Appl Microbiol Biotechnol (2000) 54: 487–493

ORIGINAL PAPER

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Effect of feeding strategy on *Zymomonas mobilis* CP4 fed-batch fermentations and mathematical modeling of the system

Received: 30 November 1999 / Received revision: 24 March 2000 / Accepted: 7 April 2000

Abstract In this work, the effect of the feeding strategy in *Zymomonas mobilis* CP4 fed-batch fermentations on the final biomass and ethanol concentrations was studied. Highest glucose yields to biomass (0.018 g/g) and to ethanol (0.188 g/g) were obtained in fed-batch fermentations carried out using different feeding rates with a glucose concentration in the feed equal to 100 g/l. Lower values (0.0102 g biomass/g glucose and 0.085 g ethanol/g glucose) were obtained when glucose accumulated to levels higher than 60 g/l. On the other hand, the highest biomass (5 g/l) and ethanol (39 g/l) concentrations were obtained using a glucose concentration in the feed equal to 220 g/l and exponentially varied feeding rates. Experimental data were used to validate the mathematical model of the system. The prediction errors of the model are 0.39, 14.36 and 3.24 g/l for the biomass, glucose and ethanol concentrations, respectively. Due to the complex relationship for describing the specific growth rate, a fed-batch culture in which glucose concentration is constant would not optimize the process.

Introduction

Gram-negative facultative *Zymomonas mobilis* bacteria have been considered a promising alternative to yeast in the synthesis of industrial ethanol. Compared with yeast, *Z. mobilis* has a higher tolerance to ethanol and better kinetic characteristics such as higher specific substrate uptake, ethanol synthesis rate and substrate yield to ethanol (Rogers et al. 1982). It has been reported that the growth rate of *Z. mobilis* is inhibited by glucose and ethanol. In continuous culture, the biomass, substrate and ethanol concentrations present an oscillatory behavior that has been attributed to the inhibition caused by ethanol (Daugulis et al. 1997). The source of this inhibition could be the rate of change of the ethanol concentration rather than a specific value thereof (Li et al. 1995). If this is the case, then fed-batch operation can be used to overcome the inhibition.

The purpose of fed-batch cultures is to control the nutrient concentration and to extend the productive phase of the batch process. Fed-batch production of the desired metabolite is generally characterized by the relationship between cell growth and nutrient consumption, the dependence of the desired metabolite synthesis dynamics on the feeding nutrient concentration (Gordillo et al. 1998), and the increase in the culture volume (Tulin et al. 1992). For the production of growth-associated products, the synthesis rate is a function of the specific growth rate. In this case it is of interest to feed the fermentor in such a way that the specific growth rate remains constant. An example of this is the production of hepatitis-B surface antigen by *Saccharomyces cerevisiae* (Agrawal et al. 1989; Alfafara et al. 1992).

In fed-batch fermentations, the feeding strategy can be defined based on an open-loop, if a mathematical model is available (Meszaros and Bales 1992), a feedback control (Kleman et al. 1991), or in another way depending on the specific kinetics of each fermentation. In fact the feeding rate can be modified depending on the cellular activity (Konstantinov et al. 1991). Recently, fed-batch fermentations have been used in the production of spectinomycin, an antibiotic whose synthesis rate is affected by the glucose and oxygen concentration (Gomes and Menawat 1998). In *E. coli* pH-stat fed-batch cultures, high levels of poly-(3-hydroxybutyrate) (PHB) have been observed (Wong and Lee 1998). The technique has also been used to overcome product inhibition in cultures of co-immobilized *Lactococcus lactis* and *Clostridium formicoaceticum* and to adapt cells to high acetate concentrations (Huang and Yang 1998).

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In this work the behavior of *Z. mobilis* CP4 fed-batch fermentations carried out using different feeding strategies is studied. The first objective of this work was to find out whether the final biomass and ethanol concentrations are affected by the feeding strategy; the second was to use the experimental data for validating the mathematical model of the system. This type of model can be used in future work in seeking the operational conditions that maximize the productivity of the system.

