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Experimental design to enhance the production of L-(+)-lactic acid from steam-exploded wood hydrolysate using *Rhizopus oryzae* in a mixed-acid fermentation

Adenise L. Woiciechowski [a], Carlos R. Soccol [a,*], Luis P. Ramos [b], Ashok Pandey [a]

[a] *Laboratório de Processsos Biotecnológicos, Departamento de Engenharia Quimica, Universidade Federal do Parana, PO Box 19011, CEP 81531-970, Curitiba, Brazil*

[b] *Laboratório de Processamento de Biomassa, Departamento de Química, Universidade Federal do Parana, CEP 81531-970, Curitiba, Brazil*

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Abstract

The fermentation of hemicellulosic hydrolysate from *Pinus taeda* chips, using the fungal culture *Rhizopus oryzae*, was carried out to produce L-(+)-lactic acid and to optimize and enhance the biological conversion of reducing sugar into L-(+)-lactic acid using the experimental design to evaluate the culture conditions. The first factorial design based on surface response with five factors (agitation level, substrate concentration, $CaCO_3$ concentration, C/N and C/P ratios) at low levels and one medium point was performed to optimize culture conditions. The second study tested two factors (substrate concentration and C/N ratio) at three levels. The statistical analysis of the data obtained from the factorial study showed that a C/N ratio of 35 and substrate concentration of 90 g/litre were the best conditions to produce L-(+)-lactic acid with *R. oryzae* on *P. taeda* hydrolysate, but in this case the statistical projection was not correct and the real optimized conditions were C/N ratio of 55 and substrate concentration of 75 g/litre of reducing sugar.

Keywords: Hemicellulosic hydrolysate; Lactic acid; Mixed-acid fermentation; *Rhizopus oryzae*; Submerged fermentation; Steam explosion

1. Introduction

Since wood and agricultural products are produced by photosynthetic processes, they are virtually inexhaustible, and provide a very attractive substrate for fermentation processes. The structure of wood is based on three main components: cellulose, hemicellulose and lignin. Cellulose is a linear β-D-glucan, associated with an amorphous matrix of lignin and hemicellulose. Hemicellulose is an heteropolysaccharide, composed of neutral sugars, uronic acid and acetyl groups [1]. Thus, the hemicellulosic hydrolysate consists mainly of xylose, glucose, mannose, arabinose, galactose and traces of other sugars, depending on the kind of the wood used. The pentosan component of hemicellulose results mainly in D-xylose and a smaller quantity of arabinose.

The hydrolysis of wood hemicellulose can be performed using acid [2–4] or steam [5]. Steam hydrolysis can be performed under high pressure with or without rapid decompression (explosion) [6]. Steam explosion is a process in which the biomass is treated with high-pressure steam under specified conditions and subsequently quenched to atmospheric pressure by adiabatic expansion of the reactor contents [7]. Steam explosion offers several advantages, one being the efficient fractionation of the three main components of lignocellulosic residues [8]. Steam explosion has been used by several workers to enhance the bioconversion of lignocellulosic substrates [9–11].

Hemicellulosic hydrolysate offers an attractive possibility to be used as substrate in fermentation processes for the production of ethanol [4,12], xylitol [5,13–15] and microbial protein [16]. During hydrolysis, however, some toxic compounds such as furan derivatives (2-furaldehyde and 5-hydroxymethyl-2-furaldehyde) and organic acids (formic acid, acetic acid, levulinic acid)

* Corresponding author. Fax: +55-41-266-0222.
E-mail address: soccol@engquim.ufpr.br (C.R. Soccol)

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are also formed, which may exert inhibitory effects on the fermentation process [17–19]. These inhibitory effects, however, could be minimized using various pre-treatments such as steam stripping, neutralization of the hydrolysate with alkali or activated charcoal, ion exclusion chromatography, solvent extraction, enzymatic detoxification and molecular sieving [20,21].

Many organic acids, such as lactic acid, fumaric acid, acetic acid and malic acid, can be produced by fungi through the aerobic fermentation of sugars [22,23]. Hemicellulosic hydrolysate could be an alternative substrate for such fermentative processes. When hemicellulosic materials are submitted to wet (steam) treatment with pressure and temperature for a period of time, they yield a water-soluble fraction with soluble sugars derived from the hemicellulose, mainly xylose, and a water-insoluble fraction formed by lignin and cellulose.

