Brazilian Journal
of Chemical
Engineering

ISSN 0104-6632
Printed in Brazil

Vol. 21, No. 04, pp. 509 - 518, October - December 2004

HYBRID NEURAL NETWORK MODEL FOR SIMULATING SORBITOL SYNTHESIS BY GLUCOSE-FRUCTOSE OXIDOREDUCTASE IN *Zymomonas mobilis* CP4

S. Bravo, M. C. Diez and C. Shene*

Department of Chemical Engineering, Universidad de La Frontera,
Fax: (56) (045) 325-053, Casilla 54-D, Temuco, Chile.
E-mail: cshene@ufro.cl

(Received: May 19, 2003 ; Accepted: June 4, 2004)

Abstract - A hybrid neural network model for simulating the process of enzymatic reduction of fructose to sorbitol process catalyzed by glucose-fructose oxidoreductase in *Zymomonas mobilis* CP4 is presented. Data used to derive and validate the model was obtained from experiments carried out under different conditions of pH, temperature and concentrations of both substrates (glucose and fructose) involved in the reaction. Sonicated and lyophilized cells were used as source of the enzyme. The optimal pH for sorbitol synthesis at 30º C is 6.5. For a value of pH of 6, the optimal temperature is 35º C. The neural network in the model computes the value of the kinetic relationship. The hybrid neural network model is able to simulate changes in the substrates and product concentrations during sorbitol synthesis under pH and temperature conditions ranging between 5 and 7.5 and 25 and 40º C, respectively. Under these conditions the rate of sorbitol synthesis shows important differences. Values computed using the hybrid neural network model have an average error of $1.7 \cdot 10^{-3}$ mole.
Keywords: sorbitol synthesis, neural network model, glucose-fructose oxidoreductase in *Zymomonas mobilis* CP4.

INTRODUCTION

Glucose-fructose oxidoreductase (GFOR) in *Zymomonas mobilis* catalyzes the simultaneous conversion of glucose and fructose into glucono-δ-lactone and sorbitol, respectively. While sorbitol is not metabolized by the bacteria, glucono-δ-lactone is hydrolyzed to gluconic acid (Chun and Rogers, 1998). In *Z. mobilis* CP4 fermentations, sorbitol production depends on glucose and fructose concentrations with yields not higher than 5% (Shene and Bravo, 2001), probably because sorbitol acts only as an intracellular osmotic protector in high sugar concentration media (Loos et al., 1994). Because of this, enzymatic sorbitol production should be separated from the growth of the bacteria.

*To whom correspondence should be addressed

Neural Networks

The neural network concept is used for describing a particular type of model that emulates the way the human brain works. In these models the basic unit is the neuron. A neuron transforms the sum of its weight inputs (activation value) through a transfer function. Parameters in the net, weight factors and bias (vector w) are fitted in the training process. If backpropagation of the error is used for training, w is determined, so the error function, E(w), is minimized:

$$E(w) = \frac{1}{2} \sum_{i=1}^{L} (td_i - f(x_i, w))^2 \qquad (1)$$

where L is the number of training data pairs {x, td}, x the input data, td the target data and f(x, w) the output of the neural network for the given input.

The prediction capability of a neural network will depend on its design; on the quality of data used for training, considering both the noise and its distribution in the space; and on the training algorithm. Neural networks are basically used as interpolating tools.

Hybrid Neural Network Models

Hybrid models combine more than one approach for simulating the behavior of a given system. In particular, in the modeling of chemical and biochemical processes hybrid models take advantage of the capabilities of neural networks for describing complex functions in the mathematical models derived from first principles. Kinetic relationships are examples of these functions.

Kinetic relationships in biological processes are in most cases nonlinear functions that involve more than one condition of the environment in which the phenomenon takes place, such as substrate and product concentrations, temperature and pH. Even in the case in which a mathematical relationship is available for describing the kinetics, this expression usually contains one or more parameters that have to be estimated. The use of neural networks for computing the kinetics avoids one of the steps in the solution of the problem, which is to establish the relationship between the different variables that affect the kinetics.

In the problem analyzed in this work, product formation takes place once the enzyme is added to the substrate solution (glucose and fructose). During the process, pH and temperature are held constant. Mass balances for the substrates and the product form a system of differential equations that are related through the kinetic relationship, r_S. In addition to these mass balance equations, a first-principle-based model has to include an equation for describing enzyme activity in terms of system conditions. Since one of the products of the enzymatic reaction (glucono-δ-lactone) is hydrolyzed to gluconic acid and the process is carried out at constant pH, a neutralizing solution has to be added. Because of this, the model has to consider an equation for computing the total mass of the system. Table 1 shows the model for describing the enzymatic sorbitol synthesis. In the equation for system volume, it has been assumed that for each mole of fructose converted into one mole of sorbitol, one mole of glucose is converted into one mole of glucono-δ-lactone that is hydrolyzed producing one mole of gluconic acid. In our experiments the acid formed was neutralized with a solution at a concentration of 0.5 N.