Materials and methods

Z. mobilis CP4 fed-batch fermentations were carried out in a laboratory fermentor (Biostat M, 2L) under controlled temperature (30 °C) and pH (6) and smooth agitation rate (150 rpm). The medium used in the batch and the feed has the following formulation: glucose 100 g/l; yeast extract 10 g/l; KH_2PO_4 1 g/l; $MgSO_4 \cdot 7H_2O$ 0.5 g/l; $(NH_4)_2SO_4$ 1 g/l. In those experiments in which a different glucose concentration was used, the concentration of the other components was changed accordingly. Feeding strategies such as constant feeding rate, exponentially varied feeding rate, and feeding rate varied so that the dilution rate of the culture is constant, were used. Different glucose concentrations in the feed were also used. In all the experiments the initial volume (500 ml, glucose concentration equal to 50 g/l) was inoculated with 50 ml (same concentration). The inoculum was prepared 24 h before the inoculation, picking up colonies of cells from plates. Feeding was started during the exponential growth rate phase once the optical density (600 nm) of diluted culture samples reached a specified value. Ethanol (NAD oxidation method; Merck), glucose (DNS method; Miller 1959) and biomass (dry weight) concentrations were determined at regular intervals. The *Z. mobilis* CP4 strain used in this work is found at Fundação Tropical de Pesquisas e Tecnologia "André Tosello", Campinas, São Paulo, Brasil (Number 2176).

Results

Fed-batch fermentations of *Z. mobilis* CP4

In fed-batch fermentations during the feeding period the synthesis rate of biomass and products either can or cannot exceed the dilution of the culture due to the fresh medium added. Dilution rate of the culture is defined as the ratio between the net flow rate through the culture and the culture volume (Yamane and Shimizu 1984). Relationships between the biomass and ethanol synthesis rates and the dilution rate are given by the mass balance equations shown in Table 1.

The results obtained in *Z. mobilis* CP4 fed-batch fermentations carried out using a constant feeding rate and glucose concentration in the feed (S_F) equal to 100 g/l are shown in Fig. 1. In the fermentation runs carried out with inlet flow rates lower than or equal to 0.22 l/h (Fig. 1a, b) the biomass concentration increases during the feeding period; thus biomass is growing at a specific growth rate greater than the dilution rate. The opposite is true when feeding rates higher than 0.22 l/h are used (Fig. 1c, d). In fed-batch fermentations substrate concentration will increase if its addition rate is higher than its uptake rate. This is the case in *Z. mobilis* CP4 fed-batch fermentations carried out using flow rates higher than 0.11 l/h. On the other hand, the ethanol concentration is not diluted if the feeding rate is lower than 0.55 l/h. Final biomass and ethanol concentrations and glucose yields obtained in fed-batch experiments carried out using constant feeding rates are given in Table 2. From these results the final biomass concentration and the glucose yield to biomass are a function of the feeding rate. On the other hand, the glucose yield to ethanol is in most cases not affected by the feeding rate. In terms of biomass and ethanol produced per unit of consumed glucose, the best operations are those carried out using feeding rates equal to 0.43 and 0.55 l/h, respectively.

In Fig. 2 results obtained in fed-batch fermentations carried out using exponentially varied feeding rates are shown; in these fermentation runs feeding was started with an initial rate equal to 0.015 l/h which was doubled at intervals of 0.5, 1.0 and 1.5 h (doubling time, DT). The results show that during the feeding period the biomass concentration is diluted, remains constant or

Table 1 Equations used for the mathematical description of *Zymomonas mobilis* CP4 fed-batch fermentations. Kinetic expressions for the specific growth rate and ethanol synthesis rate are those proposed previously (Jarzebski 1992). Parameter values proposed previously are indicated by *asterisks*

