Title: An approach for estimating the maximum specific growth rate of *Gluconobacter japonicus* in strawberry purée without cell concentration data.

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ABSTRACT

The estimation of the maximum specific growth rate (μ_{max}) for non-readily culturable bacteria, growing on complex media containing suspended solids, is a difficult task considering the important problems in obtaining reliable measures of cell concentration. An example of this situation can be a culture of *Gluconobacter japonicus* growing in strawberry purée for producing gluconic acid. Based on the dependency between energy requirements of the genus *Gluconobacter* and substrate uptake as well as its constant relationship between gluconic acid production and total substrate uptake, the total substrate concentration profile during the exponential growth phase could be used for estimating μ_{max} without cell concentration measures. In this case, the high selectivity of the strain for glucose in comparison to fructose resulted in no fructose consumption during the batch; so, just using the glucose concentrations data during the exponential phase allow us to obtain an estimation of μ_{max}. Additionally, a rough estimation of the apparent and stoichiometric yields of cell on glucose is also possible.

Key words

Maximum Specific Growth Rate; *Gluconobacter japonicus* CECT 8443; Growth Kinetics; Biokinetics; Biotransformations; Batch Processing

Mathematical notation

G: total glucose concentration, $g \cdot L^{-1}$

GA: gluconic acid concentration, $g \cdot L^{-1}$

G_i: total initial glucose concentration, $g \cdot L^{-1}$

$(G_i)_g$, initial glucose concentration to be used for growth and other uses, $g \cdot L^{-1}$

G_{ov}: glucose concentration used for gluconic acid production, $g \cdot L^{-1}$

G_g: glucose concentration for growth and other uses, $g \cdot L^{-1}$

K_G: Monod constant, $g \cdot L^{-1}$

m_G: cell maintenance coefficient, g glucose$\cdot cell^{-1} \cdot h^{-1}$

q_G: term of proportionality between the glucose uptake rate and the concentration of viable cells, g glucose$\cdot cell^{-1} \cdot h^{-1}$

t: time, h

TA: total acidity, $g \cdot L^{-1}$

X: total cell concentration, $cell \cdot L^{-1}$

X_v: viable cell concentration, $cell \cdot L^{-1}$

X_{vi}: initial viable cell concentration, $cell \cdot L^{-1}$

$Y_{GA/G}$: apparent yield of gluconic acid on total glucose, g gluconic acid$\cdot$(g glucose)$^{-1}$

$Y_{X/G}$: apparent yield of cells on total glucose, cell$\cdot$(g glucose)$^{-1}$

$Y_{X/Gg}$, yield of cells on glucose, cell$\cdot$(g glucose)$^{-1}$

μ_c: specific growth rate, h^{-1}

μ_{max}: maximum specific growth rate, h^{-1}

$(\mu_{max})_{est}$: estimated maximum specific growth rate using Eq. (6), h^{-1}

1. INTRODUCTION

The study and design of many microbiological processes use to need information about their kinetic aspects, being the maximum specific growth rate a particularly interesting parameter in this regard. Nevertheless, for non-readily culturable bacteria as well as bioprocesses in which natural media, containing solids in suspension, are used as substrate, the resulting complex bioprocess matrix complicates very much obtaining this information because of the difficulties in measuring cell concentrations. This is the case for a culture of *Gluconobacter japonicus* growing on strawberry purée in the scope of the production of a gluconic acid-based ferment. As many other acetic acid bacteria [1-3], *G. japonicus* is difficult to cultivate under standard laboratory conditions by forming colonies on an agar-based medium, so this fact could give rise to underestimation of the total and active cell concentration. The problem of "viability" and "culturability" has been often dealt with [2, 4, 5]. To overcome these disadvantages, other, chemical or physical, methods could be used to quantify bacteria [5-9]. For instance, the interest for designing on-line monitored and controlled bioprocesses has encouraged the development of, mainly physical, on-line biomass monitoring devices. From these stand out capacitance probes based on dielectric spectroscopy [8, 9]; this methodology seems to offer some advantages over others, it is able to differentiate viable and nonviable cells.

On the other side, epifluorescence microscope methods have been used for bacterial enumeration, for instance, direct epifluorescence microscope counts have proved that could be a reliable, rapid and easy to use method for quantifying total microbial biomass as well as giving an estimation of the percentage of viable acetic acid bacteria from acetators [7, 10-12]. Nevertheless, with complex partially insoluble media, this method cannot be used otherwise the significant amount of suspended solids could be partially

accounted as cells. So, among others [6], biochemical traits could be used, as an alternative, for measuring bacterial growth in these complex media; for instance, carbohydrate utilization profile could be useful in this regard for many microorganisms. In this work, an easy method for estimating the maximum specific growth rate of *G. japonicus* in strawberry purée without using cell concentration measurements is employed; the method just uses the common strategy of combining the cell and substrate balances, a direct substrate uptake and bacterial growth association is required.

2. MATERIALS AND METHODS

2.1. Raw material

As raw material, commercial strawberry purée from Hudisa S.A. (Lepe, Spain), containing approximately 50 g sugars·L^{-1} in a glucose:fructose ratio of ca. 1:1, was used.

2.2. Microorganism

A strain of *G. japonicus* (CECT 8443), which was isolated from grape must from the Mas dels Frares Experimental Cellar (Constantí, Tarragona, Spain) [13], was used.

2.3. Fermenter

Fermentation runs were conducted batch-wise in a Biostat A 5 L fermentation tank from Sartorius-Stedim Biotech (www.sartorius.us/us) properly equipped for measurement and control of pH, agitation, dissolved oxygen and temperature. An average volume of 3 L was used. Agitation at 500 rpm and a temperature of 29 ºC were set; the pH of the medium was allowed to evolve freely throughout. The experiments were carried out, in

triplicate, under sterile conditions; so, the fermenter containing the medium was sterilized at 121 ^{o}C for 15 min.

2.4. Preparation of inocula

According to Cañete-Rodriguez et al. [11], the inoculum was prepared by seeding in 250 mL Erlenmeyer flasks containing 125 mL of GYP liquid medium (50 g glucose L^{-1}, 10 g yeast extract L^{-1} and 20 g bacteriological peptone L^{-1}) that was previously autoclaved at 121ºC for 15 min. After shaking in an incubator at 29 ºC and 150 rpm for 24 h, the flask was supplied with 125 mL of sterilized strawberry purée. After 24 h of additional incubation, the inoculum was ready for addition to the fermentation tank.

2.5. Determination of cell concentration

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 [7].

2.6. Determination of sugars and gluconic acid

Sugars and gluconic acid were quantified with the following enzyme kits from Megazyme (www.megazyme.com): K-GLUC 07/11 for glucose, K-FRUGL 12/12 for fructose, K-SUCGL 09/14 for sucrose and K-GATE 12/12 for gluconic acid. All determinations were performed at least in triplicate.

2.7. Fitting experimental data

For analysing the results, regression procedures to identify the equations most closely fitting the experimental data were used. Significant differences between experimental concentrations were determined by analysis of variance (ANOVA). Computations were

done with the advanced scientific graphing and statistical analysis platform SigmaPlot ver. 11.0 (www.sigmaplot.com).

3. RESULTS AND DISCUSSION

Fig. 1 shows the total cell concentration profiles using both linear as well as common logarithmic scales. These results confirm substantial growth of the bacterium. Nevertheless, though all cell counts were performed at least in quintuplicate, the presence of solid particles from the strawberry purée may have led to overestimation and, in any case, to high standard deviations. Then, the uncertainty in using these experimental cell concentration data for estimating the maximum specific growth rate could be too high to be acceptable. In any case, the logarithmic plot in Fig. 1 is suggesting a typical cell concentration profile: data for cell concentration during an exponential phase can be fitted to a straight line; so, in this case, the exponential phase might roughly last six hours. In this regard, it is important to point out that the inoculum preparation procedure used prevented a lag phase from happening in the fermenter [14]. Although the high physico–chemical complexity of the growth medium used (strawberry purée) precluded accurate monitoring changes in bacterial concentration over a cycle, the values of the maximum specific growth rate (μ_{max}) could be estimated, without the need for biomass concentration measurements, using some of the glucose data shown in Fig. 2 as it will be proposed.

3.1. Procedure and justification

It is well known that many microorganisms of the genus *Gluconobacter* are among the most frequently used in industrial biotechnology [15] and gluconic acid one of their most important products [16]. In this case, *G. japonicus* produced gluconic acid with a

constant apparent yield ($Y_{GA/G}$) of 0.81 ± 0.01 g of gluconic acid per g of total glucose (0.74 mol of gluconic acid per mol of glucose), see Fig. 3. Additionally, it is interesting to note that sucrose was not detected and no fructose consumption was observed (see Fig. 2), therefore glucose is the only substrate that can be used for growing in these experiments, at least during the exponential growth phase; then, the decay profile for glucose could be used in the proposed method.

Before go further it is important to remind that acetic acid bacteria show the phenomenon of incomplete oxidation, or "overflow metabolism" [17]. For instance, for *Gluconobacter oxydans,* evidences have been presented of a mechanism that determines the partitioning of glucose metabolism through the direct oxidative pathway to gluconic acid and the pentose phosphate pathway [18]. The carbon flux through either one pathway or the other is strongly regulated in *G. oxydans* growing in glucose-containing media. 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^{-1}, resulting, in these cases, in an accumulation of gluconic acid in the medium.

Two different growth phases have been suggested for *G. oxydans* depending on the dominant glucose uptake pathway; in a first growth phase, the incomplete oxidation of glucose produces sugar acids leading to a fast acidification of the medium with an advantage for this acid tolerant bacterium. The short electron transport chain in the process of overflow metabolism allows fast oxidation of glucose, but its ineffective energy transduction results in low growth yields [17]. In a second growth phase, once the glucose has been exhausted, the incompletely oxidized substrates are metabolized by the pentose phosphate pathway [17-19].

In general, during the first phase, there is a clear relationship between growth and total glucose consumption for *Gluconobacter* and *Acetobacter sp*. [18-24], resulting in

constant apparent yields of cells on total glucose ($Y_{X/G}$) (see Table 1); different pH control strategies as well as initial glucose concentrations may affect the value of $Y_{X/G}$. In addition to this behaviour, no ketogluconates formation is observed during this phase [19], see Table 1. Also it is important to notice that, at the same time, a constant relationship between gluconic acid production and total glucose consumption is found in all cases, see values of $Y_{GA/G}$ in Table 1.