A diagram of the approach for simulating the sorbitol synthesis process at a constant pH using the hybrid neural network model is shown in Figure 1. Input variables of the neural network are time (t), glucose ([Glu]) and fructose ([Fru]) concentrations, pH and temperature (T). The net output variable is the value for the sorbitol synthesis rate, r_S, for the given system conditions ([E], [Glu], [Fru], pH, T). Some of the input variables of the neural network and the net output are used for integrating the differential equations.

In this work results of the experiments carried out to establish the effects of pH, temperature and the concentration of both substrates (glucose and fructose) in enzymatic sorbitol synthesis catalyzed by GFOR in *Z. mobilis* CP4 are presented. Sonicated and lyophilized cells were used as the source of the enzyme. The experimental data was used to derive and validate the hybrid neural network model that simulates system behavior under the different conditions tested.

Table 1: Mathematical model for sorbitol synthesis in glucose-fructose mixtures catalyzed by glucose-fructose oxidoreductase in *Z. mobilis* CP4.

Glucose,	$\frac{dG}{dt} = \frac{d([Glu]\cdot V)}{dt} = -r_S \cdot V$
Fructose,	$\frac{dF}{dt} = \frac{d([Fru]\cdot V)}{dt} = -r_S \cdot V$
Sorbitol,	$\frac{dS}{dt} = \frac{d([Sor]\cdot V)}{dt} = r_S \cdot V$
Enzyme,	$\frac{d([E]\cdot V)}{dt} = -k_D(T, pH, t)\cdot V$
Volume,	$\frac{d(V)}{dt} = \frac{r_S \cdot V}{0.5}$
Kinetic relationship, r_S,	$r_S = r_S([E], [Glu], [Fru], pH, T, t)$

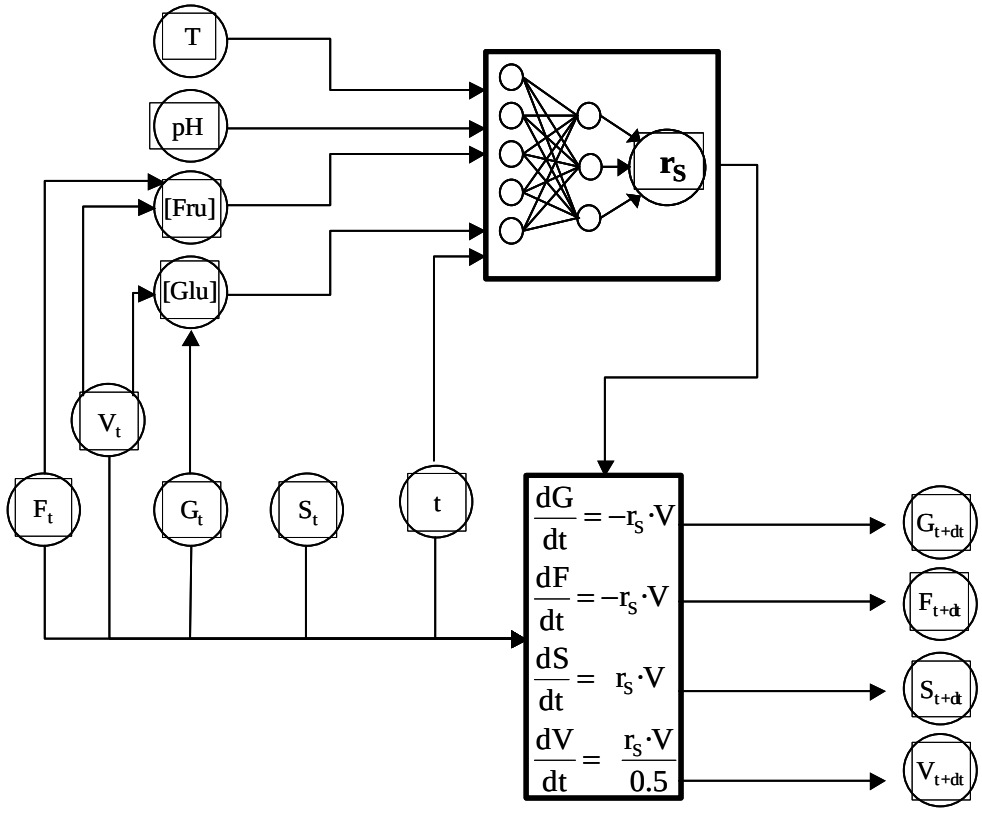


Figure 1: Diagram of the hybrid neural network model used for simulating enzymatic sorbitol synthesis using GFOR in *Z. mobilis* CP4.

MATERIALS AND METHODS

Crude Extract

Z. mobilis CP4 strain used in this work was provided by Fundação Tropical de Pesquisas e Tecnologia André Tosello, Campinas, Sao Paulo, Brazil (Number 2176). Biomass was grown for 24 h on glucose (200 g/L) (Shene and Bravo, 2001) at fixed pH (6) and temperature (30º C) (Bioflo 2000, New Brunswick, USA). Culture medium was centrifuged (10000 rpm, 10 min) and the pellet was washed twice with phosphate buffer 0.1 M (pH 6.4). Cells resuspended in the same buffer were sonicated in 10 bursts of 60 s (170 Watts) (Misonix, New York, USA) the solution was lyophilized and the powder was used as the source of the enzyme. Activity of the GFOR in the extract was quantified measuring the mass of sorbitol synthesized in the 30 min incubation of 0.5 mL of the extract solution (40 g/L), 5 mL of the glucose-fructose mixture (50 g/L glucose, 50 g/L fructose) and 5 mL of phosphate buffer at 30º C. One enzyme unit refers to 1 μmole of sorbitol produced per minute. Protein concentration in the extract was 538 mg/g and the specific activity of the GFOR was 0.036 U/mg.