Model equations	
Biomass concentration	$\frac{dX}{dt} = \left(\mu - \frac{F}{V}\right)X$
Glucose concentration	$\frac{dS}{dt} = \frac{-\mu X}{Y_{XS}} - mX + \frac{F}{V}(S_F - S)$
Ethanol concentration	$\frac{dE}{dt} = Q_E - \frac{F}{V}E$
Volume of the culture	$\frac{dV}{dt} = F$
Kinetic relationships and parameters	
Specific growth rate	$\mu = 0.993\frac{S}{36.11+S}\left(1-\frac{E}{34.67}\frac{S}{2.33+S}\right)$ $\mu = 0.25^*\frac{S}{3.0^*+S}\left(1-\frac{E}{70.0^*}\frac{S}{3.0^*+S}\right)$
Specific ethanol synthesis rate	$Q_E = 8.68\mu X + 0.0X$ $Q_E = 4.26^*\mu X + 2.6^*X$
Glucose yield to biomass	$Y_{XS} = 0.032$ $Y_{XS} = 0.235^*$
Maintenance coefficient	$m = 0.0$ $m = 4.42^*$

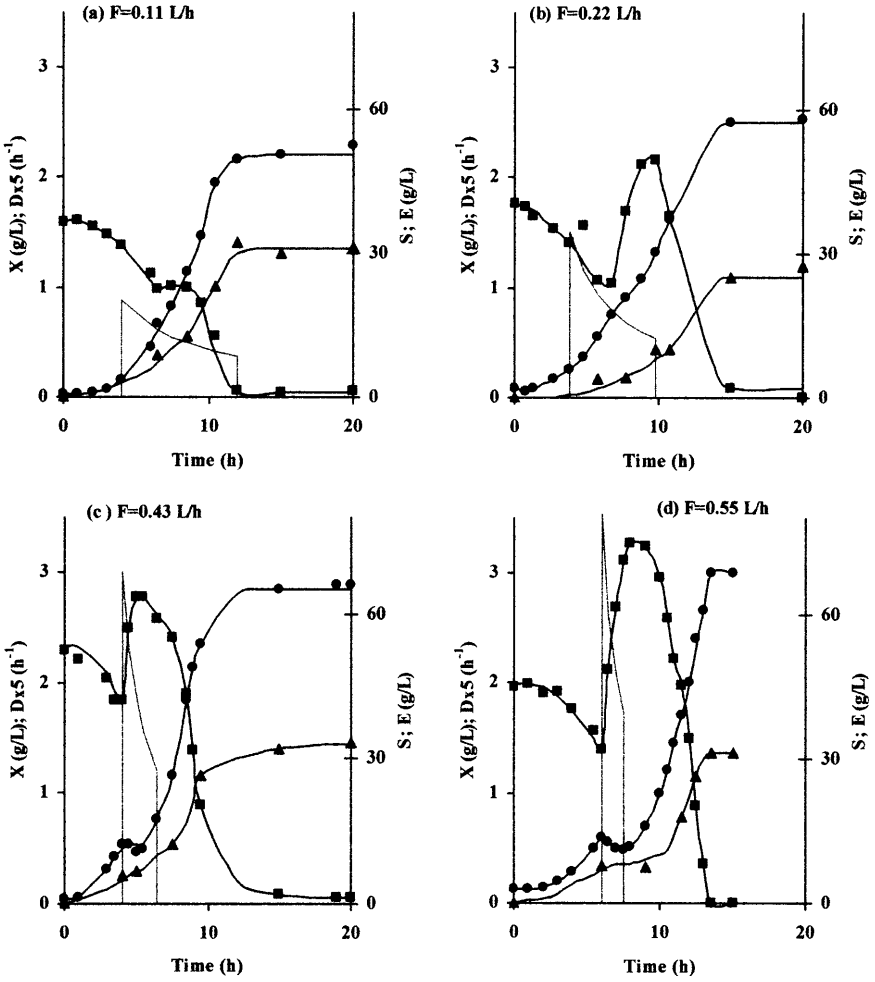


Fig. 1 Biomass (X; ●), glucose (S; ■) and ethanol (E; ▲) concentration profiles and dilution profile (D×5; —) in fed-batch fermentations of *Z. mobilis* CP4 carried out using constant inlet flow rate (F) and inlet glucose concentration equal to 100 g/l. $F = 0.11$ l/h (**a**), $F = 0.22$ l/h (**b**), $F = 0.43$ l/h (**c**), $F = 0.55$ l/h (**d**)

