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ORIGINAL PAPER

Analysis of experimental errors in bioprocesses. 1. Production of lactobionic acid and sorbitol using the GFOR (glucose-fructose oxidoreductase) enzyme from permeabilized cells of *Zymomonas mobilis*

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Abstract The proper determination of experimental errors in bioprocesses can be very important because experimental errors can exert a major impact on the analysis of experimental results. Despite this, the effect of experimental errors on the analysis of bioprocess data has been largely overlooked in the literature. For this reason, we performed detailed statistical analyses of experimental errors obtained during the production of lactobionic acid and sorbitol in a system utilizing as catalyst the GFOR (glucose-fructose oxidoreductase) enzyme from permeabilized cells of the bacteria *Zymomonas mobilis*. The magnitude of the experimental errors thus obtained were then correlated with the process operation conditions and with the composition of the culture media used for bacterial growth. It is shown that experimental errors can depend very significantly on the operation conditions and affect the interpretation of available experimental data. More specifically, in this study, experimental errors depended on the nutritional supplements added to the cultivation medium, the inoculation process, and the reaction time, which may be of fundamental importance for actual process development. The results obtained also indicate, for the first time, that GFOR activity can be affected by the composition of the medium in which cells are cultivated.

Keywords Lactobionic acid · Sorbitol · GFOR (glucose-fructose oxidoreductase) · *Zymomonas mobilis* · Reproducibility · Repeatability

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Introduction

When an experiment is repeated under what can be regarded as constant experimental conditions, the results obtained are not necessarily the same. The fluctuations detected when distinct replicates are compared with each other are due to unavoidable experimental fluctuations [3]. For this reason, the study of experimental errors is of paramount importance when the objective of the study is to analyze the performance of biochemical processes and/or build models. As it is well known, the quality of the experimental data can only be specified when experimental errors are known and subsequently properly characterized using sound statistical methods. However, the proper characterization of experimental errors and the analysis of how unavoidable experimental errors affect process analysis and model building are seldom performed in the biotechnological field [13, 15].

The proper characterization of experimental errors may be very important in bioprocesses when the researcher is interested in designing optimum operation conditions, analyzing product recovery, designing bioreactors, improving process control, and reducing process variability [1]. In particular, the control of biotechnological processes differs from traditional control systems due to the many issues related to the metabolism of living organisms [12]. For example, the proper control of the inoculum and of the inoculation is a very complex task, as it may not be possible to characterize precisely whether the microorganisms have the same level of activity from one batch to the other [21]. Therefore, characterization of process variability may greatly facilitate the proper analysis of process data. Nevertheless, the influence of experimental errors on the operation of bioprocesses has been largely overlooked in the literature.

A particular bioprocess that has attracted much attention in recent years is the production of sorbitol and lactobionic acids by the glucose-fructose oxidoreductase (GFOR) enzyme isolated from permeabilized cells of the bacterium *Zymomonas mobilis* [14]. Zachariou and Scopes [24] were the first to report the existence of an enzymatic complex containing GFOR (EC 1.1.1.99) and glucono-δ-lactonase (EC 3.1.1.17) that is present only in *Z. mobilis*. GFOR is capable of simultaneously promoting the oxidation of aldose sugars to their respective aldonic acids and the reduction of ketoses to their respective polyols. Most studies on GFOR have focused on the production of sorbitol and gluconic acid from fructose to glucose, respectively, since these sugars are "natural" substrates of the enzyme and the enzymatic activities are higher. However, the production of sorbitol and gluconic acid using free cells of *Z. mobilis* containing GFOR is not economically attractive due to the relatively low value of the products. According to Jonas and Silveira [14], in order for this process to be economically viable it is also necessary to produce an aldonic acid with a larger aggregate value, such as the lactobionic acid. The market value of this acid is about 70-fold higher than that of gluconic acid.

The worldwide production of sorbitol is larger than 500,000 ton per year and the market demand is increasing. More than 50% of this total is used as a 70% sorbitol solution and about 25% is used for the synthesis of vitamin C. Besides, sorbitol finds many applications in the food, cosmetic, pharmaceutical and textile industries and is widely utilized for formulation of toothpastes [4, 9, 10, 14].

Lactobionic acid has been largely used in the cosmetic industry. A promising application of this acid is the production of biodegradable surfactants. However, its major commercial application comes from the fact that lactobionic acid is the main constituent of fluids used for preservation of organs during transplant procedures [6, 20].

Sorbitol and lactobionic acid are normally produced by distinct and very expensive processes: sorbitol can be obtained through catalytic hydrogenation of glucose and lactobionic acid can be produced through the electrochemical oxidation of the lactose [7, 14]. However, it is possible to produce sorbitol and lactobionic acid from fructose to lactose, respectively, with the GFOR enzyme present in permeabilized cells of *Zymomonas mobilis* [14].

Although the production of sorbitol and gluconic acid by *Z. mobilis* has been intensively studied [4, 6, 7, 9, 10, 14, 20, 24], the lactobionic acid production process has not been analyzed in detail. Many questions on the influence of the composition of cultivation medium on the final enzyme activity and rate of product formation remain to be answered. Several authors [2, 4, 8–10, 14, 16, 22, 24] propose using culture media containing different nutrients at distinct compositions and ratios to improve the production of sorbitol and gluconic acid. However, it is not clear how important these nutrients are for the final enzymatic activity of GFOR, with the exception of glucose, as reported by Zachariou and Scopes [24].

The main objective of this study was to evaluate the effect of important procedural conditions (such as the composition of the cultivation medium) on the production of lactobionic acid and sorbitol using the GFOR (glucose-fructose oxidoreductase) enzyme obtained from permeabilized cells of the bacteria *Z. mobilis* as catalyst. In order to do this, we characterized the experimental errors in detail, with the aim of explaining how process variability can affect the final results. A second objective was the proper statistical characterization of the inoculation procedure, as this may be regarded as a major source of process variability. The analysis of the experimental errors performed here was based on a rigorous statistical analysis of the experimental replicates. Formal statistical treatment of the results was carried out to verify the reproducibility and repeatability of the data and correlate the magnitude of the observed experimental errors to procedural parameters and the composition of the culture medium.

