figures:

$$concentration = y_0 + \frac{a}{1+e^{-\frac{t-t_0}{b}}} \qquad (1)$$

Fig. 2 shows the fitted curves for glucose, gluconic acid and fructose in E1, E2, E3 and WI.

Table 1a shows the fitted parameter values for glucose in Eq. (1), with a and y_0 denoting the glucose concentration at time zero and infinity, respectively. As expected, there were no significant differences in either parameter between treatments. Parameters b

and t_0 are related to the falling slope of the curve, the latter representing the time at which the glucose concentration was halved. As can be seen from Fig. 2 and is confirmed by the data of Table 1a, t_0 was dependent on the experimental conditions.

Given that the experiment without inoculum (WI) additionally consumed fructose, Fig. 2 shows the corresponding fitted curve and Table 1b the fitted parameter values for Eq. (1).

Table 2 shows the fitted parameter values for gluconic acid in Eq. (1). The meaning of the fitting parameters was similar to those of b and t_0. In this case, the inverse relationship between the glucose and gluconic acid concentrations resulted in opposite slopes; also, parameters a and y_0 exchanged their meaning.

Comparing the glucose uptake and gluconic acid formation results allowed the apparent gluconic acid yield to be estimated. By way of example, Fig. 3 shows the results for E1. The slope must coincide with the gluconic acid yield from glucose, $Y_{GA/G}$ (see Table 3). There was no significant difference between gluconic acid yields; the mean value, 0.83± 0.01, was 76.1± 0.9 % of the maximum possible (stoichiometric) yield: 1.09 g gluconic acid/g glucose.

The variation of *G. japonicus* cell concentrations throughout the experiments clearly confirmed growth of the bacterium (results not shown). The special properties of the fermentation medium (a purée) led us to perform cell counts at least in quintuplicate; even so, standard deviations were high.

Fig. 4 shows the total yeast cell concentrations found in the different experiments.

4. Discussion

The results obtained differed with the inoculum preparation method. As can be seen from the glucose data in Fig. 2, allowing the microorganism a pre-cultivation in

strawberry purée (E2) provided better results in kinetic terms than using an inoculum cultured in only GYP medium (E1). In fact, the glucose curve for E2 was left-shifted with respect to E1 (i.e., E2 took less time to reach the same final results as E1). As expected, an identical conclusion was drawn from gluconic acid production. The main differences between curves were in parameter t_o (see Tables 1 and 2); thus, the time needed for half the initial amount of glucose was roughly 28 % longer in E1 than in E2.

Also, as can be seen by comparing the t_o values of Tables 1 and 2, no statistically significant differences between experiments were found; therefore, the time needed for the glucose concentration to be halved coincided with that required for half of the final gluconic acid concentration to be reached. There was thus no any delay in gluconic acid formation following glucose uptake, so glucose was very rapidly converted into gluconic acid via glucone-δ-lactone (Olijve et al., 1979a).

It is also important with a view to obtain a naturally sweet beverage that no fructose uptake was observed in E1, E2 or E3 (Fig. 2).

Taking into account that E1 and E2 were carried out with pasteurized strawberry, it is important to elucidate which are the microorganisms potentially responsible for the activity observed. As can be seen from Fig. 4, *G. japonicus* was accompanied by apparently non-growing yeasts in E1 and E2. Because yeast population size was only assessed by counting under a microscope, the presence of small solid particles from strawberry purée may have led to overestimation. The microorganisms involved in WI were identified by plating samples on GY agar medium and found to be *Pichia kudriavzevii* yeasts (yellow colonies) and, to a lesser extent, *Saccharomyces cerevisiae* yeasts (white colonies).These results are unsurprising since yeasts are usually the main contaminants in the microflora associated to spoilage of fruit and vegetable juices (Patrignami et al., 2009). *Pichia kudriavzezii* was previously isolated from various