When the previous conditions are met, a simple procedure just combining the glucose and cell mass balances during the exponential growth phase could be used for estimating the maximum specific growth rate without cell concentration data.

First

$$-\frac{dG}{dt} = q_G \cdot X_v \qquad (1)$$

where q_G is a term of proportionality between the total glucose uptake rate *(-dG/dt)* and the concentration of viable cells (X_v).

On the other side, considering that glucose uptake is dependent on the particular energy requirements of the genus *Gluconobacter* [18, 21], and the process is assumed to conform to a Monod kinetics, then q_G can be expressed as

$$q_G = \frac{\mu_c}{Y_{X/G}} + m_G = \frac{1}{Y_{X/G}} \cdot \frac{\mu_{max} \cdot G}{K_G + G} + m_G \qquad (2)$$

where μ_C is the specific cell growth rate, $Y_{X/G}$ the apparent yield of number of cells per g of total glucose, m_G the cell maintenance coefficient and K_G the Monod constant. Also, m_G and K_G could be neglected during this growth phase since, on one side, m_G will be negligible by effect of the little energy required for cell maintenance at this stage and, on the other side, based on the low values of K_G [18, 25], the substrate concentration will considerably exceed K_G; in any case, the problem can also be solved without

neglecting K_G (as will be explained later) but, in this case, the parameter is not identifiable. Therefore, Eq. (3) can be rewritten as follows:

$$-\frac{dG}{dt} \cong \frac{\mu_{max}}{Y_{X/G}} \cdot X_v \qquad (3)$$

Also, solving the equation for the viable cell balance during the exponential growth phase on the assumption of a constant specific growth rate, yields

$$X_v = X_{vi} \cdot e^{\mu_{max} \cdot t} \qquad (4)$$

where X_{vi} is the initial concentration of viable cells. Substitution into Eq. (3) yields

$$-\frac{dG}{dt} \cong \frac{\mu_{max}}{Y_{X/G}} \cdot X_{vi} \cdot e^{\mu_{max} \cdot t} \qquad (5)$$

integration of which leads to

$$G = G_i + \frac{X_{vi}}{Y_{X/G}}(1 - e^{\mu_{max} \cdot t}) \qquad (6)$$

where G_i is the initial concentration of total glucose. Regardless of the specific fluxes of glucose used by direct oxidative and pentose phosphate pathways, previous equation could be applicable as long as the ratio between glucose used for overflow and for growth remains constant.

Fitting Eq. (6) to the experimental results for total glucose concentration during the exponential phase, allowed us to estimate the maximum specific growth rate values, $(\mu_{max})_{est}$, shown in Table 1. A comparison with the experimental μ_{max} data for the microorganisms ATCC 621 H, NBIMCC 1043, IFO 12528 and ATCC 49037, showed an excellent agreement between the values.

The method, as it could be expected, seems to be also applicable to other type of bacteria in which the phenomenon of glucose overflow metabolism is present too. For instance, Xu et al. [26] presented a model to describe cell growth for *E. coli* W3110, respiration and acetate formation (the product of glucose overflow metabolism in this case) as well as acetate reconsumption during batch and fed-batch cultures; as can be

seen in Table 1, the value estimated by the used method in this work coincides with the given by the authors using experimental cell concentration data.

3.2. Estimation of maximum specific growth rate for *G. japonicus*

Once the method has been validated with the previous examples it might be applied to our results for *G. japonicus* growing in strawberry purée. From the experimental gluconic acid concentrations and the stoichiometrically required glucose uptake for its production, it was possible to estimate the glucose that was converted (G_{ov}) into gluconic acid and the glucose that went to growth and other uses (G_g), see Fig.4. During the exponential phase, we will expect that growth requirements will use most of G_g. Also it is important to notice that there is a constant relationship between G_{ov} and G_g during the first 6-7 h of the cycle (see dash line in Fig. 4). Additionally, a constant difference between total acidity and gluconic acid concentrations through the cycle (see Fig. 5) allows us to conclude that ketogluconic acids formation could be neglected.

Before applying the method it is necessary to find the final time for the exponential phase. It is well known that the inflection point for the substrate curve could be a reference to indicate that some limitations are appearing in the system, so, if a direct substrate uptake and bacterial growth association exists, the corresponding time for the inflection point might be chosen as the final limit for the exponential phase. To locate this point, glucose concentrations in Fig. 2 were fitted by least squared estimations in order to facilitate their first derivatives calculation. This procedure allowed to find a value of roughly six hours for this time. Similarly, the same result is obtained when gluconic acid data are used; this is an additional proof of the relationship between glucose and gluconic acid evolution (detailed information can be found in Appendix A).

Finally, Fig. 6 shows the results from fitting Eq. (6) to glucose concentration data during the exponential phase. As can be seen, the value for $(\mu_{max})_{est}$ is the same regardless of using either total glucose or glucose for growth data (additional information about the fitting can be found in Appendix B).

All the parameters in Eq. (6) were fitted: G_i, $(G_i)_g$, $(X_{vi}/Y_{X/G})$, $(X_{vi}/Y_{X/Gg})$ and μ_{max}. The obtained values for G_i and $(G_i)_g$ are in good agreement with the experimental ones (see Fig. 4). Furthermore, from the obtained values for $(X_{vi}/Y_{X/G})$ and $(X_{vi}/Y_{X/Gg})$ an estimation of $Y_{X/G}$ and $Y_{X/Gg}$ respectively could be possible using the initial cell concentration (see Fig. 1, Xvi = 1.4e10 ± 5.7e9 cell·L^{-1}): $Y_{X/G}$ = 3.77e9±2.03e9 cell per g of total glucose and $Y_{X/Gg}$ = 1.25e10±6.28e9 cell·per g of glucose for growth; unfortunately, the high errors obtained could be expected taking into account those of X_{vi} and the fitted parameters $(X_{vi}/Y_{X/G})$ and $(X_{vi}/Y_{X/Gg})$ (see Appendix B).

As commented previously, and aimed to estimate also the value for K_G, the substrate and cell balances can be solved without neglecting this parameter in Eq. (2), (see procedure and detailed information in Appendix C). Unfortunately, a problem of identifiability is found, so the value for K_G cannot be obtained, but at least, it could be suggested that its value is ≤1 g glucose L^{-1}.

Finally, and keeping in mind the previous comments about the reliability of the results shown in Fig. 1, Eq. (4) could be fitted to cell concentration data during the exponential phase (see Appendix D) for obtaining an additional estimation of μ_{max}= 0.25±0.03 h^{-1}; the agreement with the estimated values by Eq. (6) might also validate, in some extent, the procedure. Notwithstanding, because of the potential overestimation and high standard deviations of data in Fig. 1, we ought to be very careful assessing the validity of this last calculation (intentionally, estimated values using our cell concentrations data

have not been included in Table 1); in fact, this is the main motive for the method used in this work.

In conclusion, the use of total glucose concentration data during the exponential phase together with the integration of the cell and substrate balances allow the estimation of the maximum specific growth rate of *G. japonicus* growing in strawberry purée without using cell concentration measures. This procedure has been necessary because of the complexity of the medium, containing suspended solids, as well as the difficulties for cultivating the strain on agar-based medium in order to quantify its concentration throughout the fermentation. Also, a rough estimation of the apparent and stoichiometric yields of cell on glucose is possible. Although he Monod constant, K_G, is not an identifiable parameter in this case, a value ≤1 g glucose L^{-1} may be suggested.

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FIGURE CAPTIONS

Fig.1 Total cell concentration during the batch, with dots and bars representing mean experimental values and standard deviations respectively.

Fig.2 Experimental concentrations of glucose, gluconic acid and fructose; sucrose was not detected. Bars represent standard deviations and solid lines their trend lines. There is no a statistically significant difference among values for fructose concentrations at 95% confidence level.

Fig.3 Apparent yield of gluconic acid production on total glucose consumption. Bars represent standard deviations.

Fig.4 Estimated glucose concentrations to be used for gluconic acid production (G_{ov}) and growth and other uses (G_g), with dots and bar representing mean values and standard deviations respectively. Solid lines represent best trends and the dash one the ratio between the trend lines for G_{ov} and G_g.

Fig.5 pH, total acidity and gluconic acid concentration. Bars represent standard deviations and solid lines their trend lines. There is no a statistically significant difference among values for the difference between total acidity and gluconic acid concentrations at 95 % confidence level.

Fig.6 Experimental concentrations for total glucose and glucose for growth during the exponential phase and its best fit to Eq.(6).

Fig.A.1 Determination of the inflection point for the glucose concentration fitting. (Electronic supplementary material).

Fig.A.2 Determination of the inflection point for the gluconic acid concentration fitting. (Electronic supplementary material).

Fig.C.1 Case 1 fit to total glucose concentrations during the exponential phase. (Electronic supplementary material).