Rhizopus spp. are edible moulds, used in many countries for the preparation of fermented foods, increasing food digestibility and the protein value of the foods [24,25]. Several strains of *Rhizopus* are also used for the production of enzymes [26] and organic acids [27].

The present study was undertaken to evaluate the ability of a strain of *R. oryzae* to grow on *Pinus taeda* hemicellulose hydrolysate, prepared by steam explosion, for lactic acid production. Process optimization was carried out using two factorial designs, which were based on surface response factors.

2. Materials and methods

2.1. Micro-organism and growth media

Rhizopus oryzae NRRL 395 used in the present studies was grown on potato–dextrose–agar (PDA) medium and preserved at 4°C.

2.2. Preparation of inoculum

Inocula were prepared on cassava–agar medium as follows: 30 g of cassava flour were mixed in 1 litre of distilled water and cooked for 1 h (121°C). The mixture was filtered using a cheesecloth while hot and the filtrate obtained was mixed with 2.93 g $(NH_4)_2SO_4$, 1.5 g KH_2PO_4, 0.72 g urea and 15 g agar After adjusting the pH to 5.5 with Na_2CO_3 (3 mol dm^{-3}), the medium was sterilized at 121°C (20 min). Petri plates (10 cm diameter) were prepared using this medium (10 ml in each plate) which were inoculated with *Rhizopus formosa* (stock culture) and incubated at 32°C for 10 days. Spores were washed from plates in 10 ml of 1% (v/v) Tween-80 using a platinum loop and the suspension was used as inoculum after counting in a Malassez cell counter (Herka, France).

2.3. Preparation of wood hydrolysate

The hemicellulosic hydrolysate used for fermentation was obtained by steam explosion using chips of *Pinus taeda* (moisture content approximately 10–15%). Steam pre-treatment was performed in a steam gun (5-litre capacity). The chips were impregnated with 0 5% (w/v) sulphuric acid (final moisture content in the chips approximately 50%). These were loaded into the gun, steam treated at 205°C for 5 min and released from the gun into a steel cyclone by rapid depressurization of the vessel, causing the material to expand (explode) The resulting material was extracted with water (5%) The liquid phase was concentrated under vacuum to achieve the desired reducing sugar concentration and used as the substrate for fermentation.

2.4. Fermentation

Submerged fermentation was carried out using a medium which contained (g/litre): wood hydrolysate (reducing sugars) 56.55, KH_2PO_4 0.8, $MgSO_4$ 0.3, $ZnSO_4$ 0.06, $Fe_2(SO_4)_3$ 0.01 and $(NH_4)_2SO_4$ to give a C/N ratio of 5. After adjusting the pH to 7.2 with $CaCO_3$, the medium was filtered and autoclaved at 121°C for 15 min. For fermentation, 50 ml of the medium were placed in 250-ml flasks and inoculated with the spore suspension of *R. oryzae* at an inoculation density of 10^7 spores/ml. The flasks were incubated at 32°C on a rotary shaker at 100 rpm for the first 48 h and then at 200 rpm, after the addition of $CaCO_3$.

2.5. Assay methods

The fermented broth was centrifuged at 10,000 rpm for 10 min. Mycelia so obtained were washed with 2 N HCl and then with water to eliminate the residual $CaCO_3$ and dried at 105°C for 24 h to determine the growth of the fungal culture. Reducing sugars were determined in the supernatants according to the method of Somogyi–Nelson [28]. pH was measured at the end of fermentation. The substrates and the fermentation products were analysed in the supernatant using a Shimadzu LC-10-AD high-performance liquid chromatograph. A Bio-Rad Aminex HPX-87-H column was used at 60°C, with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 ml/min. Sugars and organic acids were detected in the column eluent by differential refractometry (Shimadzu RID 10 A).

2.6. Factorial design 1

A 2^{5-1} factorial design based on the surface response, with five experimental factors at two levels and a medium point, was used to optimize the culture conditions for the best production of lactic acid (the response

Table 1
Value (real and coded) of the experimental variables used in the experimental design

Factors	Coded terms		
	−1	0	+1
A. Substrate concentration (g/litre)	82	97	112
B. Agitation (rpm)	180	205	230
C. $CaCO_3$ (g/flask)	1	2	3
D. C/N ratio	38	47.5	57
E. C/P ratio	135	407.5	680

Table 2
Value (real and coded) of the experimental variables used in the experimental design

Factors	Coded terms		
	−1	0	+1
Substrate concentration (g/litre)	66	76	86
C/N ratio	40	55	70

variable). The real and coded experimental variables are shown in Table 1.

