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XI SINAFERM - Simpósio Nacional de Fermentações: Trabalhos selecionados

XI SINAFERM - National Fermentation Symposium: Selected papers

São Carlos, SP, Brazil
July 31 - August 2, 1996

Editors

Symposium

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Revista de Microbiologia

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Preface

The first National Fermentation Symposium, SINAFERM, was held in São Paulo, SP, in 1964 bringing together professionals from the different fields related to reactions catalysed by enzymes or living cells. Since then topics in areas such as industrial microbiology, biochemical engineering, enzymology, molecular biology, biochemistry, biophysics, wastewater treatment and pollution control have been the subjects of the works presented during its sessions.

The XI SINAFERM, held at the Universidade Federal de São Carlos in August, 1996, included four plenary lectures and 152 presented papers refereed by the Scientific Committee Board presided over by Willibaldo Schmidell Netto. It was organized on behalf of the Brazilian Society for Microbiology, SBM, and for the first time on behalf of the Brazilian Society of Chemical Engineering, ABEQ. One of the most important decision taken by the Organizing Committe, was that of paying tribute to Professor Walter Borzani as the organizer of the First SINAFERM.

Mention should be made here of the invaluable collaboration of Maria Therezinha Martins (*in memoriam*), Revista de Microbiologia Editor at the time, who made possible the present issue by promptly agreeing to publish this Supplementary Number with selected papers from the XI SINAFERM. The Scientific Committee and the Session Chairpersons were responsible for the selection of the original works.

We hope that this symposium and this representatives of all publication contribute to bringing together of professionals working in areas related to what is now known as biotechnology. We also wish to express our gratitude to all SINAFERM's participants who made possible the success of the meeting.

São Carlos, October 1997.

Carlos Osamu Hokka
for the Organizing Comitee
XI SINAFERM

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Diagram and Lay-out
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DOMINANDO O PROCESSO QUE TRANSFORMA BIOTECNOLOGIA EM COMPETITIVIDADE

INTRODUÇÃO:

A Vallée tem feito um trabalho pioneiro e exemplar na área de biotecnologia para aplicação no setor veterinário.

MATERIAL E MÉTODOS

Foram desenvolvidas algumas condições básicas para a obtenção de resultados :

1° Praticar o estado-da-arte da Gestão de Tecnologia

2° Manter uma equipe de alto nível, sempre treinada e comprometida.

3° Contar com projetos conjuntos com os melhores Centros de Pesquisa do Brasil e do Exterior.

4° Estar em perfeita sintonia com as políticas governamentais de fomento e normatização.

5° Trabalhar de acordo com os preceitos estritos da Gestão da Qualidade

RESULTADOS

- ✓ Vallée, líder no mercado de vacinas para bovinos
- ✓ Vallée, referência em gestão tecnológica
- ✓ Vallée, parceiro da academia no desenvolvimento científico

DISCUSSÃO

Sem discussão ...

CONCLUSÃO

VALLÉE : Há 37 anos, sempre com os líderes !

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Vallée Vallée Vallée Vallée Vallée

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COMPARISON BETWEEN THE BATCH PROCESS AND THE BATCH PROCESS WITH PULSES FOR THE SYNTHESIS OF GLUCOAMYLASE BY *ASPERGILLUS AWAMORI* NRRL 3112

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ABSTRACT

The goal of this work is to compare the performance of the batch process with polysaccharide pulses related to the traditional batch in the glucoamylase synthesis by *Aspergillus awamori* NRRL 3112, in a medium containing cassava flour as the main carbon source. Batch assays with pulses were conducted with a total polysaccharide concentration (S_T) equivalent to the initial polysaccharide concentration (S_o) of a reference batch. The results showed that, under the conditions studied, the batch process with pulses seemed equivalent to the traditional batch.

Key words: *Aspergillus awamori*, glucoamylase, pulse, batch

INTRODUCTION

The glucoamylase production process presents attractive characteristics in the field of biotechnological research, due to the fact that its synthesis is controlled by very complex metabolic regulatory mechanisms, such as induction and catabolite repression, which are dependent on the microorganism utilized, the culture medium composition, the cultivation conditions and also the fermentation process (batch, continuous, fed-batch, etc).

The fed-batch system presents all the characteristics which seem suited for the control of both induction and catabolite repression mechanisms but, as well as the continuous and the semicontinuous process, also the fed-batch one, when conducted in its classical form, with a continuous substrate feed stream, may impair some restrictions and operational difficulties.

Therefore, the objective of the present work is to explore the *batch process with pulses* (BP), which consists essentially in a fed-batch process with intermittent feeding. This process is conducted as a classical batch until the exhaustion of the carbon source. After this initial phase, one or more periodical polysaccharide pulses are promoted in the bioreactor, with the purpose of causing a sharp rise in the starch concentration in the reactor culture medium, in order to enhance glucoamylase synthesis, as a consequence of an estimulation on both cell growth and enzyme synthesis induction. In particular, in this work, the data concerning a batch run with a single pulse are presented and the results obtained are compared with those of a traditional reference batch.

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MATERIALS AND METHODS

Microorganism: Pure culture of *Aspergillus awamori* NRRL 3112 preserved in tubes with sterile soil (1) was used throughout this study. For the fermenter inoculation, the strain was pre-cultured in 1-liter shaken flasks (200 mL of medium, 200 rev/min, 35 °C) during 24 hours. A 10 % inoculum fraction (v/v) was employed in the fermenter cultivations.

Culture medium: The medium for inoculum preparation was prepared employing cassava flour as the main carbon source with an initial polysaccharide concentration (S_o) of 20.0 gTRS/L (TRS = Total Reduction Sugars), complemented with the following nutrients: $MgSO_4 .7H_2O$ - 0.50 g/L; $(NH_4)_2SO_4$ - 5.0 g/L; Na_2HPO_4 - 3.78 g/L; KH_2PO_4 - 3.5 g/L and yeast extract - 0.10 g/L. Culture media with higher polysaccharide concentrations were utilized for the fermenter cultivations, with a proportional increase in the concentration of the complementary nutrients.

Fermenter runs: Initially two traditional batch runs were performed, with S_0 around 40 and 120 g/L (Runs D1 and D2) in order to serve as a reference for the batch process with pulses (BP). The culture conditions fixed in these tests were: 35° C, pH 4.0, specific aeration flow rate 1 v.v.m. (v.v.m. = volume of air per volume of medium per minute), agitation frequency 700 rev/min, maintaining an internal pressure of 1.2 atm and with a working volume of 10 liters. The BP run was conducted in these same conditions and consisted of an initial batch phase with a working volume of 8.5 liters and S_o of about 80 g/L. This batch phase was prolonged until the exhaustion of the carbon source, when a 1.5-liter pulse of a concentrated cassava syrup was performed. The total polysaccharide concentration (S_T) in the BP run (given by the sum of the TRS mass of the initial batch phase and that one added with the pulse, divided by the final broth volume of 10 liters) was equivalent to the reference batch process with 120 g TRS/L (run D2). All the nutrients with respect to S_T, were added in the first batch stage of the process (before the pulse).

Analytical methodology: Samples were collected periodically in order to determine cell concentration (X), reduction sugar (RS) and total reduction sugar (TRS) concentrations (2), and enzymatic activity (A). One unit of enzimatic activity (U) was defined as the quantity of glucoamylase which releases 1 gram of glucose, in 1 hour, from a solution of soluble starch at 4% (w/v), 60° C and pH 4.2 (3).

RESULTS AND DISCUSSION

Figures 1,2,3 and 4 show the time course of total reduction sugars (TRS), reduction sugars (RS), cell concentration (X) and enzymatic activity (A) for the traditional batch runs, D1 and D2 (S_0 = 40 g/L and S_0 = 120 g/L). The results obtained confirm the typical behaviour already established in previous works (1), observing a glucoamylase production significantly superior for the test D2 (approximately 4,900 U/L), with respect to test D1 (around 2,000 U/L). This is mainly due to a much more effective induction, in virtue of a significantly higher polysaccharide concentration in the batch run with S_0 = 120 g/L.

Figures 5, 6 and 7 present the results obtained in the BP run. As can be observed from figure 6, the polysaccharide concentration is suddenly increased at the moment of the pulse, which could mean an increased induction for the synthesis of glucoamylase. However, it can also be noticed from this figure, that the polysaccharide supplied to the system by the pulse is almost immediately hydrolysed, as a consequence of the enzyme previously accumulated in the medium during the batch phase. Therefore, the desired induction effect becomes, as a matter of fact, negligible.

Nevertheless, in the post-pulse phase, cell growth seems to be even more prejudiced than enzyme synthesis. Analysing the increments in enzyme activity and in cell concentration obtained in the post-pulse phase, ΔA and ΔX (table 2), one would remark that ΔA is of the same magnitude as the quantity of enzyme accumulated in a batch equivalent to the post pulse phase (test D1), say, approximately 2,000

U/L, while the increment in X stays only around 40% regarding cell growth obtained in the batch run with an equivalent S_0.

In the same way, when the values of cell and enzyme productivities, P_X P_A, as well as the values of the respective yields, $Y_{X/S}$ and $Y_{A/S}$ (table 1) obtained in the post-pulse phase of the BP run are considered, it can be observed that the values corresponding to the cellular concentration are significantly more affected than those related to the enzyme activity.

It is important to point out that the cells in the post-pulse phase (DP') became from a previous 80g/L cultivation, standing these cells for some time period in adverse conditions, such as limiting dissolved oxygen concentrations, around 10% or even inferior (figure 7). As a consequence, a non-negligible fraction of the cell population might become non-viable. Nevertheless, although the enzyme production might continue to be growth associated, as suggested by Facciotti *et al.*(1), the conditions don't seem to affect cell growth and enzyme synthesis in the same proportion.

In spite of the above remarks, it can be concluded that, in terms of enzyme synthesis and considering both stages, anterior and posterior to the pulse (S_T = 120 g/L), the BP run presented a global performance comparable to the batch test with S_0 = 120 g/L (test D2). Therefore, it was verified, under the conditions studied, an equivalence between both strategies of process conduction.

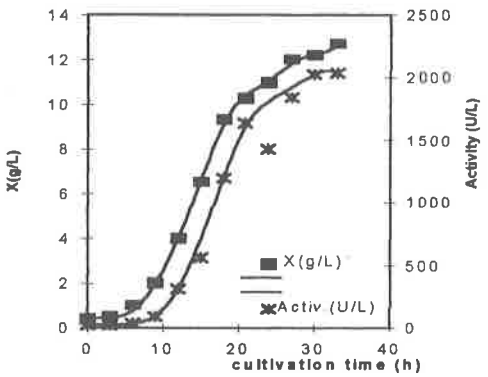

Figure 1: Time course of the cellular concentration (X) and enzymatic activity(Activ.) during assay D1.

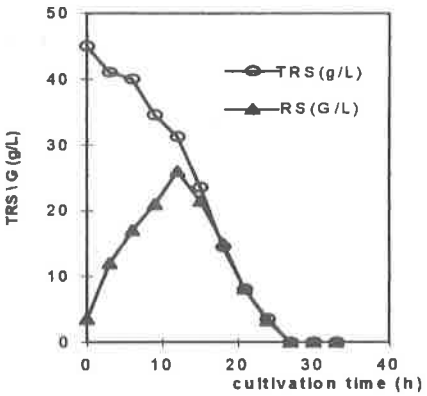

Figure 2: Time course of the total reducing sugar (TRS) and reducing sugar (RS) during assay D1.

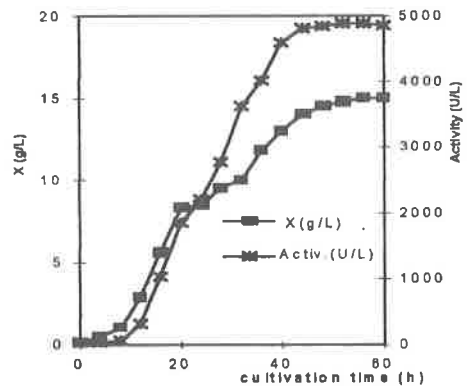

Figure 3: Time course of the cell concentration (X) and enzymatic activity (Activ.), during assay D2.

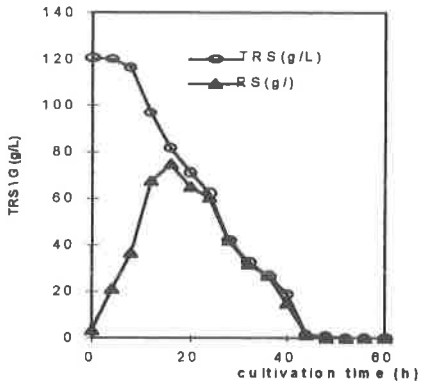

Figure 4: Time course of the total reducing sugar (TRS) and reducing sugar (RS), during assay D2.

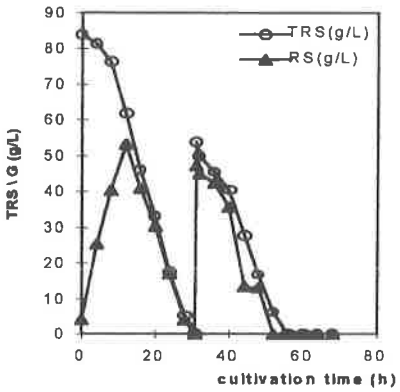

Figure 6: Time course of the total reducing sugar (TRS) and reducing sugar (RS), during assay BP.

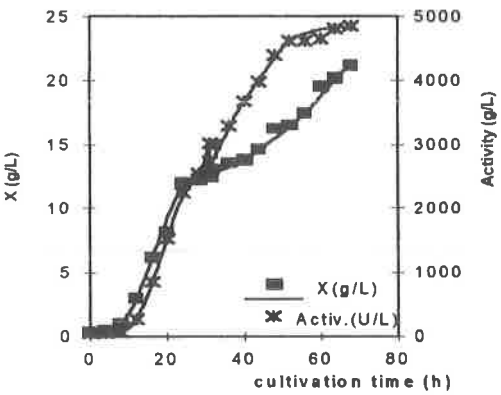

Figure 5: Time course of the cell concentration and enzymatic activity (Activ.) during assay BP.

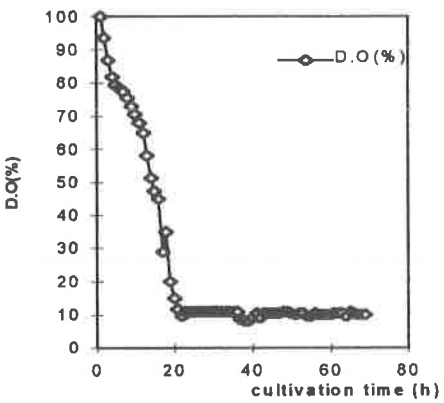

Figure 7: Time course of the dissolved oxygen (D.O), during assay BP.

Table 1 - Results obtained in the tests.

	X_O	S_O	A_O	t_f	X_f	$A_{máx}$	P_X	P_A	$Y_{X/S}$	$Y_{A/S}$
	(gL^{-1})	(gL^{-1})	(UL^{-1})	(h)	(gL^{-1})	(UL^{-1})	($gL^{-1}h^{-1}$)	($UL^{-1}h^{-1}$)	(gg^{-1})	(Ug^{-1})
D1	0.4	45	22	27	12.0	2,037	0.43	74.6	0.26	45.0
D2	0.13	120.5	39.5	52	14.5	4,890	0.28	93.3	0.12	40.3
DP	AP' 0.33	83.8	47.1	31	12.5	3,019	0.39	96.0	0.15	35.5
	DP' 12.7	53.8	2,741	25	17.4	4,850	0.19	84.4	0.09	39.2

X_O = initial cell concentration
S_O = initial polysaccharide concentration
A_O = initial enzymatic activity
t_f = time to carbon source exhaustion
X_f = cell concentration in t_f
$A_{máx}$ = maximum enzymatic activity

$P_X = (X_f - X_O)/t_f$ = cell productivity
$P_A = (A_{max}-A_O)/t_f$ = enzyme productivity
$Y_{X/S} = (X_f - X_O)/S_O$ = growth yield
$Y_{A/S} = (A_{max} - A_O)/S_O$ = enzyme yield
AP' = pre-pulse phase
DP' = post-pulse phase

Table 2 - Comparison between the enzymatic activity and the cell concentration post-pulse variations with an equivalent batch (S_0 = 40 g/L).

RUN	ΔA (U/L)	ΔX (g/L)
D1	2,015	11.6
DP	2,109	4.7

RESUMO

Comparação entre o processo descontínuo e descontínuo com pulsos para a síntese de glicoamilase por *Aspergillus awamori* NRRL 3112

Objetivou-se neste trabalho comparar o desempenho do processo descontínuo com pulsos com relação ao processo descontínuo tradicional, na síntese de glicoamilase por *Aspergillus awamori* NRRL 3112, em meio à base de farinha de mandioca. Realizaram-se ensaios descontínuos com pulsos que apresentassem uma concentração total de polissacarídeo (S_T) igual à concentração inicial de polissacarídeo (S_O) de um descontínuo referência. Os resultados mostraram que, nas condições estudadas, o processo descontínuo com pulsos mostrou-se equivalente ao descontínuo tradicional.

Palavras-chave - *Aspergillus awamori*, amiloglicosidase, pulso, descontínuo

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LIPASE PRODUCTION BY A NEW PROMISING STRAIN OF *PENICILLIUM RESTRICTUM*

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ABSTRACT

The physiological response towards cell growth and lipase production upon variable temperature and pH was studied in shaken flasks for *Penicillium restrictum*, a new strain isolated from soil and wastes of a Brazilian babassu coconut oil industry. The medium containing olive oil (C/N=9.9) at 30°C and pH 5.5 was the best for lipase poduction. The initial medium pH had no significant effect on maximum lipase concentration, but affected the time course of lipase production. Increasing on medium temperature had a positive effect on lipase production and cell growth up to 30°C, favoring only growth afterwards. The optimized lipase production in a 5-L fermenter (200 rpm, 0.75 vvm) lead to a maximum lipolytic activity of 14.000 UI^{-1}. Full lipase stability at pH 7.0 and 45° C was also observed during four hours.

Key words: *Penicillium restrictum*, lipase production, lipase stability

INTRODUCTION

Considering the application of lipases in the detergent, food and fine chemical industries the isolation of new producing microorganisms is a relevant task (10, 18). Moreover, lipases with peculiar catalytic properties would enrich its potential applications in the oil and fat industries, which process about 100 million ton. of raw materials per year (3). Also the current industrial processes often include physical and chemical transformations which offer limited options for new products, therefore more extensive and diverse lipases uses would certainly be beneficial (17, 19). Desirable features of new lipases would include a higher temperature and pH stability along with a higher specificity towards selected fatty-acids.

Considering the extensive possibilities of new producing microorganisms for the achievement of higher yields and more stable and diverse enzymes, few works have properly addressed the selection of new lipolytic strain (8, 16). This study was carried out aiming at the continuation of studies on lipase production by *Penicillium restrictum*, a newly identified lipase producer recently investigated by Freire *et al* (7), focusing on the relationship between cell growth and enzyme production in response to variable temperature and pH and preliminary crude enzyme characterization.

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MATERIALS AND METHODS

Materials - Commercial olive oil was purified with acid alumina from BDH to remove free fatty acids. Growth media components were purchased from Difco, azocasein from Sigma and other analytical grade materials from Reagen.

Shaken Flasks Fermentation Tests - Enzyme production by *Penicillium restrictum* strain was studied in 120 ml of the growth medium (olive oil 1%, peptone 2%, NaCl 0.5% and yeast extract 0.1%) at different initial pH (4.0; 5.5; 6.0, 7.0 and 8.0) and temperature (25° C, 30° C and 37° C). This medium was inoculated with 2 x 10^6 spores and incubated at 160 rpm. At selected time intervals samples were withdraw and filtered through filter paper Whatman n° 44 for the determination of enzyme activity in the culture supernatant (crude enzyme). The mycelial mass was carefully washed and dried at 75° C for dry weight determination.

Bench Scale Fermentation - The 5l instrumented fermentor (BIOFLO II-New Brunswick) containing 4l of the same growth medium was inoculated with 2 % (v/v) of its liquid volume with 24 hours grown cells. The main fermentation was carried out at the optimized pH and temperature, 200rpm and 0.75 vvm (specific air flow rate).

Enzyme Assay - The reaction mixture consisted of 19 ml of olive oil/arabic gum emulsion (5% olive oil and 5% arabic gum) in 100 mM potassium phosphate buffer, pH 7.0. This mixture was homogenized in a wiring blender for 3 min and the enzymatic reaction was started by the addition of 1 ml of the culture supernatant. The reaction mixture was incubated in a shaker at 37° C for 30 min at 200 rpm. The reaction was stopped by the addition of 20 ml of a mixture of acetone-ethanol (1:1 v/v). The amount of fatty acids produced was titrated with 0.05 M NaOH until end-point 9.5, using an automatic titration apparatus (Mettler DL21). One unit of lipase activity was defined as the amount of enzyme which liberates 1μmol of fatty acids equivalent per minute under the assay conditions.

Lipids Assay - The culture supernatant was used for gravimetric determination of lipids according to Akhtar *et al.* (1), replacing n-hexane by a mixture of petroleum-ether and ethyl-ether (1:1).

Protease Assay - Proteolytic activity was determined in the culture supernatant according to Charney and Tomarelli (5).