Enzyme Experiments

The extract (0.8 g) was dissolved in 20 mL of distilled water and added to 500 mL of the glucose-fructose solution. In order to avoid contamination, 4 mg of sodium azide were added to the mixture. The experiments were carried out in a fermentor (Biostat M, B. Braun, Germany) at constant temperature and pH; pH was controlled through addition of 0.5 N KOH. The volume added was recorded for computing the dilution of the culture.

Analytical

Glucose, fructose and sorbitol concentrations were measured by HPLC (Waters Assoc, Milford, Mass, USA). Twenty μL of the sample were injected into a Supelcogel Ca column (300x7.8 mm, Supelco, Bellfonte, PA, USA). Column temperature was maintained at 80º C. Water was used as the mobile phase at a flow rate of 0.5 mL/min. Column eluent

was detected with the refractive index (Waters Assoc, Milford, Mass, USA). Concentration of the compounds was computed using Chromatography Station for Windows (CSW v. 1.7, 1998) (DataApex, Prague, Czech Republic). Protein content was measured by the Bradford method with BSA as standard (Bradford, 1976).

Software

Computational programs for designing the neural networks were implemented in Matlab (2000). Backpropagation was used to train the net. Parameter values in the net were calculated using the Levenberg-Marquardt algorithm (Demuth and Beale, 1998).

Neural Network Design

The neural network that computes the value of the kinetic relationship has an outer layer with one neuron that computes its output using the logarithmic sigmoid transfer function. A more complicated neural network design was also tested. In this case, a hidden layer with neurons that compute their outputs using the hyperbolic tangent sigmoid transfer function was considered.

The neural network in the hybrid model computes the value of a variable that is not measured experimentally, but has to be estimated from the derivatives of the substrates and product profiles during the process (Table 1). Spline interpolating functions (Press et al., 1992) built with the experimental data were used to generate the profiles and from these the values of the derivatives were computed (for example from the sorbitol and volume profiles $r_S=(d([Sor]V)/dt)V^{-1}$). Variables for input into and output variables from the neural network were normalized (for the temperature, $T_{input} = (T-T_{min})/(T_{max}-T_{min})$; $T_{min} = 25^o$ C; $T_{max} = 40^o$ C) so the net was fed and predicted values between 0 and 1. Available data, corresponding to 263 pairs of input/output data, computed using the spline functions, were randomly separated in sets for training the neural network and validating the hybrid neural network model predictions. In order to avoid overtraining, 25% of the data in the training set was used as the neural network validating set; thus training was carried out for a number of iterations so that the error obtained with this validating set did not increase.

For the case in which the neural network has a hidden layer, the number of neurons in this layer was that for which the lowest prediction error of the hybrid model was obtained. Prediction error was defined as the square root of the average of the sum of the squared differences between experimental values and those computed by the model.

Differential Equations.

Differential equations were integrated using the fourth-order Runge-Kutta formula.

RESULTS AND DISCUSSION

Sorbitol Synthesis Experiments

Results of the experiments carried out to establish the effect of process conditions on sorbitol synthesis are shown in Figures 2 to 7. In each of these figures, the profiles of the moles of substrates and product are shown. As indicated above, when the process is carried out at a constant pH, the addition of the solution for neutralizing the acid formed changes the system volume. Because of this, product, substrate and enzyme concentrations are diluted, a fact that has to be taken into account to explain possible decreases in the rate of sorbitol synthesis.

Results obtained in the experiments carried out at 30º C and at different pH values are shown in Figure 2. In these experiments the initial concentration of the sugar mixture was 1.11 M, with glucose and fructose in the same molar fraction. Data in Figure 2 shows that the lowest rate of sorbitol synthesis was the one taking place at pH 7.5 and at optimal pH (6.5) 76% of the initial fructose was reduced to sorbitol.

The effect of temperature on the enzymatic sorbitol synthesis carried out at pH 6 in a sugar mixture with an initial concentration of 1.11 M is shown in Figure 3. The lowest sorbitol production was obtained when the process was carried out at 25º C. At 40º C sorbitol was initially synthesized at a rate close to those of processes carried out at lower temperatures (30 or 35º C). However, at this temperature after 10 h the synthesis rate decreased to zero. The effect of temperature on the residual activity of the extract kept in solution with no substrates was also determined. These results, shown in Figure 4, indicate that the activity of GFOR in *Z. mobilis* CP4 decreased when the enzyme was kept in solution (phosphate buffer 0.1 M, pH 6.5) at temperatures of 35 and 40º C.