Table 2 Final biomass and ethanol concentrations and glucose yields into biomass and ethanol in fed-batch fermentations of *Zymomonas mobilis* CP4. F Inlet flow rate, DT interval after which the inlet flow rate is duplicated, D dilution rate of the culture, S_0 initial glucose concentration, S_F inlet glucose concentration, X_f final biomass concentration, E_f final ethanol concentration, TFS total substrate used in the fermentation

Run	F (l/h)	S_0 (g/l)	S_F (g/l)	X_f (g/l)	E_f (g/l)	X/TFS	E/TFS	E/X
1a	0.11	33	100	2.3	31	0.0138	0.186	13.48
1b	0.22	40	100	2.5	27	0.0147	0.159	10.80
1c	0.43	52	100	3.0	33	0.0170	0.188	11.00
1d	0.55	45	100	3.1	31	0.0180	0.180	10.00
DT (h)								
2-a	0.5	41	108	2.6	22	0.0142	0.121	8.46
2-b	1.0	52	116	3.1	35	0.0155	0.175	11.29
2-c	1.5	47	110	2.9	18	0.0154	0.095	6.21
DT (h)								
3-a	0.5	49	220	3.6	39	0.0102	0.110	10.83
3-b	1.0	45	220	4.8	30	0.0136	0.085	6.25
3-c	1.5	46	220	5.0	34	0.0141	0.096	6.80
D (h^{-1})								
4-a	0.1	47	100	2.7	26	0.0156	0.150	9.63
4-b	0.3	47	100	2.8	18	0.0161	0.104	6.43
4-c	0.44	41	100	2.6	25	0.0152	0.147	9.62

increases if DT values equal to 0.5 h (Fig. 2a), 1.0 h (Fig. 2b) or 1.5 h (Fig. 2c) are used. On the other hand, the ethanol concentration increases during the feeding period when the operation is carried out using a DT value larger than 0.5 h. The use of a DT value smaller than 1.5 h ensures that glucose is not depleted as soon as it is fed. Final biomass and ethanol concentrations present their maximum values in the operation in which DT is equal to 1.0 h (Table 2). The worst case for the ethanol synthesis is the one in which DT is equal to 1.5 h (Table 2) fermentation in which no glucose accumulation was observed.

The same exponential feeding rates (Fig. 2) but using a higher feeding glucose concentration ($S_F = 220$ g/l)