Crude Lipase Characterization - To study the effect of pH and temperature on lipase activity, the emulsion with 1 ml of crude enzyme was incubated at variable pH values (0.1 M phosphate buffer for the pH range 4.5 to 7.0 and 0.1 M Tris-HCl buffer for the pH range 7.0 to 9.0) at 30° C and in the temperature range of 25 to 50° C at pH 7.0. The lipase activity was determined at selected time intervals for each experiment. To investigate the enzyme stability, samples of the culture supernatant were incubated at pH 7.0, 8.0 and 9.0, at 30° C and 37° C during several days. Crude lipase was also incubated at 45° C, pH 7.0, to evaluated enzyme stability at this higher temperature. The enzyme stability was followed through the measurement of its lipolytic activity and its deactivation constants and half-life time were calculated using a linear inverted model (4).

RESULTS AND DISCUSSION

Effect of temperature and pH on lipase production - Table 1 presents the peak data for biomass, lipase concentration, normalized enzyme production, productivity and specific growth rate for different conditions of pH and temperature. The best conditions for lipase production were: initial pH 5.5 and temperature of 30°C. The initial pH had no significant effect on cell growth, maximum lipase activity, normalized enzyme production and specific growth rate. However, the maximum lipolytic activity was reached when the medium pH was in the range of 6.5 to 7.5, independent of its initial pH value (results not shown). This effect lead to lower productivity results (Table 1) for the experiments carried out with higher initial

pH values. Both initial pH and time course pH variation may affect fungal lipase production as observed by Pimentel *et al.* (15), Ohnishi *et al* (13) and Asahara *et al* (2). As above mentioned the best temperature for lipase production was 30 °C (Table 1). Neither cell growth or lipase production were increased at a lower temperature 25 °C. When fermentation was conducted at 37 °C an increase on cell growth rate was observed, whereas enzyme concentration was reduced.

Table 1: Effect of different media pH and temperature on the peak data for biomass, lipase activity, normalized enzyme production, productivity and specific grow rate in shaken flasks fermentations

Initial pH (T=30°C)	Lipase activity ($U.L^{-1}$)	Biomass concentration ($mg.ml^{-1}$)	$Y_{p/X}$ ($U.mg^{-1}$)	Productivity ($U.L^{-1}.h^{-1}$)	μ (h^{-1})
5.5	11,200	21.1	0.53	155.6	0.035
6.0	10,800	17.0	0.64	90.0	0.035
7.0	11,400	20.8	0.55	95.0	0.035
8.0	10,500	17.5	0.60	72.9	0.035
Temperature pH 5.5	Lipase activity ($U.L^{-1}$)	Biomass concentration ($mg.ml^{-1}$)	$Y_{p/X}$ ($U.mg^{-1}$)	Productivity ($U.L^{-1}.h^{-1}$)	μ (h^{-1})
25°C	3,100	13.3	0.23	43.1	0.033
30°C	11,200	21.1	0.53	155.6	0.035
37°C	8,100	22.5	0.36	84.4	0.043

Lipase and Protease Production in a Bench Scale Fermenter - A typical fermentation time course for lipase production by *Penicillium restrictum* in a bench scale fermenter at 30°C, initial pH of 5.5, 200 rpm and 0.75 vvm is shown in Figure 1. The maximum lipolytic activity (14,000 $U.L^{-1}$) was observed after 80 hours of cultivation, which was coincident with the depletion of the carbon source. After 75 hours of fermentation cell lysis starts and protease concentration increases substantially.

The decrease on lipase activity after 90 hours of fermentation can be explained by pH inactivation, proteolysis or both. The results shown in Figure 3a and Table 2 indicate that *P. restrictum* lipase is not stable at pH values above 8.0. Half-life times at pH values of 8.0 and 9.0 were respectively, 20 and 1,000 times lower than that obtained at pH 7.0 (30°C). As protease production significantly increases when growth rate is declining (Figure 1) the decrease on lipase activity may also attributed to proteolysis as pointed out recently by Freire *et al* (7).

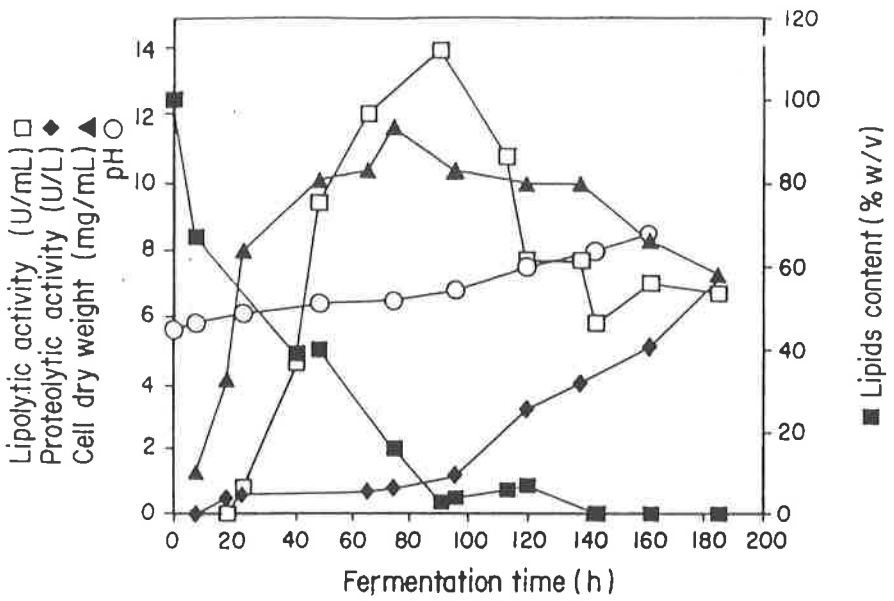

Figure 1: Typical time course for cell growth, lipase production, protease accumulation and lipids consumption by *Penicillium restrictum* in a 5 L bench scale fermenter. Experiment carried out at 200 rpm, 0.75 vvm, 30°C and initial pH 5.5.

Enzyme Optima pH and Temperature - Figures 2a and 2b presents the data for the determination of optima pH and temperature. The highest enzyme activity was observed at pH 7.0 and 37°C. Reported values for *Penicillium* lipases varies within 30°C to 40°C (7, 12, 13, 15, 16), and pH values 6.0 to 9.0.

Enzyme Stability - Data for lipase pH stability are shown in Figure 3a and 3b. Accordingly the enzyme is quite stable in the pH range 7.0 to 8.0 at 30° C (Figure 3a) although pH values above 8.0 were detrimental. The same behavior was observed at 37°C (Figure 3b). Concerning temperature stability the enzyme retained its activity several days at 30 and 37° C at pH 7.0, showing half-life times of 4,800 and 67 hours, respectively. At a higher temperature (45° C at pH 7.0) the lipase activity was fully maintained for a period of 4 hours, decreasing afterwards with a half-life time of 5.9 hours. This value is high in comparison with reported data for other *Penicillium* lipases, as follows: less than 1 hour (10); about 3 hours (7); 30 minutes (12) and 20 minutes (13).

In conclusion *Penicillium restrictum* was identified as a promising strain for lipase production due to the enzyme yields and its temperature stability. Future work will include the enzyme purification and characterization to explore its peculiar catalytic properties. Furthermore, optimization of the fermentation conditions, that can lead to an increase on enzyme yield and productivity will be also addressed.

ACKNOWLEDGMENTS

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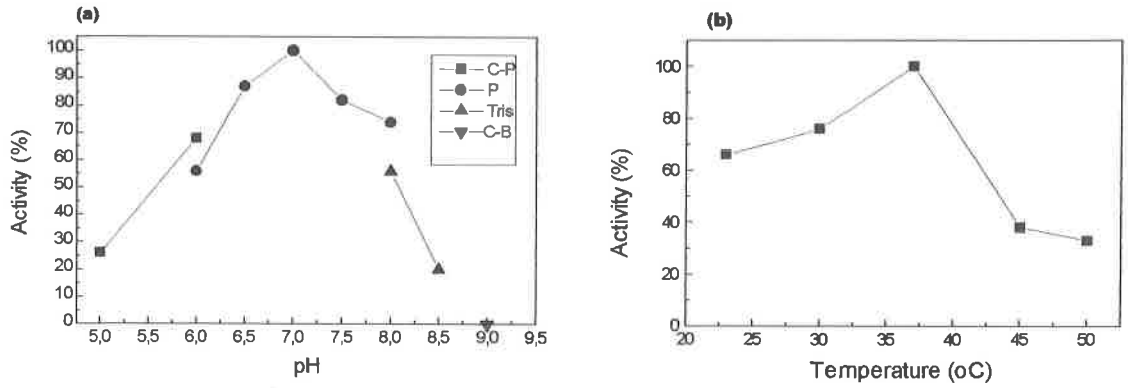

Figure 2: Variation of lipolytic activity with pH at different buffers (Citrate-Phosphate (C-P), Phosphate (P); Tris; Carbonate-Bicarbonate (C-B) at 30°C (a) and temperature at pH 7.0 (b).

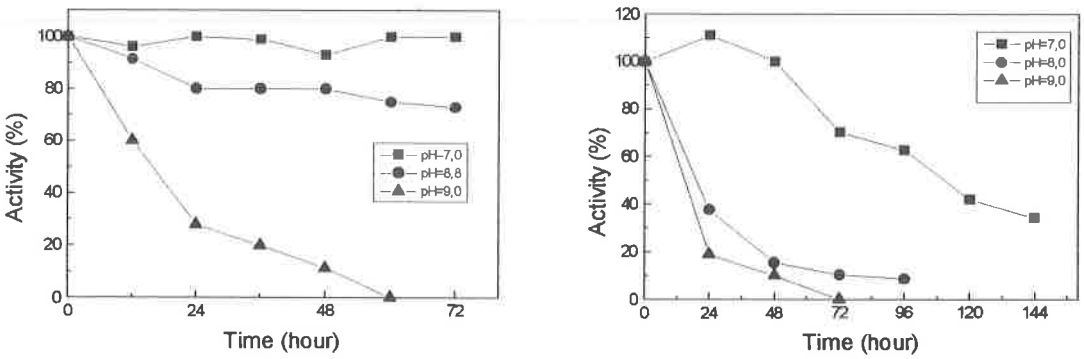

Figure 3: Enzyme stability at 30°C (a) and 37°C (b) in different pH values.

Table 2: Crude enzyme half-life time and deactivation constants at different pH and temperature values.

Assay conditions		Half-life time	Deactivation constant
Temperature	pH	(h)	(h^{-1})
30°C	7.0	4,800	2.1×10^{-4}
30°C	8.0	235	4.3×10^{-3}
30°C	9.0	4.6	2.2×10^{-1}
37°C	7.0	67	1.5×10^{-2}
37°C	8.0	6.5	1.5×10^{-1}
37°C	9.0	4.2	2.4×10^{-1}
45°C	7.0	5.9	1.7×10^{-1}

RESUMO

Produção de lipase por uma nova e promissora cepa de *Penicilium restrictum*

Um prévio trabalho de seleção de microrganismos produtores de lipase conduziu a uma cepa extremamente promissora de *Penicillium restrictum*. Foi investigada a resposta fisiológica desse fungo cultivado em meio contendo oleo de oliva (relação C/N=9.9) em condições variaveis de temperatura e pH. A maior atividade lipolitica foi detectada em cultivos conduzidos a 30°C e pH inicial de 5.5. O pH inicial do meio não afetou significativamente a máxima concentração de lipase obtida, mas causou uma mudança no perfil de produção dessa enzima. O aumento da temperatura de produção foi benéfico até 30°C, favorecendo somente o crescimento após esse valor. A produção de lipase em condições otimizadas foi investigada em fermentador de bancada de 5 L (200 rpm, 0,75 vvm) conduzindo a uma atividade máxima de 14.000 $U.L^{-1}$. Na temperatura de 45°C e no pH 7,0 a preparação bruta dessa enzima manteve sua atividade durante cerca de 4 horas.

Palavras-chave: lipase, *Penicillium restrictum*, atividade lipolitica

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PURIFICATION AND DETERMINATION OF SOME PROPERTIES OF CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *BACILLUS CIRCULANS* Nº 76 USING RESPONSE SURFACE ANALYSIS

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ABSTRACT

Cyclodextrin glycosyltransferase (EC 2.4.1.19, CGTase) from an alkalophilic *Bacillus circulans* strain was purified about 85-fold by starch adsorption and DEAE-Sephadex A-50 chromatography. The molecular weight of the enzyme was estimated to be 72,000 by SDS-PAGE. The single factor analysis for the effect of the pH and temperature on the enzyme activity revealed that at 50°C the optimum pH was around 5.7 and little activity was lost until pH 8.5. This analysis also showed that optimum temperature was about 65°C. Multiple factor and response surface analysis however showed that the optimum for enzyme activity was at a pH range 6.5-8.5 and 62°C. The purified enzyme was more stable at pH 8.5 than at pH 5.7 and its incubation at temperatures between 35 and 55°C increased the activity.

Key words: experimental design, *Bacillus circulans*, cyclodextrin glycosyltransferase

INTRODUCTION

Cyclodextrin glycosyltransferase (cyclomaltodextrin glucanotransferase, [EC. 2.4.1.19], CGTase) is the bacterial enzyme which converts starch and other α-1,4 glucans to cyclodextrins (CD). Cyclodextrins are closed-ring structures of 6 to 12 glucose units jointed by α-1,4-glucosidic bounds (6). The main cyclodextrins are cyclohexa-, cyclohepta- and cyclooctamylose which are designated as α-, β- and γ-cyclodextrin, respectively. These homogeneous cyclic compounds have the ability to form inclusion compounds changing physical and chemical properties of the guest molecules. Such cyclodextrins characteristics make them extensively used in food, cosmetic, pharmaceutical, and plastic industries as emulsifiers, anti-oxidants, and stabilizing agents (9, 23).

Since 1939, when Tilden and Hudson announced a cell free enzyme preparation from culture of *Bacillus macerans* (BMA), which had the ability to form cyclodextrin from starch, several different CGTase have been discovered from *Bacillus* species (11, 19), *B. circulans* (18), *B. ohbensis* (26), *Thermoanaerobacter* (21, 27), *B. stearothermophilus* (12), *Klebsiella pneumoniae* (5, 15), *B. megaterium* (11), *B. lentus* (25), *B. coagulans* (1), *B. macerans* (14) and *B. antolyticus* (29).

CGTases have been purified and characterized as proteins with molecular weight varying from 65,000 (1) up to 139,000 (4) with optimum pH

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and optimum temperature for the activity over the ranges 4.5 to 10.5 and 45 to 60°C, respectively. An exception is the CGTase from genus *Thermoanaerobacter* with optimum temperature at 95°C (21). Normally both optimum pH and optimum temperature have been determined by a single factor analysis in which all reactions parameters are fixed excepting pH or temperature, respectively. Here we report the purification and some properties of the CGTase from an alkalophilic *Bacillus circulans* strain isolated from Brazilian soil. The optimum pH and optimum temperature for the enzyme activity were examined by single and multiple factor analysis and the results for both methodologies were compared.

MATERIALS AND METHODS

Microorganism and fermentation conditions: Culture of *Bacillus circulans* n° 76 isolated from Brazilian cassava crop soil was grown for 5 days at 37°C in the maintenance medium composed of soluble starch 1%, peptone (Difco) 0.5%, yeast extract (Difco) 0.5%, K_2HPO_4 0.1%, $MgSO_4.7H_2O$ 0.02%, Na_2CO_3 1% and agar 1.5%. A loopful of the culture was inoculated into 500 ml Erlenmeyer flasks containing 100ml of a broth composed as the maintenance medium added of $CaCl_2$ 0.05%. The flasks were incubated at 37°C for 24 hours at 150 rpm. Twenty five mililiters of the cultured medium were inoculated into 1000 ml Erlenmeyer flasks containing 200 ml of fresh broth. Flasks were incubated for 5 days at the same conditions. The cells were harvested from the cultured broth by centrifugation at 10,000 rpm.

Analytical methods: CGTase activity was assayed using soluble starch as substrate by measuring the decrease in iodine-staining power. The reaction mixture containing 0.1 ml of enzyme solution suitably diluted, 0.5 ml of 1% soluble starch, 0.3 ml of distilled water, 0.1 ml of 0.1 M citric acid buffer, pH 6.0, was incubated at 50 °C for 10 minutes. The reaction was stopped by the addition of 0.5 ml of 1 N HCl. After addition of 0.1 ml of iodine solution, and dilution of the mixture to 15 ml with distilled water, the absorbance was measured at 620 nm. One unit of enzyme activity (DU) was defined as that which brings about the hydrolysis of 1 mg starch per minute in the presence of 5.0 mg of the substrate. Protein in the purification steps was measured by the method of Lowry et. al. (16). The protein concentration of column fractions was determined by measuring the absorbance at 280nm.

Determination of molecular weight: The molecular weight of the enzyme was determined by SDS-polyacrilamide gel electrophoreses. PhastGel gradient 8-25 and PhastGel SDS strips were used in Pharmacia PhastSystem equipment (Pharmacia LKB-Biotechnology, Uppsala-Sweden). Gel was silver stained according to File n° 210 from users manual. Phosphorilase b (MW 94,000), bovine serum albumin (MW 67,000), ovalbumin (MW 43,000), carbonic anhydrase (MW 30,000), tripsin inhibitor (MW 20,000) and α-lactoalbumin (MW 14,400) were used as protein standards. Samples and the mixture of standard proteins were prepared as recommended in the molecular weight calibration kit supplied by the equipment manufacturer.

Enzyme purification: All operations were carried out at room temperature unless otherwise specified. Four hundred mililiters of centrifuged culture broth were mixed with corn starch (3%) and ammonium sulfate (20%) under mild stirring at 5°C for 60 minutes. The starch was collected by filtration on Whatman n° 1 filter paper under vacuum and washed with 300 ml of 10 mM phosphate buffer (pH 7.0) containing ammonium sulfate (20%) and 1 M NaCl. The elution was carried out with 10 mM phosphate buffer (pH 7.0), containing 3 mM NaCl and 0.1 M maltose, under mild stirring, for 60 minutes at 40°C. The enzyme solution was dialyzed for 20 hours at 5°C against 10mM phosphate buffer (pH 7.0), and then concentrated 10 times by polyethyleneglicol (PEG) 20,000. Concentrated solution was applied to a DEAE-Sephadex A-50 column (20x250mm) equilibrated with 50 mM tris-HCl

buffer (pH 8.5). The column was washed with the same buffer and the enzyme was eluted with a linear gradient of sodium chloride from 0 to 0.5 M. Fractions of 3 ml were collected. The active fractions (75 ml) were pooled and concentrated 5 times by PEG 20,000.

Effect of pH and temperature on the enzyme activity: The effect of the pH on the purified CGTase activity was examined by measuring the enzyme activity under standard conditions over the pH range from 3.7 to 10.7. The effect of the temperature was examined by the CGTases activity measured at pH 5.7 or pH 8.5 over the temperature range 40-75°C. Multiple factor and response surface design was used to determine the optimum pH and optimum temperature values for the enzyme activity. In order to define on which way the enzyme activity was affected by the pH and temperature of the reaction mixture a complete factorial design of experiments followed by response surface analysis were first defined for an acid pH region and for an alkaline pH region individually (Table 1).

Table 2 shows the coded variables temperature and pH used for the enzyme activity measurements, as well as results for the activity at the conditions defined on the previous table. The three first tests at the center point were done in order to estimated the experimental error.

Table 1: Natural and coded variables pH and temperature for multiple factor analysis for acid and alkaline pH regions.

Acid pH Region				Alkaline pH Region			
T (°C)	X_1	pH	X_2	T (°C)	X_1	pH	X_2
58.0	-1.4	4.39	-1.724	58.0	-1.4	7.76	-1.396
60.0	-1	4.94	-1	60.0	-1	7.97	-1
65.0	0	5.70	0	65.0	0	8.50	0
70.0	+1	6.51	+1.07	70.0	+1	8.87	+0.698
72.0	+1.4	6.70	+1.316	72.0	+1.4	9.30	+1.509

Table 2: Coded variables and enzyme activity for multiple factor analysis for the acid and alkaline regions.

	Acid pH Region			Alkaline pH Region		
Exp.	X_1	X_2	Activity (DU/ml)	X_1	X_2	Activity (DU/ml)
1	0	0	85.1	0	0	86.8
2	0	0	84.3	0	0	88.1
3	0	0	81.3	0	0	92.2
4	-1	-1	58.6	-1	-1	78.4
5	+1	-1	11.8	+1	-1	46.8
6	-1	+1.07	89.2	-1	+0.698	69.3
7	+1	+1.07	96.0	-1	+0.698	48.1
8	-1.4	0	74.6	-1.4	0	76.6
9	+1.4	0	60.6	+1.4	0	77.0
10	0	-1.724	19.2	0	-1.395	88.3
11	0	+1.316	96.7	0	+1.509	21.9

Later, we applied a complete factorial design of experiments followed by response surface methodology for a unique region of pH covering both acid and alkaline values. Table 3 shows the natural and coded variables pH and temperature as well as the enzyme activity values for that statistical design. Central composed design and analysis of variance calculations were performed to fit the results to empirical second-order equation (Equation 1) and statistical F-Test was performed to determine the significance of the second-order model

$$y = b_0 + \sum_{i=1}^{2} b_i X_i + \sum_{i=1}^{2} b_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=1}^{2} b_{ij} X_i X_j + \varepsilon \quad i \prec j \quad (1)$$

Table 3: Natural and coded variables pH and temperature and enzyme activity for multiple factor analysis for the unique pH region.