2.7. Factorial design 2

A 3^2 factorial design, with two factors at three levels, based on the surface response was used to optimize the culture conditions for best production of lactic acid, the response variable. The real and coded experimental variables are shown in Table 2. The experiment was carried out in duplicate.

The experimental results were analysed according to Eq. (1):

$$Y_i = \beta_0 + \sum_i \beta_i X_i + \sum_i \sum_j \beta_{ij} X_i X_j + \varepsilon \quad (1)$$

where Y_i is experimental response (lactic acid, g/litre), β_0 is response coefficient for the experimental design central point, β_i is response coefficient for factor i, X_i is experimental factor (coded unit), ε is error

The evaluation of the conversion efficiency of sugars into lactic acid ($Y_{P/S}$) and into biomass ($Y_{X/S}$), was carried out according to Eqs. (2) and (3):

$$Y_{P/S} = \frac{P_f}{S_i - S_f} \quad (2)$$

$$Y_{X/S} = \frac{X_f}{S_i - S_f} \quad (3)$$

where P_f is final lactic acid content, S_i and S_f are initial and final reducing sugar content and X_f is final biomass content.

3. Results and discussion

Table 3 shows the results of cultivation of *R. oryzae* on wood hydrolysate. The addition of $CaCO_3$ allowed the pH to be maintained in the desired range during the course of fermentation. Apart from lactic acid, malic and succinic acids were also produced by the strain, but no acetic and fumaric acids were produced. A moderate concentration of ethanol was also obtained. In earlier studies [23,27] this strain did not show the production of acetic acid.

3.1. Experimental design 1

In order to obtain higher lactic acid productivity, the culture conditions were optimized through a factorial design. Five factors, namely substrate concentration, agitation level, added $CaCO_3$, carbon/nitrogen (C/N) ratio and carbon/phosphorus (C/P) ratio, distributed in two levels and one medium point were evaluated. The response measured was lactic acid concentration along with biomass and ethanol produced (Table 4).

Results of lactic acid were analysed using the statistical program STATISTICA to evaluate the more important factors associated with the response variables. The results were submitted to analysis of variance (ANOVA) and by eliminating no significant effects the data obtained for lactic acid production are shown in Table 5.

The correlation coefficient value obtained ($R^2 = 0.9675$) was considered satisfactory for this kind of experiment. The significant factors at the level of 5% were the substrate concentration, the C/N ratio and its synergistic interaction, but the other effects analysed by ANOVA were also considered for the regression analy-

Table 3
Submerged fermentation of *Pinus taeda* hemicellulosic hydrolysate with *R. oryzae*

Strain	Final pH	Biomass (g/litre)	Substrate/biomass conversion ($Y_{M/S}$)	Residual reducing sugar (g/litre)	Products (g/litre)	Substrate/product conversion ($Y_{P/S}$)
R. oryzae	7.5	8.38	0.176	8.98	Lactic acid 12.00	0.252
					Ethanol 5.61	0.118
					Malic acid 2.93	0.062
					Succinic acid 2.70	0.057

Table 4
Experimental design used to optimize lactic acid production from hemicellulosic hydrolysate by *R. oryzae* 395