Materials and methods

Microorganism and maintenance

The strain *Z. mobilis* CP4 (ATCC 31821) was provided by the Departamento de Antibióticos, Universidade Federal de Pernambuco (UFPE, Brazil). The maintenance was carried out in a culture medium containing 5 g l^{-1} of yeast extract and 20 g l^{-1} of glucose. Following inoculation, cells were grown for 24 h at 30°C and then stored at 4°C.

Eight culture media were prepared and used for assessing cell growth. The composition of the various media are given in Table 1 and were defined in accordance with published data [2, 4, 8–10, 14, 16, 22, 24]. Both rich and defined composition media were prepared. Media containing glucose concentrations >150 g l^{-1} were not used because of the known substrate inhibition at high sugar concentrations [8].

Before to preparing the cultivation media, solutions containing each component were sterilized separately and then aseptically mixed to give the final concentrations shown in Table 1. The pH of ferrous sulfate solutions was adjusted to 3.0 to avoid precipitation.

Cultivation

Cells of *Z. mobilis* were initially cultivated in a medium having the same composition as that used for cell maintenance at 30°C. After 24 h, an inoculum (5% v/v) was

Table 1 Media composition

Nutrients (g l^{-1})	Medium							
	1 [8]	2 [22]	3A	3B [9]	4A [10]	4B	5A [2]	5B
Glucose	100	100	100	150	100	150	100	150
Yeast extract	5.00	5.00	5.00	5.00	5.00	5.00	–	–
$(NH_4)_2SO_4$	1.00	1.00	2.00	2.00	–	–	–	–
$MgSO_4 \cdot 7H_2O$	0.50	1.00	1.00	1.00	–	–	2.00	2.00
KH_2PO_4	1.00	–	3.50	3.50	–	–	3.50	3.50
Calcium pantothenate	–	0.01	–	–	–	–	0.005	0.005
$FeSO_4 \cdot 7H_2O$	–	–	0.01	0.01	–	–	0.005	0.005
Sodium citrate	–	–	0.20	0.20	–	–	–	–
NH_4Cl	–	–	–	–	–	–	1.00	1.00
K_2HPO_4	–	–	–	–	–	–	1.75	1.75
$ZnSO_4 \cdot 7H_2O$	–	–	–	–	–	–	0.0012	0.0012
$MnSO_4 \cdot 7H_2O$	–	–	–	–	–	–	0.0007	0.0007
KCl	–	–	–	–	–	–	0.008	0.008
NaCl	–	–	–	–	–	–	0.008	0.008

A, media containing 100 g l^{-1} of glucose; B, media containing 150 g l^{-1} of glucose

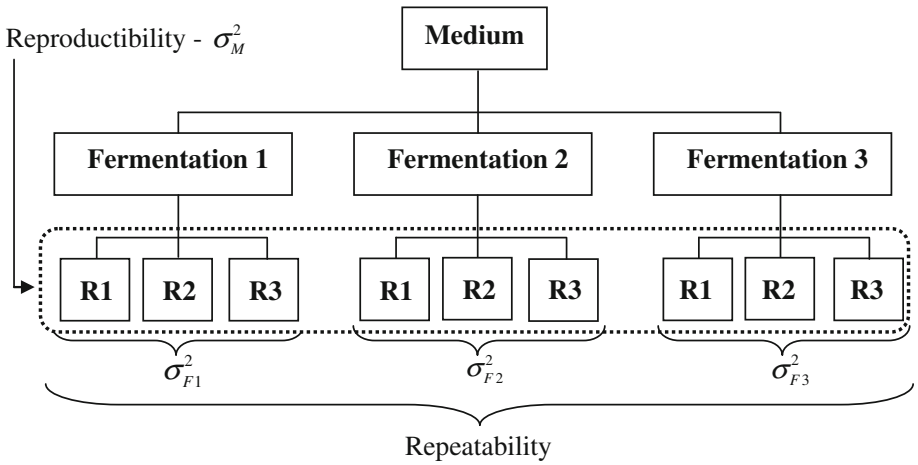

Fig. 1 Diagram for evaluation of the reproducibility and repeatability. *R1*, *R2*, *R3* Enzymatic reactions; σ_{F1}^2, σ_{F2}^2, σ_{F3}^2 represent the variances of the enzymatic reactions for each fermentation; σ_M^2 represents the variances of the enzymatic reactions for the cultivation medium

transferred to a second cultivation medium with the desired composition, as described in Table 1. After 18 h of growth, a second inoculation was performed in cultivation media containing the desired composition, as described in Table 1. The volume concentration of inoculum was equal to 5% v/v for solutions containing 100 g l^{-1} of glucose, performed after 30 h of cultivation, and 10% v/v for solutions containing 150 g l^{-1} of glucose, performed after 40 h of cultivation. The cells were then cultivated at 30°C.

Evaluation of reproducibility and repeatability

To investigate the influence of medium composition on lactobionic acid and sorbitol formation by the enzyme produced during the fermentation, we performed kinetic studies according to the experimental design presented in Fig. 1. The proposed experimental design enables the reproducibility and repeatability of the experiments to be assessed under all procedural conditions. The proposed experimental design also allows for the statistical characterization of experimental fluctuations induced by the inoculation step.