foods and fruit sources such as fermented pineapple juice (Chanprasartsuk et al., 2010), orange juice (Arias et al., 2002) and grapes (Zott et al., 2010). Hsu et al. (2014) conducted controlled laboratory studies of strawberry purée with initial microbial counts in untreated purée of 2.9–3.4 $\log_{10}$ CFU/g and found no survivors of yeasts and moulds (YM) to be recovered from samples after heating at 60 ºC for more than 80 s; however, the degree of inactivation was affected by other factors including the sugars —log counts of surviving YM increased slightly with increasing sugar concentration. Interestingly, Tournas et al. (2006) found industrial pasteurized fruit juice samples to contain YM at concentrations from 1 to almost 7 $\log_{10}$ CFU/mL, and yeasts to be the main contaminants present. As stated in Section 2.5, however, the data of Fig. 4 are total concentrations of cells as determined by direct counting under a microscope. In fact, no yeast colonies were recovered from the original raw medium (pasteurized strawberry purée) plated on GY. This, however, only means that any yeasts present — and visible under the microscope— were unable to grow in the culture media. It has been well established that these yeasts in viable but not cultivable statuses could grow once the conditions are favourable (McDougald et al., 1998) as it could be the WI fermentation, where no competitors were present. Clearly, after growing for 22 h, viable populations were large enough to start using available sugar in measurable quantities. The significance of this work arises from the fact that industrial purée instead of laboratory preparations was used. In fact, industrial preparations are very likely to contain unwanted microorganisms, then, allowing the *G. japonicus* inoculum a pre-cultivation in industrial media efficiently helps to prevent growth of such unwanted microorganisms and expedites the desired conversion of glucose into gluconic acid.

Regarding the previously addressed question of whether part of the glucose uptake in E1 and E2 was due to the presence of yeasts, it can be found some clues by comparing

the results of E2 and E3. Thus, as can be seen from Fig. 4, sterilizing the strawberry purée in the fermenter before inoculation (E3) prevented the presence of yeasts; therefore, the activity observed in this experiment can only be ascribed to *G. japonicus*. The absence of statistically significant differences between E2 and E3 (see Fig. 2 and Tables 1–3) suggests that yeast activity in E2, if any, was negligible. This assumption is supported by the fact that no significant differences in gluconic acid yield from glucose ($Y_{GA/G}$) were found with similar total glucose uptake and gluconic acid production values (Table 3). The yields were similar to those previously obtained by other authors using *G. oxydans* in sterilized synthetic media (Velizarov and Beschkov, 1998; Velizarov and Beschkov, 1994).

Based on the results of WI (Figs. 2 and 4), which used pasteurized strawberry purée but no *G. japonicus* inoculum, no glucose or fructose consumption occurred before about 25 h —the time needed for the non-cultivable yeast to reach an adequate population size.

Then, medium-adapted *G. japonicus* was able to rapidly convert glucose into gluconic acid and the biological activity in E1 and E2 was primarily due to *G. japonicus*.

5. Conclusions

The results obtained in this work suggest that the *G. japonicus* strain used (CECT 8443) is effective in selectively converting glucose in strawberry purée into gluconic acid while preserving the original fructose content of the substrate —a crucial requirement with a view to developing new naturally sweet products containing no glucose when industrial strawberry purée is used.

Additionally, preserving the sensory properties of the raw material requires using pasteurized rather that sterilized strawberry purée so that the AAB strain must be able to

prevail over other, unwanted, microorganisms inevitably present in industrial pasteurized strawberry purée. Thus, the inoculum must be prepared in two stages involving: (1) allowing the microorganism to grow in GYP medium for 24 h and (2) adding an identical volume of sterilized strawberry purée to the medium and continuing incubation for a further 24 h (i.e., the procedure used in E2).

Although an inoculum prepared as in E1 could be equally useful, fermentation would take more than about 20 h, thereby increasing the risk of yeasts, surviving industrial pasteurization, playing a substantial role in the observed bio-transformations.

Further research is required to check the stability of the produced gluconic acid and of the remaining fructose content.

6. Acknowledgements

The authors are grateful to Spain's Ministry of Science and Innovation (Projects AGL2010-22152-CO3-02 and AGL2010-22152-CO3-03) and to Junta de Andalucia and University of Cordoba (Program: "Fortalecimiento de las Capacidades de I+D+I de la UCO", RNM271).