Results of the experiments carried out to test the effect of the initial concentration of the equimolar mixture of sugars (1.11, 1.67, 2.22 and 3.33 M) are

shown in Figure 5. According to these results, in the period between 0 to 5 h the initial concentration of the mixture had no effect on sorbitol synthesis. Figure 6 shows the results of the experiments carried out using different molar ratios of glucose and fructose (G:F) but keeping the same initial concentration of mixture (1.11 M). The lowest sorbitol production took place when the G:F ratio was 9:1.

The effect of enzyme concentration is shown in Figure 7; here the results obtained with 1.5 and 3.0 g/L of the cell extract are compared. These results indicate that not only did the amount of fructose reduce to sorbitol increased from 69 to 94%, but also the rate of the process increased when the concentration of enzyme was doubled.

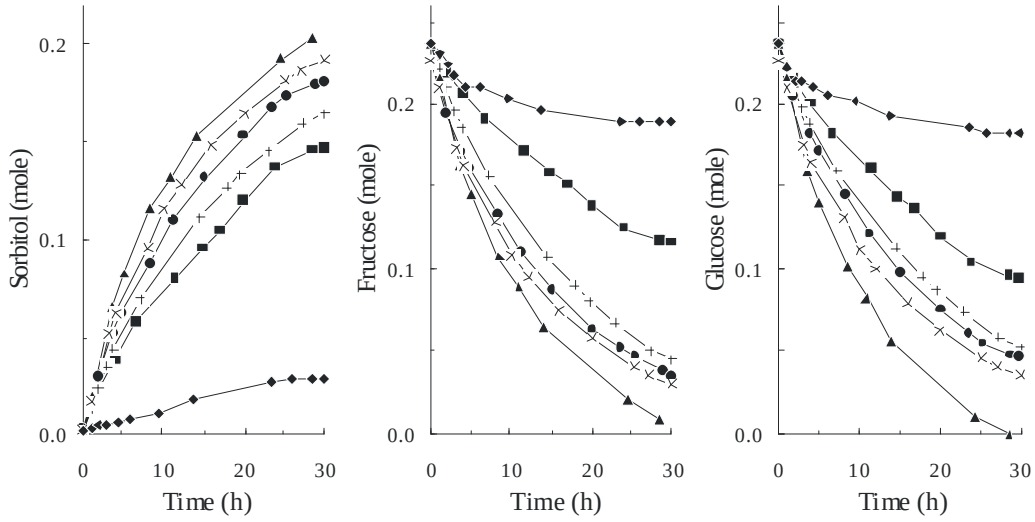


Figure 2: Effect of pH on sorbitol synthesis catalyzed by GFOR in *Z. mobilis* CP4 at 30 ºC (■ 5.0; +, 5.5; ●, 6.0; ▲, 6.5; ´, 7.0; ♦, 7.5).

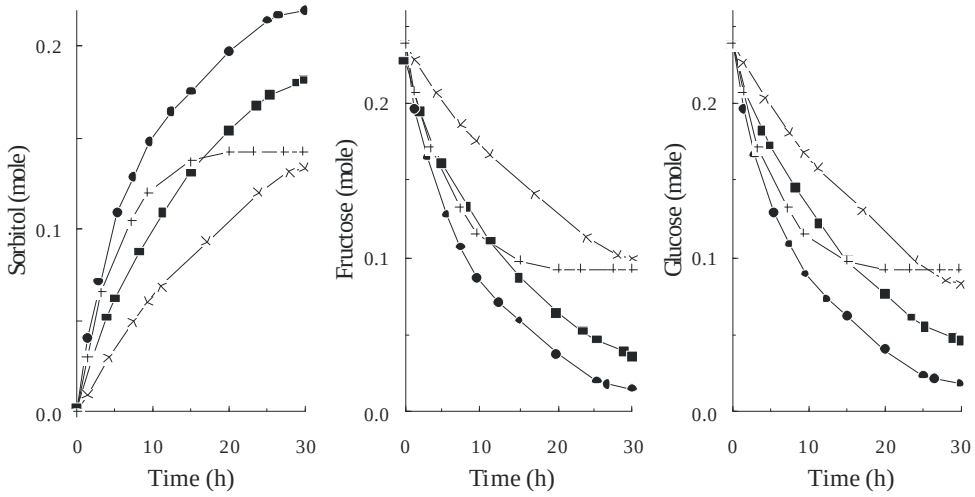


Figure 3: Effect of temperature on sorbitol synthesis catalyzed by GFOR in *Z. mobilis* CP4 at pH 6 (´, 25 ºC; ■, 30 ºC; ●, 35 ºC; +, 40 ºC).

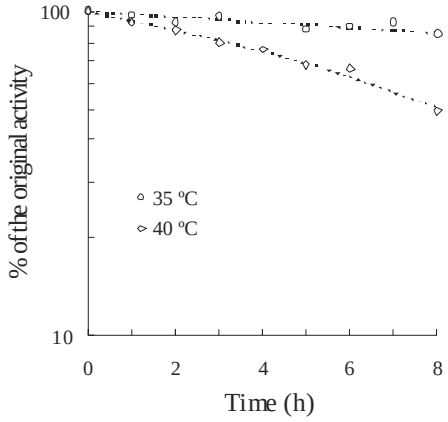


Figure 4: Effect of temperature on the stability of GFOR in *Z. mobilis* CP4.