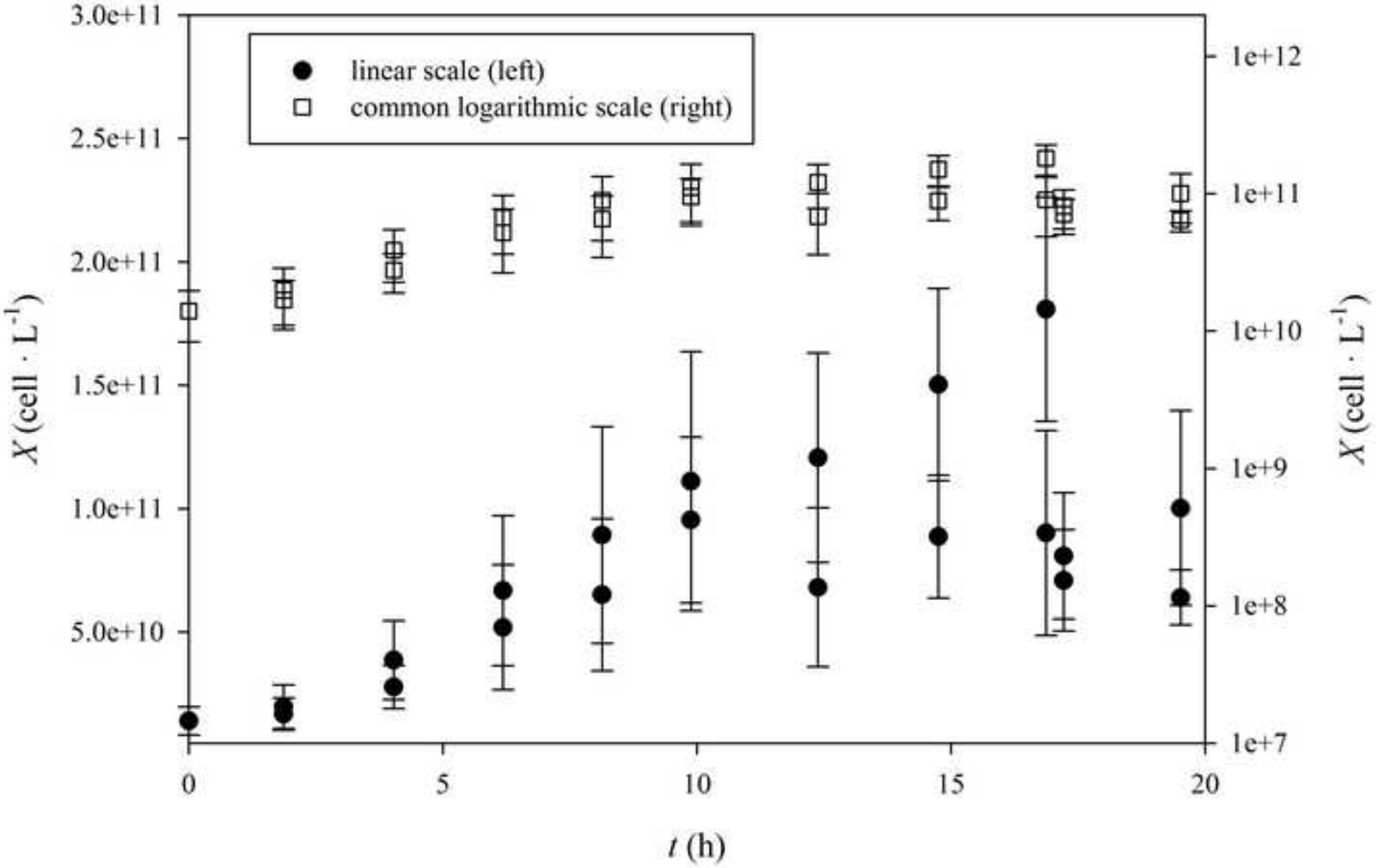
Fig.C.2 Case 9 fit to total glucose concentrations during the exponential phase. (Electronic supplementary material).

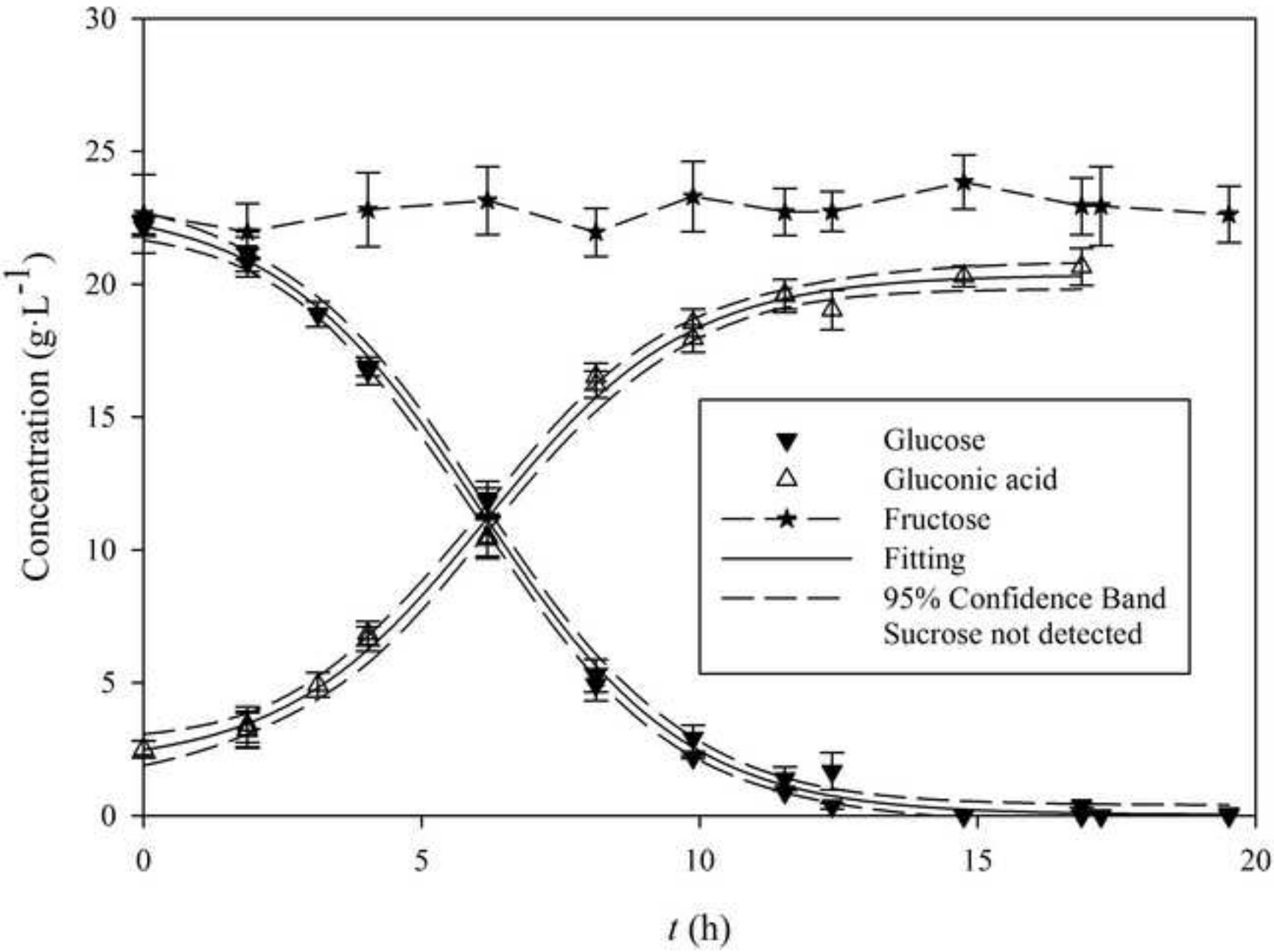
Fig.D.1 Fitting of Eq. (4) to cell concentrations data during the exponential phase. (Electronic supplementary material).

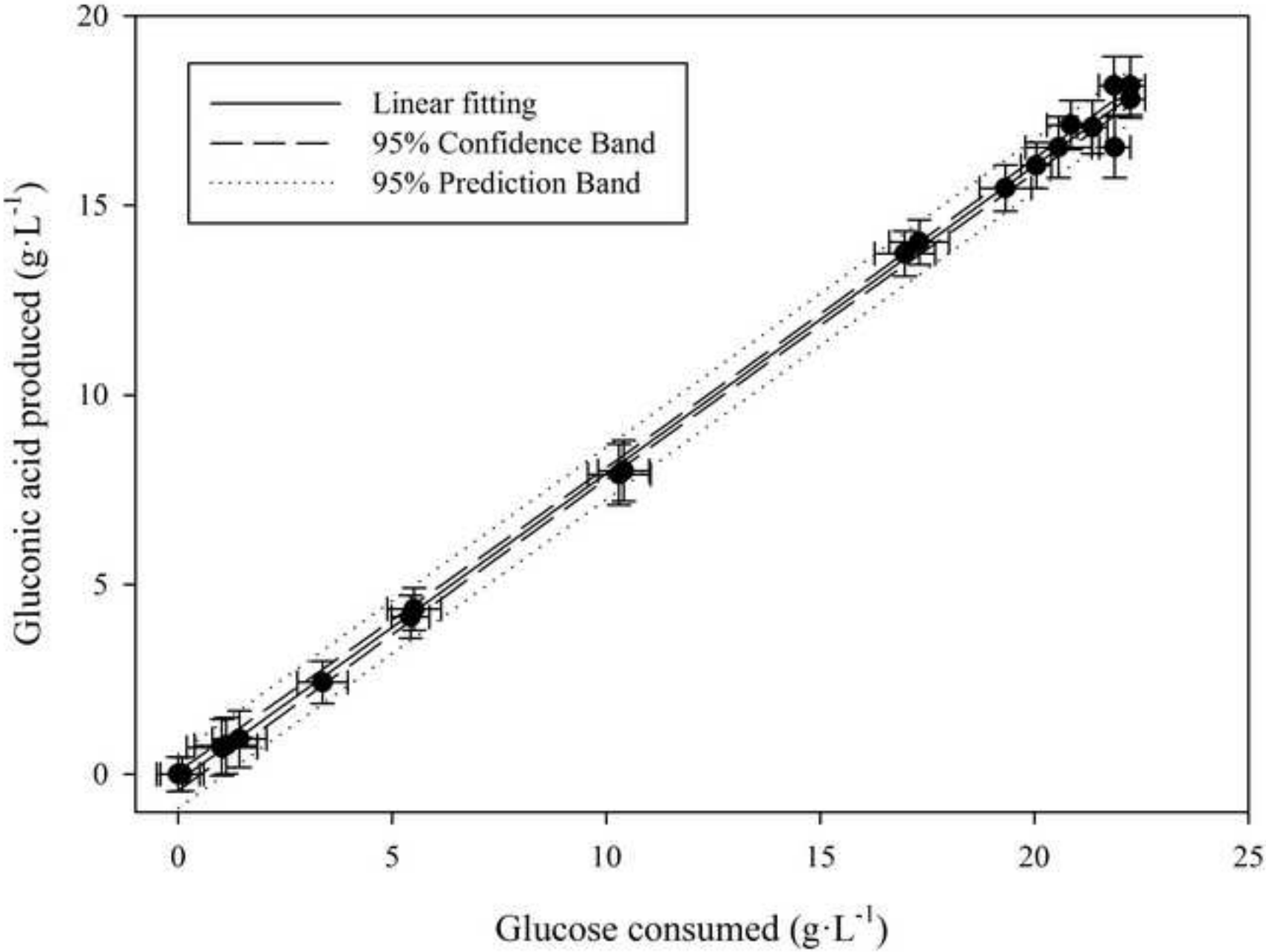
Table 1
Comparison between experimental and estimated by Eq. (6) specific growth rates for different microorganisms and experimental conditions.