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Table 1a. Parameter values for fitting the experimental glucose concentration data.

Exp.	*a*		*b*		t_o		y_o		R	P
	Value	SE	Value	SE	Value	SE	Value	SE		
E1	22.075	0.610	-2.257	0.144	8.117	0.168	0.480	0.223	0.996	0.1079
E2	22.287	0.829	-1.880	0.212	6.359	0.206	0.269	0.345	0.997	0.1913
E3	24.007	0.810	-1.985	0.136	5.733	0.181	-0.011	0.213	0.998	0.0010
WI	22.109	0.879	-1.070	0.184	29.486	0.253	0.119	0.803	0.989	0.0365

SE: standard error
R: regression coefficient
P: Constant variance test
t: time, h
G: glucose concentration, g/L
Function: $G = y_0 + a/\{1 + exp[-(t - t_0)/b]\}$

There is no a statistically significant difference among values for parameters "a" and "y_o" at 95 % confidence level.

There is no a statistically significant difference among values for parameter "b" at 95 % confidence level for experiments E1, E2 and E3; nevertheless, statistically significant differences were found when comparing these experiments with WI.

For experiments E1, E2 and E3, statistically significant differences were found for all pairwise multiple comparisons of values of parameter "t_o" (Student-Newman-Keuls Method; $p<0.05$). Nevertheless, by using the Tukey Test (a more conservative test than the Student-Newman-Keuls Method), there is no a statistically significant difference for this parameter when comparing experiments E2 vs. E3.

Statistically significant differences were found among values for parameter "t_o" when comparing experiment WI vs any of E1, E2 or E3.

Table 1b. Parameter values for fitting the experimental fructose concentration data.

Exp.	*a*		*b*		t_o		y_o		R	P
	Value	SE	Value	SE	Value	SE	Value	SE		
WI	23.850	0.330	-0649	0.071	31.277	0.072	-0.226	0.310	0.998	0.4050

For experiment WI, there is no a statistically significant difference among values for parameters "a", "b" and "y_o" at 95 % confidence level when comparing the fitting for glucose and fructose. At the same time, a statistically significant difference was found for parameter "t_o"

Table 2. Parameter values for fitting the experimental gluconic acid concentration data.

Exp.	*a*		*b*		t_o		y_o		R	P
	Value	SE	Value	SE	Value	SE	Value	SE		
E1	17.9202	0.9301	1.9621	0.2749	8.7550	0.3011	2.1377	0.6087	0.9908	0.0439
E2	17.0830	1.1340	1.2850	0.2900	6.6480	0.300	2.8320	0.7370	0.9860	0.3558
E3	16.5320	0.6290	1.1070	0.1550	6.017	0.1730	3.1590	0.4900	0.9980	0.7418

SE: standard error
R: regression coefficient
P: Constant variance test
t: time, h
GA: gluconic acid concentration, g/L
Function: $GA = y_0 + a/\{1 + exp[-(t - t_0)/b]\}$

There is no a statistically significant difference among values for parameters "a", "b" and "y_o" at 95 % confidence level.

For all pairwise multiple comparisons of values of parameter "t_o" (Student-Newman-Keuls Method; $p<0.05$), statistically significant differences were found only when comparing experiments E1 vs. E2 and E1 vs. E3.

Table 3. Parameter values for the linear fitting of gluconic acid formation versus glucose uptake data.

Exp.	$Y_{GA/G}$)		R
	Value	SE	
E1	0.838	0.019	0.9786
E2	0.849	0.031	0.9799
E3	0.789	0.023	0.9823
Mean	0.825	0.014	

$Y_{GA/G}$: gluconic acid yield from glucose, g gluconic acid/g glucose
SE: standard error
R: regression coefficient
There is no a statistically significant difference among values for parameter "$Y_{GA/G}$" at 95 % confidence level.