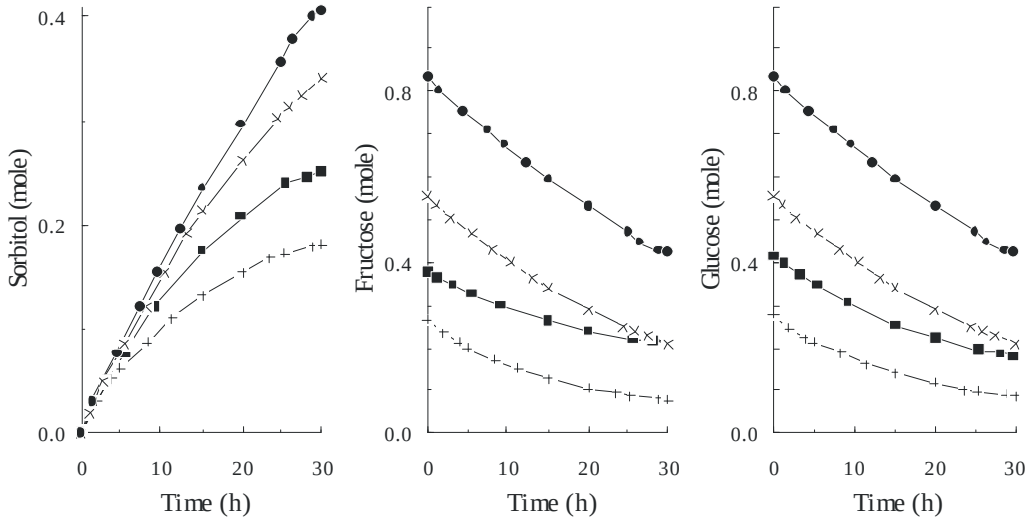


Figure 5: Effect of the initial concentration of sugar mixture on sorbitol synthesis catalyzed by GFOR in *Z. mobilis* CP4 at 30 ºC and pH 6. (+, 1.11 M; ■, 1.67 M; ´, 2.22 M; ●, 3.33 M).

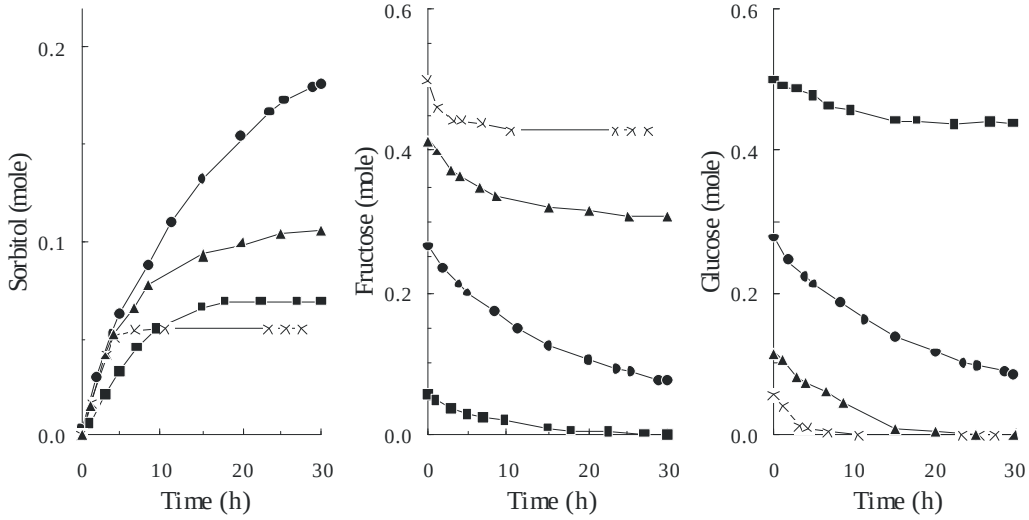


Figure 6: Effect of the glucose-to-fructose molar ratio, G:F, in the sugar mixture on sorbitol synthesis catalyzed by GFOR in *Z. mobilis* CP4 at 30 ºC and pH 6. (●, G:F = 1:1; ■, G:F = 9:1; ´, G:F = 1:9; ▲, G:F = 1:3).