In order to evaluate the reproducibility and repeatability of the experimental data, three fermentations were carried out for each cultivation medium analyzed and the variability of the enzymatic reaction rates determined. Therefore, for each distinct medium, three fermentation runs and nine enzymatic reaction tests (3 reaction tests for each of the 3 fermentation runs) were performed. Repeatability refers to the agreement among the results for distinct enzymatic reactions under similar reaction conditions, using cells cultivated from a single fermentation run. In practical

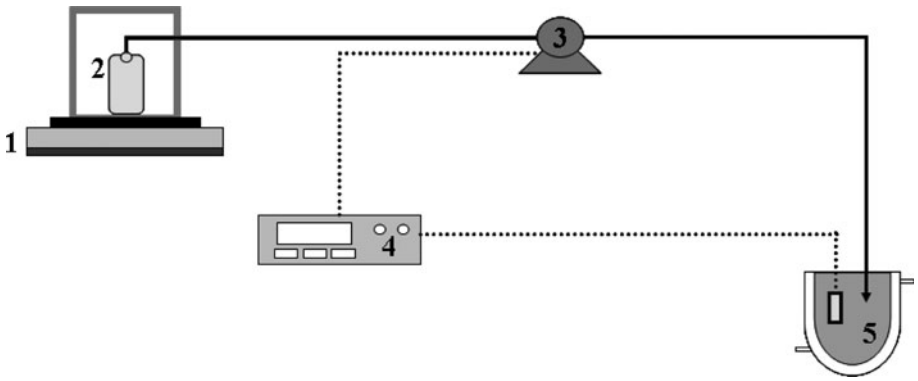

Fig. 2 Experimental setup used to perform enzymatic reactions: *1* Balance, *2* NaOH solution, *3* pump, *4* pH controller, *5* jacketed reactor

terms, the analysis of repeatability was performed when the three variances calculated with data obtained at particular fermentation runs (σ^2_{F1}, σ^2_{F2}, σ^2_{F3}) were compared to each other. Reproducibility also refers to the agreement among the results for distinct enzymatic reactions performed under similar reaction conditions, using cells cultivated from all fermentation runs. In practical terms, analysis of reproducibility is performed when the three variances calculated with data obtained at particular fermentation runs (σ^2_{F1}, σ^2_{F2}, σ^2_{F3}) are compared to the variance calculated when all reaction data are grouped into a single data set (σ^2_M).

According to Fig. 1, σ^2_M represents the variances of all enzymatic reactions carried out for a specific cultivation medium, while σ^2_{F1}, σ^2_{F2}, σ^2_{F3} represent the variances of the enzymatic reactions carried out for specific fermentation runs. Thus, if the variances of each individual run (σ^2_{F1}, σ^2_{F2}, σ^2_{F3}) are statistically similar to the variance of the whole group (σ^2_M), the data are reproducible. In this case, if all calculated variances are similar to each other, the obtained average rate values can also be regarded as similar, because the distinct calculated variances provide information on how individual measurements are distributed around the respective averages.

Cell permeabilization

After the fermentation step, cells were centrifuged at 11,700*g* for 10 min (centrifuge: Jouan CR3i) and resuspended in distilled water. Following the addition of cetyltrimethylamonium bromide (CTAB) (0.04 $g_{CTAB}.g^{-1}_{cell}$) [16, 22], the suspension was homogenized, kept under constant stirring for 30 min at room temperature, and centrifuged one more time. Permeabilized cells were resuspended in water, with the pH adjusted to 6.2.

Kinetic studies

The enzymatic reactions performed with the permeabilized cells were carried out at 39°C and a pH of 6.2 [24]. The pH was controlled with the automatic addition of NaOH. An aliquot (25 ml) of a solution containing lactose (50 g l^{-1}) and fructose (25 g l^{-1}) was initially added to the jacketed reactor, followed by the addition of 25 ml of a solution containing the permeabilized cells (20 g l^{-1}) to the reaction solution, under constant stirring, in order to reach a final cell concentration of 10 g l^{-1}. Figure 2 illustrates the experimental setup used to carry out the experiments.

The rate of product formation was calculated on the basis of hydroxide consumption rate, recorded using an analytical balance, as:

$$\text{Rate}\left(\text{mol } g^{-1}_{\text{cell}}\ h^{-1}\right) = \frac{1}{X_{\text{cell}}}\left[\left(\frac{m_{\text{NaOH}} \cdot C_{\text{NaOH}}}{d \cdot t}\right) + \left(\frac{m_{\text{NaOH}} \cdot C_{\text{NaOH}} \cdot F}{d \cdot V}\right)\right] \quad (1)$$

where X_{cell} is the mass of cells (g_{cell}); m_{NaOH} is the mass of hydroxide consumed (g); C_{NaOH} is the concentration of the hydroxide solution (mol l^{-1}); t is the reaction time (h); d is the density of the hydroxide solution (g l^{-1}); F is the volumetric flow rate (l h^{-1}); V is the reaction volume (l).

Analytical methods

Cell concentration was determined spectrophotometrically after measuring the optical density of the reactio mixture at 600 nm. Lactose concentration was determined enzymatically according to the Sanchez-Manzanares method [17]. The enzyme β-galactosidase (Sigma G5160; Sigma, St. Louis, MO) was used to convert lactose to galactose and glucose, and then the SL Glucose kit [Companhia Equipadora de Laboratórios Modernos (CELM), SP, Brazil], containing the enzymes glucose-oxidase and peroxidase, was used to evaluate the lactose concentration. The solution to be analyzed contained 80 µl of sample, 20 µl of distilled water, 200 µl of a citrate buffer, pH of 6.6, and 50 µl of a 3,000 U ml^{-1} β-galactosidase solution, maintained at constant agitation at 400 rpm in an orbital shaker (Certomat MOII; B. Braun Biotech International, Allentown, PA) at room temperature. After 1 h of reaction,

1.0 ml of the solution from the SL Glucose kit was added to the analyzed solution, which was incubated at 37°C for 10 min, followed by the determination of absorbance at 510 nm.

Results and discussion

Quantification of lactose

As the reaction between glucose and fructose is equimolar, the use of the hydroxide mass consumed during the reaction to estimate the concentration of all reagents and products has been well documented [9, 10, 19, 22]. In order to check if this assumption remains valid for the reaction of fructose and lactose, we used a second, independent methodology to quantify lactose, as described in the previous section.