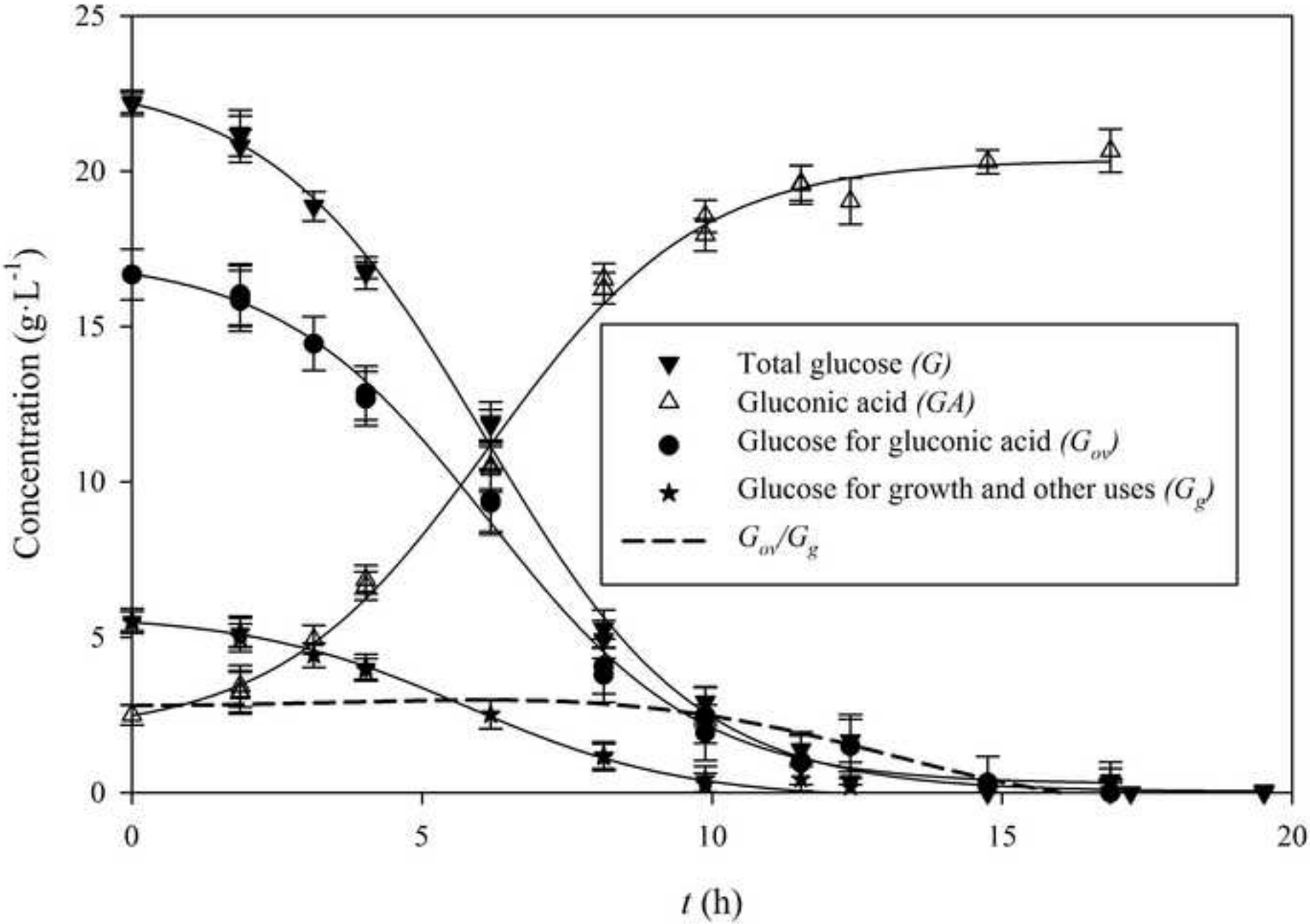
Microorganism	Growth conditions			$Y_{X/G}$*	$Y_{GA/G}$* ($mol_{GA} \cdot mol_{G}^{-1}$)	Production of keto-gluconates**	μ_{max} (h^{-1})	$(\mu_{max})_{est}$ (h^{-1})***	Reference
	Temperature (°C)	G_i ($g \cdot L^{-1}$)	pH						
Gluconobacter oxydans ATCC 621 H	28[a]	10	From 6.5 to 3.5	$8.27 \cdot 10^{-3}$ $OD_{640} \cdot mM^{-1}$	0.74	No	0.45[b]	0.47±0.02	[18]
			2.5[a]	$4.26 \cdot 10^{-3}$ $OD_{640} \cdot mM^{-1}$	0.69	No	0.19[b]	0.17±0.02	
		16	5.5[a]	Cell data not available	0.78	No	0.33[d]	0.35±0.03	[19]
Gluconobacter oxydans NBIMCC 1043	32[a]	90	From 4.2 to 2.6	$5.55 \cdot 10^{-3}$ $OD_{600} \cdot mM^{-1}$	0.93	No	0.39[d]	0.42±0.03	[21]
		210	From 4.5 to 2.0	$2.56 \cdot 10^{-3}$ $OD_{600} \cdot mM^{-1}$	0.95	No	0.17[d]	0.16±0.01	
	28[a]	90	5.5[a]	$6.4 \cdot 10^{-3}$ $g_{Dry\ cell} \cdot g^{-1}$	0.96	No	0.40[d]	0.41±0.02	[22]
Gluconobacter suboxydans IFO 12528	30[a]	10[c]	From 6.5 to 3.8	$6.3 \cdot 10^{-1}$ Klett unit$\cdot mM^{-1}$	0.67	No	0.05[b]	0.04±0.01	[24]
		20	From 6.5 to 3.0	$5.9 \cdot 10^{-1}$ Klett unit$\cdot mM^{-1}$	0.47	No	0.04[b]	0.05±0.01	
			5.0[a]	$6.5 \cdot 10^{-1}$ Klett unit$\cdot mM^{-1}$	0.61	No	0.06[b]	0.06±0.01	
Acetobacter diazotrophicus ATCC 49037	30[a]	2.7	From 5.6 to 3.0	$2.0 \cdot 10^{-2}$ $OD_{420} \cdot mM^{-1}$	0.82	No	0.07[b]	0.09±0.02	[23]
		153	3.5[a]	$1.24 \cdot 10^{-2}$ $OD_{420} \cdot mM^{-1}$	0.89	No	0.17[d]	0.16±0.01	
Escherichia coli W 3110	35[a]	14	7.0[a]	$5.2 \cdot 10^{-1}$ $g_{Dry\ cell} \cdot g^{-1}$	0.24[e]	-	0.55[d]	0.53±0.05	[26]
Gluconobacter japonicus CECT 8443	29[a]	25	From 3.5 to 3.05	-	0.79	No	-	0.22±0.04	This work

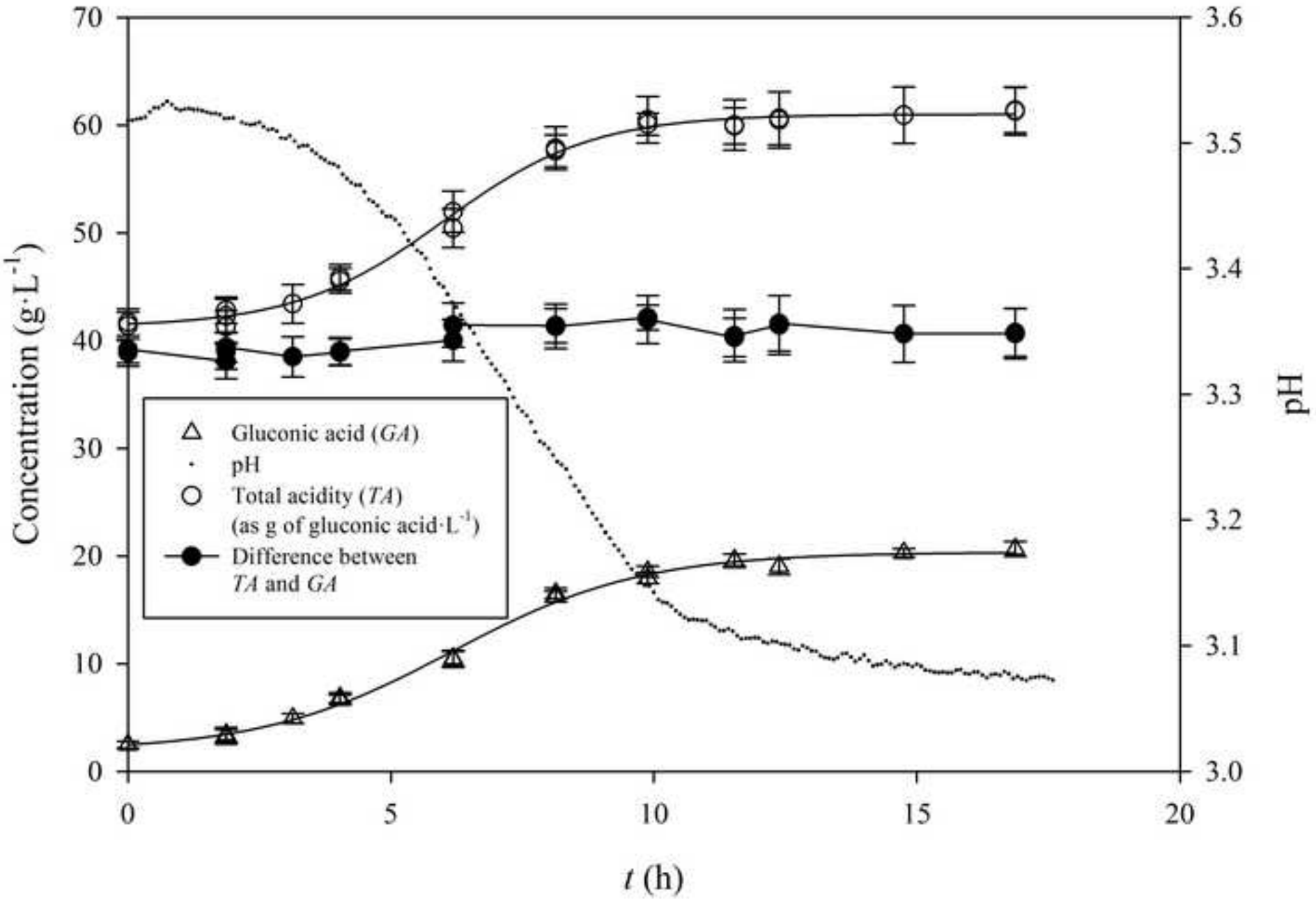
* Constant value during exponential growth phase
** During exponential growth phase
*** Estimated values by the proposed method
[a] Controlled variable
[b] Calculated value from cell growth data
[c] Additionally, the medium has an initial GA concentration of 10 $g \cdot L^{-1}$
[d] Given by the authors
[e] ($mol_{Acetate} \cdot mol_{Glucose}^{-1}$)