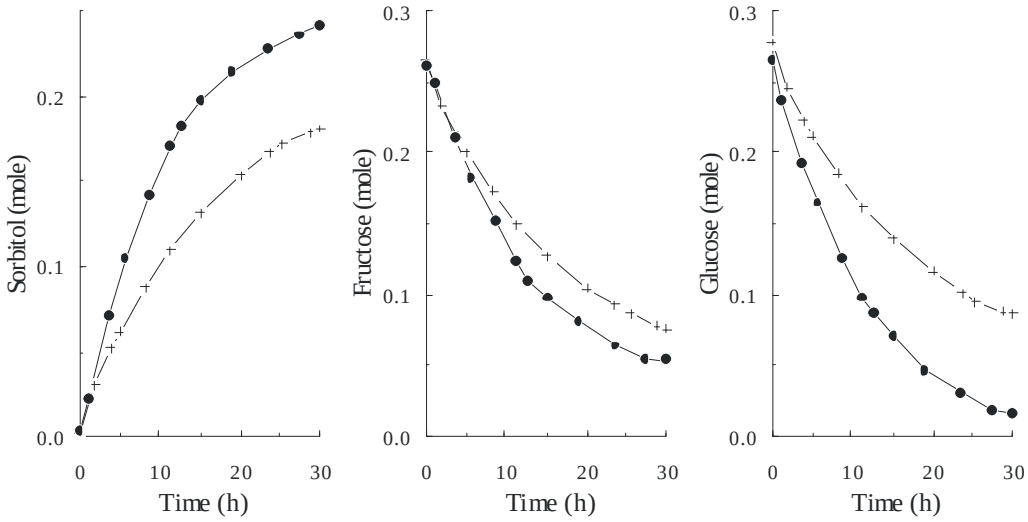


Figure 7: Effect of concentration of the extract used as the enzyme source on sorbitol synthesis at 30 ºC and pH 6 (+, 1.5 g/L; ○, 3.0 g/L).

Hybrid Neural Network Model for Simulating Enzymatic Sorbitol Synthesis

The hybrid neural network model for simulating enzymatic sorbitol synthesis is formed of a four differential equation system (Table 1) and a neural network that computes the kinetics. When the process is simulated using this model, the equation for enzyme activity is not considered because the net was trained with values of the instantaneous rate of sorbitol synthesis in which the state of the enzyme is taken into account.

The prediction capability of the hybrid model, in which the neural network that computes the value of the kinetic relationship, r_S, has no hidden layer, was tested. The error of the model obtained with neural networks trained with training data sets of different sizes is shown in Table 2. Total prediction error of the hybrid model was 21.74, 21.71 and 22.14 when the neural network was trained with 40, 50 and 60% of the available data, respectively. The variable that most contributed to the prediction error was volume of the system.

A neural network with no hidden layer is the simplest net design and from the results obtained it was not possible to improve its performance by changing the size of the training data set. However, using a neural network having a hidden layer with 30 neurons, the total prediction error of the hybrid model decreased from 10.96 to 9.82 when the size of the training set increased from 50 to 60%. When a smaller training set (40% of the available data) was used for training the neural network a prediction error of 10.93 was obtained with 35 neurons in the hidden layer. An error on the same order (10.96) was obtained using a neural network having 30 neurons in the hidden layer that was trained with 50% of the data (Table 2).

Results in Table 2 show that if the neural network that computes the value of the kinetics has a hidden layer it is possible to reduce the prediction error of the hybrid model, unlike what occurs in the case in which the neural network has no hidden layer.

Graphs in Figure 8 compare the experimental values of the moles of sorbitol (not used for training) and those values computed by the hybrid model in which the neural network that computes the kinetics has a hidden layer with 30 neurons trained with 60% of the available data. From the comparisons shown in Figure 8, the hybrid neural network model derived in this work has the capability of simulating the enzymatic sorbitol synthesis (at constant pH) for operational conditions that significantly affect the rate of sorbitol synthesis.

Although neural networks are commonly used as interpolating tools, some simulations were carried out and these results are presented next. The neural network having 30 neurons in the hidden layer trained with 60% of the experimental data was used to compute the initial values (t = 0 h) of the kinetic relationship for all the temperatures and pH values tested. The surface generated with this data is shown in Figure 9. According to these results for each pH there is a temperature for which the maximum rate of sorbitol synthesis is obtained.

Graphs in Figure 10 compare the sorbitol profiles (time step 2.5 h) in the synthesis processes carried out at different temperatures (25, 30, 35 and 40º C) and pH values (6 and 6.5) computed by the hybrid neural network model. In these graphs the experimental profiles are also plotted. Simulation results suggest that at pH 6.5 sorbitol synthesis is improved when the process is carried out at temperatures of 25 and 30º C. At higher temperatures (35 and 40º C) best results are obtained when the pH is 6.

Table 2: Effect of the number of neurons in the hidden layer (N) and the percentage (%) of data points used for training the neural network that computes the value of the kinetics in the prediction error of the hybrid neural network model that simulates sorbitol synthesis using GFOR in Z. mobilis CP4.

N	%	Error ´ 10^3				
		G	F	S	V	Total
No hidden layer	40	4.08	4.24	4.13	9.29	21.74
	50	4.06	4.22	4.10	9.33	21.71
	60	4.12	4.33	4.22	9.47	22.14
30	50	1.67	1.84	1.41	6.04	10.96
30	60	1.43	1.55	1.19	5.65	9.82
35	40	1.74	1.72	1.67	5.65	10.93