Run	Experimental factors					Response variable		
	Substrate concentration (g/litre)	Agitation (rpm)	$CaCO_3$ added (g/flask)	C/N ratio	C/P ratio	Lactic acid (g/litre)	Biomass (g/litre)	Ethanol (g/litre)
1	97	205	2	47.5	407.5	7.85	7.986	11 68
2	82	180	1	38.0	680.0	5.33	10.612	6.62
3	112	180	1	38.0	135.0	0.00	6.076	1.13
4	82	230	1	38.0	135.0	15.39	10.442	9.62
5	112	230	1	38.0	680.0	0.00	0.00	0.00
6	97	205	2	47.5	407.5	3.93	8.31	13.505
7	82	180	3	38.0	135.0	6.29	11.298	5.54
8	112	180	3	38.0	680.0	0.00	6.224	4.33
g	82	230	3	38.0	680.0	7.01	13.184	2.76
10	112	230	3	38.0	135.0	0.00	0.00	0.00
11	97	205	2	47.5	407.5	7.60	9.758	7.94
12	82	180	1	57.0	135.0	0.00	8.092	11.607
13	112	180	1	57.0	680.0	0.00	0 00	11.00
14	82	230	1	57.0	680.0	7.88	9.578	11.50
15	112	230	1	57.0	135.0	0.00	0.00	0.00
16	97	205	2	47.5	407.5	7.20	10.142	5.905
17	82	180	3	57.0	680.0	0.00	8.298	7.60
18	112	180	3	57.0	135.0	0.00	0.00	0.00
19	82	230	3	57.0	135.0	0.00	0.00	0.00
20	112	230	3	57.0	680.0	0.00	0.00	0.00
21	97	205	2	47.5	407.5	8.48	8.532	4.56

Table 5
ANOVA for lactic acid produced response from the optimization design

Effect	Sum of squares	Degrees of freedom	Mean squares	*F*-ratio	*P* value[a]
A: substrate concentration	109.726	1	109.726	34.46	0.0042
B: agitation	21.762	1	21.762	6.83	0.0592 S
C: $CaCO_3$ addition	14.631	1	14.631	4.59	0.0987 NS
D: C/N ratio	42.706	1	42.706	13.41	0.0215 S
AB	21.762	1	21.762	6.83	0.0591 NS
AC	14.631	1	14.631	4.59	0.0987 NS
AD	42.706	1	42.706	13.41	0.0215 S
BC	18.533	1	18.533	5.82	0.0734 NS
DE	18.533	1	18.533	5.82	0 0734 NS
Lack of fit	74.870	7	10.696	23.09	0.0086 NS
Pure error	12.712	4	3.178		
Total (corr.)	392.557	20			
R^2	0.9675				

[a] NS, not significant; S, significant.

sis, so the resulting regression equation fitted to the experimental data was:

$$\text{Lactic acid (g/litre)} = 27.9197 - 0.3026A + 0.3619B + 0.3939C - 1.4537D - 0.0026AB + 0.0637AC + 0.0115AD - 0.0359BC + 0.0004DE$$

where *A*, *B*, *C*, *D* and *E* are the coded levels for the factors (see Table 1).

For the first experimental design, the response surfaces obtained are shown in Figs. 1 and 2. From the statistical analysis for lactic acid, it was concluded that the substrate concentration and the C/N ratio were the important factors to define the best conditions for lactic acid production, and that these factors acted synergistically.

The best agitation level was 230 rpm. Since the amount of added $CaCO_3$ and the C/P ratio were not significant for the lactic acid production, only two other remaining factors, viz. substrate concentration and C/N ratio, distributed in three levels, were evaluated at the second experimental design. The response measured was lactic acid concentration along with biomass and ethanol production.

3.2. Experimental design 2

The experiment was carried out in duplicate and the results for both experiments of each response measured are shown in Table 6. The results obtained for lactic acid production was submitted to ANOVA. The differences detected in lactic acid production for each of the experiments showed that the substrate nature largely affected the fermentation results (Table 7).

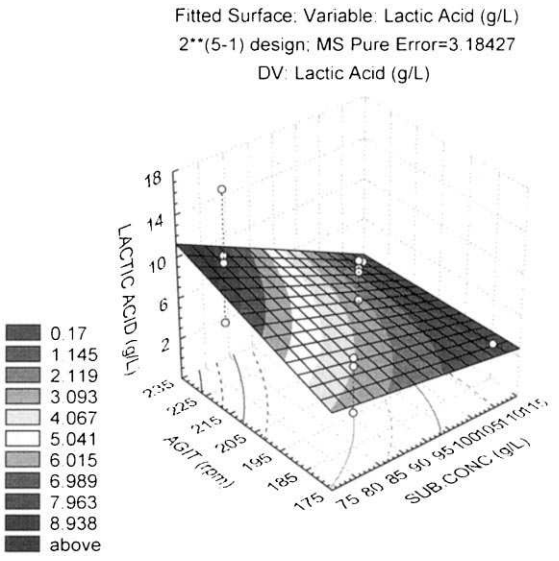

Fig. 1. Response surface for lactic acid production by *R. oryzae* 395 in *P. taeda* hydrolysate as a function of agitation level and substrate concentration.