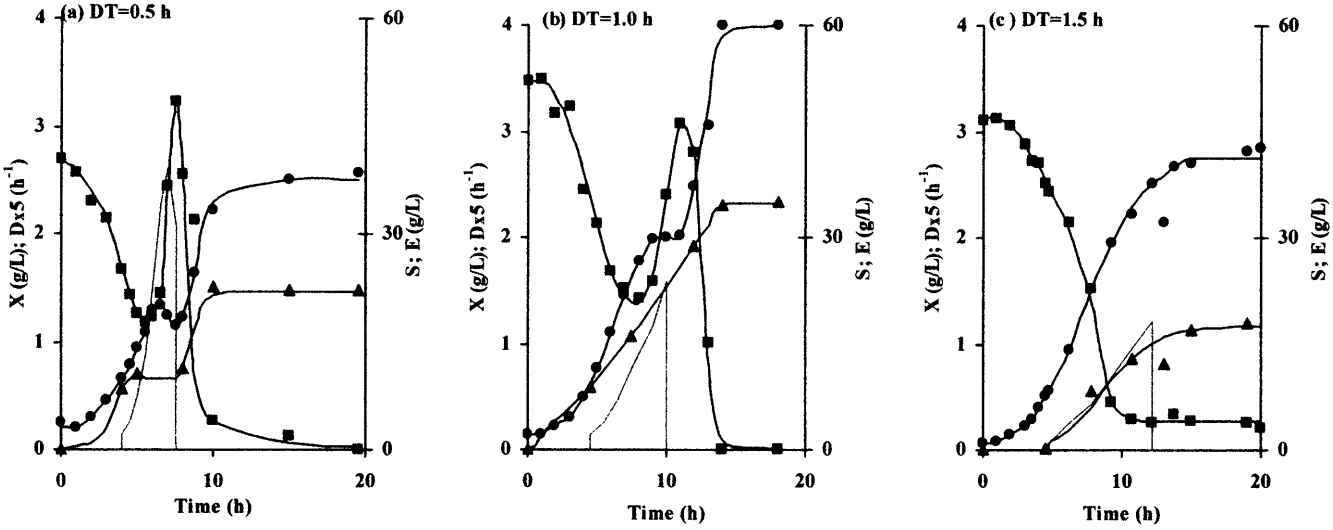


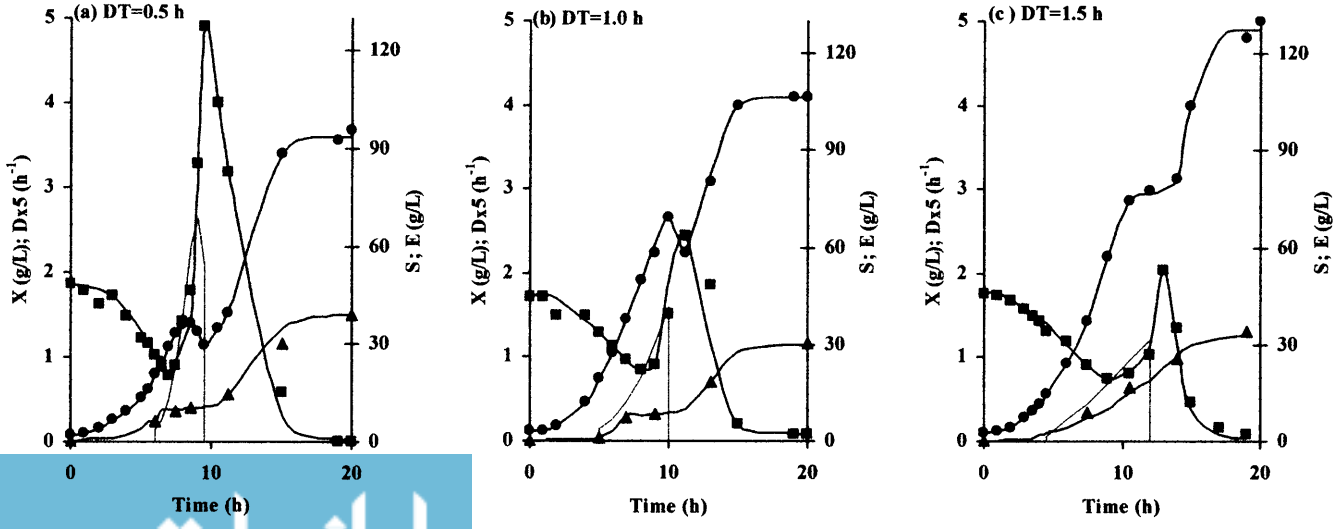
Fig. 2 Biomass (X; ●), glucose (S; ■) and ethanol (E; ▲) concentration profiles and dilution profile (D×5; —) in fed-batch fermentations of *Z. mobilis* CP4 carried out duplicating the inlet flow rate at different intervals (DT) and inlet glucose concentration equal to 100 g/l. DT = 0.5 h (**a**), DT = 1.0 h (**b**), DT = 1.5 h (**c**)

were also carried out (Fig. 3). In these experiments, the glucose concentration reaches higher values; information related to this effect can be deduced by comparing the results shown in Figs. 2 and 3. In the experiments in which a DT value equal to 1.0 h was used, whether the biomass concentration is diluted or not depends on whether S_F is equal to 220 (Fig. 3b) or to 100 g/l (Fig. 2b). A similar case is that found in the operation carried out using a DT value of 1.5 h. Here, the biomass concentration can either be kept constant (Fig. 3c) or increased (Fig. 2c) depending on the feed concentration.