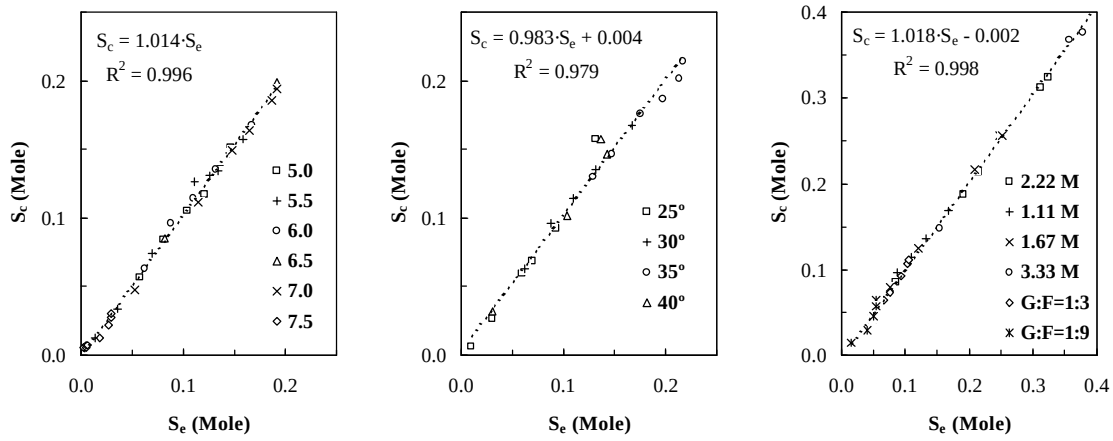
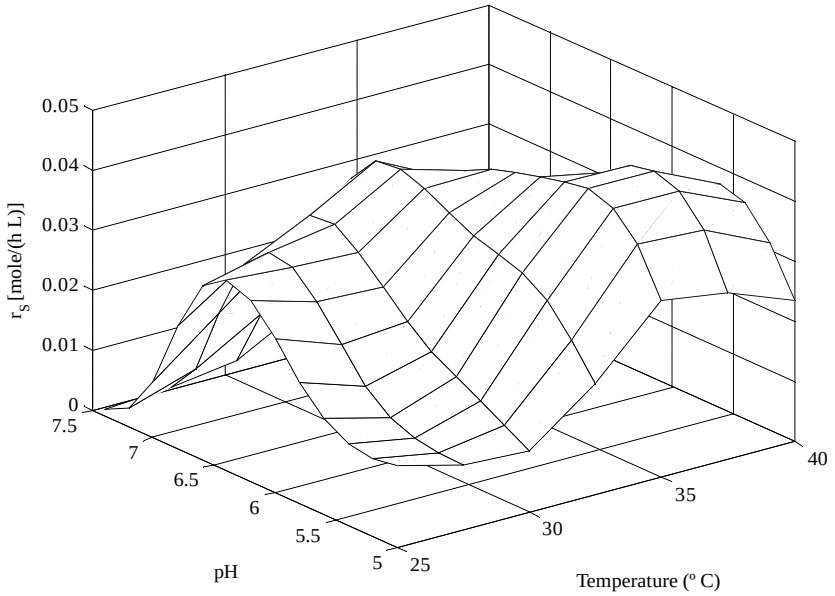


Figure 8: Comparison between the experimental moles of sorbitol and the values computed by the hybrid neural network model for different operational conditions (a) effect of pH, (b) effect of temperature, (c) effect of concentration of sugar mixture.



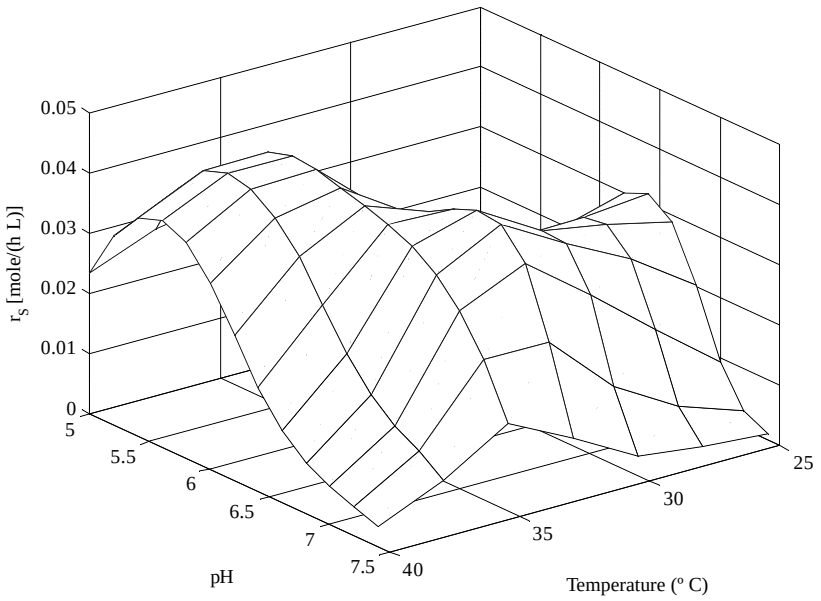


Figure 9: Values of the rate of sorbitol synthesis computed by the neural network for the different pHs and temperatures.