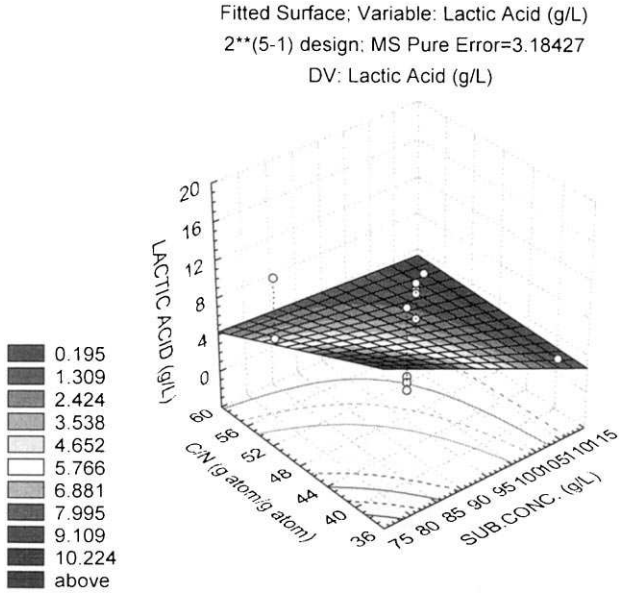

Fig. 2. Response surface for lactic acid production by *R oryzae* 395 in *P. taeda* hydrolysate as a function of C/N ratio and substrate concentration.

The correlation coefficient value obtained ($R^2 = 0.90684$) was considered satisfactory for this kind of experiment. The significant factors at the level of 5% were the substrate concentration, the C/N ratio and its interaction. Only the quadratic effect of the factor B (C/N ratio) was not significant. Hence, the resulting regression equation, fitted to the experimental data of the experimental design 2 was:

$$\text{Lactic acid (g/litre)} = 23.8029 - 7.2039A + 0.537A^2 + 8.0873B - 0.566B^2 + 0.0248AB$$

where A and B were the coded levels for the factors (see Table 2).

The response surface for lactic acid production using the experimental design 2 is shown in Fig. 3. Clearly the statistical projection for the best lactic acid production was reached in the direction of lowest C/N ratio (35) and highest substrate concentration (90 g/litre), but when this region was tested for the fermentation no fungal growth was observed (data not shown). At 86 g/litre substrate concentration, the fungal growth was inhibited in two of the three treatments tested (see Table 6). Thus, the analysis of graphics in Fig. 3 (in relation to experimental results) showed that, for the best production of lactic acid, the fermentation should be carried out at a C/N ratio of 55 and substrate concentration of 75 g/litre.

3.3. Process kinetics

In order to characterize the process related to kinetic parameters, a study was performed to verify the evolution of substrate consumption and biomass and lactic acid production. The initial substrate concentration was 75 g/litre of reducing sugar and C/N ratio of 55. The results of fungal growth, substrate consumption and lactic acid production as a function of time are shown in Fig. 4. Apparently, the fermentation was characterized by a lag phase of 48 h. The biomass concentration continued to increase until the fourth day, which apparently was due to the addition of $CaCO_3$ maintaining suitable pH for the growth of the culture. The lactic acid production reached a maximum (19.13 g/litre) after 120 h.

One important concern of the wood hydrolysate fermentation to desirable products has been the presence of inhibitory compounds in the hydrolysate [17–19]. This has been particularly true in the case of ethanol fermentation from hemicellulosic hydrolysate. Even if the strain used has the capacity to resist the inhibitory effect of toxic compounds (e.g. a strain of *Saccharomyces cerevisiae* resistant to inhibitors [29,30]), detoxification may be still necessary to achieve maximum