Exp.	T (°C)	X_1	pH	X_2	Activity (DU/ml)
1	67.0	0	7.56	0	31.2
2	67.0	0	7.56	0	32.0
3	67.0	0	7.56	0	31.4
4	62.0	-1	6.95	-1	33.5
5	72.0	+1	6.95	-1	24.5
6	62.0	-1	8.13	+0.934	30.6
7	72.0	+1	8.13	+0.934	21.9
8	60.0	-1.4	7.56	0	28.2
9	74.0	+1.4	7.56	0	6.1
10	67.0	0	6.56	-1.639	34.0
11	67.0	0	8.51	+1.557	25.4
12	60.0	-1.4	6.56	-1.639	29.0
13	74.0	+1.4	6.56	-1.639	17.0
14	60.0	-1.4	8.51	+1.557	26.3
15	74.0	+1.4	8.51	+1.557	8.3

Thermal stability of the enzyme: The purified enzyme in buffer solutions at pH 5.7 or 8.5 was incubated at 65 °C for 1 hour. Other experiments in which the enzyme solutions were incubated for 30 minutes over the temperature range 40-65°C were also done. Standard method assayed in buffer solutions at pH 5.7 or 8.5 was used to measure the remaining activity.

RESULTS AND DISCUSSION

The results for each purification step are summarized in Table 4. The specific enzyme activity increased around 28-fold after starch desorption and at the end of the purification process the enzyme was purified approximately 85-fold with a 14 % yield.

Table 4: Purification of the CGTase from *Bacillus circulans* n° 76.

Step	Total Activity (DU)	Total Protein (mg)	Specific Activity (DU/mg)	Purification (fold)	Yeld (%)
Cell free extract	33,460	1,768	18.9	1.0	100
Starch adsorp-tion	23,440	45.0	520.9	27.6	70
DEAE-Sephadex A-50	4,660	2.9	1,600	84.6	14

A single protein peak was observed in the chromatography, as shown in Figure 1. The sample eluted from starch showed 2 protein bands whereas the sample eluted from the DEAE-Sephadex A-50 column presented only one protein band (Fig. 2). The molecular weight of this protein, estimated as 72,000 by SDS-PAGE, was very similar to that determined for CGTase from *B. circulans* E192 (3). Other *B. circulans* and alkalophilic *Bacillus* sp produce CGTase with molecular weight varying from 70,500 up to 103,000 (7,10,17,19,20,22, 24).

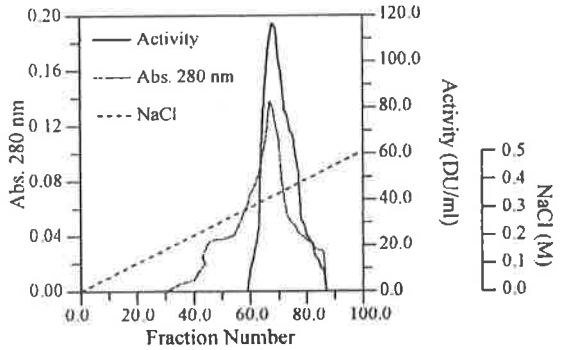


Figure 1: Elution profile of CGTase from *Bacillus circulans* n° 76 from a DEAE-Sephadex A-50 column (20x250 mm) equilibrated with 50 mM Tris-HCl buffer, pH 8.5. The column was eluted with a 0-0.5 M gradient of sodium chloride in 50 mM Tri-HCl, pH 8.5, at flow rate of 0.4 ml/min. The volume of each fraction was 5 ml.

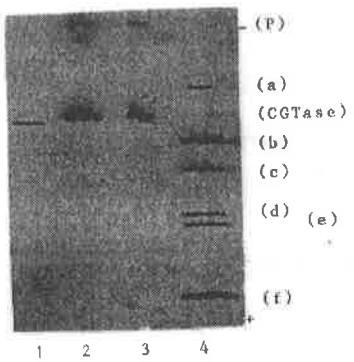


Figure 2: SDS-PAGE pattern of purified enzyme (line 1), enzyme eluted from starch (lines 2 and 3) and standard proteins (line 4)on PhastGel gradient 8-25. Four micrograms of protein were applied for both samples. Gel was silver stained. The standard protein used were: (a) phosphorilase b (MW 94,000), (b) bovine serum albumin (MW 67,000), (c) ovalbumin (MW 43,000), (d) carbonic anhydrase (MW 30,000), (e) tripsin inhibitor (MW 20,000) and (f) α-lactoalbumin (MW 14,400).

Similarly to CGTase from *Bacillus* sp IT 25 (2) and *B. alkalophilus* (18, 8) the CGTase from *B. circulans* n° 76 exhibited high activity over a broad pH range (Fig. 3). The maximum enzyme activity was around pH 5.7 but another peak of activity was observed at pH 8.5, in which the activity was around 88 % of the maximum. The highest activity of the enzyme in buffer solutions at both pH 5.7 and pH 8.5 was around 65°C, but at 70 °C the enzyme was more active at pH 8.5 than at pH 5.7. (Fig. 4). The results suggested that the enzyme would be less thermoresistant in acid than in alkaline solutions.

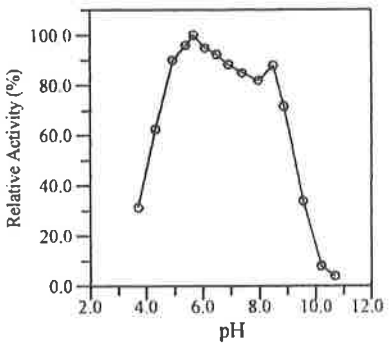


Figure 3: Effect of pH on purified CGTase activity. Reaction were performed at 50°C in the following buffer solutions: acetate (pH 3.7-5.4), phosphate (pH 5.7-8.0), borate (pH 8.5-8.9), sodium carbonate-sodium bicarbonate (pH 9.6-10.7).

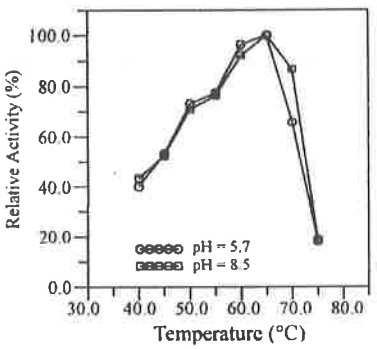


Figure 4: Effect of temperature on purified CGTase activity. Reactions were performed at different temperatures in buffer solutions at pH 5.7 or pH 8.5.

For multiple factor analysis 3 different pH regions were considered: a region of acid pH values, a region of alkaline pH values and a third region which covered both acid and alkaline pH values. The linear terms in the model Equation (1) (Table 5) had significant negative b_1 coefficient for temperature in acid region (-8.0) and for alkaline region (-7.0) indicating that an increase in the reaction

mixture temperature decreases the enzyme activity regardless the pH region considered. The linear term b_2 in the model for acid region (+25.9) has a significant positive value indicating that increases in the reaction pH values resulted in increases on the enzyme activity. On the other hand the negative b_2 coefficient for the alkaline pH region (-16.0) shows that increases in the reaction pH values resulted in contributions that lowered enzyme activity. These results suggested that optimum pH value for the enzyme activity was in the confluence of the acid pH region with the alkaline pH region. The significant positive b_{12} (+13.0) and b_{12} (+5.0) coefficients of the cross terms of reaction mixture temperature with reaction mixture pH for acid and alkaline pH region, respectively, indicate the high interaction between these two factors.

Table 5: Regression coefficients for second-order model, standard errors and coefficient of determination for multiple factor analysis for the acid, alkaline and unique pH regions.

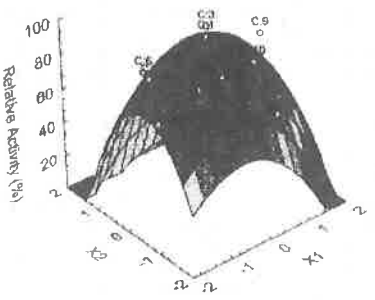
Parame-ters	Acid Region	Alkaline Region	Unique Region
b_0	+85 ±1	97 ±2	93 ±1
b_1	+8.0 ±0.7	−7 ±1	−17.0 ±0.9
b_2	+25.9 ±0.7	−16 ±1	−5.8 ±0.9
b_{11}	+8.9 ±0.9	−10 ±1	−17 ±1
b_{22}	−8.7 ±0.7	−21 ±1	0.1 ±0.8
b_{12}	+13 ±1	5 ±2	2 ±1
Analysis of Variance			
F-exp.	46.3	3.96	25.2
F-tab	11.0 (99 % confidence level)	3.45 (90 % confidence level)	6.06 (99 % confidence level)
Coefficient of determination			
R^2	97.9	79.8	93.3

Second-order equation for the unique pH region has negative b_1 coefficient for temperature (-17.0) indicating that increases in the reaction mixture temperature decrease the enzyme activity. Negative b_2 value coefficient for pH (-5.8) indicates that increases in the reaction mixture pH decrease the enzyme activity. The lower negative b_{12} (-2.0) reveals less interaction between temperature and pH factors for the unique region than for either acid or alkaline region.

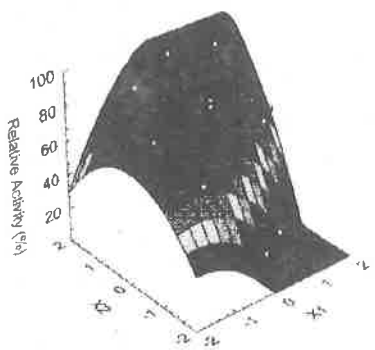
Figure 5 and Figure 6 show response surfaces for acid, alkaline and unique pH regions. Figure 5(a) shows that for acid pH region the maximum enzyme activity occurs at the highest pH values near the alkaline region. Figure 5(b) shows that in the alkaline region the maximum enzyme activity was at a pH value lower than that used as center point, near the acid region. These results suggested that there was not an optimum pH value for acid region and another one for alkaline region but only one optimum value for pH range from 5.7 to 8.5. Possibly this unique optimum pH for the enzyme activity was at the region of alkaline pH values near the region of acid pH. Figure 6 shows that the activity of the CGTase from *Bacillus circulans* n° 76 is independent of the reaction mixture for the pH range 6.5 to 8.5 and that the optimum temperature for activity was clearly defined as being 62°C. Thus multiple factor analysis revealed that there is not an maximum activity at pH 5.7 as revealed by single factor analysis, probably that maximum activity observed was a result of an interaction between the temperature and pH of the assay.

The enzyme was more stable at pH 8.5 than at pH 5.7 so that after 10 minutes incubation at 65°C the remaining activity was around 10% at the acid pH and around 81% at the alkaline pH (Fig. 7(a)). Increasing temperatures in the range between 35 to 55°C increased the enzyme activity at both pH 5.7 and 8.5 (Fig. 7(b)). Although enzyme activity normally decreases with heat treatment some few enzymes like ADL acilase and cephalosporin-C deacetylase (13, 28), presented the same behavior here observed. As suggested by the results of the effect of temperature on the enzyme activity (Fig. 4) this CGTase was less stable at pH 5.7 so that after 30 minutes incubation at 60°C the

enzyme activity was around 134% at pH 8.5 and around 40% at pH 5.7.



(a)



(b)

Figure 5: Response surface for acid (a) and alkaline (b) regions. Statistical analysis presented in table 8.

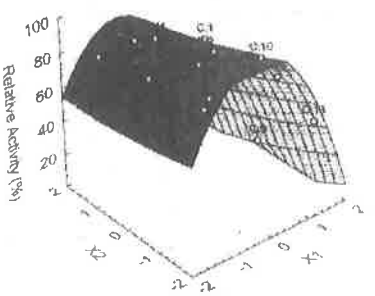
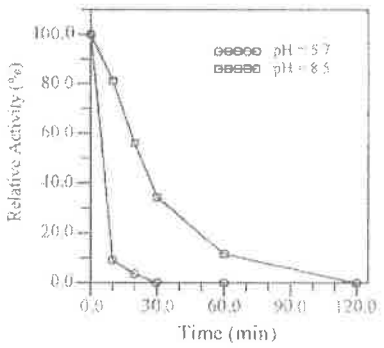
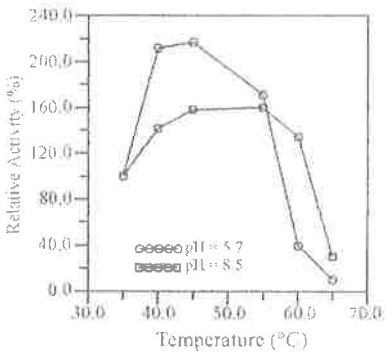


Figure 6: Response surface for unique region. Statistical analysis presented in table 8.



(a)



(b)

Figure 7: Effect of the temperature on the thermal stability of the purified CGTase. Enzyme in buffer solutions at pH 5.7 and pH 8.5 was incubated at 65°C for 1 hour (a). Enzyme in buffer solution at pH 5.7 or pH 8.5 was incubated for 30 minutes on a temperature range 35-65°C (b).

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RESUMO

Purificação e determinação de algumas propriedades de ciclodextrina glicosiltransferase de *Bacillus circulans* n°76 usando análise de superfície de resposta

A enzima ciclodextrina glicosiltransferase (EC 2.4.1.19, CGTase) produzida por uma linhagem de *Bacillus crculans* alcalofílica foi purificada cerca de 85 vezes por adsoração em amido e cromatografia em coluna de DEAE-Sephadex A-50. O peso molecular da enzima foi estimado em 72.000 por SDS-PAGE. A análise univariada do efeito do pH e da temperatura sobre a atividade enzimática revelou que a 50°C o seu pH ótimo estava ao redor de 5,7 e que pouca atividade era perdida até o pH 8,5. Esta análise também mostrou que a temperatura ótima da enzima estava ao redor de 65°C. A análise multivariada seguida de análise de superfície de resposta do efeito do pH e da temperatura sobre a atividade da enzima mostrou, entretanto, que a atividade máxima estava na faixa de pH 6,5-8,5 e a 62°C. A enzima purificada se mostrou mais estável a pH 8,5 do que pH 5,7 e quando incubada a temperaturas entre 35 e 55°C houve um aumento de sua atividade.

Palavras-chave: planejamento experimental, *Bacillus circulans*, ciclodextrina glicosiltransferase

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XYLAN-DEGRADING ENZYME PRODUCTION BY SOLID-STATE CULTURES OF AEROBIC FUNGI

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ABSTRACT

The aerobic fungi *Aspergillus fumigatus* Fresenius, *Humicola grisea* var. *thermoidea*, *Trichoderma harzianum* and *Acrophialophora nainiana* produce xylan-degrading enzyme activities when grown by solid-state cultivation on media containing wheat bran as carbon source. For convenience, cultivation conditions (other than temperature) and enzyme assays were the same for all fungi, i.e., no attempt at optimization of individual yields was made. Fractionation of the crude extracts on gel filtration chromatography and ion-exchange chromatography showed enzyme multiplicity. Some of these enzymes were purified by ultrafiltration and gel filtration, ion-exchange and hydrophobic interaction columns. Each enzyme preparation yielded a single band when stained for protein following electrophoresis. The molecular weight values were estimated by SDS-PAGE and gel filtration on HPLC and Sephacryl S-100. The purified enzymes were most active at pH range of 4.0-5.0 and 70 ^{O} C. Most of the purified enzymes were glycoprotein. The apparent K_m values was much lower than some K_m values reported in the literature. Some enzymes were very active against some p-nitrophenylglucosides and xylans, respectively. A synergistic effect was observed when some xylan-degrading enzyme activities were incubated with pre-treated arabinoxylan.

Key words: xylan, xylan-degrading enzyme activities, purification, characterization

INTRODUCTION

Xylan is the most abundant of the hemicelluloses (7, 12). It has a linear backbone comprised of β-1,4-linked D-xylopyranose residues which, depending on the origin, may be substituted with branches containing mainly acetyl, arabinosyl and glucuronosyl residues (9, 12). There is a great interest in its enzymatic hydrolysis because of possible applications in ruminal digestion, waste treatment, fuel and chemical production, and paper manufacture (6, 12, 22).

As xylans are structurally of mixed constitution, its complete hydrolysis requires the synergistic actions of β-xylanase and β-xylosidase with other enzymes such as α-arabinosidase, acetyl xylan esterase and α-glucuronidase (5, 12, 19).

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Solid-state cultivation is defined as the controlled growth of microorganisms on moist solid substrate in the absence of water (4). It has some advantages over liquid-state fermentation. The cultivation conditions are close to some extent the natural habitat of the microorganism; lower costs and improved enzyme stability (4, 20). Because of the above, we have begun investigation of individual component of the xylan-degrading system produced by aerobic fungi when grown on solid-state cultivation. This work reports the production, purification and some properties of xylan-degrading enzymes from *Aspergillus fumigatus* Fresenius, *Humicola grisea* var. *thermoidea*, *Trichoderma harzianum* strains and *Acrophialophora nainiana*.

MATERIALS AND METHODS

Enzyme Production: The fungi were maintained at 4 ^{O} C after growing for 7 days in MYG medium (0.2% malte extract, 0.2% yeast extract, 2% glucose and 2% agar) at 28 ^{O} C. The procedure for enzyme production (13, 14, 24) was as follows: a 250 ml flask containing sufficient agar to cover the bottom of the flask was inoculated with spores from a routine subculture and incubated at 28 °C (*Trichoderma harzianum*), 40°C (*Acrophialophoria nainiana*) and 42°C (*Humicola grisea* var. *thermoidea* and *Aspergillus fumigatus* Fresenius) until the agar was covered with growth for 7 days. Then, 12.5 g of presterilized supplemented substrate (wheat bran) was added. The flask was then shaken briefly to ensure dispersion of spores through of the added substrate and incubation was continued for 7 days. The contents (other than agar) were transferred to a 1-litre flask containig 25 g of presterilized supplemented substrate (wheat bran). Growth was allowed to proceed for 7 days. The enzyme extraction procedure was carried out as follows: the content of the flasks was extracted with 10 volumes of 25 mM sodium acetate buffer, pH 5.0, containing 0.1% (v/v) tween 80, by blending for 20 sec in a homogenizer and incubating with shaking at room temperature for 2 h. The crude extracts were centrifuged for 30 min at 5000 rpm. The supernatants obtained were filtered and stored at 4 ^{O} C for subsequent use as enzyme assay solutions.

Enzyme Assays: In experiments involving p-nitrophenylglycosides, enzyme activities were determined by measuring the amount of p-nitrophenol released from the substrates. The assays were carried out at 50 ^{O} C in 100 mM sodium acetate buffer, pH 5.0, using p-nitrophenyl-β-D-xyloside (PNPX) and p-nitrophenyl-α-L-arabinofuranoside (PNPA). The final concentration of these substrates in reaction mixtures was 0.25 mM. An appropriately diluted enzyme solution (10 - 100 µl) was mixed with each substrate in a total volume of 1.0 ml. The p-nitrophenol released was measured by monitoring the increase in A_{410} nm following 10 min (β-xylosidase activity) and 15 min (α-arabinofuranosidase activity) incubation time. The reaction was stopped by the addition of 1.0 ml of 1.0 M sodium carbonate. One unit of activity was defined as the amount of enzyme that catalyzed the release of 1 µmol of p-nitrophenol per min at 50 ^{O} C (IU ml^{-1}) and IU ml^{-1} mg^{-1}. The hydrolysis of xylan was determined by measuring the release of reducing sugars in 100 mM sodium acetate buffer, pH 5.0, at 50 ^{O} C, for 30 min. The assay volume was 600 µl. Reducing sugars were measured using the dinitrosalicylic reagent (17). Xylanase activity were expressed as µmol reducing sugar formed min^{-1} ml^{-1} enzyme solution, i.e., as IU ml^{-1} and IU ml^{-1} mg^{-1}. Protein concentration was measured by the method of Bradford (3), using bovine serum albumin as standard. Carbohydrate content was determined by the phenol-sulfuric acid method (8).

Electrophoresis: Sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) and polyacrylamide gel electrophoresis in the absence of sodium dodecyl sulfate (non-denaturing conditions) were carried out by as described by Laemmli (16) using 12% gels. After electrophoresis, protein bands were silver

stained by the method of Blum *et al.* (2). The low and high molecular weight standards were obtained from Sigma Chemical Co. Samples with β-xylanase activity were electrophoresed on polyacrylamide gel under non-denaturing conditions and containing oat spelts xylan (1, 11, 18). After electrophoresis, the polyacrylamide gel was stained in a Congo red solution (1 mg/ml) for 1 h at room temperature, destained with 1 M NaCl and fixed with 0.5% acetic acid. Clear areas in a dark blue background indicated xylanase activity.

Enzyme Purification: All steps were carried out at 4 ^{o} C unless otherwise specified. The crude extract was concentrated by ultrafiltration using an Amicon system with a 10 kDa cut-off point membrane (PM 10). Most of the enzyme activities were found in the retentate. Xylanase activity was also found in the permeate. Aliquots of the retentate or permeate were fractionated by gel filtration (Sephacryl S-100), anion-exchange (Q-Sepharose, DEAE-Sepharose and Econo Pac High Q) cation-exchange (S-Sepharose, CM-Sepharose and Econo Pac High S) and hydrophobic interaction (Phenyl Sepharose) columns in appropriate conditions (13, 24).