Figure 3 shows the variation in lactose concentration over time as determined with the two methodologies. Measurements were performed in triplicates. As shown in Fig. 3, the two methodologies led to similar results.

Consequently, we concluded that the quantification of enzyme activity based on rates of hydroxide consumption is very reliable, as described and recommended by several authors [9, 10, 19, 22]. In addition, preliminary tests carried out by our group with standard solutions prepared with known enzyme compositions indicated that the precision of reaction rate measurements was better than 1.0×10^{-4} g l^{-1} h^{-1}, which is much lower than the precision reported here when distinct fermentation runs and reaction tests were performed. It can therefore be concluded that the quantification of reaction rates through the rates of hydroxide consumption is sufficiently precise and that the main sources of variability in the analyzed problem are related to the fermentation and reaction runs and not to the determination of reaction rates.

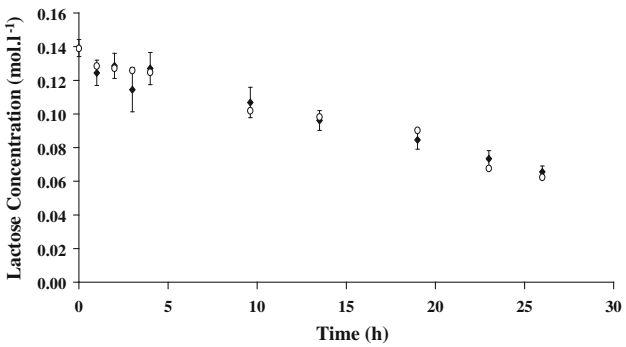

Fig. 3 Monitoring of the lactose concentration through the consumption of NaOH (*open circle*) and use of the enzymatic kit (*filled diamond*). Reaction temperature 39°C, pH 6.2, sugar concentration 0.138 mol l^{-1}, cell concentration 10 g l^{-1}

Evaluation of the cultivation medium

Determination of the average specific rate of reaction

Figure 4 shows the rates of lactobionic acid formation with cells containing the enzyme GFOR produced for all investigated media. The vertical bars show the confidence intervals for the averages, as computed with the well-know *t* test with 95% of confidence, based on the standard deviations of the three replicates of enzymatic reactions carried out for each fermentation run [18, 23]. The results presented in Fig. 4 show that the rate profiles obtained for reactions carried out with permeabilized cells from the same fermentation cannot be considered to be statistically different at a 95% level of confidence. The exceptions are the specific rates obtained with the cells from media 4A and 4 B, as shown in Fig. 4. As media 4A and 4B only contained glucose and yeast extract, this result probably indicates that the addition of nutritional supplements is of fundamental importance to remove the natural variability that can be attributed to yeast extract composition and, consequently, to the performance of cells produced in complex media and used during the reaction. To the best of our knowledge, this is the first report that the addition of nutritional supplements can positively affect the variability of biotransformations.

Figure 4 shows that the initial enzymatic activities were very high in all cases and that activities decreased smoothly as the reaction continued. This result is likely related to the mass transfer limitation of sugars from the bulk solution to the interior of the permeabilized cells containing the enzyme GFOR, because in the beginning of the reaction the resistance to mass transfer is lower. Although product inhibition by lactobionic acid can also be considered as an explanation of the decrease in the enzymatic activity, this assumption is not supported by the very small conversions observed in all experiments.

Figure 4 shows that the variability of the reaction rate values at the beginning of the reactions is very large. After about 0.1 h, variability diminishes and measurements become more precise. Table 2 shows the average specific rates calculated for each medium after 0.1 h, which was the time interval required to reach the desired set point. The large variability in the beginning is probably related to the larger reaction rates and, consequently, to the larger rates of consumption of NaOH, which in turn leads to larger variations in the weighed mass of soda for fixed intervals. This result may be regarded to have important practical implications as the best discrimination of enzymatic activities is therefore not necessarily obtained when the measured values are very high, given the much higher experimental uncertainties at these conditions.