Table 6
Response variable for the experimental design

Run	Experimental factors		Response variable			
	Substrate concentration (g/litre)	C/N ratio	Lactic acid (g/litre)	Biomass (g/litre)	Ethanol (g/litre)	Residual reduced sugar (g/litre)
1	65	40	15.08, 8.76	3.90, 4.29	0.54, 0.00	7.66, 5.32
2	65	55	14.89, 13.69	3.01, 4.02	2.91, 0.67	9.91, 7.76
3	65	70	8.99, 8.12	3.65, 3.29	0.00, 0.00	7.76, 8.58
4	76	40	13.19, 14.87	4.34, 3.78	4.66, 2.5	10.00, 11.75
5	76	55	16.57, 13.29	3.03, 2.80	3.36, 5.06	22.29, 19.04
6	76	70	10.31, 19.09	2.42, 2.32	5.69, 7.76	20.87, 10.43
7	86	40	19.78, 16.56	3.59, 3.83	6.75, 1.15	9.61, 9.01
8	86	55	0.00	0.00	0.00	85.90, 80.43
9	86	70	0.00	0.00	0.00	86.30, 86.10

Table 7
ANOVA for lactic acid produced response from the optimization design

Effect	Sum of squares	Degree of freedom	Mean squares	*F*-ratio	*P* value[a]
A: Substrate concentration L	91.8865	1	91.8865	11.5382	0.00791 S
A: Q	131.4386	1	131.4386	16.5048	0 00283 S
B: C/N ratio L	145.0883	1	145.0883	18.2188	0 00209 S
B: Q	8.8705	1	8.8705	1.1139	0.31875 NS
AB	320.3671	4	80.0918	10.0572	0 00223 S
Pure error	71.6730	9	7.9637		
Total (corrected)	769.3239	17			
R^2	0.9068				

[a] NS, not significant; S, significant.

productivity. In the present studies, however, no inhibition was observed in the growth and fermentative activity of the fungal culture.

4. Conclusions

The *P. taeda* hemicellulosic hydrolysate produced by steam explosion was shown to be fermentable and a good substrate for lactic acid production in submerged fermentation. The statistical analysis of the data obtained by the factorial study showed that the C/N ratio of 35 and substrate concentration of 90 g/litre were the best conditions to produce lactic acid with *R. oryzae* in *P. taeda* hydrolysate, but in this case the statistical projection was not true and the real optimized conditions were a C/N ratio of 55 and substrate concentration of 75 g/litre of reducing sugar. The toxic compounds present in the hydrolysate affected neither the growth of the strain nor lactic acid production.

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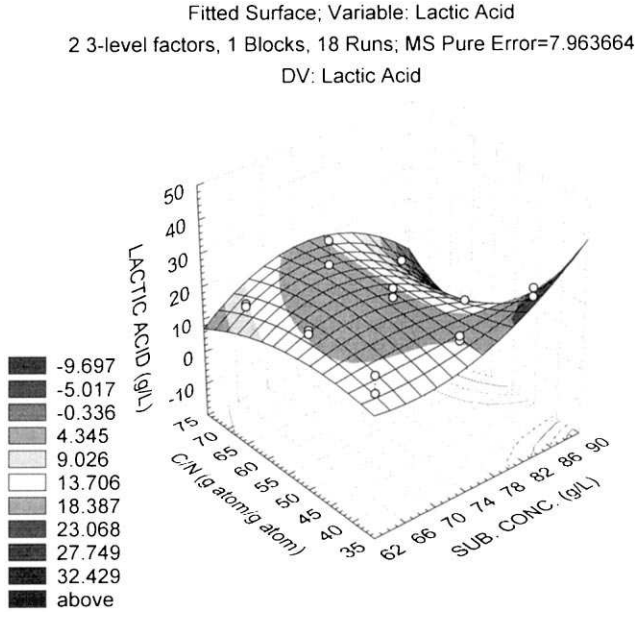

Fig. 3. Response surface for the lactic acid production with experimental design 2.

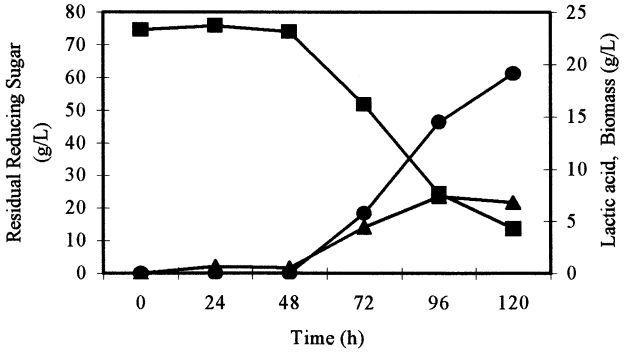

Fig. 4. Kinetics of the process of lactic acid fermentation. ■, sugar; ●, lactic acid; ▲, biomass.

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