RESULTS AND DISCUSSION

The production of several xylanolytic enzymes was studied in aerobic fungi using enzyme activity assays. During growth on media containing wheat bran *Aspergillus fumigatus* Fresenius, *Humicola grisea* var. *thermoidea*, *Trichoderma harzianum* and *Acrophialophora nainiana* produced considerable amounts of β-xylanase, β-xylosidase and α-arabinofuranosidase activities. We have noticed that even on the same cultivation conditions there may be considerable variation in the production of xylanolytic enzymes. A variety of enzymes are involved in the breakdown of xylan (12). The complexity and heterogeneous nature of xylan require the presence of multiple xylan-degrading enzyme forms for efficient biodegradation of the polimer (22, 23). A multiplicity of xylan-degrading enzymes have been reported in bacteria and fungi (12, 21, 23, 24). The occurrence of multiple xylan-degrading enzymes was found in *Humicola grisea* var. *thermoidea*, *Aspergillus fumigatus* Fresenius and *Acrophialophora nainiana* cultures. This enzyme multiplicity can be caused by several factors such as differential mRNA processing, partial proteolysis or differences in the degree of amidation and glycosylation (21). The functional and genetic basis of xylan-degrading enzyme multiplicity has not been completely elucidated (23).

Xylan-degrading enzymes purification procedures have generally used standard chromatographic methods such as gel filtration, ion-exchange and hydrophobic interaction (23). Gel filtration chromatography on Sephacryl S-100 resulted in the separation of several protein peaks with xylan-degrading enzyme activities (Fig. 1). Some of these were used for further purification by anion-exchange, cation-exchange and hydrophobic interaction chromatography (Fig. 1). The purification step procedure provided an apparently homogeneous preparation of some xylan-degrading enzyme activities, as determined by sodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE) (result not shown). The molecular weight range by SDS-PAGE was 16-97 kDa (Table 1) . The single band staining for β-xylanase activity on non-denaturing electrophoresis was coincident with the staining for activity (result not shown). The molecular weight was also determined by HPLC (Protein-Pak 300 SW). However, the M_r values were below 30 kDa, suggesting that the enzyme interacted with the resin (Table 1) (24). Very low values generally have been obtained by gel filtration and should be viewed with caution because they are probably due to anomalous behavior during gel filtration, i.e., physical interactions between the enzyme and chromatography resin, leading to a retention of the proteins (10, 11, 23). Ultrafiltration can be used for separation of xylanases from others proteins (23). Some xylanases from *Aspergillus*

fumigatus Fresenius and *Trichoderma harzianum* were able to diffuse through the pores of the ultrafiltration membrane. Perhaps this ability to penetrate the ultrafiltration membrane is due to their compact structures or to the nonuniformity of pore sizes in the membrane (10, 21). The ability of some xylanases to penetrate small pores would facilitate the interaction between the enzyme and the complex hemicellulose-lignin-cellulose in wood (10, 15, 21).

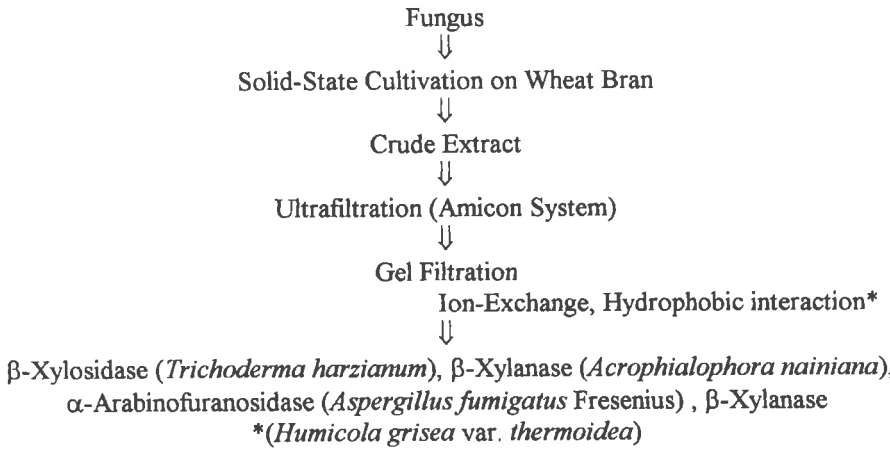

Figure 1: Purification scheme of some xylan-degrading activities produced by fungi.

The purified xylan-degrading enzymes were thermostable, having a temperature optimum at 70 ^{O} C, pH 5.0 (Table 1). A small amount of carbohydrate was detected when β-xylosidase from *Trichoderma harzianum and* α-arabinofuranosidase from *Aspergillus fumigatus* Fresenius were submitted to phenol-sulfuric acid method. The carbohydrate in question may be non-covalently rather covalently bound.

The kinetic parameters of some xylan-degrading enzymes are listed in Table 1. The apparent K_m values were examined from a Michaelis Menten plot using a non-linear regression data analysis program. The K_m values were much lower than the K_m of the xylan-degrading enzymes from *Penicillium capsulatum* and *Myrothecium verrucaria* (10,11).

A number of non-specific xylanases have been characterized (11, 22). Substrate cross-specificity should be considered for certain xylanase preparations. β-Xylanase from *Humicola grisea* var. *thermoidea* was active against xylan from oat spelts and birchwood, p-nitrophenyl-glycoside (PNPG), β-1,4-glucan, laminarin and CM-celullose. α-Arabinofuranosidase from *Aspergillus fumigatus* Fresenius was only active against PNPA and PNPG. Cellulose and xylan may compete for the same enzyme active site (10, 11, 23).

A synergistic effect was observed when β-xylanase from *Aspergillus fumigatus* Fresenius was incubated with arabinoxylan after pre-treatment of the relevant substrate with α-arabinofuranosidase from the same fungus (24).

The amount of reducing sugar released was increased when β-xylosidase from *Trichoderma harzianum* and β-xylanase from *Aspergillus fumigatus* Fresenius were used simultaneously. The action of a side chain enzyme (α-arabinofuranosidase) facilitated the release of products by the main chain enzymes (β-xylanase and β-xylosidase) (24).

Table 1: Some properties of xylan-degrading enzymes produced by fungi.

M_r (SDS-PAGE)*	16 kDa
M_r (SDS-PAGE)**[a]	97 kDa
M_r (SDS-PAGE)***	60 kDa
M_r (SDS-PAGE)****	66 and 70 kDa (subunits)
M_r (HPLC)***	< 20 kDa
M_r (HPLC)****	< 30 kDa
M_r (Sephacryl S-100)**[b]	28 kDa
Optimum Temperature (^{O} C), pH 5.0**	70
Optimum Temperature (^{O} C), pH 5.0****	70
Optimum Temperature (^{O} C), pH 5.0***	70
Optimum pH at 50 ^{O} C***	4.0-4.5
K_m (mM)***	0.053 (PNPX)
K_m (mg/ml)****	7.233 (oat spelts xylan)
	1.805 (birchwood xylan)

*β-xylanase from *Acrophiolophora nainiana*; **[a] α-arabinofuranosidase from *Aspergillus fumigatus* Fresenius; **[b] β-xylanase from *Aspergillus fumigatus* Fresenius; ***β-xylosidase from *Trichoderma harziamum*; **** β-xylanase from *Humicola grisea* var. *thermoidea*; ***** β-xylanase from *Acrophiolophora nainiana*

CONCLUSION

Hemicellulose is one of the main polymeric constituents in plant cell walls. Xylan-degrading enzymes play an important role in recycling carbon fixed by photosynthesis, in agriculture and in the pulp, paper, textile and food industries. Therefore, we must have an understanding of the enzyme systems required for the hydrolysis of xylan and of microorganisms that produce these enzyme systems. The finding above showed *that Aspergillus fumigatus* Fresenius, *Humicola grisea* var. *thermoidea*, *Trichoderma harzianum* and *Acrophialophora nainiana* produces at least three kinds of xylan-degrading activities: β-xylanase, β-xylosidase and α-arabinofuranosidase.

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RESUMO

Produção de Enzimas Xilanolíticas por Fungos Aeróbicos Crescidos em Meio Sólido

Os fungos aeróbicos *Aspergillus fumigatus* Fresenius, *Humicola grisea* var. *thermoidea*, *Trichoderma harzianum* and *Acrophialophora nainiana* produzem enzimas xilanolíticas quando crescidos em meio sólido contendo farelo de trigo como fonte de carbono. Por conveniência, as condições de cultivo, com exceção da temperatura de crescimento e ensaios enzimáticos, foram as mesmas para todos os fungos. O fracionamento de extratos brutos por cromatografias de filtração em gel e troca iônica demonstrou que as enzimas xilanolíticas apresentam multiplicidade de forma. Algumas dessas enzimas foram purificadas por técnicas de ultrafiltração e cromatografias de filtração em gel, troca iônica e interação hidrofóbica. As amostras enzimáticas apresentaram uma única banda protéica quando submetidas a eletroforese e coradas para proteína. Os pesos moleculares foram estimados por SDS-PAGE e filtração em gel (HPLC e Sephacryl S-100). As enzimas purificadas foram mais ativas no intervalo de pH 4.0-5.0 e 70 ^{o}C. A maioria dessas enzimas foram coradas para glicoproteína. Os valores de K_m aparente foram muito menores de que alguns valores descritos na literatura. Algumas dessas enzimas foram muito ativas contra p-nitrofenilglicosídeos e xilanas, respectivamente. Um efeito sinérgico foi observado quando algumas enzimas xilanolíticas foram incubadas com arabinoxilana pré-tratada.

Palavras-chave: fungos aeróbicos, xilana, enzimas xilanolíticas, purificação, caracterização

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COMPARISON OF MYCELIAL AND SPORE INOCULUM ALTERNATIVES IN *MUCOR BACILLIFORMIS* FOR RENNIN OBTAINMENT IN SOLID STATE FERMENTATION

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ABSTRACT

Rennin is obtained in wheat bran by solid state fermentation using spores of *Mucor bacilliformis* as inoculum. The spores are produced on tomatoe juice agar or on the same substrate used to obtain the enzyme. In industry, productivity optimization is based on by minimization of the lag phase. As an alternative procedure, mycelial cells10% ($^{w}/_{w}$), grown in wheat bran, were utilized as inoculum. Compared to spore inoculum, mycelial inoculum presented higher productivity (Qp = 310 $\pm$ 20 EU/gds.h and 410 $\pm$ 30 EU/gds.h, respectively) but the R coefficients (activity ratio) were the same (R= 11 $\pm$ 2). Furthermore, these results were also obtained in a vertical cylindric acrylic column. Besides, biomass reuse eliminated previous spore production and minimized the lag time of spore germination.

Key words: solid state fermentation, *Mucor bacilliformis*, rennin

INTRODUCTION

Solid state fermentation (SSF) is generally defined as the growth of microorganisms on solid materials in the absence of free water (7). It is not widely used because substrate is not homogenous throughout the fermentation time, microbial heat generation and heat transfer limitations cause heat buildup and parameters such as pH, a_w, dissolved O_2, cell content and substrate concentration in the solid state are difficult to measure. In spite of this, the SSF process has the advantages of high productivity, simpler techniques, reduced energy requirement, low waste water output and improved product recovery (4,5,8).

Rennin, the milk curdling enzyme used in cheese making, has been obtained from the fungus *Mucor bacilliformis* by SSF (1,3). Sporangiospores are used as inoculum in the fermentation, which usually takes around 12 hours for germination and initiation of the growth phase. In detriment to the maximum productivity of rennin obtained at industrial scale. The purpose of this article is to report the use of mycelium instead of spores as an alternative inoculum preparation in a SSF for maximization of the enzyme productivity in a pilot plant scale reactor and for the reuse of the waste material obtained at the end of fermentation.

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MATERIALS AND METHODS

Culture. The microorganism employed in this study was a strain of *Mucor bacilliformis*. Test stock culture was maintained on potato dextrose agar slants. The culture was preserved at 4°C and renewed every six months.

Substrate and Inoculum. Sporangiospores were obtained from *Mucor bacilliformis* culture slants on tomato juice agar incubated for 14 days at 21°C.

Spores were suspended in sterilized distilled water and counted in Neubauer chamber following proper dilution. A commercial quality wheat bran with 12 % humidity was used as substrate. This was moistened at 120 % ($^{v}/_{w}$), dry basis, with HCl 0.1 N and autoclaved for 30 minutes at 121 °C. After cooling, 90 grams of substrate were tranferred to a pilot plant scale reactor with pack density of 0.5 gms/cm^3. A schematic diagram of the bioreactor is shown in Fig. 1. The bioreactor consisted of a vertical, cylindrical acrylic column as fermentor (length/diameter = 5.3; volume 180 cm^3), with four temperature probing points to monitor the temperature of the fermenting bed.

The medium was inoculated with 5 x10^5 spores/gds. Cultivation was carried out at 28°C in a temperature controlled chamber and the aeration rate was kept at 5.56 mL/gds.min. The gas flow rate was measured by using a Cole Palmer flowmeter, model N092-04. Since CO_2 evolution should be indicative of growth, the inlet gas was bubbled through a 2 N NaOH solution to remove CO_2. To prevent drying, the inlet gas was passed through a humidifier and then was sterilized by an hydrophobic membrane of 0.22 µm pore size. After 22 hours of fermentation, the medium was homogenized and used as inoculum in Erlenmeyer flasks of 250 mL containing 10 gms. Flasks were inoculated with 10, 15 and 20% (g inoculum/gds) and incubated for 40 hours at 28 ± 1 °C. Samples were withdrawn intermittently. Identical flasks containing 5 x 10^5 spores/gds were used as control.

Extraction of Enzyme. A known quantity of the fermented dough containing enzyme was treated with deionized water in 5:1 (water mL/gds) and mixed thoroughly in a reciprocal shaker at 200 rpm during 2 hours. Solids were separated by centrifugation at 15000 rpm for 20 minutes at 5°C. Supernatants were adjusted to pH 5 for milk clottting and proteolytic activities.

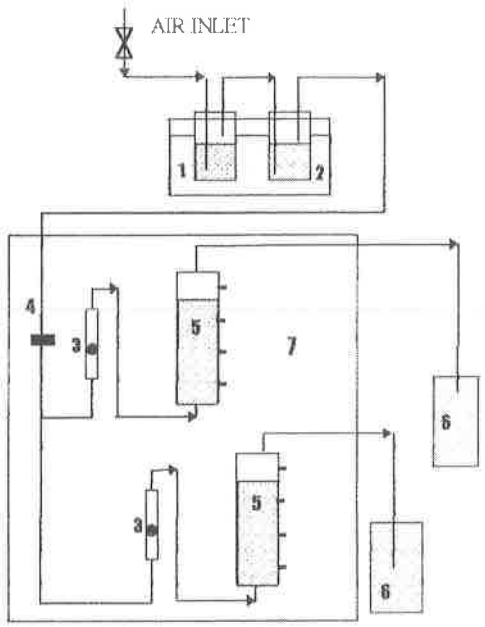

Figure 1. Schematic diagram of the bioreactor. 1.-NaOH 2N; 2.-humidifier; 3.-flowmeter; 4.-hydrophobic membrane 0.22 µm; 5.-acrylic packed bed reactor ; 6.-NaOH 0.3 N; 7.- temperature controlled chamber.

Assay of Enzyme Activity. Milk clotting activity was determined according to the method of Arima *et al.* (9). 400 EU were defined as the amount of enzyme contained in 1 mL extract which clots 5 mL of 0.01 M $CaCl_2$ solution containing 0.5 g of skim milk powder in 60 seconds at 35 °C. The correlation used, valid between 35 and 85 seconds, was:

$$EU/mL = 2 \times (878.2 - 7.62 \times t_c) \times d$$

The proteolytic activity (PA) was assessed by a photometric assay using modified Anson´s method (6). Test tubes with 1.0 mL solution of

2% casein in phosphate buffer at pH 6 were incubated in a water bath with reciprocal shaker at 35 °C during 15 minutes. Culture filtrates obtained as above (1.0 mL diluted if necessary) were added to each test tube and stabilized for 10 minutes. Then 2.0 mL of trichloroacetic acid 0.4 M were added, vortex stirred and centrifuged for 25 minutes at 15000 rpm and 5 °C. Supernatants were measured at 280 nm by triplicate using a blank for the absorbance zero adjustment. The PA was expressed as miligrams of tyrosine released within 1 min.
The correlation used was:

$$PA = 4 \times [38.93 - \frac{(22.63 - 24.44 \times A) \times 0.5}{0.1222}] \times d$$

Growth Rate. CO_2 evolution was used as an indirect measurement of the growth rate. The method relied on the collection of CO_2 from the effluent gas using a 0.03 N NaOH solution for its absorption (2). The variation in the electric conductivity measured is proportional to the amount of CO_2 absorbed in the solution.
To determine the maximum productivity, milk clotting activity was measured at 0, 20, 24, 29 and 40 hours of fermentation by triplicate. Control flasks were measured at 36 and 48 hours by triplicate.

Pilot Plant Scale Reactor. Results obtained in Erlenmeyer flasks were confirmed in two pilot scale reactors, one of them inoculated with spores as described above. Mycelial inoculation ratio used was 10% (g inoculum/gds). The fermentation was monitored by the CO_2 evolution per unit time and the temperature evolution. When the maximum CO_2 production was obtained, the fermentation was finished and milk clotting and proteolytic activities were determined.

RESULTS AND DISCUSSION

The evolution of the culture on wheat bran with different mycelial and spore inoculum is shown in Fig. 2.

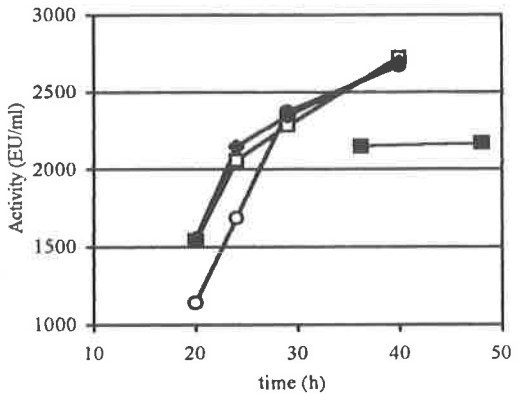

Figure 2. Milk clotting activity (EU/mL) of 10% (o), 15% (□), 20% (♦) ($^{w}/_{w}$) mycelial inoculum and spore inoculum (■) versus time.

Milk clotting enzyme and proteolityc activity increased with time for all the different inoculum percentage, remaining R coefficient constant, as shown in Table 1.

No statistical difference waas observed between R coefficients obtained for mycelial or for spore inocula. Thus, it is not possible to define, whether mycelial inoculum fermentation is advantageous compared with the spore inoculum fermentation.

Table 1. Productivity Qp (EU/gds.h) and R coefficient versus fermentation time (h) with different inocula.

Inoculum %	time	Qp	R
10	20	285 ± 20	7 ± 2
	24	350 ± 25	8 ± 2
	29	410 ± 30	11 ± 3
	40	335 + 25	11 + 2
15	20	385 ± 30	10 ± 2
	24	430 ± 30	10 ± 2
	29	395 ± 30	9 ± 2
	40	340 ± 25	10 ± 2
20	20	390 ± 30	9 ± 2
	24	445 ± 30	9 ± 2
	29	405 ± 30	8 ± 2
	40	335 ± 25	9 ± 2
spore inoculum	36	310 ± 20	11 ± 2
	48	230 ± 15	10 ± 2

As mycelial inoculum percentage increased,

maximum Qp was obtained earlier and its value was always higher than the one obtained with spore inoculum. The absence of lag time with mycelium inoculum, which takes 12 hours for spore germination, was considered one of the main reasons for this improvement.

Table 2. Productivity Qp (EU/gds.h), R coefficient and μ_{max} (h^{-1}) in pilot plant scale reactor inoculated with $5x10^5$ spores/gds (A) and 10 % ($^w/_w$) mycelial inoculum (B) at 17.5 and 21.0 h.

Inoculum	time	Qp	R	μ_{max}
(A)	21.0	195 ± 15	4 ± 1	0.14 ± 0.03
(B)	17.5	263 ± 20	5 ± 1	0.19 ± 0.02

In the pilot scale reactor, the R coefficient was significantly lower than the one obtained in flasks fermentation mainly because of the lowest milk clotting activity reached in this scale, as seen in Table 2.

Heat and mass transfer phenomena ocurring at this level were responsible for these events leading to a higher bulk temperature which could denaturate partially the enzyme. The high temperature reached might also affect the microorganism development. However, temperature evolution shows a better fermentation perfomance in comparison with spore inoculum fermentation (Fig. 3).

This evolution correlates well with CO_2 development, as shown in Fig. 4. μ_{max} obtained whith mycelial inoculum was higher than that one obtained with spore inoculum, which means a better fungal fermentation. Concomitantly, a better maximum Qp was obtained (33 % higher) when mycelial inoculum was used instead of spore inoculum.

The final choice of the mycelial inoculum ratio in pilot plant scale reactor considers economic aspects (data not shown) and for this reason only 10 % ($^w/_w$) inoculum reuse was assessed in this scale.