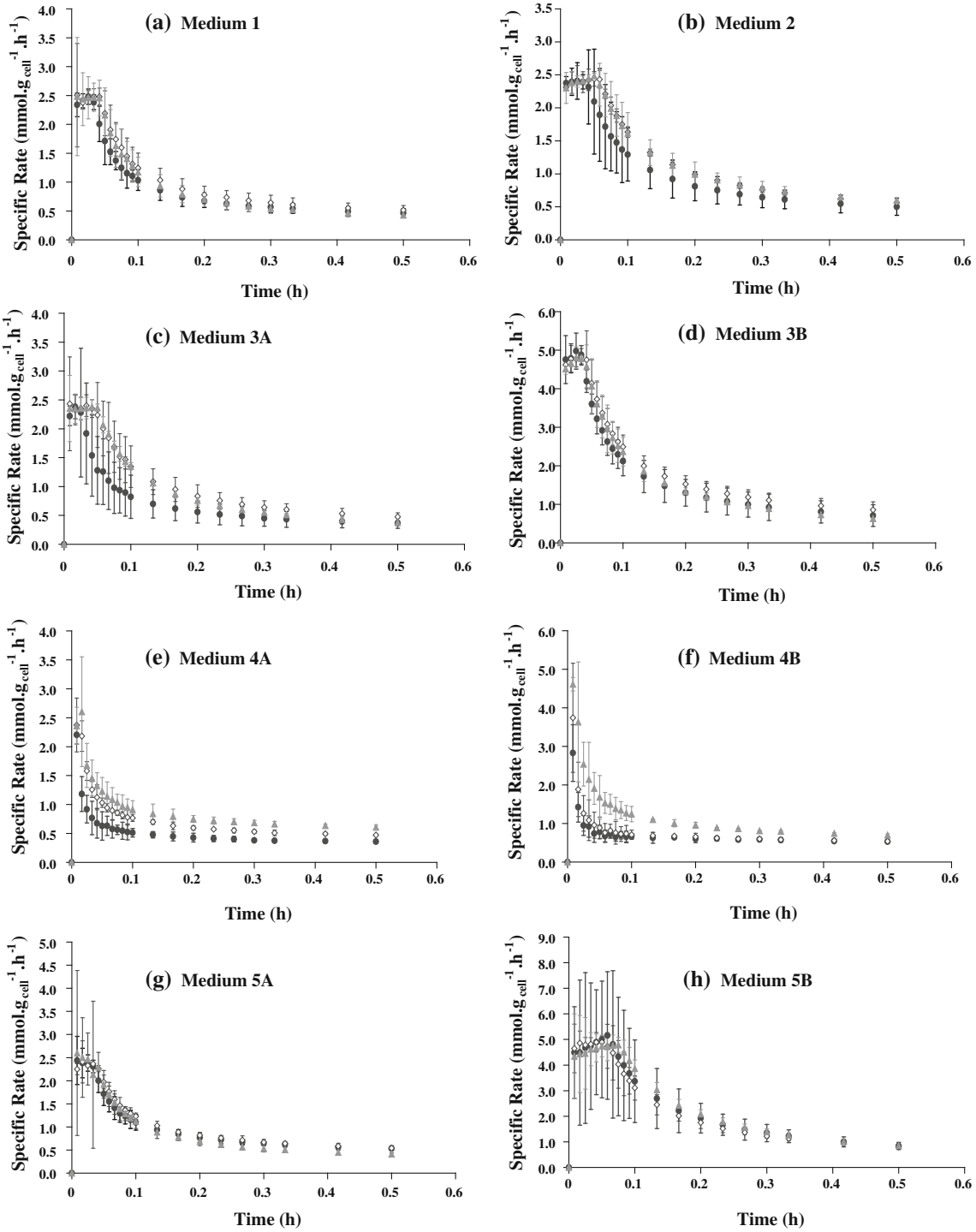

Fig. 4 Rates of lactobionic acid specific formation rate. Cells from fermentation media 1 (**a**), 2 (**b**), 3A (**c**), 3B (**d**), 4A (**e**), 4B (**f**), 5A (**g**), and 5B (**h**). *Vertical bars* Confidence intervals, *symbols* cultivated cells in the first (*filled circle*), second (*open diamond*), and third (*gray filled triangle*) fermentations

It can be observed that cells from medium 5B, a minimal medium with 150 g l^{-1} of glucose, led to the highest reaction rates. When compared to results obtained with cells from medium 5A, which had the same composition but a lower glucose concentration (100 g l^{-1}), the influence of the initial glucose concentration on the specific rate of the GFOR enzyme becomes evident. This influence had already been reported by Zachariou and Scopes [24]. In that study, the authors showed that the highest activity of GFOR was obtained when a high glucose concentration was added to a

Table 2 Average rates of lactobionic acid production (mmol g_{cell}^{-1} h^{-1}) utilizing permeabilized cells from several cultivation media

Medium	Fermentation 1	Fermentation 2	Fermentation 3	All fermentations
1	0.65 ± 0.05	0.77 ± 0.06	0.68 ± 0.04	0.70 ± 0.07
2	0.77 ± 0.08	0.94 ± 0.02	0.95 ± 0.05	0.90 ± 0.10
3A	0.53 ± 0.07	0.78 ± 0.07	0.72 ± 0.02	0.68 ± 0.13
3B	1.23 ± 0.14	1.45 ± 0.08	1.26 ± 0.05	1.32 ± 0.14
4A	0.42 ± 0.02	0.58 ± 0.02	0.73 ± 0.03	0.58 ± 0.13
4B	0.60 ± 0.02	0.62 ± 0.02	0.91 ± 0.02	0.71 ± 0.15
5A	0.73 ± 0.02	0.78 ± 0.03	0.65 ± 0.03	0.73 ± 0.07
5B	1.78 ± 0.23	1.55 ± 0.15	1.95 ± 0.13	1.76 ± 0.23

medium in which glucose was the sole carbon source. However, glucose concentrations >150 g l^{-1} cause an inhibition of the cellular growth [8].

Similar behavior was observed for cells cultivated in media 3A and 3B, which contained, respectively, 100 and 150 g l^{-1} of glucose. Again, higher specific reaction rates were obtained for the highest analyzed glucose concentration. The behavior is the same in media 4A and 4B, although the observed reaction rates were smaller in this case. As these media contained only glucose and yeast extract, this result suggests that any improvement in enzyme activity depends not only on the glucose concentration, but also on the presence of others nutrients in the culture medium.

Figure 5 shows the average dynamic reaction rate profiles for each medium, taking into account all nine reactions. Figure 5 indicates that the analyzed media form four distinct sets of statistically equivalent reaction rate values. The worst results were those obtained for media 4A and 4B, which contained glucose and yeast extract only. This result clearly shows that the addition of nutritional supplements can be extremely important when the aim is to improve the production rates of lactobionic acid by permeabilized cells, due to the production of enzymes with enhanced activity. Figure 5 shows very clearly that proper formulation of the cultivation medium is fundamental for the production of lactobionic acid by permeabilized cells of *Z. mobilis* containing the enzyme GFOR.