Fig. 3 Biomass (X; ●), glucose (S; ■) and ethanol (E; ▲) concentration profiles and dilution profile (D×5; —) in fed-batch fermentations of *Z. mobilis* CP4 carried out duplicating the inlet flow rate at different intervals (DT) and inlet glucose concentration equal to 220 g/l. DT = 0.5 h (**a**), DT = 1.0 h (**b**), DT = 1.5 h (**c**)

Although these behaviors can be explained in terms of a depression in the specific growth rate due to the glucose concentration in the medium, this could not be the only reason. As reported previously, *Z. mobilis* specific growth rate is negatively affected by the ethanol concentration (Lee et al. 1979) and in at least one of these fermentations ethanol concentration is higher when a higher glucose concentration in the feed is used. On the other hand, ethanol concentration remains constant during the feeding period no matter what the S_F value is (Figs. 2a and 3a) if a DT value of 0.5 h is used. However, using a DT value equal to 1 h, ethanol concentration increases (Fig. 2b) or remains constant (Fig. 3b) depending on the feeding concentration. From the results given in Table 2, glucose yield to biomass and that to ethanol are lower when glucose accumulates in the culture.

Profiles obtained in fed-batch fermentations in which the feeding rate was changed so that the dilution rate of the culture was constant are shown in Fig. 4. When the culture is diluted at a rate of 0.10 h^{-1} (Fig. 4a) the biomass concentration increases. A small increase and a decrease in this concentration are obtained when dilu-