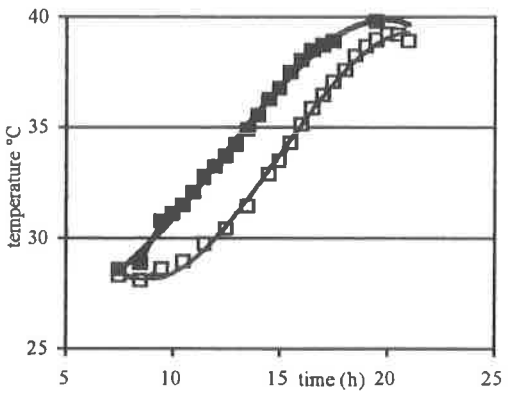

Figure 3. Temperature evolution with time in $5x10^5$ spores/gds (□); and 10% ($^w/_w$) mycelial inoculum (■).

CONCLUSIONS

Mycelial inoculum fermentation is an interesting alternative for spore inoculum fermentation in pilot plant scale reactor, because of the improvement in productivity. Heat and mass transfer phenomena in pilot plant scale have shown to be critical factors for the fermentation performance, affecting directly the enzyme production. This limitation should be overcome to increase enzyme production.

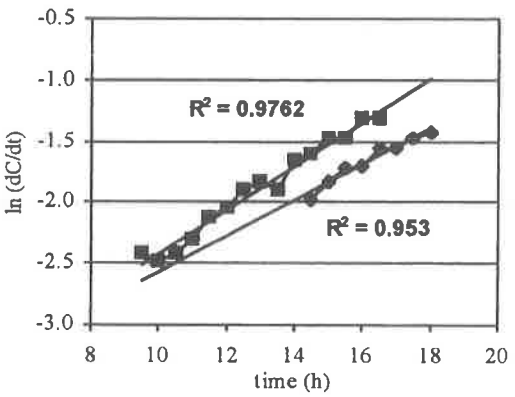

Figure 4. CO_2 evolution with time using different inoculum.

NOMENCLATURE

gds grams of dried solid
gms grams of moist solid
PA proteolytic activity in (mg Tyrosine/L.min).
d dilution
A Absorbance
R $\frac{\text{Milk Clotting Activity}}{\text{Proteolytic Activity}}$
Qp Productivity(EU/gds h)
tc milk clotting time

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RESUMO

Comparação entre duas alternativas de preparo de inóculo no processo de obtenção de renina em fermentação em estado sólido por *Mucor bacilliformis*

Renina é obtida por fermentação em estado sólido de farelo de trigo utilizando esporos de *Mucor bacilliformis* como inóculo. Os esporos são produzidos em agar suco de tomate ou com o mesmo substrato usado na obtenção de enzima. A produtividade é otimizada minimizando-se a fase de adaptação do microrganismo. Utilizando-se, como alternativa, inóculo composto de células na forma de micélios, na proporção de 10% (p/p), foi obtida uma produtividade em enzima Qp de 410 ± 30 (EU/gds.h) e razão de atividades R=11 ± 2. O valor de Qp foi maior que o obtido com esporos como inóculo, enquanto que o valor de R permaneceu o mesmo. Os resultados foram confirmados em escala piloto com reator cilíndrico vertical em acrílico. A reutilização da biomassa eliminou a etapa de produção de esporos e minimizou o tempo de adaptação relativo a germinação de esporos.

Palvras-chave: fermentação em estado sólido, *Mucor bacilliformis*, renina

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SPORE PRODUCTION BY ENTOMOPATHOGENIC FUNGUS *BEAUVERIA BASSIANA* FROM DECLASSIFIED POTATOES BY SOLID-STATE FERMENTATION

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ABSTRACT

Several agroindustrial substrates were tested to produce spores from the fungus *Beauveria bassiana*, which is used for the biological control of pests of banana, sugar cane, soybean and coffee cultures. Solid state fermentation was carried out using 50 mL natural media (integral wheat, rye, cassava, rejected potato and rice flours, and cassava bagasse), in erlenmeyer flasks, inoculated with 10^6 spores and incubated at 28° C for 10 days. The best results for spore production ($3.92 .10^9$/g dry substrate) were obtained by using declassified potato flour. After culture condition optimization, spore production raised to $3,0.10^{10}$ spores/ g dry substrate in column bioreactor, with 87 % viability. Results reported in this article show the potential of using agroindustrial residues, namely declassified potato flour, as substrate for spore production by entomopathogenic fungi like *Beauveria bassiana*.

Key words: solid state fermentation, agroindustrial substrates, *Beauveria bassiana*

INTRODUCTION

Cultivation of potato (*Solanum tuberosum*, L.) is essentially limited to South and South-West Brazil. Paraná State is an important supplier for other Brazilian regions and recently became the greatest national potato producer, contributing with 29 % of the total production of the country, which was around 2,330,000 tons in the last 10 years. This amount placed Brazil as the eigteenth largest world producer (8), but also generated high amounts of residues, namely declassified potatoes, which normally are not profitable. Consequently, the development of processes to add value to potato wastes is important to reduce pollution and to take advantages of the high potato production of Parana State.

Recently, the use of the entomopathogenic fungi for biological control of insects and pests has received increasing attention (7, 8,10), since fungi are the main pathogens of insects. Fungi of 80 genera and approximately 700 species were involved in more than 80 % of diseases of

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the insects (1, 16). Among them, *Beauveria bassiana* is widely distributed in nature and infects over 70 species of insects. This fungus is reported as the most common species isolated from dead and moribund insects (25). Therefore, the development of processes to produce this fungus in large scale will be of considerable interest for immediate industrial application.

Nowadays, the most well-developed mass production techniques involve a surface cultivation or a two-stage cultivation in which fungi are first grown in submerged conditions, and are then allowed to sporulate in a surface culture in still-liquid or on a semi-solid medium. This method yields elevated amounts of conidia, characterized by high virulence and good resistance to adverse environmental conditions. However, these cultivation processes take time and require intensive labour, and are therefore too expensive (25).

Recently, among the different methods of biomass production, solid state fermentation (SSF) has gained importance due to several advantages related with industrial strategies, namely high productivity, simplicity of the technique, low energy requirements and waste water output, and facility of product recovery (15, 19, 22). High yields of entomopathogenic fungi can be obtained using natural solid substrates such as bean flours, molasses, beef extract, and flour prepared from potato residues (10, 11). Rice grain flour is widely utilised in Brazil to produce elevated amounts of conidia from *Metarhizium anisopliae* and *Beauveria bassiana* (4, 12).

The objective of this article is to evaluate the potential of using agroindustrial residues, such as declassified potato flour, as a cheap and efficient substrate to produce spores from the entomopathogenic fungus *Beauveria bassiana*.

MATERIALS AND METHODS

Raw material. The following raw materials for preparation of the natural substrates were provided by local industry or bought in the local market: integral wheat, rice and rye flour, cassava (*Manihot esculenta* Crantz) bagasse and cassava flour, and declassified potato (*Solanum tuberosum*, L.) flour.

The physico-chemical analysis of the substrates were carried out according to the techniques recommended by Instituto Adolfo Lutz (21).

Microorganism. The strain *Beauveria bassiana* was obtained from Departamento de Fitotecnia e Fitossanitarismo, Universidade Federal do Paraná. The microorganism was maintained on potato dextrose agar (PDA) plates, at 4° C.

Culture media. The following commercial media were used: malt agar, Sabouraud agar, and PDA. The manually selected materials were washed, sliced and dried in a stove (50 to 60°C) for 24 h. The dry material was then crushed in a grinder, sifted, ground and selected for the fraction retained between 0.84 mm and 2 mm sieves.

Inoculum production. Inoculum production was carried out on PDA medium (39 g/L), which was sterilized (121° C, 20 min.), poured into Petri dishes (90 mm diameter) and incubated for 10 days at 28° C. Spores were collected by adding 10 mL of a Tween 80 solution (0.1 %) to the dishes, and counted in a Malassez chamber.

Spore production in erlenmeyer flasks

Spore production on commercial agar. The effect of three different culture media on spore production were investigated: malt-agar (20 g/L), Sabouraud-agar (65 g/L) and PDA (39 g/L). 50 mL of each of the media were poured into separate 250 mL erlenmeyer flasks, sterilized, and cooled to 50-55 °C. The flasks were inoculated with a spore suspension (10^6 spores/mL) and incubated for 10 days at 28° C. The spores were collected by adding 80 mL of Tween 80 solution (0.01 %) and glass balls (30 g), under shaking for 20 min. The spore solution was filtered on a 200 μm mesh nylon bolter sieve. Spores were counted in a Malassez

chamber, with the results from three flasks being averaged (23,24).

Spore production on natural agar. Flours of different natural substrates prepared as described before (usually 40 g/L), were cooked in an autoclave for 1 h and filtered. The volume was made up to 1 L and 15 % (w/v) agar was added. 50 mL of the media were then poured into 250 mL erlemeyer flasks. After sterilization, the inoculation and cultivation were done as described before.

Effect of pH. The effect of pH on spore production was studied using declassified potato flour. Flasks were inoculated with a spore suspension (10^7 spores/mL). The pH was adjusted (4.0, 5.0, 6.0, 7.0, 8.0) by adding 10 % (w/v) tartaric acid and NaOH (3 M).

Effect of temperature and lighting: The effect of different temperatures (24° C, 28° C, 35° C, 37.5° C) and of a 12 h photoperiod on spore production were studied, using declassified potato flour as substrate, pH 6.0 and 28 °C. Substrate preparation, inoculum, and other culture conditions were the same as described before.

Kinetics of spore production. The kinetic experiments were carried out in 250 mL erlenmeyers flasks containing 50 mL of sterilized medium prepared from declassified potato flour (40 g/L) and 1.5 % (w/v) agar. The substrate was inoculated with 1 mL of a spore solution (10^7 spores/mL) and incubated at 28° C. Spore extraction and counting were done every 2 days, from the 6^{th} to 26^{th} day of culture.

Solid State Fermentation in Bioreactor Columns. The studies of spore production in bioreactor columns were carried out with declassified potato flour as the substrate, with a granule size between 0.8-2.0 mm. The initial substrate moisture was adjusted to 55 %, pH was adjusted to 6.0 and incubation was at 28° C. The humidified material was autoclaved (15 min., 121 ° C), cooled to room temperature, inoculated with 10^5 spores/g dry substrate and placed in column bioreactors (4 cm x 20 cm, working volume 251 cm^3). This equipment consisted of 16 columns immersed in water, with temperature and air flux control (0-20L/h/column). The bioreactor was connected to a gas chromatograph, which allowed the measuring of the outlet gases (23). Spore production was evaluated after 10 days of culture, by drying the material in a vacuum oven, and counting the produced spores in a Malassez chamber. The conidia viability was done according to Soccol (23).

The effect of the following parameters on spore production were investigated: inoculation rate, initial moisture content of the substrate and aeration. The kinetics of spore production were also characterized. Inocula preparation, inoculation, culture conditions and counting were done as described before.

Effect of inoculation rate and of initial moisture: the range studied for inoculation was 10^3 to 10^7 spores/g dry substrate; the initial moisture varied in the range of 30 to 70 % (w/w) of the dry substrate.

Effect of aeration: this experiment was done in order to establish the best air flow condition for the growth of *Beauveria bassiana* in bioreactor columns. The rates of air flow studied were 0.0 to 80 $mL.min^{-1}.column^{-1}$.

Kinetics of spore production: the substrate was inoculated with 1 mL of a spore solution (10^7 spores/mL) and incubated at 28 °C. Spore extraction and counting were done every 2 days, from the 6^{th} to 22^{nd} day of culture.

Spore viability. In order to evaluate spore viability, 1 mL of spore suspension (10^7 spores/mL) was diluted with 20 mL of potato-dextrose broth (PDB) to a final concentration of 10 to 15 spores/mL. The medium was then incubated (7 h, 30° C, 125 rpm) and samples were taken at each hour of the incubation period. The viability of spores was calculated by the ratio between the germinated spores number and the total spores number, according to Soccol (24).

RESULTS AND DISCUSSION

Table 1 shows the results of the physico-chemical analysis of declassified potato flour used in this work.

Table 1: Physico-chemical composition of declassified potato flour.

Composition	%[a]
Moisture	8.5
Protein	10.64
Lipids	0.75
Starch	76.5
Fiber	2.91
Ashes	0.7

a) %: dry basis, according to the techniques recommended by Instituto Adolfo Lutz (21).

The high starch and protein contents (76.5 % and 10.64 %, respectively) make this residue a good alternative as a natural substrate in SSF.

Spore Production in Erlenmeyer Flasks. To investigate spore production on commercial agar, three media were studied: malt agar, PDA and Sabouraud-agar. As shown in Figure 1, there was a significant difference in spore production amongst the studied media. The best result was obtained with PDA medium ($3.6\pm0.15 \times 10^9$ spores/g medium). This result is similar to that reported by many authors (2, 14, 5) for spore production by *B. bassiana*.

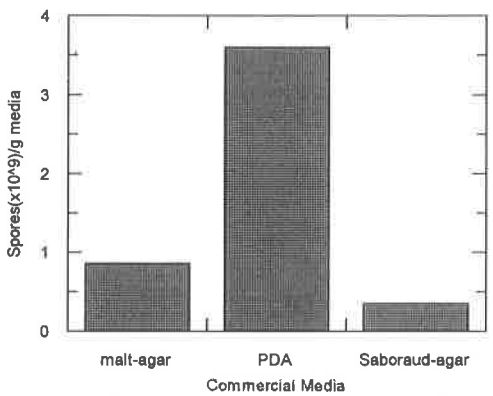

Figure 1. Production of spores by *Beauveria bassiana* on commercial media: malt-agar (20 g/L), Sabouraud-agar (65 g/L) and PDA (39 g/L). Culture conditions: inoculum with a suspension of 10^6 spores/mL; incubation for 10 days at 28° C.

Amongst the natural substrates studied (integral wheat, rice and rye flour, cassava bagasse and cassava flour, and declassified potato flour), the best level of spore production was obtained with declassified potato and wheat flour (3.92×10^9 and 3.12×10^9, respectively), as shown in Figure 2.

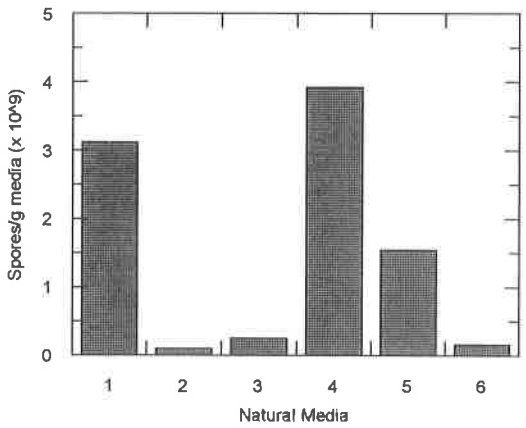

Figure 2. Production of spores by *Beauveria bassiana* on natural media: 1) wheat, 2) cassava bagasse, 3) cassava, 4) declassified potato, 5) rye and 6) rice flours. Culture conditions: inoculum with a suspension of 10^6 spores/mL; incubation for 10 days at 28° C.

Initial pH of the media is a very important factor in spore production by *B. bassiana*, as shown in Figure 3. The mould presented a good development in the range of pH 5.0 to 7.0, with the maximum of spore production at pH 6.0. In the extremes of the studied pH, the growth was diminished, but still significant, suggesting a large range of pH tolerance by the fungus. Similar results for *B. bassiana* were reported by Nahas and Arai (17).

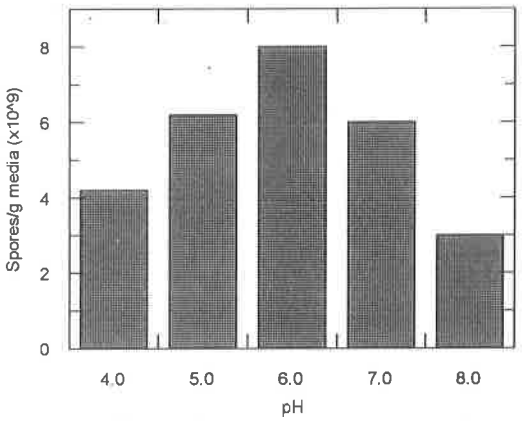

Figure 3. Effect of pH on spore production by *Beauveria bassiana* on potato-agar medium prepared from declassified potato flour (40 g/L) and 15 % (w/v) agar. Culture conditions: inoculum spore suspension (10^7 spores/mL), incubation for 10 days at 28° C.

The effect of incubation temperature on spore production by *B. bassiana* cultured in declassified potato flour is shown in Table 2. Temperatures from from 25 to 37.5° C were tested. The fungus grew well in the range from 25°C to 28°C, with the maximum at 28°C ($7.95\pm0.25 \times 10^9$ spores/g medium). Higher temperatures reduced considerably the amount of spore production, with a decrease of approximately 90 % at 35°C.

The effect of the lighting on the spore production by *B. bassiana* was tested at 25 °C, by performing the experiment in the absence of light (Table 2). There was a significant decrease of spore production (40 %) in comparison with the experiment done with a photoperiod of 12 h, indicating that light is important for the growth of the fungus, as demonstrated before by many authors (6, 13, 25).

Table 2. Effect of temperature and lighting on spore production by *Beauveria bassiana* on potato-agar medium prepared from declassified potato flour (40 g/L) and 15 % (w/v) agar. Culture conditions: inoculum spore suspension (10^7 spores/mL), incubation for 10 days at 28 °C.

Temperature (°C)	Spores/g Media x 10^{9} a)	Photoperiod (h)
25	2.9±0.19	no light
25	4.76±0.13	12
28	7.95±0.25	12
35	0.78±0.029	12
37.5	0.02±0.001	12

a) average of triplicates

The kinetics of spore production by *B. bassiana* grown on potato-agar medium were studied by measuring spore production from the 6th to the 26th day, using the optimized culture condition (pH 6.0, 28 °C, 12 h photoperiod). Figure 4 shows that spore production was approximately exponential throughout 0-18 days; at 18 days, the production reached a maximum (12×10^9 spores/g medium), and subsequently, it decreased to 75 % of the maximum production on the 26th day of cultivation.

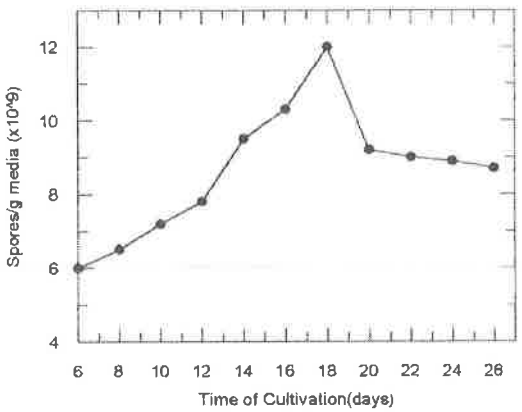

Figure 4. Kinetics of *Beauveria bassiana* sporulation on potato-agar medium prepared from declassified potato flour (40 g/L) and 15 % (w/v) agar. Culture conditions: inoculum spore suspension (10^7 spores/mL), pH 6.0, 28°C, 12 h photoperiod.

Solid State Fermentation in Column Bioreactor. The studies of spore production in bioreactor columns were carried out in order to simplify the process optimized in erlenmeyer flasks on potato agar medium and to maximize spore production by *B. bassiana*. Declassified potato flour was used as substrate, with a granule size of 0.8-2.0 mm, as described in the Materials and Methods section.

Figure 5 shows the effect of initial moisture (30 to 70 %) of the substrate on conidia production by *B. bassiana*. At 50 % moisture the spore production by the fungus was maximal ($7.0x10^9$ spores/g dry substrate). Values above and beyond 50 % led to a decrease of spore production.

The spore inoculation rate is an important parameter in SSF and should be enough to ensure homogeneous substrate inoculation and fast initial growth, to avoid incidental competition with contaminants (23, 24). Many authors have demonstrated that the optimal inoculation rate is between 10^6 and 10^7 spores per gram of dry support (18, 20, 23, 24). Inoculation rates over 10^8 spores per gram of dry support can inhibit spore germination (3).

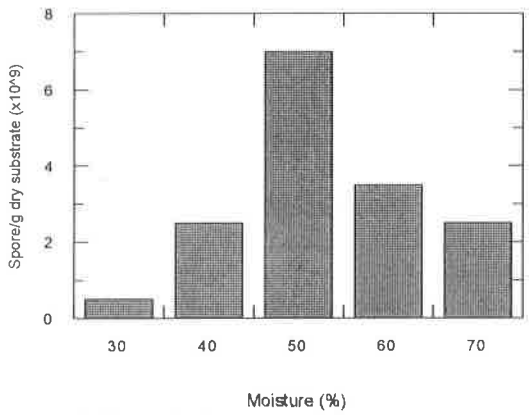

Figure 5. Effect of substrate initial moisture on spore production by *Beauveria bassiana* using SSF in column bioreactors (4 cm x 20 cm), and declassified potato flour as substrate, pH 6.0 and 28° C, inoculum 10^5 spores/g dry substrate, 10 days of culture.

The results presented in Figure 6 confirm these statements, showing that the best inoculation rate for spore production by *B. bassiana* is 10^6 spores per gram of dry support.

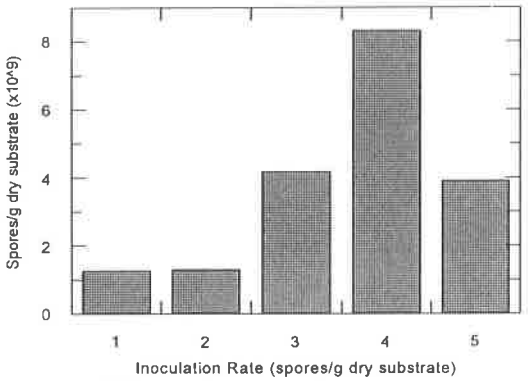

Figure 6. Effect of inoculation rate on spore production by *Beauveria bassiana* using SSF in column bioreactors (4 cm x 20 cm), and declassified potato flour as substrate, pH 6.0 and 28° C, 50 % initial moisture, 10 days of culture.