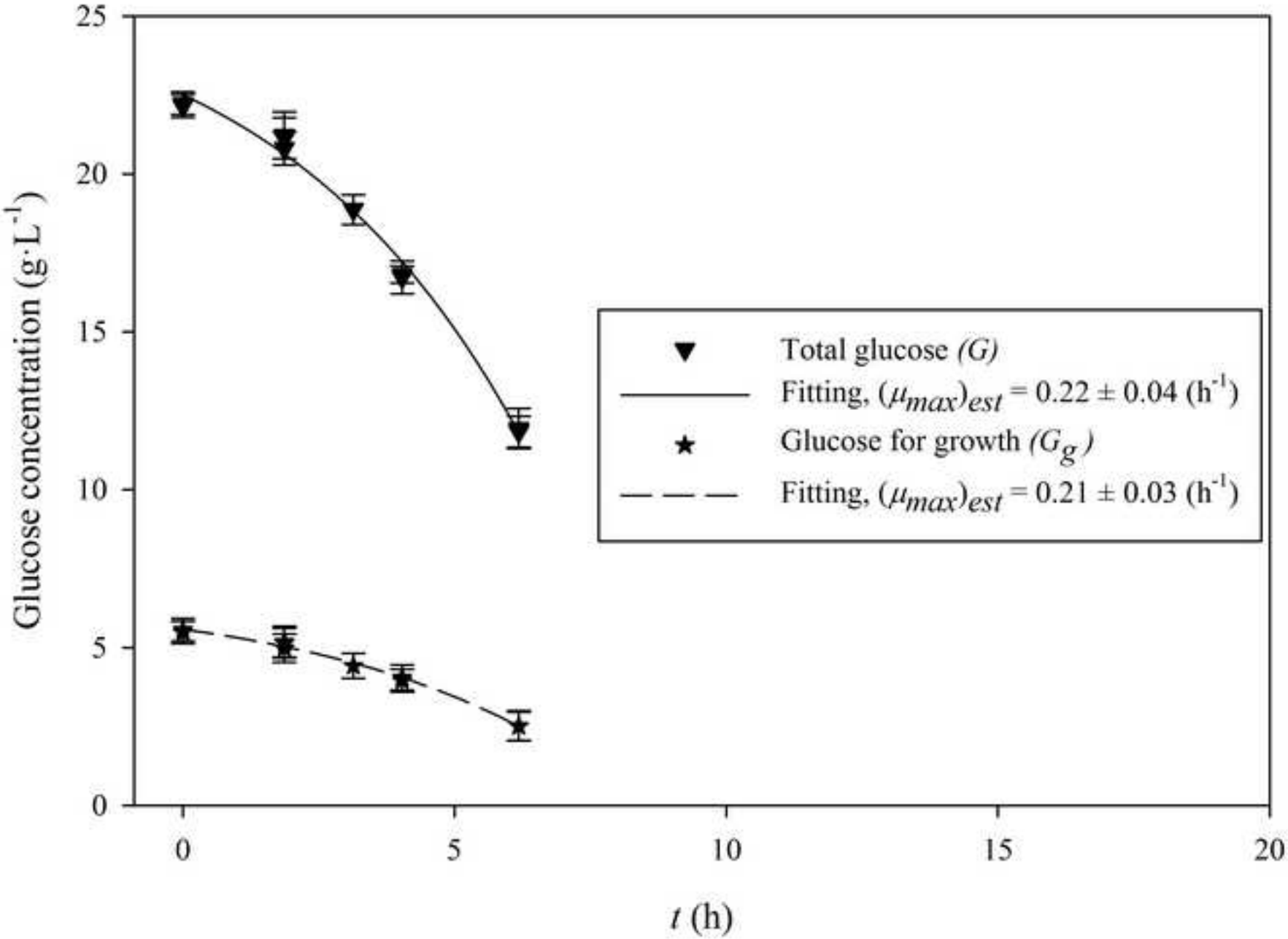

linear scale (left)
common logarithmic scale (right)
3.0e+11
2.5e+11
2.0e+11
1.5e+11
1.0e+11
5.0e+10
1e+12
1e+11
1e+10
1e+9
1e+8
1e+7
0
5
10
15
20
X (cell · L-1)
X (cell · L-1)
t (h)


30
25
20
15
10
5
0
0
5
10
15
20
Concentration (g·L-1)
t (h)
Glucose
Gluconic acid
Fructose
Fitting
95% Confidence Band
Sucrose not detected


Linear fitting
95% Confidence Band
95% Prediction Band
Gluconic acid produced (g·L-1)
Glucose consumed (g·L-1)
0
5
10
15
20
25


25
20
15
10
5
0
0
5
10
15
20
Concentration (g·L-1)
t (h)
Total glucose (G)
Gluconic acid (GA)
Glucose for gluconic acid (Gov)
Glucose for growth and other uses (Gg)
Gov/Gg


Concentration (g·L-1)
pH
t (h)
Gluconic acid (GA)
pH
Total acidity (TA)
(as g of gluconic acid·L-1)
Difference between
TA and GA
0
10
20
30
40
50
60
70
3.0
3.1
3.2
3.3
3.4
3.5
3.6
0
5
10
15
20


25
20
15
10
5
0
0
5
10
15
20
Glucose concentration (g·L-1)
t (h)
Total glucose (G)
Fitting, (μmax)est = 0.22 ± 0.04 (h-1)
Glucose for growth (Gg)
Fitting, (μmax)est = 0.21 ± 0.03 (h-1)

Appendix A

The glucose and gluconic acid concentrations in Fig. 2 were fitted as follows using the general equation:

$$Concentration = y_0 + a/\{1 + exp[-(t - t_0)/b]\}$$

For glucose data, the next fitting parameters were obtained:

a		***b***		***t_o***		***y_o***		**R**	**P**
Value	**SE**	**Value**	**SE**	**Value**	**SE**	**Value**	**SE**		
22.946	0.475	-1.833	0.093	6.064	0.112	0.037	0.174	0.999	0.555

Being: *Concentration*= Total glucose concentration *(G)*, $g \cdot L^{-1}$

For gluconic acid data, the next fitting parameters were obtained:

a		***b***		***t_o***		***y_o***		**R**	**P**
Value	**SE**	**Value**	**SE**	**Value**	**SE**	**Value**	**SE**		
18.463	0.569	1.807	0.135	6.155	0.150	1.890	0.426	0.998	0.100

Being: *Concentration*= Gluconic acid concentration *(GA)*, $g \cdot L^{-1}$

t: time, h

SE: standard error

R: regression coefficient

P: Constant variance test

Fig.A.1 shows the location of the inflection point for the glucose concentration fitting:

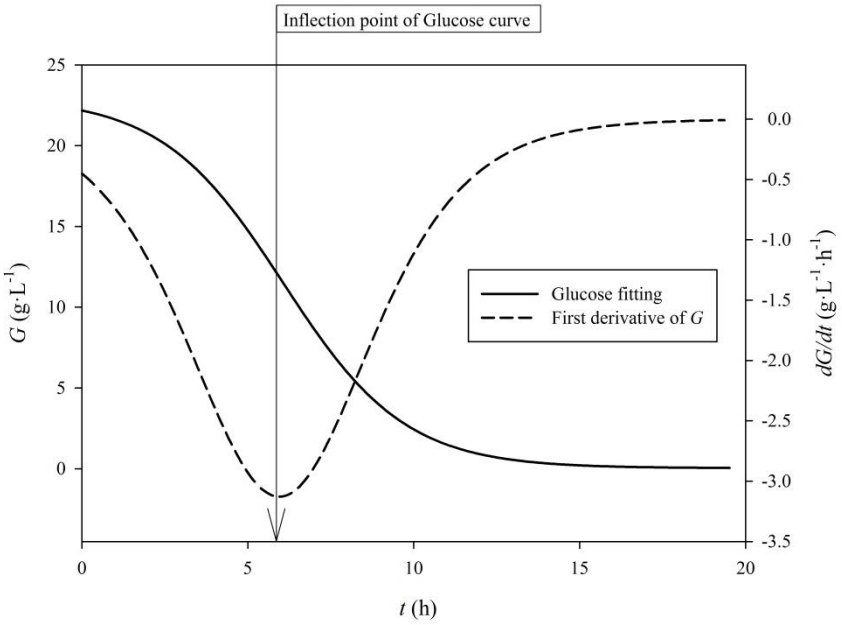
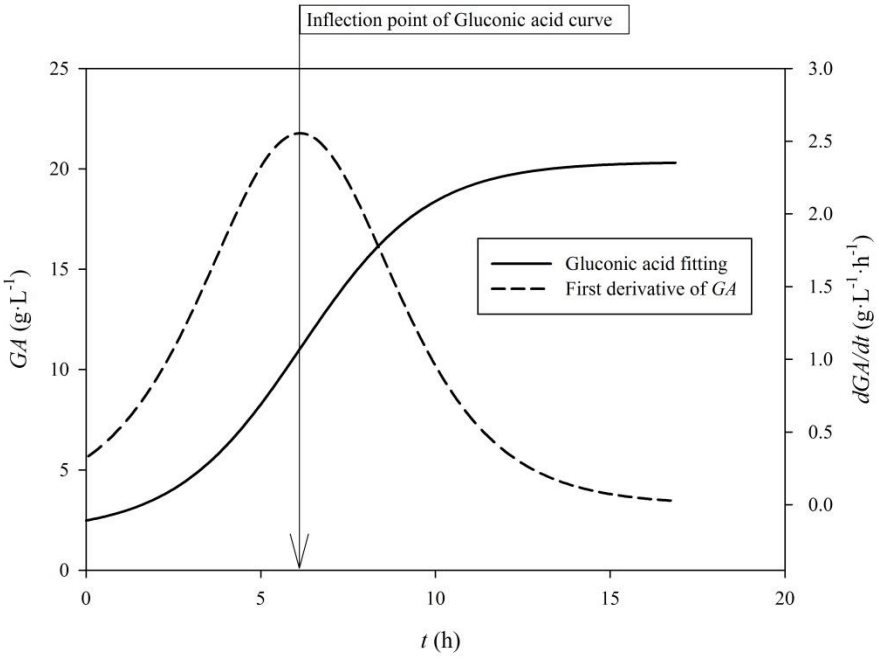