In this study, particular attention was paid to the initial rates of product formation for two reasons. First, the GFOR enzyme can be inhibited by the product lactobionic acid, as reported in the literature [10, 11]. This phenomenon can be particularly important for reactions performed with permeabilized cells, as the acid accumulation effect may be magnified in the micro-environment in which the GFOR enzyme is found [16]. The second reason (dependent on the first) is related to the fact that in situ separation of the product (for instance, through electrodialysis) may be necessary for the minimization of product concentration (lactobionic acid). Based on these two factors, reaction

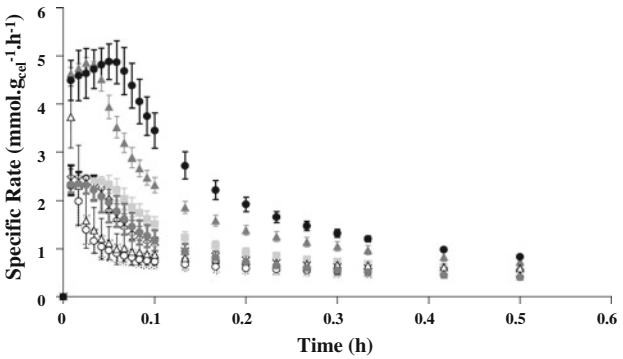

Fig. 5 Profile of specific formation rates of lactobionic acid for the nine reported reaction runs. *Vertical bars* Confidence intervals, *symbols* the different cultivation media: 1 (*open diamond*), 2 (*light-gray square*), 3A (*dark-gray circle*), 3B (*dark-gray triangle*), 4A (*open circle*), 4B (*open triangle*), 5A (*asterisk*) and 5B (*black triangle*)

medium 3B would appear to be the best one to produce cells containing an enzyme with enhanced activity.

The data in Table 2 verify that medium 5B presented with a higher specific rate than medium 3B. However, these data also show that the variability was much higher for medium 5B than for medium 3B, resulting in the average reaction rate values being not so different when these two reaction media are compared to each other. However, medium 3B can still be regarded as the best medium in terms of specific production rate.

Evaluation of the experimental repeatability

The repeatability of the experiments was analyzed as summarized in Fig. 6. The repeatability test was based on the analysis of the variance and aimed at determining whether the variability of the reaction rates performed with cells containing GFOR produced in fermentations with a specified medium (σ_{F1}^2, σ_{F2}^2 and σ_{F3}^2) were equivalent or not (variances were calculated with values obtained for different samples). First, the F value was calculated according to Eq. 2:

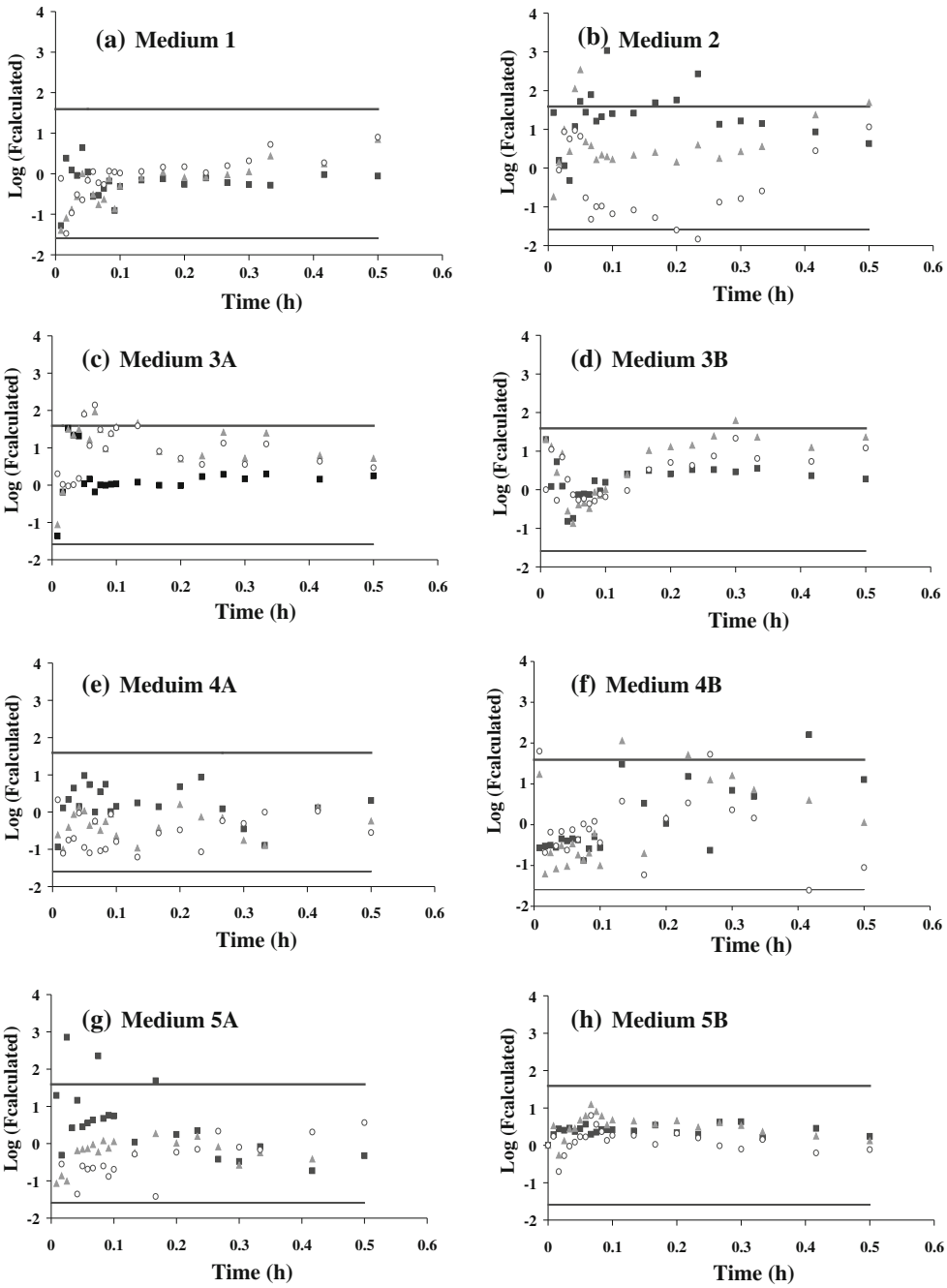

Fig. 6 *F* test values for pairs of reactions carried out with cells from fermentation media 1 (**a**), 2 (**b**), 3A (**c**), 3B (**d**), 4A (**e**), 4B (**f**), 5A (**g**), and 5B (**h**) and confidence limits for the *F*-test [Log ($F_{0,025}$) (*thick solid line*) and Log ($F_{0,975}$) *(thin gray solid line*)]. *Symbols F* test values for fermentations 1 and 2 (*black square*), 1 and 3 (*gray triangle*), and 2 and 3 (*open circle*)

$$F^*_{xy} = \frac{\sigma_x^2}{\sigma_y^2} \quad (2)$$

where σ_x^2 and σ_y^2 are variances obtained for measurements of different samples. When variances can be regarded as statistically equivalent, F^*_{xy} must lie between the established confidence limits at a specified confidence level, as described by the well-known *F* distribution [18, 23].