The effect of air flow rate on spore production by *B. bassiana* is presented in Figure 7. At an air flow of 60 mL. min^{-1}. $column^{-1}$, maximal production of spores was observed ($9.7x10^9$ spores/g dry substrate).

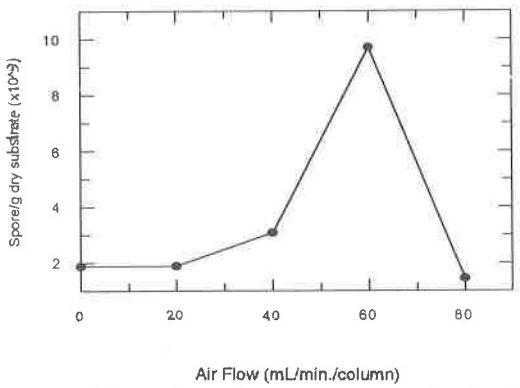

Figure 7. Effect of aeration on spore production by *Beauveria bassiana* using SSF in column bioreactors (4 cm x 20 cm), and declassified potato flour as substrate, pH 6.0 and 28° C, 50 % initial moisture, 10 days of culture.

At an aeration rate of 80 mL. min^{-1}.$column^{-1}$, a lower amount of spores is produced as compared with the control experiment (without

aeration). These results could be related to the control of the temperature in the core of the columns. Besides, volatile metabolites produced by the fungus could be easily eliminated under such aeration conditions, avoiding degradative metabolism of fungi, like oxidative processes. Replacement of O_2 into the solid substrate seems to stimulate fungal growth (data not shown). The kinetics of spore production by *B. bassiana* were studied using the optimized conditions for SSF, using declassified potato flour. Spore production was followed by sampling every 2 days, from the 6th to the 24th day of culture. There was a small spore production in the period from 6 to 14 days of culture; the production reached a maximum on the 20th. day ($3.0x10^{10}$ spores/g dry substrate), and then slightly decreased to $2.6x10^{10}$ on the 24th. of culture (Figure 8).

In this experiment, the viability of spores was also tested in two different samples: before and after dehydration of the spores.

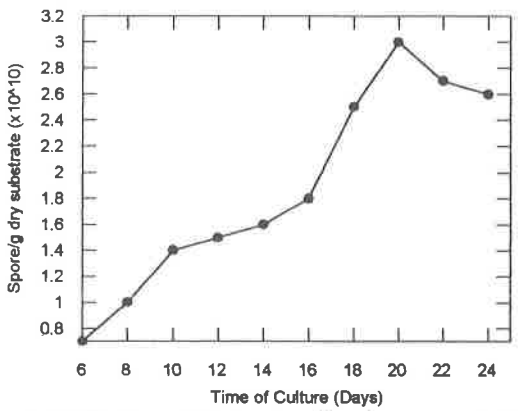

Figure 8. Kinetics of *Beauveria bassiana* sporulation using SSF in column bioreactors (4 cm x 20 cm), and declassified potato flour as substrate, pH 6.0 and 28° C, 50 % initial moisture, 10 days of culture. Air flow: 60 mL. min^{-1}.$column^{-1}$.

The viability for the non-dehydrated spores was 95 % and for dehydrated spores was 87 %, showing that even after dehydration, the viability of the produced conidia was high.

CONCLUSIONS

The present study constitutes an attempt to improve the production of the entomopathogenic fungus *Beauveria bassiana* by solid state fermentation (SSF) using agroindustrial residues, namely declassified potato flour as substrate.

The comparison between the studies in erlenmeyer flasks and agar-based media, showed that SSF in bioreactor columns is a good alternative, since the spores production was higher than that obtained for the cultivation in erlenmeyer flasks. The time-course of the fermentation showed that after 18 days of culture in erlenmeyer flasks the spore production reached a maximum, in comparison the 20 days obtained for SSF.

ACKNOWLEDGEMENTS

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RESUMO

Produção de esporos pelo fungo entomopatogênico *Beauveria bassiana* por fermentação no estado sólido utilizando batata-refugo

Vários substratos naturais (farinhas integrais de trigo, centeio, mandioca e arroz e bagaço de mandioca) foram testados quanto à produção de esporos pelo fungo *Beauveria bassiana*, que normalmente é utilizado para controle biológico de pragas nas culturas de banana, cana-de-açúcar, soja e de café. Inicialmente, utilizou-se fermentação no estado sólido em erlenmeyers, com o meio natural adicionado de agar. 50 mL do meio foram colocados em erlenmeyers e inoculados com 10^6 esporos e incubados a 28 °C por 10 dias. O melhor resultado ($3.92x10^9$/g do meio) foi obtido para o meio agar-batata. Depois da otimização das variáveis em erlenmeyers (pH, temperatura, iluminação), as condições

otimizadas foram testadas em reatores de colunas, utilizando como substrato a farinha obtida a partir de batata-refugo. A produção de esporos aumentou para $3.0x10^{10}$ esporos/g substrato seco, com 87 % de viabilidade.

Os resultados apresentados neste trabalho mostram a viabilidade de utilização de substratos naturais, especialmente aquele obtido a partir de batata-refugo e da fermentação no estado sólido na produção de esporos pelo fungo entomopatogênico *Beauveria bassiana.*

Palavras-chave: fermentação no estado sólido, substratos naturais, *Beauveria bassiana*

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PRODUCTION OF P3HB-co-3HV BY SOIL ISOLATED BACTERIA ABLE TO USE SUCROSE

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ABSTRACT

Two bacterial strains isolated from soil of sugar cane plantations were characterized as regards to the production of poly-3-hydroxybutyrate-co-3-hydroxyvalerate (P3HB-co-3HV) in 5 L bioreactors. Dissolved O_2 (DO) evolution was used as the criterion to determine the complete consumption of carbon source between cultivation steps 1 and 2, when all nutrients were available in the medium allowing cell multiplication. The inversion of pH pattern tendency was used to define the transition from step 2 to 3 of cultivation, when nitrogen was exhausted, impeding the cell multiplication to continue and allowing an increasing of P3HB-co-3HV accumulation. Both strains were compared concerning their volumetric productivities and efficiencies in converting substrates in cells or polymer.

Key words: *Burkholderia*, P3HB-co-3HV, biodegradable plastics

INTRODUCTION

Poly-3-hydroxybutyrate (P3HB) belongs to the group of polyhydroxyalkanoates (PHAs), polyesters accumulated by different bacteria as intracellular granules. Accumulation of these polymers usually is obtained using media limited in one nutrient essential to cell multiplication (N, P, Mg, Fe, etc.) and containing an excess of carbon source (2). The industrial interest on P3HB started in the 1960's when its thermoplastic properties were first described (1). During the 70's this polymer was considered a potential substitute for conventional petroleum based plastics, since P3HB is produced from renewable carbon sources such as carbohydrates (3). However, after characterization, P3HB was evidenced as a very hard and brittle polymer with limited applications (5). Since 1981 there has been an increasing interest on this material based on the following discoveries: (a) *Alcaligenes eutrophus* is able to synthesize the copolymer poly-3-hydroxybutyrate-co-3-hydroxyvalerate (P3HB-co-3HV) when supplied with carbohydrate and substances such as propionic acid as precursor of 3HV units; (b) the copolymer composition can be controlled by adjusting the ratio between carbohydrates and propionic acid supplied; (c) the malleability and resistance of the copolymer gradually increase depending on the 3HV content (3).

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Recently, in Brazil, a research project to develop the technology of P3HB-co-3HV production using sugar cane derivatives has been sponsored by the Programa de Apoio ao Desenvolvimento Científico e Tecnológico (PADCT). As part of this project, a number of bacteria able to produce those polymers were isolated and characterized (4). Some of these isolates are able to grow on sucrose, unlike *Alcaligenes eutrophus*, the most studied microorganism producing P3HB-co-3HV. In this paper, we present the results obtained in the evaluation in bioreactor of two strains isolated from sugar cane plantation soil. Since substrates represent one of the main items in the cost of PHAs production, the bacterial evaluation was based on aspects related to volumetric productivity and efficiency in the conversion of substrates into cells or product.

MATERIALS AND METHODS

Microrganisms - *Burkholderia cepacia* IPT-044 (DSMZ 9241) and *Burkholderia* sp IPT-101.

Inocula Preparation And Cultivation Conditions - Cells of each strain were cultivated during 24 h (30°C, 250 rpm) in nutrient broth (5 g/L peptone and 3 g/L beef extract) and used to inoculate at 10% (v/v) a mineral salts medium (7) containing sucrose as the sole carbon source and incubated for 24 h (30°C, 250 rpm). A volume of 150 mL of this culture was then utilised to inoculate 5 L of mineral salts medium in a BBraun Biostat ED bioreactor (pH 7.0 and DO > 20% of saturation). Cultivation in the bioreactor was performed in 3 steps. In the first step all nutrients were available in the medium allowing cell growth until total consumption of carbon source. In the second step, the bioreactor was fed, at a constant rate, with a sucrose solution. Under these conditions, cell multiplication proceeded until all nitrogen source was exhausted. In the third step, the bioreactor was supplied with a solution containing sucrose and propionic acid at a feeding rate controlled by its demand to control pH.

Analytical Methods - Total biomass dry weight was determined gravimetrically by filtering a known volume of culture through a 0.45 μm pore membrane and drying at 100 °C. Dissolved Oxygen was measured using a polarographic electrode. Carbohydrates were determined by HPLC (4). Ammonium concentration was measured by a specific electrode (Orion mod. 95-12). Phosphate was determined by a colorimetric method (8). Propionic acid was measured after acidification of the sample with HCl 0.2 N by gas chromatography (GC) using a HP-FAAP column. PHAs amount and composition (3HB and 3HV) were determined by GC of their propil-esters (9).

Yield Calculations - Residual biomass yields from carbohydrates, nitrogen and phosphate ($Y_{Xr/C}$, $Y_{Xr/N}$ and $Y_{Xr/P}$), as well as PHA yields from carbon sources ($Y_{3HV/PR}$ and $Y_{PHA/C+PR}$) are expressed in grams of residual biomass or PHA per gram of substrate.

RESULTS AND DISCUSSION

Results obtained during cultivation of both bacterial strains are shown in Figure 1 and Table 1.

Using On Line Measurements for Steps Transition - The cultivation was performed in three steps. In the first one, all nutrients were available and exponential cellular multiplication occurred until the carbon source was exhausted. At this point, dissolved oxygen (DO) in the culture medium increased as a consequence of the reduction in the respiratory rate. The measurement of DO was very useful to define the transition between steps 1 and 2 for the strain *Burkholderia cepacia* IPT-044. The strain *Burkholderia* sp. IPT-101 hydrolyses sucrose extracellularly and uses the glucose and fructose produced.

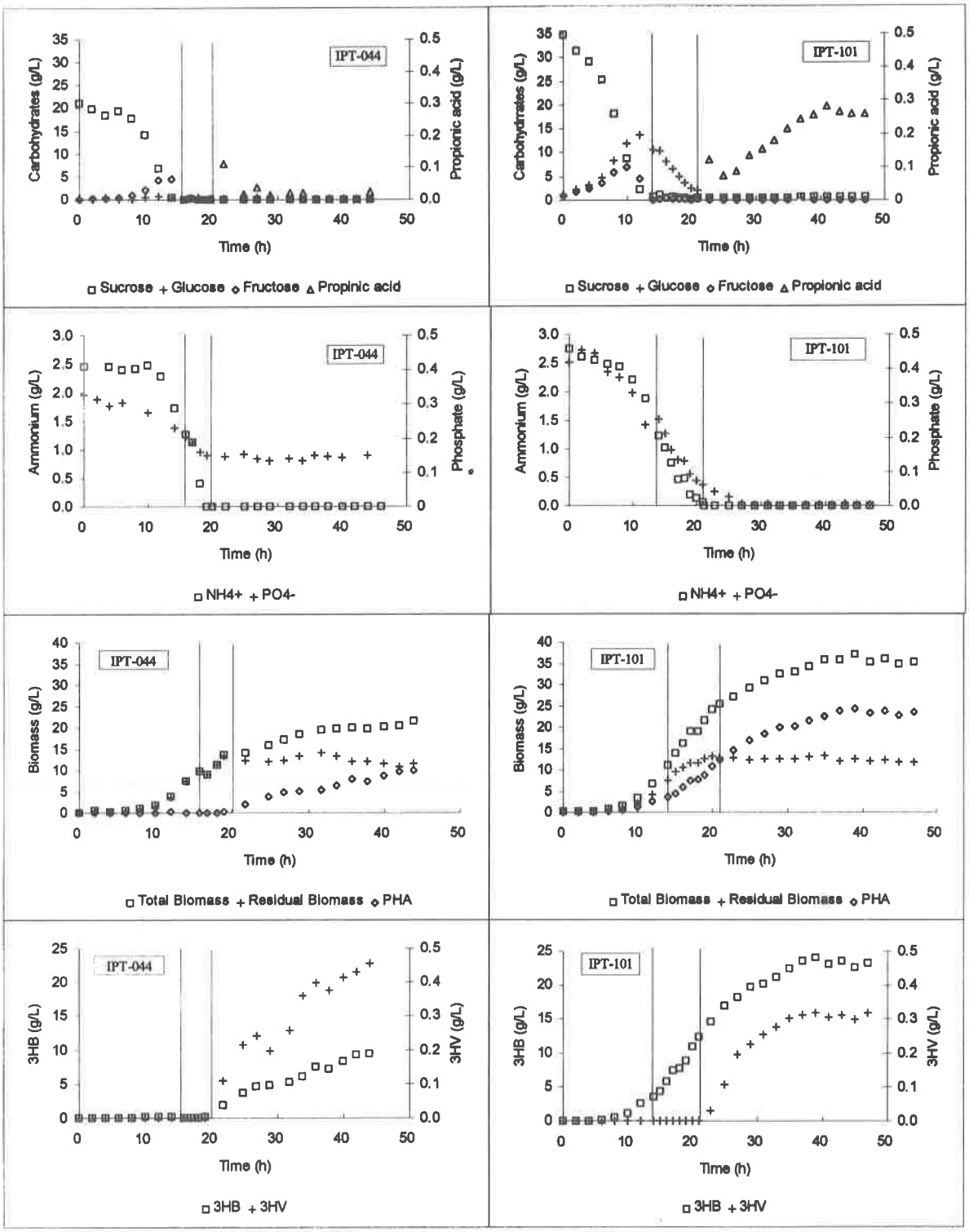

Figure 1. Results obtained during the cultivation of the strains IPT-044 and IPT-101.

IPT-101 consumed fructose faster than glucose and a significant reduction in the respiratory rate was observed after fructose exhaustion, though about 10 g/L of glucose was still available. Therefore, at the first sight, the evolution of DO was less useful to define the

transition between steps 1 and 2 for the strain IPT-101. However the knowledge of this behaviour should permit the use of this strategy in an efficient way. During step 1, some P3HB was produced by both strains, though the nitrogen and phosphate concentrations were still high. This growth-associated production of PHA was more important for strain IPT-101 than for IPT-044.

Table 1: Results obtained in the cultivation of the strains IPT-044 and IPT-101.

Strain	Steps of cellular multiplication				
	Xr (g/L)	μ_{Xrmax} (h^{-1})	$Y_{Xr/C}$ (g/g)	$Y_{Xr/N}$ (g/g)	% PHB
IPT-044	12.5	0.34	0.38	6.87	1.5
IPT-101	13.3	0.33	0.30	6.62	49.0
Strain	Steps of polymer accumulation				
	%PHA	3HV (mol%)	P_{PHA} (g/L.h)	$Y_{3HV/PR}$ (g/g)	$Y_{PHA/C+PR}$ (g/g)
IPT-044	46.2	3.95	0.23	0.045	0.18
IPT-101	65.9	1.12	0.63	0.033	0.37

Xr - residual biomass (total biomass minus PHA biomass).
$Y_{Xr/C}$ - residual biomass yield from carbon source.
$Y_{Xr/N}$ - residual biomass yield from nitrogen source.
P_{PHA} - volumetric productivity of PHA.
$Y_{3HV/PR}$ - 3HV yield from propionic acid.
$Y_{PHA/C+P}$ - PHA yield from carbon sources.

In the second step, a sucrose solution was supplied at a constant feeding rate which was smaller than the one demanded by the culture to grow exponentially. The aim at this step was to impose a linear growth by carbon limitation in order to allow the utilization of any P3HB accumulated in the previous step. A carbon limitation was indeed obtained for the strain IPT-044 (Figure 1), but, as the amount of P3HB accumulated in the previous step was less than 6% of cell dry weight, the polymer consumption was inexpressive (Figure 1). For strain IPT-101, the second step started with a high concentration of glucose, that decreased but did not disappear during this step. Thus, a carbon source limitation was not achieved and more P3HB was accumulated, reaching 50% of cell dry weight. The second step continued up to nitrogen source was exhausted. Following the total consumption of nitrogen, an inversion in pH tendency was observed (in presence of NH_4^+, pH showed a decreasing tendency and when it was exhausted, pH tended to increase). This observation was used as a criterion to define the end of the second step.

In the third step, the bioreactor was fed with a solution containing sucrose and propionic acid allowing the synthesis of the copolymer P3HB-co-3HV. The feeding pump was connected to the pH control of the bioreactor. In this way, as propionic acid was consumed, an increase in the pH occurred and more of the feeding solution was automatically pumped to bring the pH value to its set point. In the case of the strain IPT-044 besides glucose and fructose, gluconic acid was also detected as a transient product of the sucrose assimilation. This could be a problem for the pH-based feeding strategy, since the consumption of gluconic acid could determine an increasing on pH similar to propionic acid consumption, possibly leading to an overfeeding of substrate mixture. However, this was not observed and the feeding rates during step 3 were very similar for strain IPT-044 and IPT-101.

Substrate Conversion Yields and Productivity - Both strains studied were able to use sucrose as the sole carbon source by extracellular hydrolysis of the substrate, but they differ somehow in the subsequent steps of the carbohydrate assimilation. Fructose produced by the hydrolysis of sucrose, was consumed faster than glucose by strain IPT-101. The strain IPT-044 oxidizes the produced glucose to gluconic acid, which can transiently accumulate in the medium. Knowing the different products generated from sucrose and the efficiency of their consumption are essential to design a feeding strategy to control the relation between carbon source and other nutrients supplied, allowing the establishment of conditions for accumulation, non-accumulation

or consumption of the polymer. Moreover, it should permit to control the carbohydrates and propionic acid ratio effectively consumed, which is important to define the composition of the polymer synthesized (3).

Strain IPT-044 showed higher residual biomass yield from carbohydrates than strain IPT-101. This apparent superiority is not true and could be explained by observing that strain IPT-101 used an important part of the carbohydrates for the synthesis of P3HB, that reached 49% of the cell dry weight (Table 1). The performance of strain IPT-101 was similar to that observed in a fermentation process using *Alcaligenes eutrophus* DSM 545, when a lower amount of P3HB was accumulated (10).

The residual biomass yield from nitrogen ($Y_{Xr/N}$) observed for the strains IPT-044 e IPT-101 were 6.87 and 6.62 g/g, respectively (Table 1). These values are slightly lower than 7.02 g/g observed with *Escherichia coli* B/r (6). The determination of $Y_{Xr/N}$ values is very important to define the extent of the multiplication steps and this is fundamental in a process that has to proceed with a more expressive polymer accumulation under conditions of nutrient limitation.

Regarding to phosphate consumption, different behaviors were observed. The residual biomass yield from phosphorus ($Y_{Xr/P}$) observed for the strains IPT-044 and IPT-101 were 33.6 and 72.3 g/g, respectively. The value described for *E. coli* B/r was 37.6 (6). As showed in Figure 1, strain IPT-044 stopped phosphate consumption after NH_4^+ exhaustion. The strain IPT-101 continued to consume phosphate even after no more NH_4^+ was available. Since cell growth in this condition is impossible, the phosphate consumed by strain IPT-101 in this step is probably stored as granules of polyphosphate. The accumulation of polyphosphate is not desired in a process for P3HB-co-3HV production, since the polyphosphate granules could use the intracellular space for PHA granules. However, knowing $Y_{Xr/P}$ values, it is possible to supply amounts of phosphate in a more controlled way and to reduce the amount of polyphosphate generated.

Considering only the accumulation step (third step), the strain IPT-101 showed higher PHA yield from carbon sources than IPT-044 and similar values to those obtained with *A. eutrophus* DSM 545 (10). Since the feeding rate of carbon source was very similar for strain IPT-101 and IPT-044, the last one should excrete some intermediate metabolite or another product. An increasing in the viscosity of the medium was observed that might be attributed to polysaccharide formation. The PHA volumetric productivity showed by strain IPT-101 was considerably higher than the value reached by the strain IPT-044. The specific rate of PHA synthesis (qPHA) observed for IPT-101 was 47.7 mg/g.h. *A. eutrophus* DSM545 showed a value significantly higher, 86.7 mg/g.h (10).