Figure 1

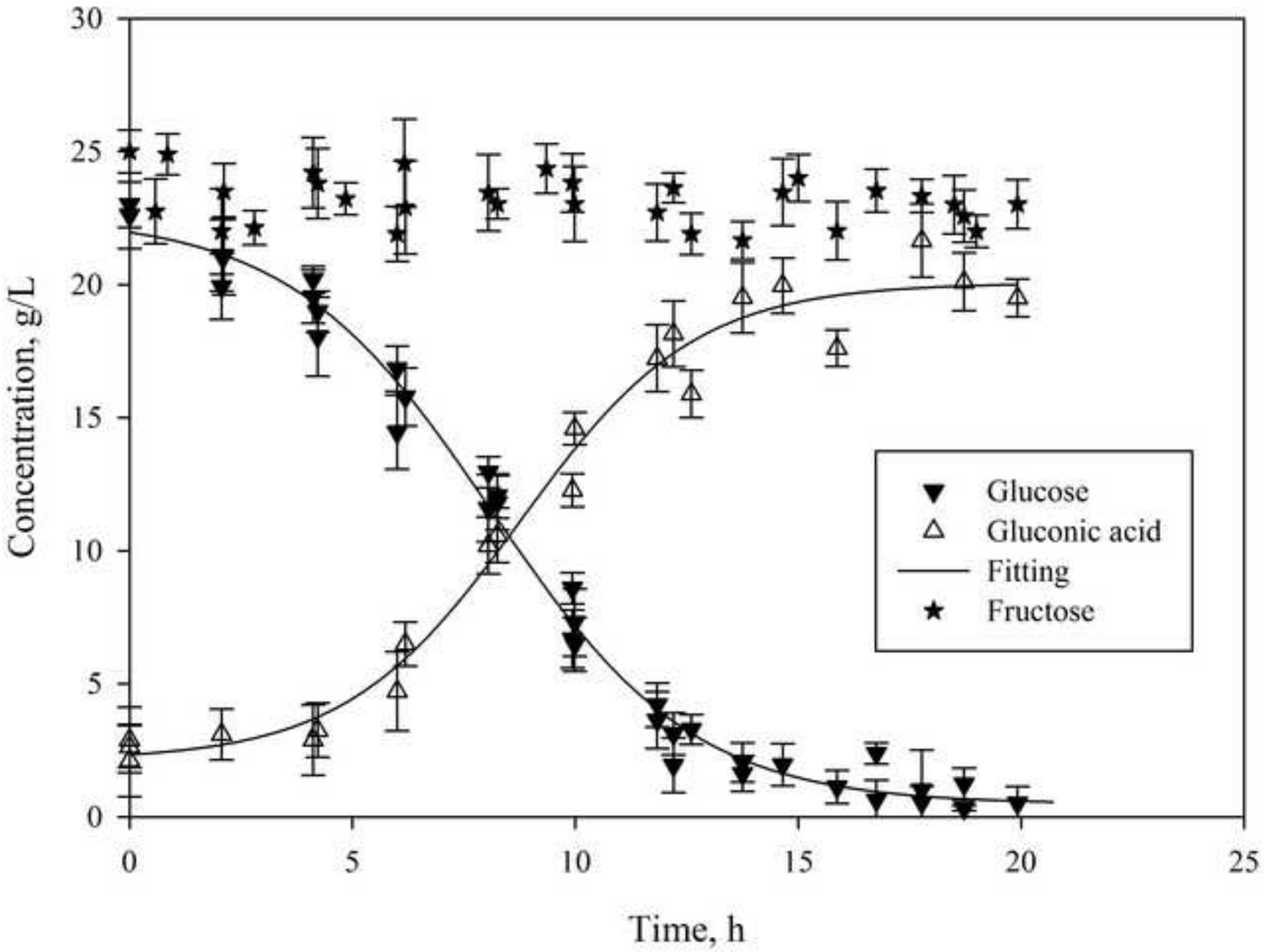

Concentration, g/L
Time, h
0
5
10
15
20
25
30
Glucose
Gluconic acid
Fitting
Fructose