Figure 6 shows the *F* values, as calculated for pairs of fermentation runs performed with similar cultivation media, and the confidence intervals at a 95% level of confidence, as represented by the horizontal bars. Figure 6 also shows that, for all analyzed media and at a 95% level of confidence, variances in enzymatic activities obtained from single runs can be considered statistically equivalent (only few points for media 2, 3A, 3B, 4B and 5A lie outside the confidence region). Therefore, the *F* test shows that variances of the enzymatic reactions performed with cells containing GFOR produced in similar fermentation media had a similar variability, indicating that variances of enzymatic activity were always very similar and that sampling and reaction rate evaluation were reproducible in

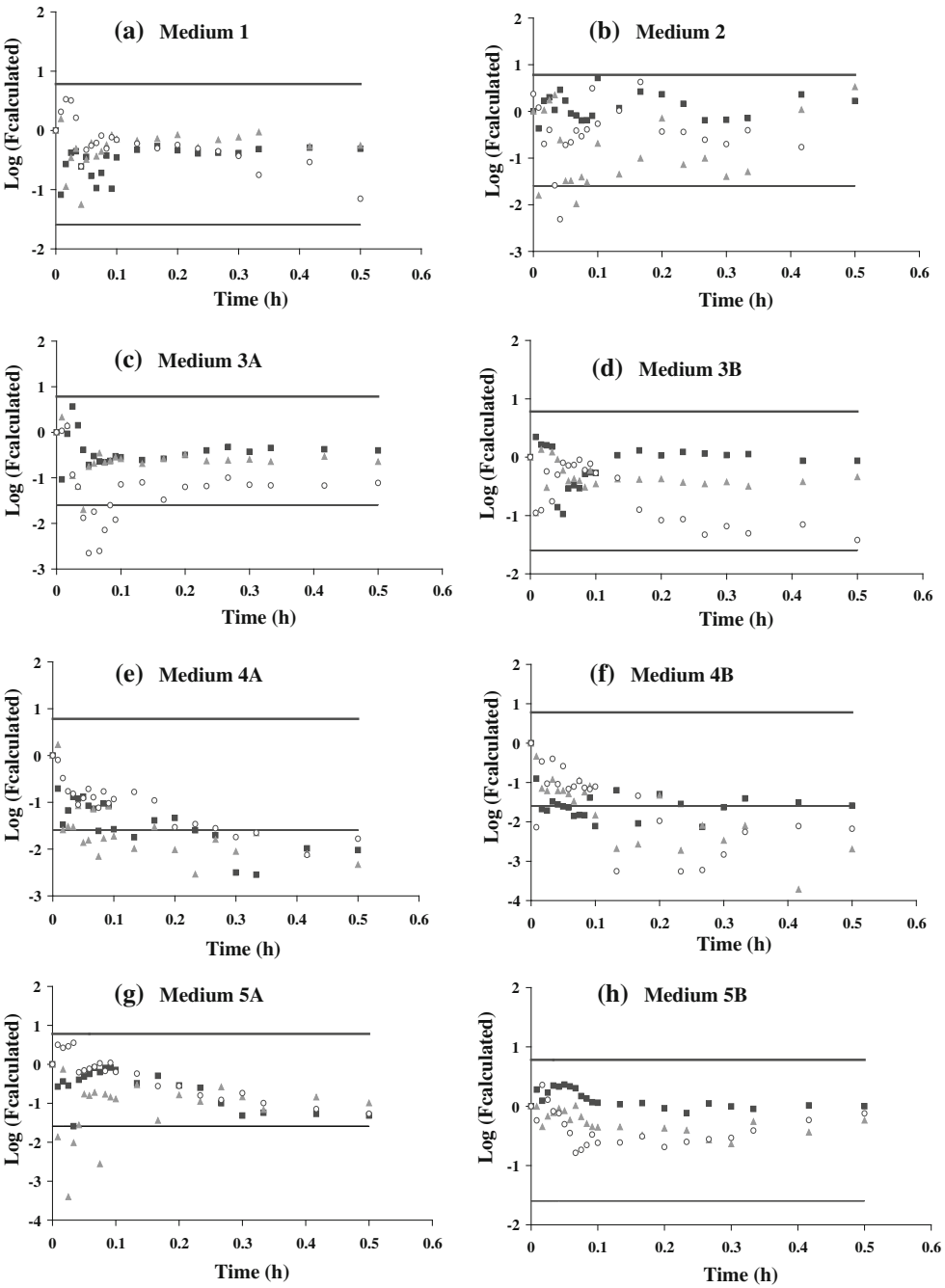

Fig. 7 F test values obtained when σ_M^2 is compared to σ_{F1}^2 (*black square*), σ_{F2}^2 (*gray triangle*) and σ_{F3}^2 (*open circle*) for distinct fermentation media. Confidence limits are represented by [(Log ($F_{0,025}$) (*thick solid line*) and Log ($F_{0,975}$) (*thin gray solid line*)]

the analyzed case. Despite this result, it must be emphasized that variances change with the reaction time and are larger at the beginning of the reaction, as shown in Fig. 4 and as already discussed.