The 3HV yield from propionic acid was very small for both isolated strains, when compared to *A. eutrophus* DSM545 (0.34 g/g) (10). It was verified that these strains operate at less than 10% of the maximum theoretical yield, calculated as 1.35 g/g (4). Presently, it is in development a program for the obtaining of mutants from strain IPT-101 unable to use propionic acid for growth but able to use this substrate for the synthesis of 3HV units. These mutants should make the conversion of propionic acid into 3HV units in a more efficient way.

CONCLUSION

Dissolved oxygen and pH monitoring could be used as important tools in the control of process for P3HB-co-3HV synthesis, specially if the behavior of the strain concerning to the utilization of the substrates is known.

The strain IPT-101 showed, in different aspects, a performance comparable to *A. eutrophus* used for P3HB-co-3HV production. As an advantage IPT-101 hydrolyses sucrose efficiently.

ACKNOWLEDGEMENTS

This work was supported by PADCT-FINEP as a part of the project "Production of biodegradable plastics from sugar cane by biotechnological process".

RESUMO

Produção de P3HB-co-3HV por bactérias isoladas de solo capazes de utilizar sacarose

Duas linhagens bacterianas isoladas de solo de canaviais foram caracterizadas com relação à produção de P3HB-co-3HV em fermentadores de 5 L. A evolução dos valores de O_2 dissolvido foi utilizada como critério para se definir o esgotamento da fonte de carbono entre as etapas 1 e 2 do cultivo, nas quais todos os nutrientes estão disponíveis no meio de cultura, favorecendo a multiplicação celular. A inversão na tendência do pH foi utilizada para definir a transição entre as etapas 2 e 3 do cultivo, quando ocorre a exaustão do nitrogênio, impedindo que a multiplicação celular prossiga e o acúmulo de polímero se torna mais expressivo. As duas linhagens foram comparadas com relação à eficiência na conversão de substratos em células ou produto, bem como com relação à produtividade.

Palavras-chave: *Burkholderia*, P3HB-co-3HV, plásticos biodegradáveis

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POLY-3-HYDROXYBUTYRATE-CO-3-HYDROXYVALERATE ACCUMULATION AT DIFFERENT STRATEGIES OF SUBSTRATE FEEDING

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ABSTRACT

Two different strategies of culture medium feeding (step and pH control addition) were tested in order to produce poly-3-hidroxybutyrate-co-3-hydroxyvalerate from propionic acid, glucose and fructose with *A. eutrophus*. When substrate was fed by pH control, the copolymer content in biomass, its 3-HV content (although decreasing along the time) and also the yields of substrate in products were higher. The best results obtained with the feeding by pH control were attributed to a lower substrate limitation resulting of this strategy.

Key words: biopolymer, poly-3-hydroxybutyrate-co-3-hydroxyvalerate, *Alcaligenes eutrophus*

INTRODUCTION

The industrial application of the copolymer Poly-3-hydroxybutyrate-co-3-hydroxyvalerate (P(HB-co-HV)) is related to its thermoplastics properties and biodegradability that turns it potential substitute of traditional plastics. The hydroxyvalerate units (HV) are essential to confer mechanical properties and processability to the copolymer.

After bacterial growth in a balanced medium, the process of P(HB-co-HV) production is performed from carbohydrates and organic acids as co-substrate at nitrogen and/or phosphorus limited conditions.

A. eutrophus has been studied most extensively due to its ability to rapidly accumulate large amounts of P(HB-co-HV), reaching about 80% (w/w) of cell dry mass (5).

Nowadays, different strategies of feeding substrate have been described for these bacteria with glucose and propionic acid (6, 7, 11) or glucose and valeric acid (8).

Though sucrose is a cheap and abundant carbon source in our country, *A eutrophus* naturally does not have the capacity to metabolize this carbohydrate (14). However, glucose and fructose, obtained from sucrose hydrolysis, have been successfully used to cultivate this strain (9).

In the present work, the results of two different ways of feeding a mixture of hydrolyzed sucrose (glucose and fructose) and propionic acid as accumulation medium (addition by steps and addition by pH control) are presented.

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MATERIALS AND METHODS

Strain - *Alcaligenes eutrophus* DSM 545, maintained by periodical transfer to Nutrient Broth Agar medium (Difco).

Media - The medium was composed of a mixture of hexose (glucose and fructose) and propionic acid, in a relation of 2.25 g hexose/g propionic acid.

Experimental Procedure - The inocula were obtained from a cellular growth on an initial mineral medium batch followed by a fed-batch limited on the carbon source as described by Piccoli (9). After exhausted the nitrogen source, polymer accumulation was carried out in two different ways. In the first, the medium (glucose, fructose and propionic acid) was supplied at a rate of 0.23 g substrate/g dry cell.h and readjusted during the experiment by steps in order to avoid substrate accumulation. In the second, the pH control was utilized. This strategy of feeding is possible due to the medium alcalinization tendency during the copolymer accumulation and the acid characteristic of the feeding medium.

Determinations - Periodic sampling were made along the assays. The cell dry weight was determined by membrane filtration (0.45 μm , Millipore) and dried at 105°C to constant weight. The residual biomass concentration (X) was obtained subtracting the PHB quantity (4). The polymer was estimated by gas chromatographic method (1). A modification was done in the method, changing the methanolysis by propanolysis of the samples as described by Riss and Mai (12). Glucose and fructose (hexoses) concentrations were determined by HPLC using a Schodex SC1011 column. Propionic, acetic and piruvic acids were determined by a gas chromatographic method as described by Gomez *et al* (3), using a megabor FFAP column.

RESULTS AND DISCUSSION

Figure 1 illustrates the two strategies of feeding. On the first, the feeding rate of 0.23 g substrate/g dry cell.h was adjusted in steps to avoid substrate accumulation in the medium (assay A). On the assay B, the medium with the same composition was fed by the pH control resulting in a initial rate of about 0.25 g substrate/g dry cell.h. Considering that hexoses and propionic acid accumulation in the culture medium was not significant (unpublished dates), it is possible that assay B operation was in a less substrate limitation condition than assay A.

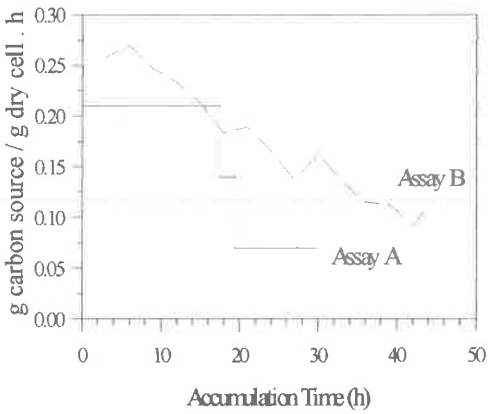

Figure 1: Specific feeding rate of assay A (addition by steps) and assay B (by pH).

The copolymer content is presented in Figure 2. When substrate feeding was by pH control and copolymer content reached about 50 - 55% of cellular biomass an increasing of about 30% in the HV copolymer content was shown, resulting in best yield of HV from propionic acid. It can also be seen that in assay B the copolymer content was always higher than in A. About 30 h were needed to accumulate 60% of polymer on assay A whereas this time was only 17 hours in assay B.

Specific polymer synthesis rate and specific substrate consume rate (Figure 3) were calculated by polynomial adjustment to the experimental curve with the aid of a microcomputer software (13).

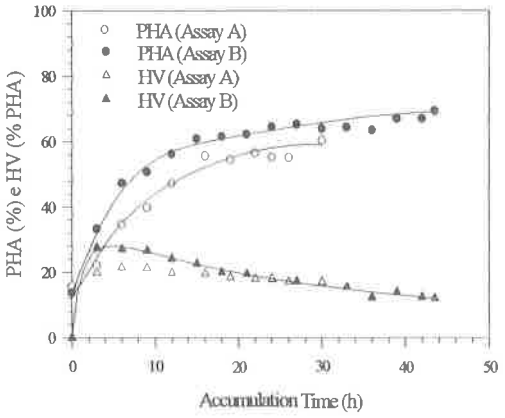

Figure 2: Polymer content on assay A (addition by steps) and assay B (by pH).

Alcaligenes eutrophus DSM 545 when cultivated on the tested conditions presented a decreasing HV synthesis rate (μ_{HV}). HB synthesis rate (μ_{HB}) increased until about 40% of polymer content and after the polymer content reached 60% both HB and HV synthesis rates tended to zero or negligible values. The hexoses and propionic acid uptake had a similar tendency. On the other hand, values of μ_{HB} and μ_{HV} and substrate consumption rates were always higher on assay B compared to assay A.

Figure 3 shows that the yields of hydroxyvalerate from propionic acid ($Y_{HV/prop}$) and hydroxybutyrate from hexoses ($Y_{HB/hex}$) were higher for assay B, tough they had the same decreasing tendency with polymer content in cellular biomass. The $Y_{HV/prop}$ values from assay B were significant higher than those reported by Ramsay *et al* (10) of 0.1 g/g in a fed batch process with constant feeding, and similar to Kim *et al* (6) that utilized propionic acid as the sole carbon source during the accumulation.

The polymer accumulation by *Alcaligenes eutrophus* DSM 545 occurs with the consumption of a carbohydrate and an organic substrate correlated to the HV structure like propionic acid. The biosynthetic pathway of accumulation indicates that the majority amount of propionic acid supplied was directed to the descarboxilation to acetil-CoA, resulting in HB units. The ratio of carbohydrate and organic acid uptake influences the HB and HV content of the synthesized copolymer (2). Therefore, it is desirable to set up a production process in which the substrates uptake rates could be controlled. Besides, the conversion yields, especially the organic acid in HV moiety, is fundamental in the process due to its impact on the copolymer cost of production.

The addition of substrate by pH control seems to be an adequate technique to make the substrate feeding. It is simpler than the regulation by substrate analysis off-line, and the substrate limitation for the microbial population seems to be lower, giving a contribution to a better performance on copolymer synthesis

However, the feeding control by pH was not efficient enough to avoid the significant decreasing on substrate consumption and copolymer synthesis rate, when PHA content in biomass reached 55 - 60 %. More detailed studies on carbon sources feeding policy have been carried out to check if this is a characteristic of the microorganism or it can be avoided by a more sophisticated substrate feeding control.

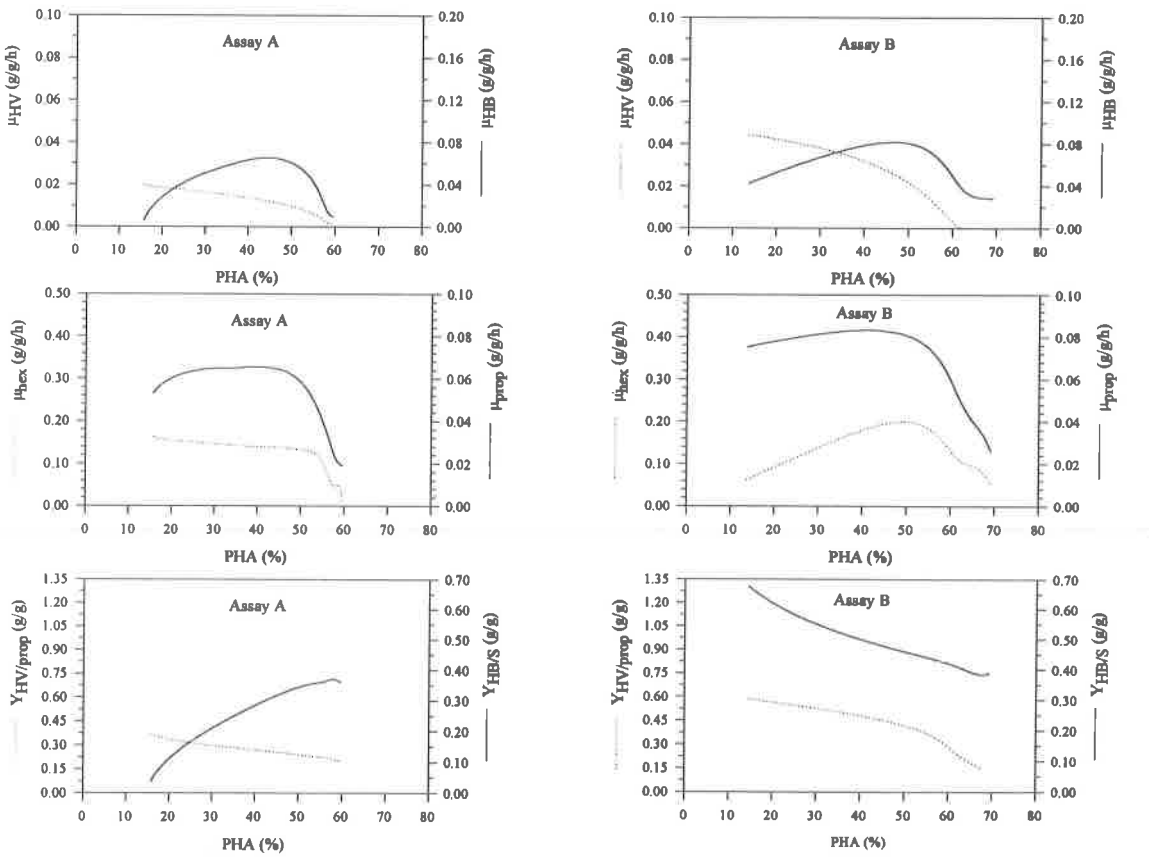

Figure 3: Specific synthesis rates of polyhydroxybutyrate (μ_{HB}) and hydroxyvalerate (μ_{HV}), specific consumption rates of hexoses (glucose + fructose) and propionic acid and yields of HV from propionic acid and HB from hexoses as a function of polymer content (PHA) of assays A (addition by steps) and B (by pH).

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RESUMO

Acúmulo de poli-3-hidroxibutirato-co-3-hidroxivalerato em diferentes formas de oferta de substrato

Duas estratégias de oferta de meio de cultura (adição em degraus e adição via controle de pH) foram testadas utilizando-se *Alcaligenes eutrophus* para síntese de poli-3-hidroxibutirato-co-3-hidroxivalerato a partir de ácido propiônico, glicose e frutose. Quando a oferta de substrato foi realizada via controle de pH, o teor do copolímero na biomassa foi sempre mais elevado, o teor de HV no copolímero, embora decrescente no tempo, foi significativamente maior no início do ensaio e os fatores de conversão dos substratos a produto também foram superiores. Os melhores resultados encontrados com a adição de meio de cultura via controle de pH foram atribuídos a uma menor limitação de substratos conseguida com essa metodologia.

Palavras-chave: poli-3-hidroxibutirato-co-3-hidroxivalerato, biopolímeros, *Alcaligenes eutrophus*

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STABILITY OF THE STRAIN S93 OF *BACILLUS THURINGIENSIS* SUBSP. *KURSTAKI*, STORED AT -18^{0}C

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ABSTRACT

Laboratory experiments showed that a Brazilian isolate of *Bacillus thuringiensis* subsp. *kurstaki* (S93), has a high larvicidal potential against *Spodoptera frugiperda*, a very important corn pest. Aiming the production of a bioinsecticide based on strain S93, the stability of the microorganism during conservation in inclined test tubes containing nutrient agar at -18^{0}C was studied. The bacterial stability was evaluated by storing the inclined test tubes up to 90 days in freezer and taking off a test tube each 10 (+/- 2) days, when the bacterium was cultivated and submitted to growth and toxicity analysis against *S. frugiperda*. The cultures were grown in a rotary incubator for 12 and 36 h at 30^{0}C/200 rpm. Determinations of pH, dry mass, cells and spores concentrations were performed. The culture of 36h was also used in bioassays against *S. frugiperda*, to verify the toxicity of *B. thuringiensis* along the period. The results showed that the growth and virulence of strain S93 were not affected by the freezing time. The freezing method, for this strain, showed to be efficient for the period of 90 days.

Key words: Biological control, bioinsecticide, stability, freezing, *Spodoptera frugiperda*

INTRODUCTION

The use of microorganisms in biotechnology and the need to maintain strains of interest have created an increased request for preservation and conservation of valuable genetic materials (2). Microorganisms are usually preserved because some strain characters are scientifically and industrially significant (3). Report from World Data Center of Microorganisms, 1986, shows that approximately 500.000 cultures would be stored in 52 countries (2). The conservation of cultures has, generally, the following objectives: (a) preservation of the culture as close to the original as possible; (b) preservation of genetic integrity of the culture without any loss of biochemical properties and (c) preservation of the culture in a way that it is possible to easily transport and handle (1). The methods used should minimize the occurrence of events as loss of viability, contaminations, mutations or loss of plasmids (3). There are many conservation methods used but we can distinguish those of short and medium time (drying, freezing, refrigeration, conservation in soil, silica-gel, etc.) and those of long time

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(freeze-drying, cryopreservation, etc.). often made by using the method of strips or pieces of paper, full with bacterial spores and stored in glass tubes, sealed by fire. Conservation of *Bacillus sp.* for short periods (up to a year) is done by keeping the culture well grown (with many spores) in slants, inside a domestic freezer.

A strain of *B. thuringiensis* subsp. *kurstaki*, S93, was isolated at CENARGEN. This strain shows high activity against larvae of the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae) (4, 5) and it is under development as a bioinsecticide. So, it was necessary to make the evaluation of the freezing conservation method, in terms of maintenance of viability, growth characteristics and toxicity along 90 days of storage. These informations are very important for process development, product storage and industrial production, where the microbial stability is fundamental for uniformity of the process and homogeneity of the product.

MATERIALS AND METHODS

The microorganism *B. thuringiensis* subsp. *kurstaki*, strain S93, was stored in inclined test tubes (slants) containing nutrient agar and kept at -18^{0}C in domestic freezer. Each 10 (+/-2) days, one test tube was retrieved from the freezer, cultivated and submitted to analysis to evaluate stability.

Two ml of saline solution were added to the slant retrieved from the freezer. It was homogenized in vortex for a few seconds and 0,5 ml of its content was added to an erlenmeyer containing 50 ml of nutrient broth. The procedure was made in duplicate. The flasks containing the microorganism were put into a rotary incubator at 30°C and 200 rpm. One flask was taken off the rotary incubator, 12h later, submitted to pH analysis, dry mass and cell concentration (CFU). A second sample was taken 36h of cultivation time and submitted to pH analysis, dry mass, cell and spores concentrations determination, as well as bioassays against *S. frugiperda*. To count Conservation of *Bacillus sp.*, for a long time, is spores, the sample was submitted to heat shock (80°C/12 minutes) before plating. For bioassay, the sample was diluted from 10^{-1} to 10^{-5} times. Small blocks of an artificial diet were put into 50 ml plastic cups and 50 µl of the sample dilutions were applied over each block. After the surface of the diet has dried, a 2nd/3nd stage larvae of *S. frugiperda* was put into each cup, which was closed with an acrylic cover. Replicates were made for each dilution and also one control (without treatment) was made, with the same number of larvae. The insects were incubated at 26 °C and 70-80% RH. The artificial diet was replaced by a no-treated diet 48h later. The mortality evaluation was made every two days, during 8 days.

RESULTS AND DISCUSSION

Aiming to evaluate the viability of the strain S93, kept at -18°C for 90 days, analysis of pH, dry mass and cell concentration, were done in samples cultivated for 12h. For 36h of cultivation, the analysis included, besides those mentioned, determination of spore concentration. Tables 1 and 2 show the results.

Table 1 shows that for 12 h of cultivation, pH and cell concentration measured by dry mass, although with variations, was not significantly affected by storage time. On the other hand, cell concentration (Table 1) increased about ten times from the thirtieth first day at -18°C, regarding to initial storage time. For 36 h of cultivation (Table 2), the results obtained for pH, dry mass, cell and spores concentration were nearly constant along the storage time. It can be also observed (Table 2) that the cell concentration obtained for 36 h of cultivation in a shaker is about ten times higher than spore concentration, obtained for the same time of cultivation which shows a sporulation percentage near 10% along all observed time. The results obtained with isolate S93, stored at -18°C for 90 days, show that the conservation method used does not affect significantly the

viability of the culture, nor the growth sporulation characteristics.

Table 3 shows the results of bioassays using serial decimal dilution of *B. thuringiensis* (S93) samples removed from the freezer and cultivated for 36 hours. The data of Table 3 refer to results of 8 days cumulative mortality of *S. frugiperda* larvae.

Table 1. Results obtained with *Bacillus thuringiensis* S93 stored at -18°C. Samples with 12h of cultivation.

Storage time (days)	pH	Dry mass (g/L)	Cell concentration (10^8 cell/mL)
11	7,74	2.63	0.25
19	7,92	2.92	0.28
31	7,54	2.77	1.84
40	7,65	3.35	1.77
52	7,64	3.06	1.72
61	7,75	3.47	2.11
73	7,69	3.64	2.10
82	7,71	2.83	2.31
90	7,65	2.90	2.04

Table 2. Results obtained with *Bacillus thuringiensis* S93 stored at -18°C. Samples with 36h of cultivation.

Storage time (days)	pH	Dry mass (g/L)	Cell concentration (10^8 cell/mL)	Spores concentration (10^7 spores/mL)
11	8,68	1.73	2.08	2.50
19	8,55	1.56	2.02	2.68
31	8,52	1.89	1.74	2.75
40	8,43	2.09	1.13	2.99
52	8,40	1.96	1.97	2.93
61	8,34	1.73	1.28	2.80
73	8,38	2.15	1.49	2.99
82	8,43	1.66	2.13	2.95
90	8,43	1.67	2.00	2.97

Table 3. Total number of dead larvae, obtained in bioassays for serial dilutions of *B. thuringiensis* S93 against *S. frugiperda*. Samples were removed from the freezer each 10 (+/- 2) days and cultivated for 36h in shaker at 30°C and 200 rpm.