Figure 2

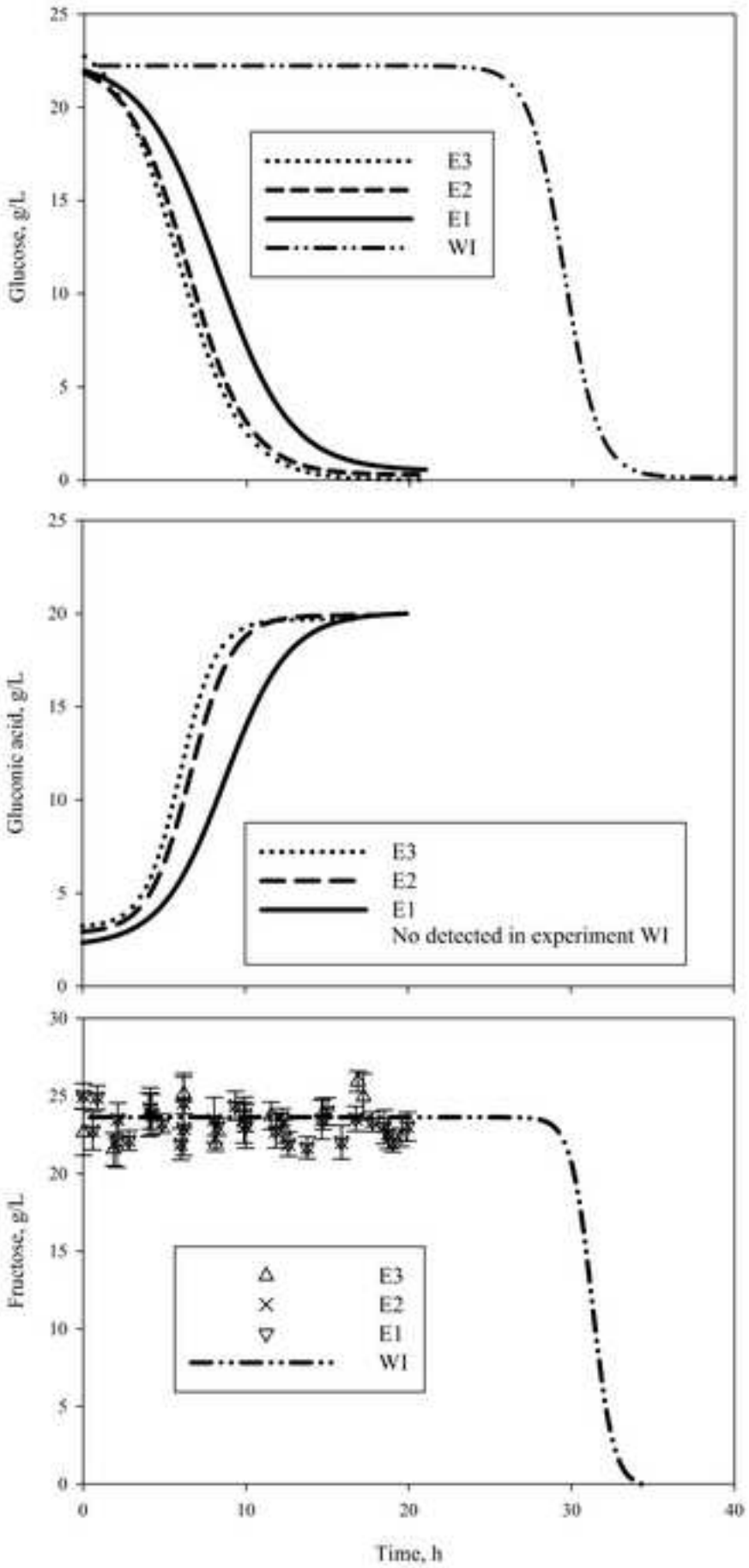

Glucose, g/L
25
20
15
10
5
0
E3
E2
E1
WI
Gluconic acid, g/L
25
20
15
10
5
0
E3
E2
E1
No detected in experiment WI
Fructose, g/L
30
25
20
15
10
5
0
E3
E2
E1
WI
0
10
20
30
40
Time, h