Fig.A.2 shows the location of the inflection point for the gluconic acid concentration fitting:



Appendix B

Parameter values to fit total glucose concentration data during the exponential phase

G_i		$X_{vi}/Y_{X/G}$		μ_{max}		R	P
Value	**SE**	**Value**	**SE**	**Value**	**SE**		
22.51	0.27	3.71	1.31	0.22	0.04	0.995	0.292

Being:

Function: $G = Gi + (Xvi/Y_{X/G}) [1 – exp(\mu_{max} \cdot t)]$

G: total glucose concentration, $g \cdot L^{-1}$

t: time, h

SE: standard error

R: regression coefficient

P: Constant variance test

Parameter values to fit glucose for growth data during the exponential phase

$(G_i)_g$		$X_{vi}/Y_{X/Gg}$		μ_{max}		R	P
Value	**SE**	**Value**	**SE**	**Value**	**SE**		
5.57	0.06	1.12	0.33	0.21	0.03	0.997	0.346

Being:

Function: $G_g = (Gi)_g + (Xvi/Y_{X/Gg}) [1 – exp(\mu_{max} \cdot t)]$

G_g: glucose concentration for growth, $g \cdot L^{-1}$

Appendix C

If K_G is not neglected, equations AC1 and AC2 must be solved:

(Eq. AC1)

(Eq. AC2)

To estimate the parameters: $Y_{X/G,}$ μ_{max} and K_G, a least squares optimization of glucose concentration residuals has been carried out using the following objective function [see below reference R1]:

where are the experimental data, are the model predictions, θ are the parameters and the data samples. Predictions $\hat{y}_i$ are been obtained through numerical integration with a 4th order variable step size Runge-Kutta. All computations have been performed using Matlab.

For the estimation, a global stochastic optimization algorithm based on evolutionary computation was used [see below reference R2], specifically an evolutionary strategy (ES), which allows the use of restrictions as penalty functions. The configuration of this algorithm was as follows:

- Population of each generation was 100 individuals

- Selection method was uniform stochastic
- 80% of next generation individuals was obtained through recombination and remaining 20% through mutation.

Next stop criteria were used (optimization ended when any one was achieved):

- Objective function tolerance: 10^{-15}.
- Maximum number of generations: 5000
- Maximum allowed time without improvement of the objective function: 300 s.

The used parameter intervals were: $\mu_{max} \in [0.1,0.25]$, and]; also, only the exponential phase has been considered, then

Given the stochastic nature of the algorithm, 50 different optimizations were carried out. The results yielding the lowest sum squared errors (SSE) can be found in the next table:

Table C.1. Parameters values for the best fittings to total glucose concentrations during the exponential phase.

Cases	μ_{max}	$Y_{X/G}$	K_G	SSE
1	0.2166	3.6725e9	0.02212	0.4818
2	0.2167	3.6724e9	0.04349	0.4816
3	0.2141	3.6028e9	0.01725	0.4808
4	0.2284	3.7606e9	0.7551	0.4718
5	0.2258	3.6945e9	0.7294	0.4717
6	0.2273	3.6391e9	0.9923	0.4715
7	0.2348	3.8741e9	0.9659	0.4714
8	0.228	3.7422e9	0.7799	0.4713
9	0.2314	3.7885e9	0.9213	0.4699

Obtaining similar values for SSE means that similar predictions are being made by the different cases. Just as examples, see Fig. C.1 and Fig. C.2 for the Cases 1 and 9 respectively. In all the Cases, values for μ_{max} and $Y_{X/G}$ are around 0.22 and 3.7e9 respectively, similar to those obtained if K_G is neglected. Nevertheless, the so different

values obtained for K_G, means that SSE values are not sensible to this parameter and then K_G is not identifiable.

Fig. C.1:

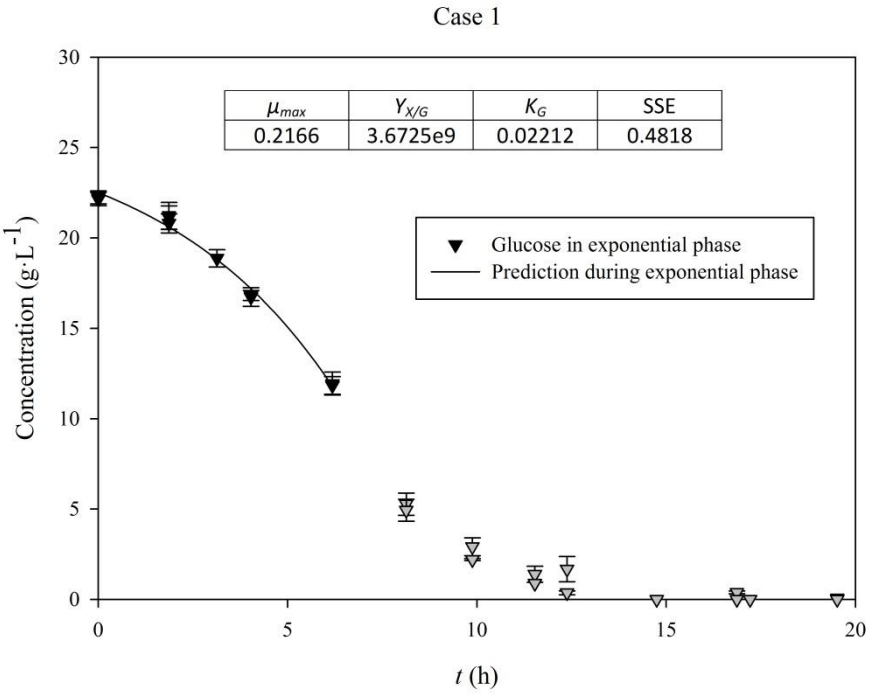
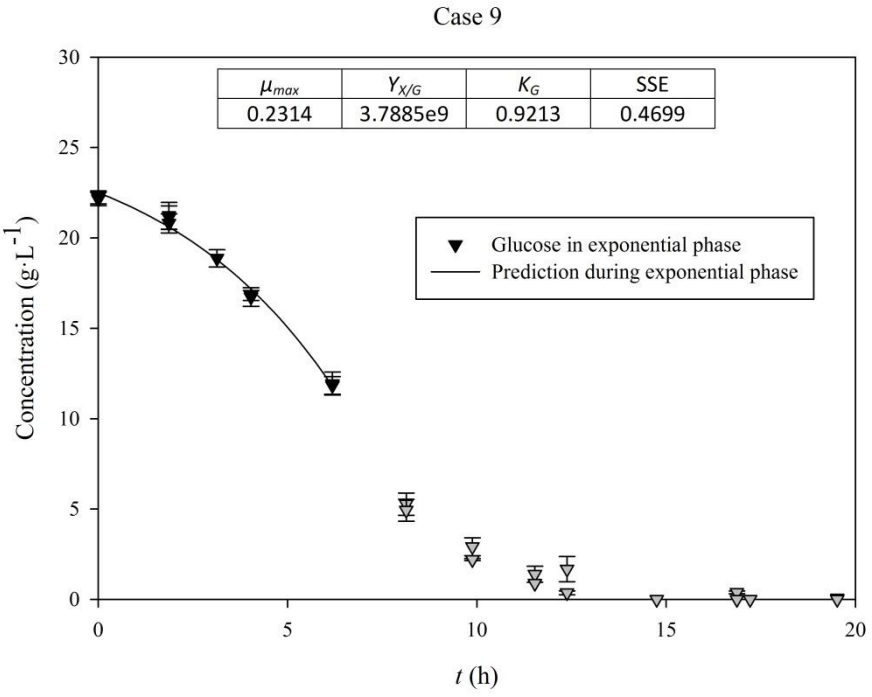


Fig. C.2:



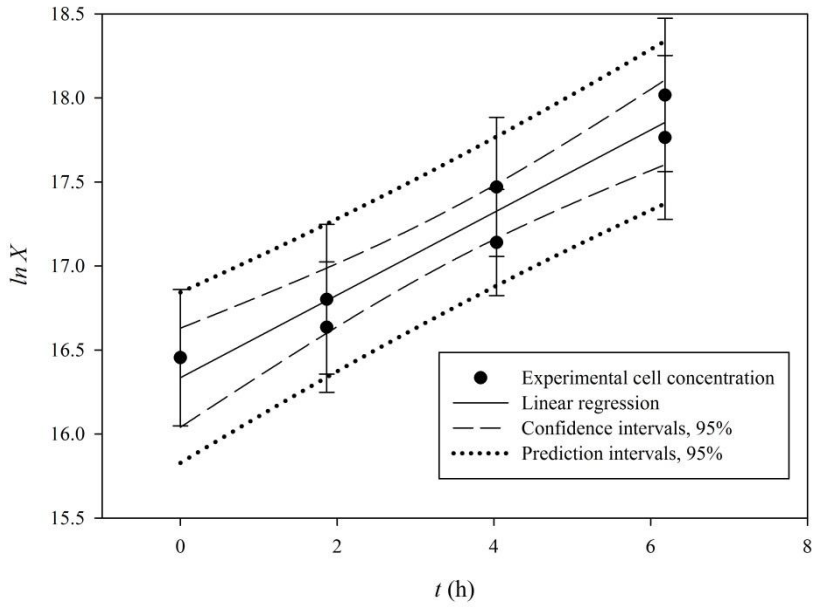
In any case, although the specific value for K_G cannot be identified, roughly a value ≤ 1 g glucose L^{-1}, it could be assumed.

[R1] O. Nelles, Nonlinear system identification, Springer, New York (2001). ISBN: 3-540-67369-5.

[R2] Z. Michalewicz, Genetic algorithms + Data structures = Evolution programs, Springer, New York (1992). ISBN: 3-540-60676-9.