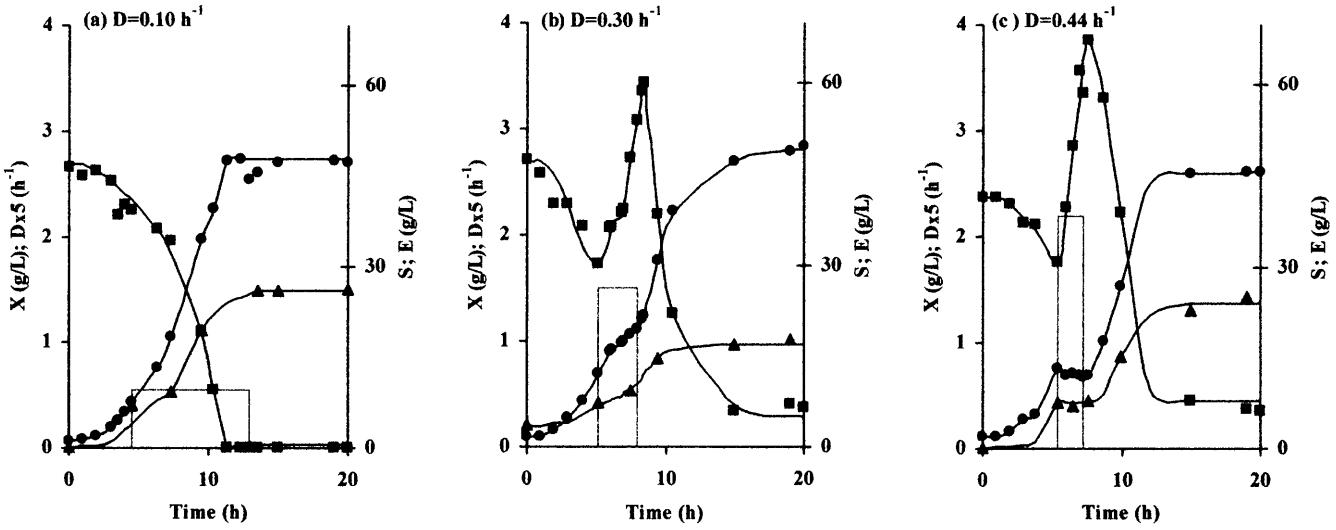


Fig. 4 Biomass (X; ●), glucose (S; ■) and ethanol (E; ▲) concentration profiles and dilution profile (D×5; —) in fed-batch fermentations of *Z. mobilis* CP4 carried out changing the inlet flow rate so the dilution rate of the culture (D) remains constant and inlet glucose concentration equal to 100 g/l. $D = 0.10\ h^{-1}$ (**a**), $D = 0.30\ h^{-1}$ (**b**), $D = 0.44\ h^{-1}$ (**c**)

tion rates are equal to 0.30 h^{-1} (Fig. 4b) and 0.44 h^{-1} (Fig. 4c), respectively. In this way and for the conditions tested, specific growth rate is between these values. Minimum changes in the final biomass concentration were obtained in these experiments (Table 2). A more significant change is that of the final ethanol concentration, which presents its lowest value in the operation carried out at a dilution rate of 0.30 h^{-1} (Table 2).

Mathematical modeling of *Z. mobilis* CP4 fed-batch fermentations

Differential equations used for computing biomass, glucose and ethanol concentrations in *Z. mobilis* CP4 fed-batch fermentations are obtained from the mass-balance equations (Table 1). Kinetic expressions for the specific growth rate and ethanol synthesis rate are those used by Jarzebski (1992) to model a *Z. mobilis* continuous culture. Parameters in the model were estimated using non-linear optimization techniques (Matlab 1998) in order to minimize the error between the experimental data and that computed using the model (Shene et al. 1999).

The parameter values obtained in this work are different from those reported previously (Table 1). The estimated value for the Monod constant (K_{S1}) is equal to 36.1 g/l; a value that is larger than that reported (3 g/l). On the other hand, if the proposed kinetic relationship for the specific growth rate is compared with the Monod equation, the Monod μ_{max} value is lower than 0.993 h^{-1}; here Monod's maximum specific growth rate depends upon glucose and ethanol concentrations. This can explain why values as high as 0.993 h^{-1} were not reached (data not shown). In the experiments described here, the inhibition effect due to the glucose concentration is not as important as that due to the ethanol concentration. As soon as this concentration differs from zero, the specific growth rate is reduced and, for values close to E_{max} (34.67 g/l), bacteria grow at very low specific rates. Glucose yield to biomass (Y_{XS}) that models the experimental data is closer to the values given in Table 2 than the reported value. In this model ethanol synthesis rate is described as growth-rate associated, which agrees with the experimental data. A comparison between the experimental values and those computed using the mathematical model is shown in Fig. 5 for two of the fermentation runs.

Discussion

The experiments have demonstrated that glucose yield to biomass and that to ethanol are negatively affected by glucose accumulation. Thus, the productivity of *Z. mobilis* CP4 fed-batch fermentation depends on the feeding strategy. The experiments have also shown that ethanol synthesis is growth-rate associated. Because of this, a feeding strategy designed to keep the culture growing at a constant high rate could improve the ethanol productivity.

Differences between reported and estimated parameter values in the mathematical model could be due to the different *Z. mobilis* strain and the culture conditions (pH and temperature) used in the fermentation runs. Moreover, in the present work a different mode of operating the culture has been used. In the model the maximum specific growth rate depends on the glucose and ethanol concentrations, with the contribution of the latter being more significant. Since the model is able to simulate the evolution of the system under different feeding strategies with a certain precision (0.39, 14.36 and 3.24 g/l for the biomass, glucose and ethanol concentrations, respectively), it can be used to define those feeding strategies that