Figure 3

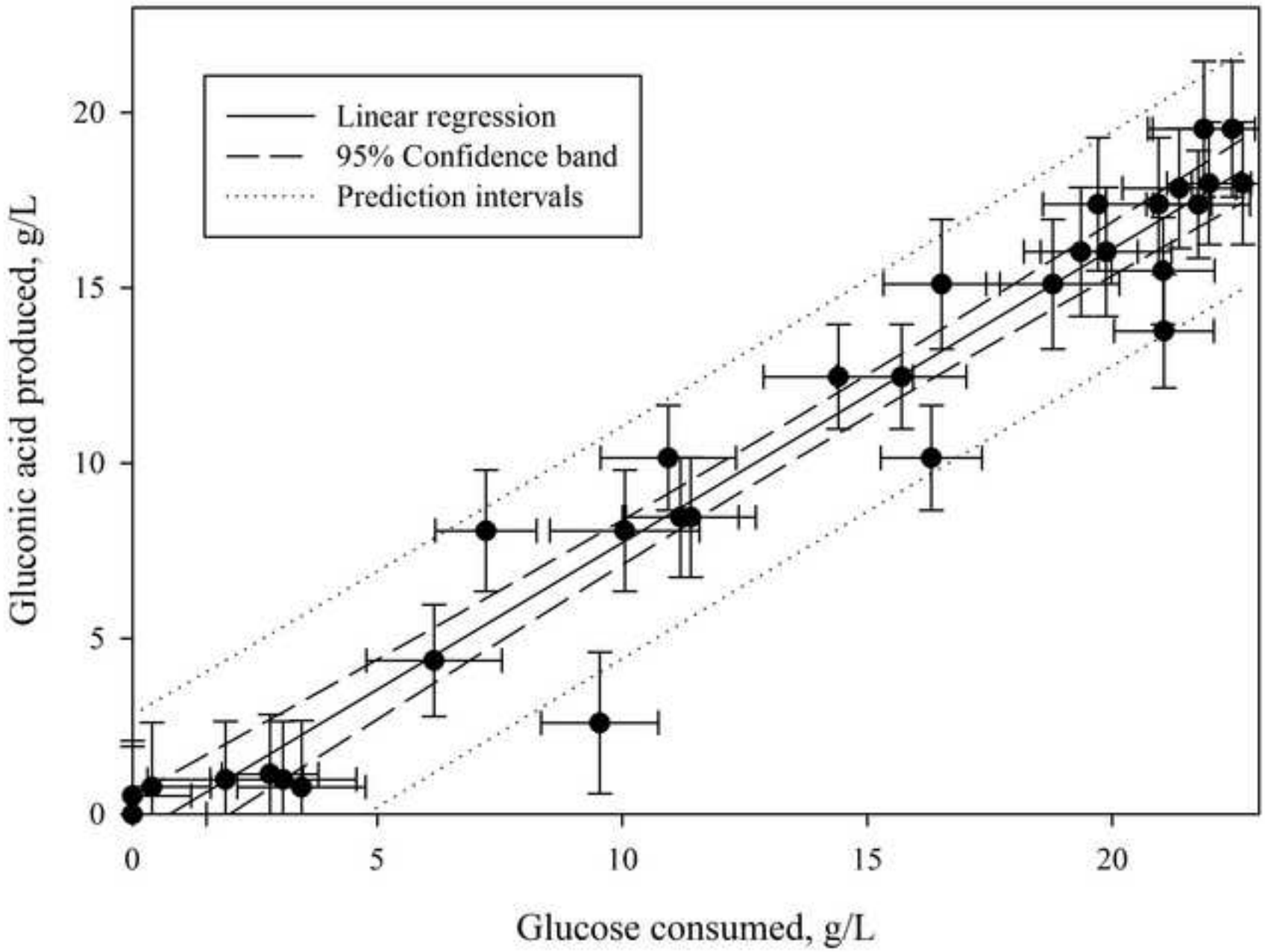
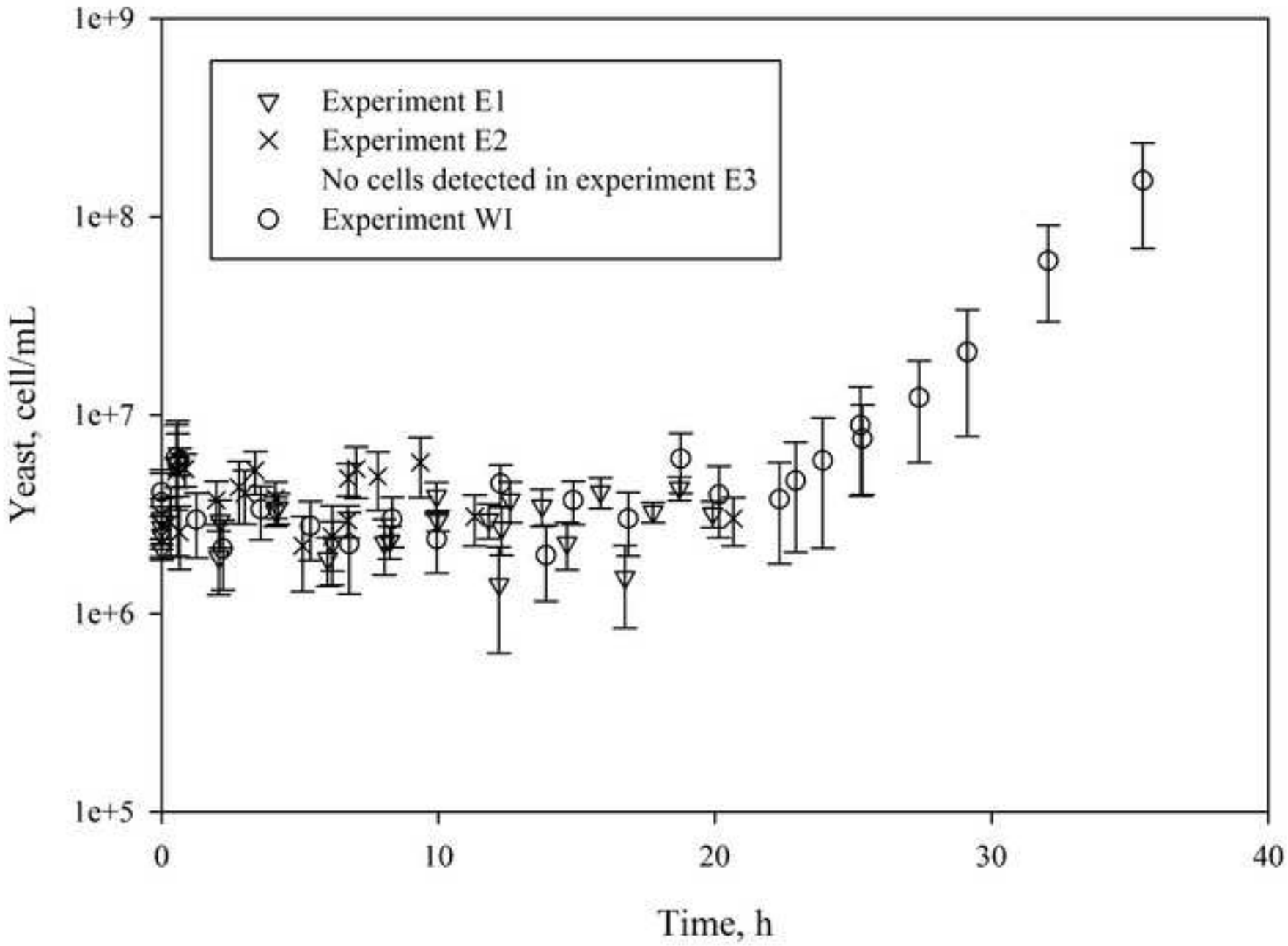

Linear regression
95% Confidence band
Prediction intervals
Gluconic acid produced, g/L
Glucose consumed, g/L
0
5
10
15
20

Figure 4


1e+9
1e+8
1e+7
1e+6
1e+5
Yeast, cell/mL
0
10
20
30
40
Time, h
Experiment E1
Experiment E2
No cells detected in experiment E3
Experiment WI