Appendix D

Fig.D.1 shows the fitting of Eq.(4) to cell concentrations data during the exponential phase:



The parameter values for the previous fitting are:

$ln\ X_{initial}$		μ_{max}		R	P
Value	**SE**	**Value**	**SE**		
16.335	0.115	0.25	0.03	0.969	0.491

Being:

Function: $ln\ X = ln\ X_{initial} + \mu_{max} \cdot t$

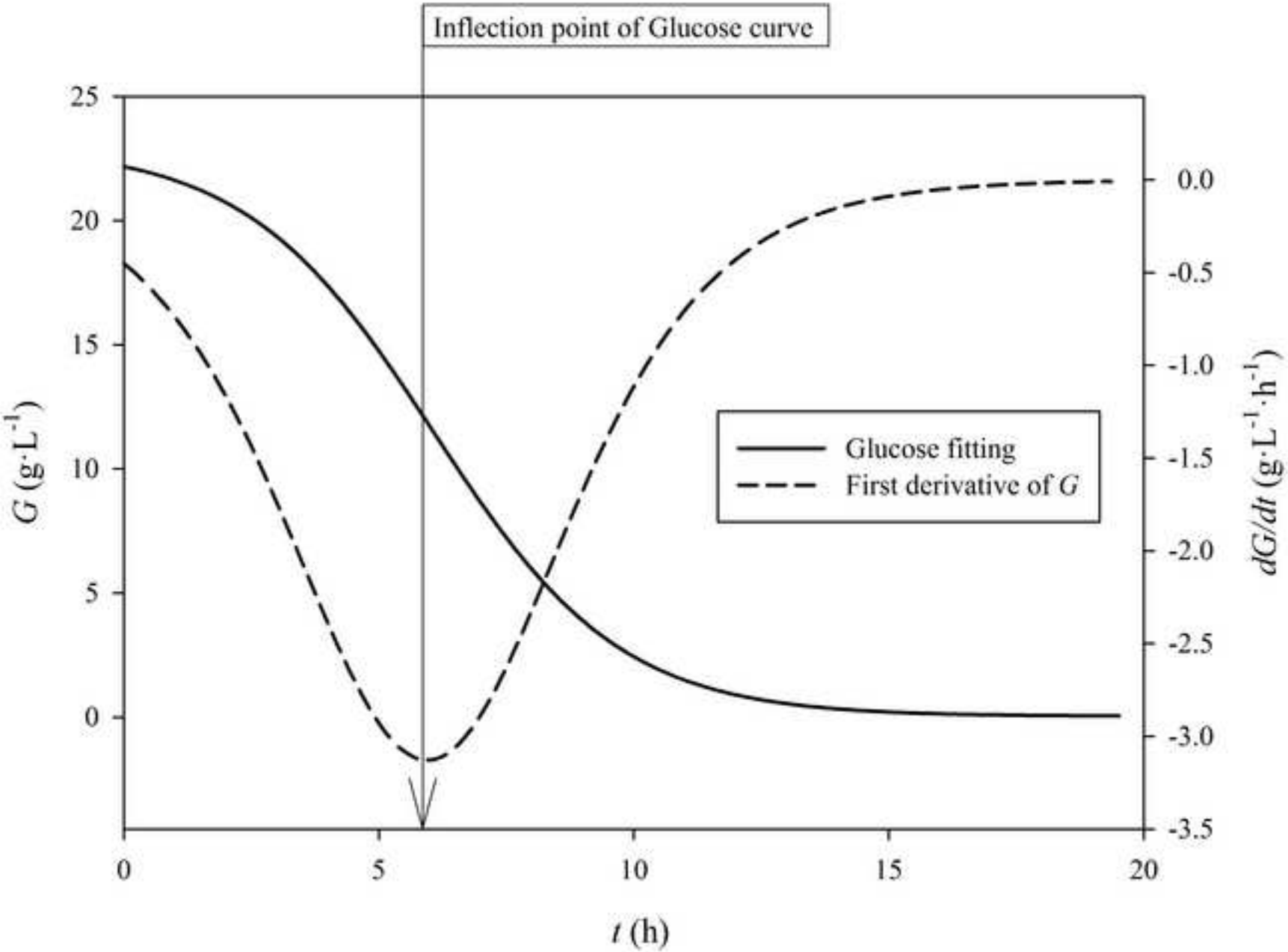
X: total cell concentration, cell·mL^{-1}