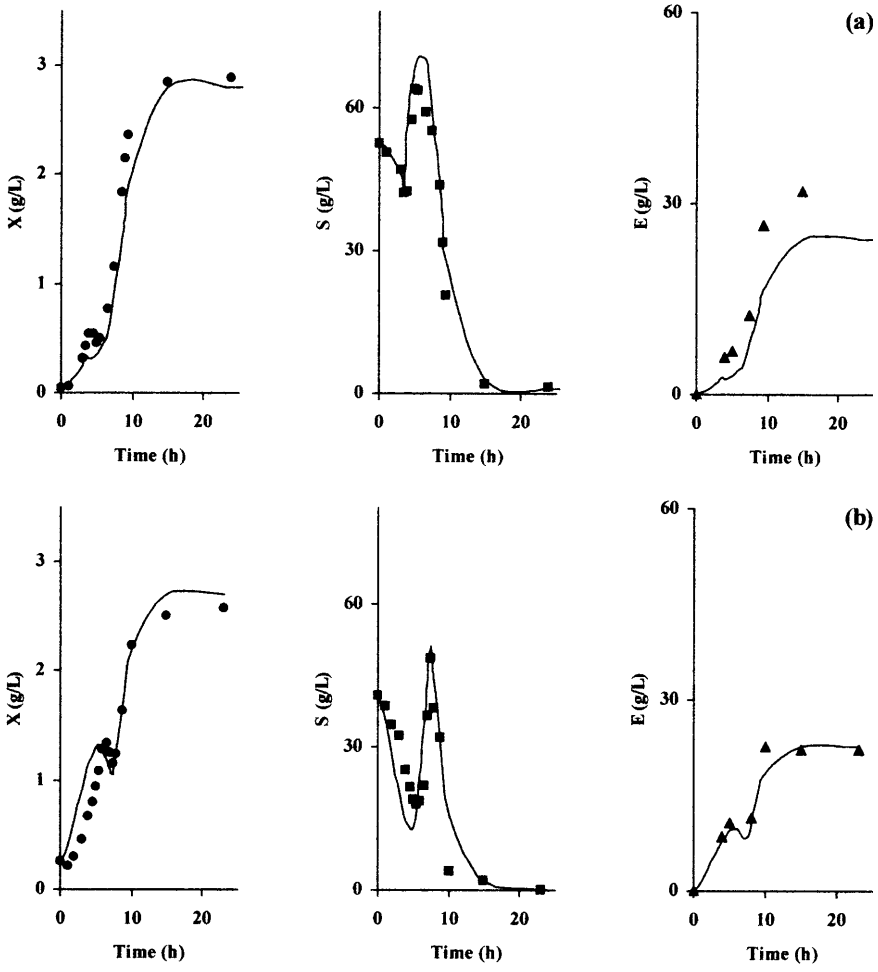


Fig. 5 Comparison of the experimental data and the computed values using the mathematical model of *Z. mobilis* CP4 fed-batch fermentations (Table 1). Biomass (*X*; ●), glucose (*S*; ■) and ethanol (*E*; ▲) concentrations in fed-batch experiments carried out using inlet glucose concentration equal to 100 g/l and inlet flow rate equal to 0.44 l/h (**a**), duplicating the inlet flow rate each 0.5 h (DT = 0.5 h) (**b**). *Continuous lines* are the predictions of the model

improve the system productivity. However, from the kinetics proposed here in which the specific growth rate is negatively affected by the ethanol concentration that increases during the process, a constant glucose concentration in the culture would not result in a constant specific growth rate. Thus, computational simulations should be carried out to find out which are the best feeding strategies. At present, this mathematical model coupled with constrained optimization algorithms (Shene et al. 1998) is being used to derive the feeding strategies that maximize the final biomass and ethanol concentrations.

Acknowledgments The authors wish to thank Direccion de Investigacion de la Universidad de La Frontera and Fondo Nacional de Ciencia y Tecnología for their financial support through projects 9907 and 1000344, respectively.

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