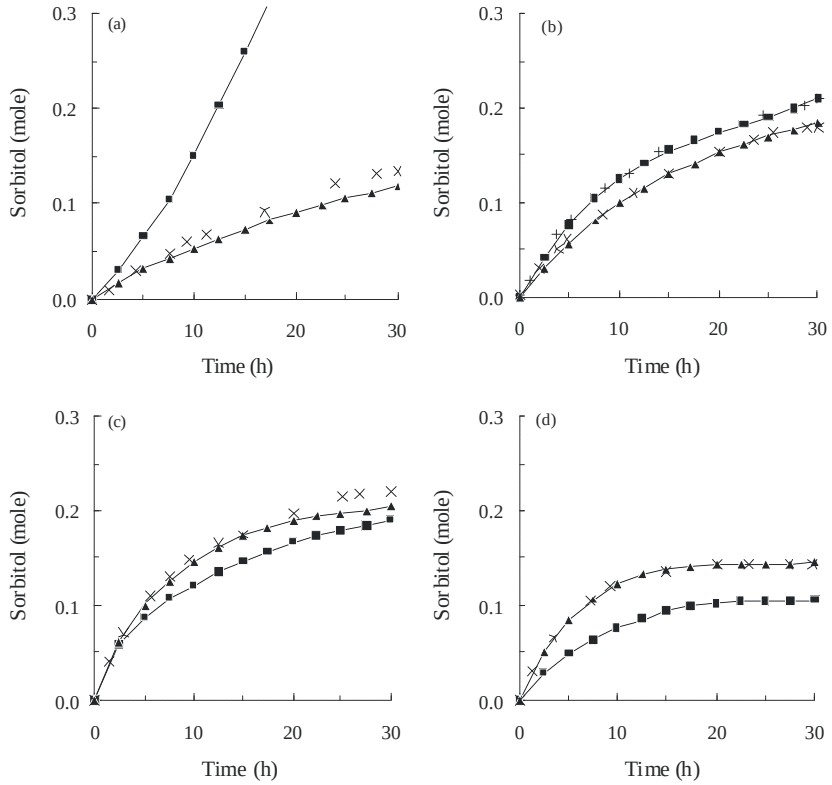


Figure 10: Simulated and experimental (´,+) values of the sorbitol profile at different pHs (▲, pH = 6.0; ■, pH = 6.5) and temperatures (a) 25º C (b) 30º C (c) 25º C (d) 40º C.

CONCLUSIONS

The main disadvantage of enzymatic sorbitol synthesis using GFOR in *Z. mobilis* CP4 at a constant pH value is the simultaneous production of gluconic acid that has to be neutralized through addition of an alkali solution. The controlled addition of this solution increases system volume with the consequent dilution of the substrates and the enzyme.

The complex relationship between the system conditions (temperature, pH and substrate concentrations) and the sorbitol synthesis kinetics is not easy to describe through a simple mathematical equation. Moreover, experimental results presented here show that the residual activity of the enzyme is a function of time and temperature. Without an expression for the kinetics of the process, the mathematical model cannot be solved. However, a properly designed neural network can be used to compute the values of the sorbitol synthesis kinetics. This neural network can be used to solve the substrate, product and system mass balances allowing simulation of the process under all the operational conditions tested. Due to the complexity of the sorbitol synthesis kinetics, the neural network has to contain at least one hidden layer and more than 30 neurons should be used in this layer.

ACKNOWLEDGMENTS

The authors wish to thank Fondo Nacional de Ciencia y Tecnología for its financial support under project 1000344.

NOMENCLATURE

[E]	enzyme activity	(U/L)
F	moles of fructose	(mole)
[Fru]	fructose concentration	(M)
G	moles of glucose	(mole)
[Glu]	glucose concentration	(M)
k_D	rate constant for enzyme decay	(h^{-1})
L	number of training data pairs	(-)
N	number of neurons in the hidden layer	(-)
r_S	sorbitol synthesis rate	($mole(L \cdot h)^{-1}$)
S	moles of sorbitol	(mole)
[Sor]	sorbitol concentration	(M)
t	time	(h)
T	temperature	(^{o} C)
td	target data	(-)
V	culture volume	(L)
w	parameters in the neural network	(-)
x	input data	(-)

REFERENCES

Chun, U.H. and Rogers, P.L., The Simultaneous Production of Sorbitol and Gluconic Acid from Glucose Using an Oxidoreductase of *Zymomonas mobilis*, Appl Microbiol Biotechnol 29: 19-24 (1998).

Bradford, M.M., A Rapid and Sensitive Method for the Quantification of Microgram Quantities of Protein Using the Principle of Protein-dye Binding, Anal Biochem 72: 248-254 (1976).

Demuth, H. and Beale, M., Neural Networks for Use with Matlab. The Math Works, Natick, Mass. (1998).

Loos, H., Kramer, R., Sahm, H. and Sprenger, G.A., Sorbitol Promotes Growth of *Zymomonas mobilis* in Environments with High Concentrations of Sugar: Evidence for a Physiological Function of Glucose-Fructose Oxidoreductase in Osmoprotection, J Bacteriol 176: 7688-7693 (1994).

Matlab, The Language of Technical Computing. The MathWorks, Natick, Mass. (2000).

Press, W.H., Teukolsky, S.A., Vetterling, W.T. and Flannery, B.P., Numerical Recipes in Fortran. 2nd Ed. Cambridge University Press, Cambridge, UK (1992).

Shene, C. and Bravo, S., *Zymomonas mobilis* CP4 Fed-batch Fermentations of Glucose-Fructose Mixtures to Ethanol and Sorbitol, Appl Microbiol Biotechnol 57: 323-328 (2001).