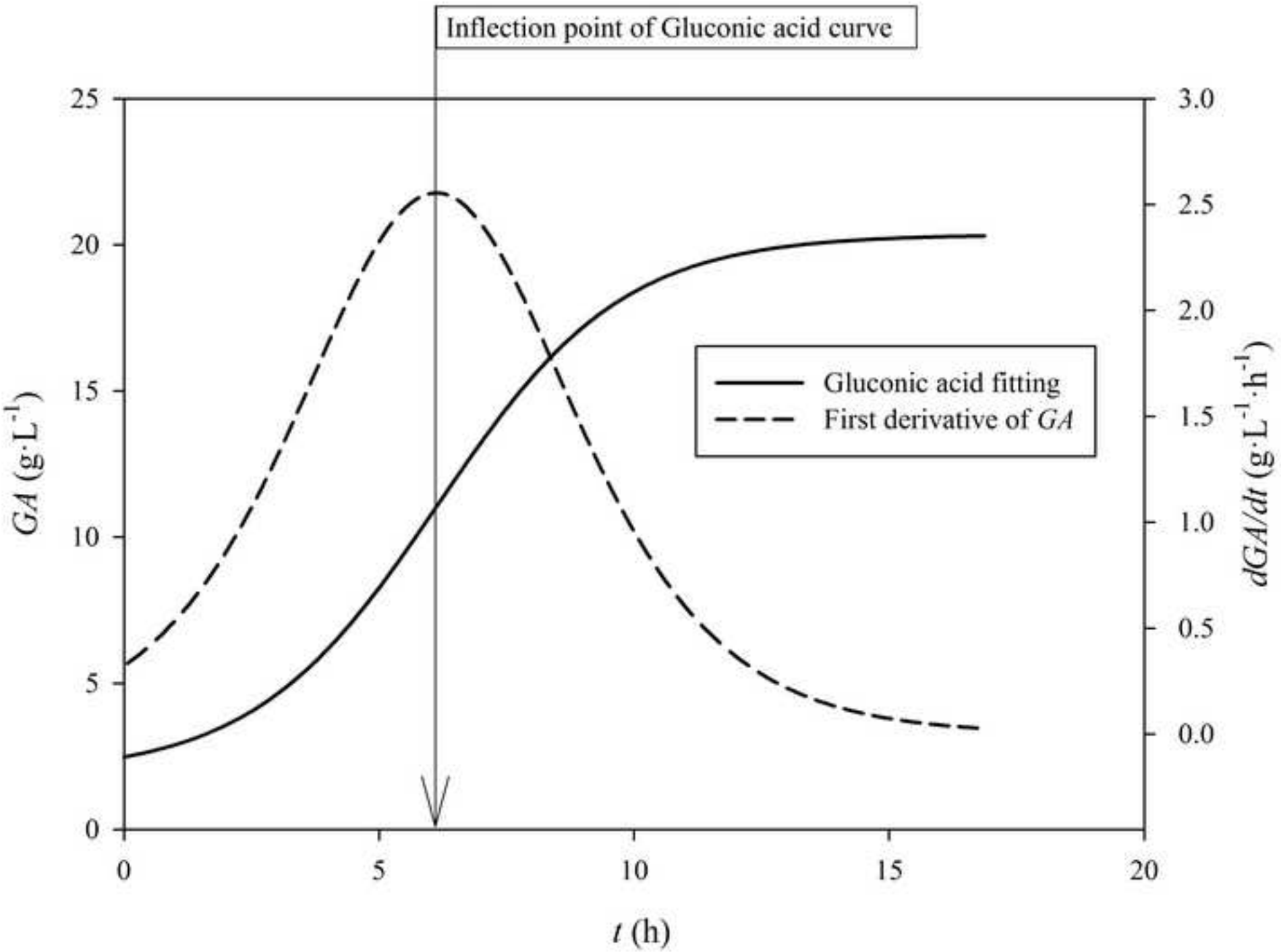
t: time, h

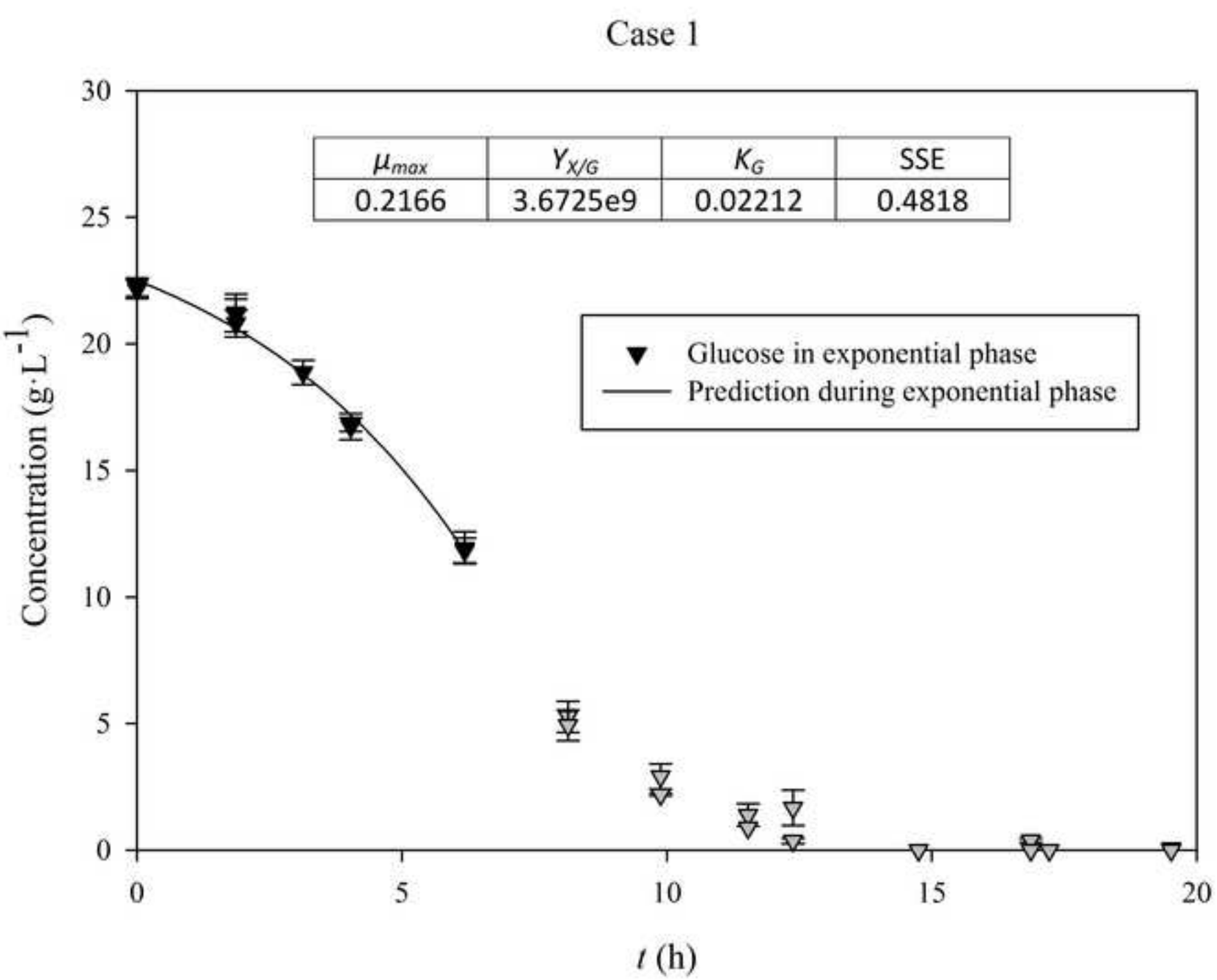
SE: standard error

R: regression coefficient

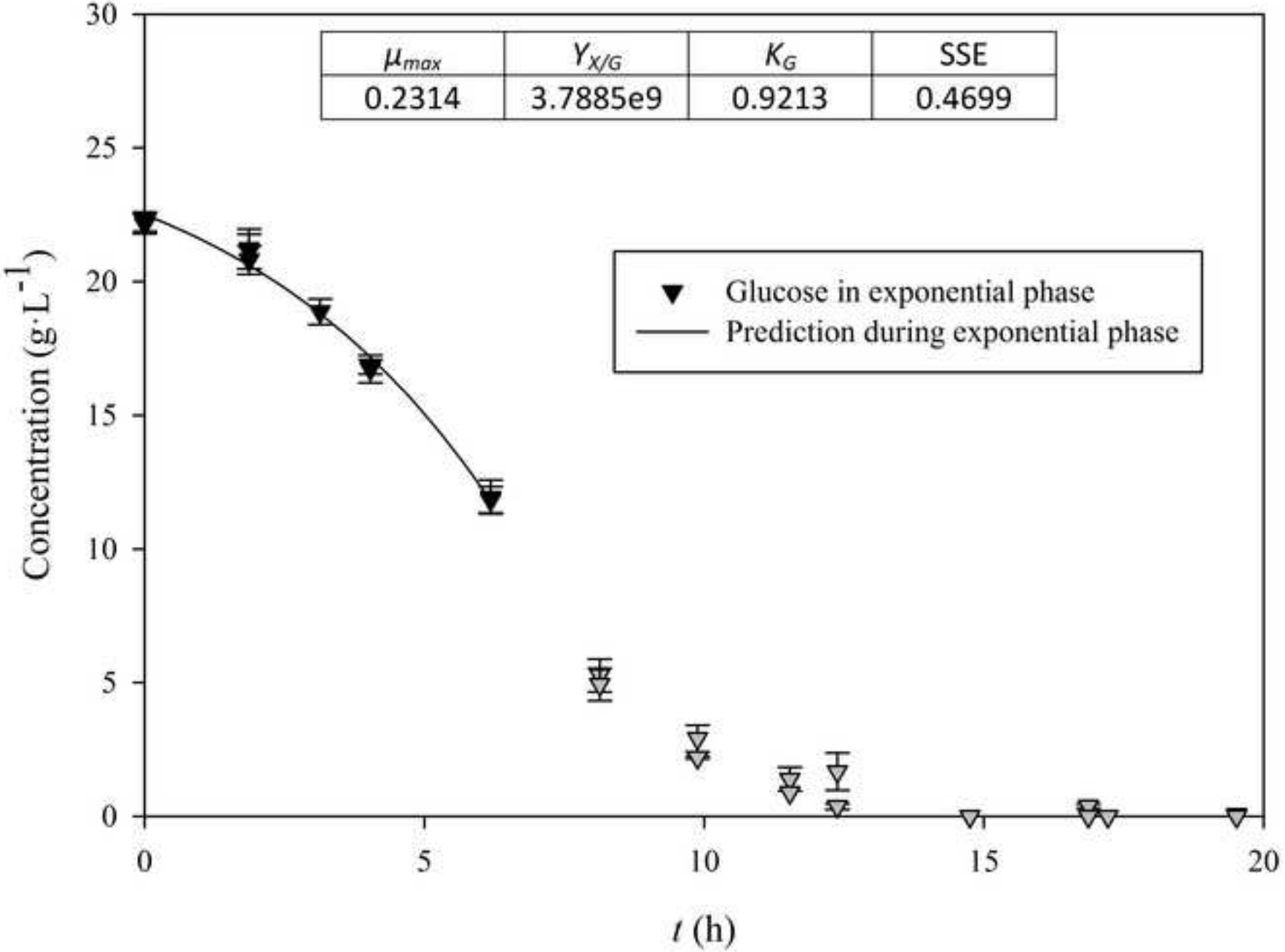
P: Constant variance test

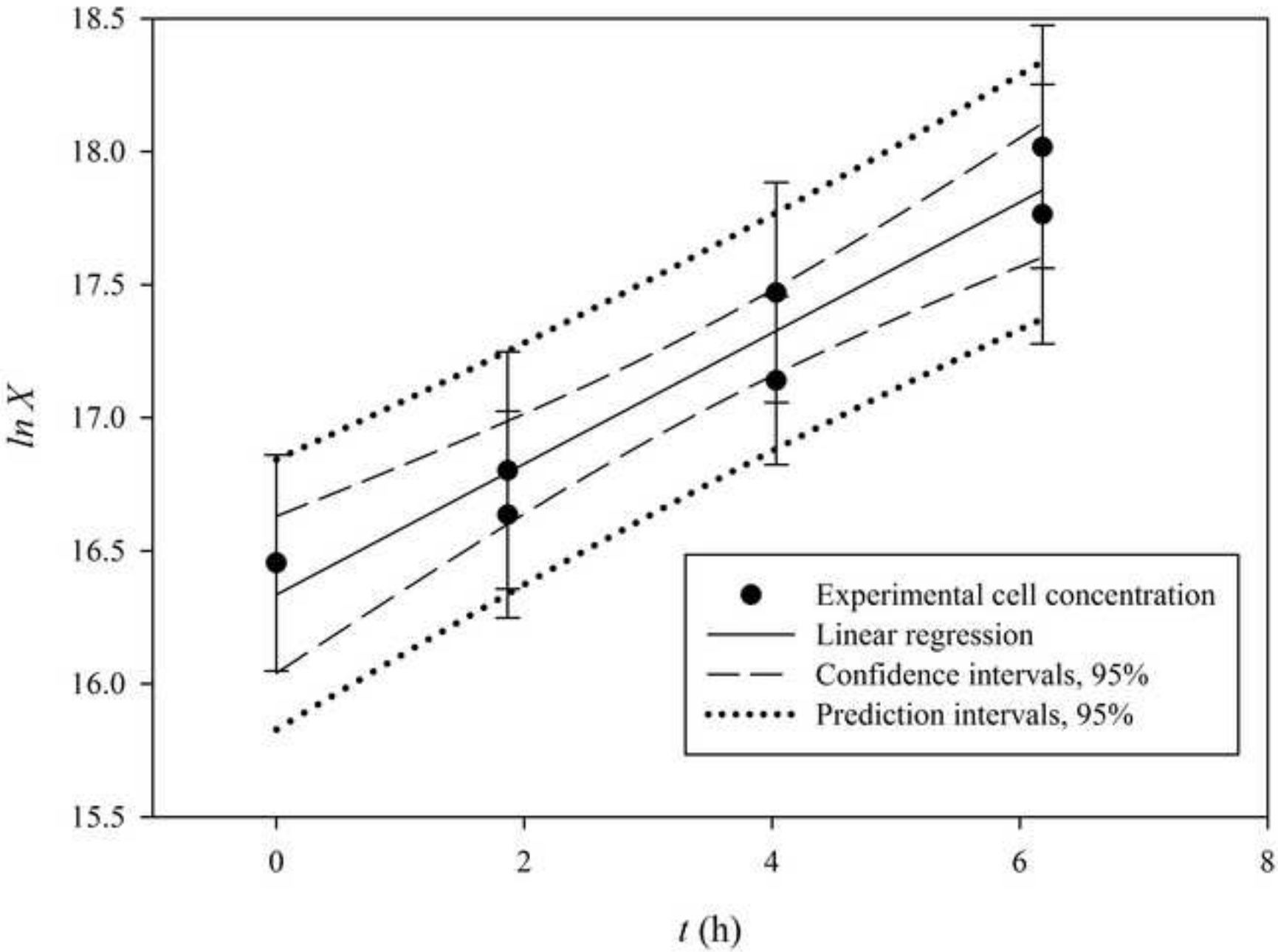

Inflection point of Glucose curve
25
20
15
10
5
0
0
5
10
15
20
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
G (g·L-1)
dG/dt (g·L-1·h-1)
t (h)
Glucose fitting
First derivative of G


Inflection point of Gluconic acid curve
Gluconic acid fitting
First derivative of GA
GA (g·L-1)
dGA/dt (g·L-1·h-1)
t (h)
0
5
10
15
20
25
0.0
0.5
1.0
1.5
2.0
2.5
3.0


Case 1
μ_{max}
$Y_{X/G}$
K_G
SSE
0.2166
3.6725e9
0.02212
0.4818
Glucose in exponential phase
Prediction during exponential phase
Concentration (g·L^{-1})
t (h)
0
5
10
15
20
25
30

Case 9


μ_{max} | $Y_{X/G}$ | K_G | SSE
0.2314 | 3.7885e9 | 0.9213 | 0.4699
Glucose in exponential phase
Prediction during exponential phase
Concentration (g·L^{-1})
t (h)
0
5
10
15
20
25
30


18.5
18.0
17.5
17.0
16.5
16.0
15.5
ln X
0
2
4
6
8
t (h)
Experimental cell concentration
Linear regression
Confidence intervals, 95%
Prediction intervals, 95%