Evaluation of the experimental reproducibility

In order to evaluate the reproducibility, the F test was applied to the variances calculated with all results available for all enzymatic reactions performed with cells obtained from similar cultivation media (σ_M^2) and variances calculated with results available for the enzymatic reactions carried out with cells from a single fermentation run (σ_{F1}^2, σ_{F2}^2 and σ_{F3}^2). The results are shown in Fig. 7. It can be seen that only media 4A and 4B presented results outside the confidence interval, indicating that specific reaction rates obtained with cells cultivated in these media were not reproducible. This result also indicates that cellular growth and enzyme production may be subject to much larger variations in media 4A and 4B, once more emphasizing that the addition of nutritional supplements may be of paramount importance during the production of

the GFOR enzyme. Therefore, the presence of nutritional supplement seems fundamental both to guarantee high enzymatic activities and the reproducibility of the fermentation runs. In all other cases, the distinct runs were similar to each other, demonstrating that medium preparation and cell inoculation were performed appropriately and did not introduce significant errors on the operation.

It is important to emphasize that repeatability and reproducibility tests are seldomly performed in enzymatic studies, although a fair repeatability and reproducibility of fermentations tests should not be taken for granted, given the large number of potential process perturbations. It is also important to emphasize that analyses of variances may indicate relevant mechanistic effects in the systems analyzed [5, 15]. For example, our results indicate the importance of nutritional elements in the lactobionic acid process. The poor reproducibility may be due to the complex and unknown composition of the yeast extract, which may affect the cell growth. When additional nutrients are added, a constant supply of nutrients is provided for cellular growth and reproducibility is improved. The statistical analysis also showed that the inoculation procedures adopted for cultivation of the *Z. mobilis* allowed for the production of enzyme with reproducible activity.

Analyses of inoculation

In order to verify whether the preparation of the inoculum might constitute a significant source of experimental error, we monitored the kinetics of cellular growth for each of the analyzed media, as shown in Fig. 8. Figure 8a and b shows the growth curves for media containing 100 and 150 g l^{-1} of glucose, respectively, with the vertical lines indicating the moment when the cells were harvested for inoculation of the medium used for cell generation and the production of GFOR. It is clear that after 18 h, cells in medium 4A were in the late stationary phase, while cells in medium 4B were in the deceleration phase.

As discussed in the previous section, it was verified that the specific reaction rates obtained with cells from media 4A and 4B were not reproducible, indicating the existence of at least one additional source of error. One possible source of error can be related to the growth phase at the moment of inoculation. It has already been reported by our group [1] that, during the inoculation step, the initial cell concentration and the physiological state of the inoculated cells can exert a large influence on microorganism growth, significantly affecting the reproducibility of the bioprocess and causing a larger dispersion of results. Figure 8a and b shows that in all of the cases analyzed, with exception of reaction media 4A and 4B, inoculated cells were in the exponential growth phase during inoculation. According to Abud [4] and Webb and Atkinson [21], inoculation should

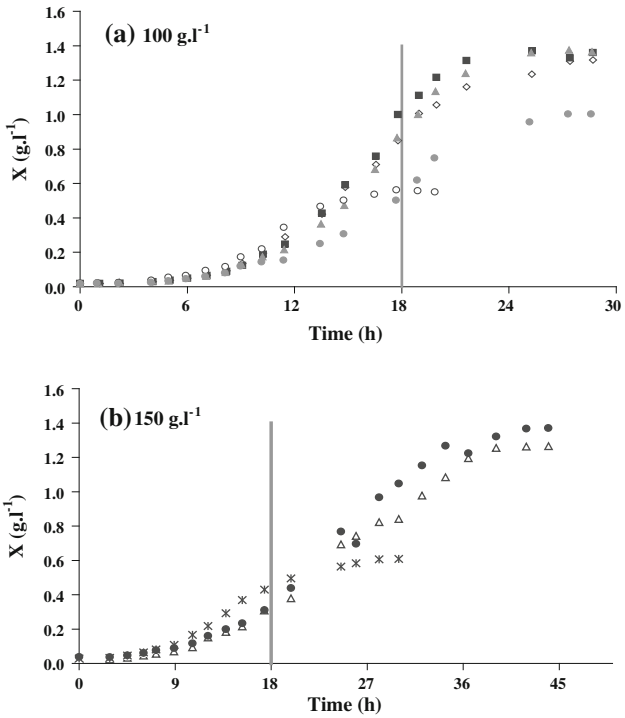

Fig. 8 Accompaniment of cellular growth of the *Z. mobilis* cells cultivated in several media with 100 (**a**) and 150 g l^{-1} (**b**) of glucose. The different media are indicated by *symbols*: 1 (*open diamond*), 2 (*black square*), 3A (*gray triangle*), 4A (*open circle*), 5A (*gray circle*), 3B (*open triangle*), 4B (*asterisk*) and 5B (*black circle*)

be performed in the exponential growth phase, with similar specific growth rates. Therefore, one can possibly conclude that the abnormal behavior observed in fermentations 4A and 4B was related to the inappropriate harvesting of cells used for subsequent inoculation or to the absence of nutritional components in the fermentation media. Therefore, one can possibly conclude that the abnormal behavior observed in fermentations 4A and 4B was related to the inappropriate harvesting of cells used for subsequent inoculation or to the absence of some salts and other nutrients in the media.

Conclusions

The results obtained in this study show that the proper formulation of culture medium is of fundamental importance if the optimization of sorbitol and lactobionic acid production is sought. It was observed that high glucose concentrations and the addition of nutritional supplements are necessary to guarantee high rates of product formation and that the proposed process is repeatable and reproducible, although the addition of nutritional supplements is also required to guarantee process reproducibility. The statistical analysis also showed that the inoculation step

adopted for the growth of the *Z. mobilis* allowed for production of the enzyme with reproducible activity. As shown experimentally, this is probably related to the fact that the exponential growth phase is very short when nutritional supplements are not added to the fermentation medium. Dynamic experiments showed that both reaction rates and measurement variances change along the time. As observed experimentally, reaction rate variances seem to be related to reaction rate values, as has also been observed for other chemical reaction systems.

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