Storage days	11	19	31	40	52	61	73	82	90
Dilution									
10^{-1}	8	7	9	10	10	9	10	10	10
10^{-2}	8	7	8	9	9	9	9	10	9
10^{-3}	6	4	6	6	5	5	5	6	6
10^{-4}	2	0	5	4	3	3	3	4	5
10^{-5}	1	0	4	3	3	2	3	4	5
control	1	0	0	0	1	1	1	1	0

The samples that caused high mortality, (70-100%) against larvae were 10^{-1} and 10^{-2} dilutions. Mortality varying from 40% to 60% was observed for the dilution 10^{-3}, for almost all storage time of the slants. It can still be observed that the lower mortality in larvae of *S. frugiperda* was obtained at 19days of storage in all dilutions.

Based on these results, we can conclude that the growth of *B. thuringiensis* (S93) was not affected by the storage period of 30 to 90 days. The toxic activity, when kept for 90 days at -18°C was also retained. Therefore, the conservation of inclined test tubes of strain S93 of *B. thuringiensis* in domestic freezer is very efficient.

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RESUMO

Avaliação da estabilidade do isolado de *Bacillus thuringiensis* subsp. *kurstaki* S93, armazenado a -18°C

Testes realizados em laboratório mostraram que um isolado brasileiro de *B. thuringiensis* subsp. *kurstaki*, denominado S93, apresenta elevada potência larvicida contra a lagarta do cartucho do milho (*Spodoptera frugiperda*). Visando a produção de um bioinseticida à base de S93, estudou-se a estabilidade do microrganismo, conservado em tubos inclinados contendo meio ágar nutritivo, à temperatura de -18°C. A avaliação da estabilidade bacteriana foi feita conservando-se os tubos por até 90 dias no congelador e retirando-se um tubo, de 10 em 10 (+/- 2 dias), que era cultivado e submetido a análises de crescimento e toxicidade contra o inseto mencionado. Os cultivos foram feitos em incubador rotativo por 12 e 36h a 30°C / 200 rpm e submetidos a análises de pH, massa seca, concentração de células e de esporos. O cultivo de 36h também foi utilizado em bioensaios contra *S. frugiperda*, com o objetivo de verificar a toxicidade do *B. thuringiensis* ao longo do período de congelamento. Os resultados obtidos demonstraram que o crescimento do isolado S93 não foi afetado pelo tempo de congelamento, bem como a sua atividade larvicida, que permaneceu constante. O método de congelamento para este isolado mostrou-se eficaz no periodo considerado.

Palavras-chave: controle biológico, bioinseticida, estabilidade, congelamento, *Spodoptera frugiperda*

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INFLUENCE OF TEMPERATURE ON THE GROWTH AND PRODUCTION OF TOXINS BY THE STRAIN S93 OF *BACILLUS THURINGIENSIS* SUBSP. *KURSTAKI*

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ABSTRACT

The effects of temperature in the production of biomass and toxins by an isolate (S93) of *Bacillus thuringiensis* subsp. *kurstaki* with a high larvicidal activity against *Spodoptera frugiperda*(fall armyworm) were studied. The microorganism was grown in a nutrient broth medium, in a shaker at 200 rpm during 48h, at 25, 27, 29, 31, 33, 35 and 37°C. Samples were taken in intervals of 2 h up to 12h and then at intervals of 12h up to 48h. Dry mass, pH, protein, cell and spore concentration were determined. Bioassays against *S. frugiperda* were also carried out with the 36 and 48h samples. The results indicated that the optimal temperature for strain S93 growth was between 29 and 31°C. The highest specific growth rate was obtained at 29°C ($0.41h^{-1}$). The highest mortality of *S. frugiperda* larvae was obtained at 25 and 27°C using 48h broth while in the other temperatures tested, the 36h broth was more efficient against *S. frugiperda*.

Key words: Biological control, bioinsecticide development, *Spodoptera frugiperda*

INTRODUCTION

Among the biological control agents utilized throughout the world, the entomopathogenic bacilli proved to be of special importance (2). *Bacillus thuringiensis* is currently the most used pathogen in the microbial control of insect pests and vectors of tropical diseases (4). Commercial products based on *B. thuringiensis* have nearly 90% of the bioinsecticide market, being the most diffused and used on the world (1). Much progress has been made in the production of bacterial biopesticides, specially regarding the development of fermentation processes (culture media and cultivation conditions) and formulations with higher stability and persistency (2).

The fall armyworm (*Spodoptera frugiperda*) is the principal corn pest under field conditions in Brazil (8) and it is little susceptible to commercial strains of *B. thuringiensis* subsp. *kurstaki* (5). As a result of a screening of 218 bacillary isolates, one, called S93, showed a high larvicidal activity against *S. frugiperda* (6,7).

The objective of this study was to develop a fermentative process to produce a bacterial bioinsecticide based on the strain S93 of *B. thuringiensis* subsp. *kurstaki*. Initial studies were performed to test the influence of temperature in the production of biomass and protoxins, the active ingredients of the bioinsecticide.

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MATERIALS AND METHODS

The strain S93 of *B. thuringiensis* subsp. *kurstaki* was utilized and grown in nutrient agar in inclined test tubes and kept at -18 °C. Starting with the frozen tube the pre-inoculum was cultured for 12h in rotatory shaker at 30°C and 200 rpm. Next, 1% of the volume was transferred to 300 mL erlenmeyers containing 50 mL of nutrient broth. Flasks were then incubated at 200 rpm and different temperatures, i.e., 25, 27, 29, 31, 33, 35 and 37°C.

The influence of temperature was evaluated by analysis of variation in pH, dry mass and protein concentration (biuret). At 12, 24, 36 and 48 h of culture, the concentration of cells and spores (CFU/mL) was also determined. To count spores, the sample was submitted to heat shock (80°C/12 minutes) before plating. Bioassays against *S. frugiperda* were realized utilizing the 36 and 48h broths The sample was diluted from 10^{-1} to 10^{-5}. Small blocks of an artificial diet were put into 50 mL plastic cups and 50 µL of the sample dilutions were applied over each block. After drying the surface of the diet, a 2^{nd} /3^{rd} stage larva of *S. frugiperda* was put into each cup, which was closed with an acrylic cover. There were ten replicates for each dilution and one for the control (without treatment), with the same number of larvae. The insects were incubated at 26 °C and 70-80% RH. The artificial diet was replaced by a non-treated diet 48h later. The mortality evaluation was made each two days, during 8 days. Specific growth rate (µ) of strain S93 was also calculated for each temperature tested, during exponential growth phase.

RESULTS AND DISCUSSION

Cellular concentration is affected by temperature. At 12h cultivation, cellular concentration tended to increase when the temperature of the culture was raised from 25 to 33°C (table 1). An inverse trend occurred when temperature increased from 33 to 37°C. Due to bacterial sporulation which begins at the end of the exponential growth phase, the cellular concentration continuously decreased after 12h of culture for all temperatures, except for 25°C. At this temperature, it appeared to have occurred a slow increase in cellular concentration (or a phase of constant cellular concentration) between 12 and 24h. It was also observed that the greatest biomass concentration for all culture times, were obtained at 29, 31 and 33°C(table 1).

A remarkable increase in values of µ between 25 and 29°C was also observed. Specific growth rate (µ) reached a maximum of 0.409 h^{-1} and begun to decrease moderately at temperatures above 29°C, reaching values close to 0.31 h^{-1} at 37°C.

Specific growth rate values found for growth of *B. thuringiensis* S93 are much lower than those found by the other authors reviewed by Ertola (3): from 0.47 to 1.90 h^{-1} in cultures of *B. thuringiensis* in broths rich in sugars and yeast extract. The differences between the values found in this study and those previously published show that various possibilities still exist for optimization and improvement of the fermentation process for the production of bacterial bioinsecticides.

Table 2 presents the results of the total mortality after 8 days, in the bioassays performed using a serial decimal dilution of samples of *B.thuringiensis* S93 cultured for 36 and 48h, at the different temperatures tested. Toxicity of strain S93 was influenced by temperature and time of cultivation. It was verified that the highest rates of mortality of *S. frugiperda* were recorded from 29 to 31°C, for 36h of culture. However, there were dilutions at other temperatures which also caused a high mortality rate.

Table 1. Determination of cellular concentration (X in g/L) and specific growth velocity (μ) of *Bacillus thuringiensis* S93 cultured for 12, 24, 36 and 48h at various temperatures.

Cultivation temperature (°C)	12 hours X	24 hours X	36 hours X	48 hours X	μ (h^{-1})
25	1.33	1.37	1.21	0.92	0.185
27	1.92	1.56	0.95	0.80	0.345
29	3.12	2 .40	1.90	1.39	0.409
31	3.29	2.04	1.56	1.63	0.357
33	2.97	2.35	1.65	1.41	0.359
35	2.39	1.59	1.16	1.14	0.290
37	2.14	-	1.21	0.62	0.307

For 48h culture, the highest mortality rates were found at temperatures of 25 and 31°C, in all dilutions and mortality rates higher than 60% were also found at temperatures of 27, 29 and 35°C. When comparing 36 and 48h cultures, we can notice that for 25 and 27°C the highest mortality rate of larvae occurred for 48h broth, while for other temperatures tested, the 36h broth was more efficient against *S frugiperda*.

Table 2. Total number of dead larvae obtained in bioassays using serial decimal dilutions of *B. thuringiensis* S93 against *Spodoptera frugiperda*, at different temperatures. Cultures of 36 and 48h.

Dilution	Time (h)	Temperature (°C)						
		25	27	29	31	33	35	37
10^{-1}	36	7	9	10	10	7	9	10
	48	10	10	10	10	6	9	5
10^{-2}	36	6	9	9	10	7	9	8
	48	9	9	8	10	7	9	8
10^{-3}	36	8	5	7	8	6	8	4
	48	8	5	7	8	6	8	6
10^{-4}	36	2	6	6	7	3	6	2
	48	9	5	2	6	2	4	2
10^{-5}	36	2	1	5	5	2	7	1
	48	1	3	0	5	2	3	1
Control	36	0	1	1	1	1	1	0
	48	0	0	1	0	1	0	0

CONCLUSIONS

The optimal growth temperature, in the conditions of culture established for isolate S93, ranges from 29 to 31°C, with the highest specific growth rate at 29°C (0.41 h^{-1}). At this interval the highest toxicity levels of *B. thuringiensis* S93 against larvae of the 2nd/3rd stages of the fall armyworm were observed.

At temperatures of 25 and 27°C the highest mortality of larvae of *S. frugiperda* occurred when bioassays were carried out with the 48h culture. For other temperatures tested (29-37°C), the 36h culture was the most efficient.

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RESUMO

Influência da temperatura de cultivo no crescimento e produção de toxinas pelo isolado S93 de *Bacillus thuringiensis* subsp. *kurstaki*

Em um "screening" envolvendo 218 isolados bacilares, foi obtido um *Bacillus thuringiensis* subsp. *kurstaki* que apresentou elevada potência larvicida contra *Spodoptera frugiperda*, principal praga da cultura do milho no Brasil. Após diversas avaliações de sua toxicidade em comparação com estirpes comerciais, decidiu-se desenvolver um bioinseticida, utilizando-se este novo isolado, denominado S93, como princípio ativo (pró-toxinas). O microrganismo foi cultivado em meio caldo nutritivo, em incubador rotativo a 200 rpm por até 48h, nas temperaturas de 25, 27, 29, 31, 33, 35 e 37°C. As amostras foram retiradas em tempos preestabelecidos e submetidas a análises de pH, massa seca, absorbância, concentração de células, esporos e proteína. Bioensaios por diluições seriadas contra *S. frugiperda* (2°/3° estágio) também foram realizados com as amostras de 36 e 48h. Os resultados obtidos, para as condições de cultivo, mostram que a temperatura ótima de crescimento, para o isolado S93, situa-se entre 29 e 31°C, tendo-se obtido a 29°C a mais elevada velocidade específica de crescimento (0,41 h^{-1}). Para os bioensaios realizados em laboratório contra *S. frugiperda*, verificou-se que a 25 e 27°C a maior mortalidade de larvas deu-se com o cultivo de 48h, enquanto que para as demais temperaturas ensaiadas o cultivo de 36h foi mais eficaz contra *S. frugiperda*.

Palavras-chave: controle biológico, desenvolvimento de bioinseticida, *Spodoptera frugiperda*

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ETHANOL PRODUCTION FROM PINEAPPLE RESIDUES BY GENETICALLY MODIFIED *KLEBSIELLA OXYTOCA*

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ABSTRACT

Brazil is among the first five world pineapple producers. The pineapple residue from cannery industry is composed by skin, crown and bagasse with lignocellulose as the main component. Moreover, a pulp fraction containing sucrose is retained when the skin is extracted. Acid hydrolysis of hemicellulose from pineapple residue generates pentoses and hexoses. Genetically modified *Klebsiella oxytoca,* used in this work, is able to efficiently convert sugars like cellobiose, pentoses and hexoses to ethanol. The utilization of this cheap and pollutant residue to produce ethanol is the goal of this study. The results point to good possibilities of ethanol production from pineapple residues by *K. oxytoca*. A genetic microorganism improvement or an adequate fermentation media treatment are possible ways to overcome the observed partial inhibition on sugar consumption and, consequently, increase ethanol production.

Key words: *Klebsiella oxytoca*, ethanol, pineapple

INTRODUCTION

The current interest in locating new or alternative sources of energy added to the cumulative effects on environment pollution by industrial and agricultural waste has focused attention on solar energy capture by crops that can be subsequently utilized as a substitute for fossil fuels (1, 2).

The conversion of agricultural waste to ethanol is an attractive option for the utilization of ligno-cellulosic biomass. Besides being abundant (4) and cheap (3), the ligno-cellulosic biomass is the main component of most industrial and agricultural residues. Thus, the production of ethanol from this biomass is also a manner for recycling this pollutant (5).

Brazil is among the first five pineapple producers being responsible for about 15% of the world production. According to data published by the Food and Agricultural to Organization of the United Nations, up to 1992, brazilian production was the biggest in South America with 800,000 metric tons. The pineapple residue, composed by skin, crown and bagasse, is a serious pollutant since it presents a high chemical and biochemical oxygen demand (6).

The idea of ethanol production from a profitable pineapple cannery residue is based on its chemical composition (% dry weight): 9.4% cellulose, 22.4% hemicellulose, 4.7% lignin and 4.4% protein (7). Cellulose is a glucose homopolymer, hemicellulose is a complex heteropolymer containing mainly xylose and significant quantities of arabinose, mannose, glucose and galactose (8). No natural microorganism has been isolated with

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fermentative characteristcs as to efficiently convert pentoses to ethanol (9). Improvements with genetic technics made it possible to isolate new strains of bacteria capable of synthesizing the enzymes responsible for the conversion of these sugars to ethanol. Thus, the insertion of *Zymomonas mobilis* genes that code for the enzymes alcohol desidrogenase (**adhB**) and piruvate decarboxilase (**pdc**) into another chromossomal gene of *Klebsiella oxytoca* resulted in a recombinant strain that has its metabolism mainly directed to ethanol production from piruvate (5,10). That gram negative bacteria have the natural capability of transfering and metabolizing cellubiose (a sub product of cellulose), lowering the need for an extra cellubiase addition (5). Moreover, these bacteria have the transfered genes chromossomaly inserted which assures more stability to the strain. Microorganisms carrying these metabolic patterns are very interesting because they produce ethanol from substrates that are not profitable for yeasts, the greatest ethanol producers from sugar-cane bagasse.

Potencially, cellulose and hemicellulose can be converted to ethanol (11). Nevertheless, these polymers can only be fermented by bacteria in the soluble form; for that, they must be hydrolysed. There are basically two ways for that hyidrolysis: enzimatically and chemically (hot acid hydrolysis). The first alternative is particularly attractive for its specificity (5) and for not producing toxic derivatives, in spite of its high cost. Acid hydrolysis is cheaper but presents some disadvantages as the production of toxic derivatives that inhibit microbial fermentation and being not efficient for cellulose breakdown (12). Cellulose hydrolysis can only be achieved with enzymatic treatment which is much more expensive. The reasons mentioned above explain why hemicellulose is the biomass fraction of our interest susceptible to breakdow by hot acid- treatment.

This paper presents an evaluation of the potential of pineapple waste as a substrate for ethanol fermentation by *Klebsiella oxytoca*.

MATERIALS AND METHODS

Microorganism - *Klebsiella oxytoca* was kindly provided by L.O.Ingram.

Culture medium - tryptone(10g/L), yeast extract(5g/L), NaCl(5g/L) and sugar (concentrations mentioned in Tables 1, 2 and 3). Chloranfenicol was added to all media (40mg/L). Agar (1,5%) was added to solid media. Yeast autolysate: 13g of dry weight baker's yeast were suspended with 100mL aqueous solution containing NH_4Cl_2 (2%) and chloroform (1mL). After 1 hour incubation with shaking (200rpm at 30°C) the mixture was centrifuged for 10 minutes at 7.000rpm. The supernatant was pasteurized for two hours at 60°C and added to the fermentation media following the proportions mentioned in Table 2. Industrial yeasts: crushed dry yeast from industrial ethanol fermentation.

Hydrolysis - Dry pineapple residue: pineapple bagasse (skin with the pulp linked to it) was minced with a mixer and squashed to remove the liquid fraction. Squashed bagasse (pineapple residue) was maintained at 60°C until constant weight. For each 30g of dry bagasse 80mL sulfuric acid (1%) were added and maintained in wet acid conditions for 24 hours before hydrolysis. The acid residue was submited to 125°C for 40 minutes (hot acid hydrolysis) and then squashed again. The liquid fraction obtained was the hydrolysate medium to be fermented.

Neutralization with simultaneous pasteurization - pH was elevated until 10 with powder calcium hidroxide, at 80°C for approximately 3 hours and lowered to pH 6,0-7,0 with sulfuric acid (1N). During incubation time at 80°C the hydrolysate was simultaneously pasteurized.

Inocula - *K. oxytoca* was maintained in stock suspensions containing glycerol (40%) at −10°C. An aliquot of this stock suspension was spread on LB solid glucose (2%) media to obtain isolated colonies. Plates were incubated overnigt at 30°C. Three isolated colonies were inoculated for each 10mL LB media (2%

glucose). After incubation for 24 hours with shaking at 30°C cell suspension was centrifuged at 12,100 g for 10 minutes and the cellular sediment was suspended again in distilled sterile water (20% of the inicial inocula volume). Inocula was uniformly distributed to fermentation flasks. For hydrolysate inocula, the same process was adopted with colonies being subcultured in medium containing pineapple hydrolysate supplemented with LB.

Fermentation - fermentation was performed in volumetric 100mL flasks containing 40 mL of fermentation medium. Flasks were closed with rubbers with an inserted inox needle to permit the exit of gas fermentation. Fermentation media including pineapple hydrolysate were supplemented with LB components and/or phosphate buffer (0.2M–pH6.5). Different sugars were added to LB fermentation media, according to concentrations specified in Tables 1, 2 and 3. Ethanol fermentation was carried out at 30°C in a rotatory New Brunswick shaker (200rpm). Immediately after inoculation, 3 mL samples were taken from fermentation medium (time 0), then after 12 hours (time 1) and there after each 24 hours until the end of ethanol production. Samples were collected with a 5mL syringe to prevent loss of ethanol, centrifuged (12,100 g) for 10 minutes and the supernatant kept in an iced penicillin bottle closed with rubber stopper and plastic film. Flasks with samples were incubated at -10°C for subsequent analysis.

Analytical procedures - ethanol was measured with distillation and subsequent titration or by gas-chromatography using a chromosorb 101 (8/100) column (150°C - 50mL/min flux). Total reducing sugars were measured according to the Somogyi method (14). Acetic acid and different sugars from pineapple hydrolysate were measured with a HPLC equiped with a BioRad Aminex column (fermentation monitoring - refractive index detector-flow rate of 0,5 $mL.min^{-1}$ at 65°C. Total bacteria count was estimated by the Alternative Most-Probable-Number method (15). Ethanol production was evaluated by the following parameters: maximum ethanol produced ($g . L^{-1}$) and volumetric productivity ($g.ethanol.L^{-1}h^{-1}$).

RESULTS AND DISCUSSION

Utilization of *K. oxytoca* as a microorganism able to produce ethanol using pineapple residues from cannery industry is related to two important features that differenciate this organism from others genetically modified with the same goal (9,16): high efficiency on the utilization of sucrose and great ability to convert to ethanol, besides xylose, high concentrations of xylobiose and xylotriose. The later feature is related to efficient synthesis of intracellular xylanases (5,17). In 1990, Ban-Koffi *et al.* (7) suggested the utilization of *Z. mobilis* and *Saccharomyces cerevisiae* to produce ethanol from pineapple residue from cannery industry. Sugar analises of pineapple residues indicate sucrose as 5.2 % of 11.7 % total sugars for the whole fruit. Results presented by those authors clearly show the efficiency of the two microorganisms in converting pineapple sucrose and other hexoses to ethanol. Moreover, these organisms are not able to ferment pentoses, the main sugars obtained after hydrolysis of hemicellulose which comprises 20% and 22.1% of pineapple skin and pulp dry matter. Chemical analises presented by Chan *et al.* (18) indicate hemicellulose as the polymer responsible for 10% of commercial pineapple juice underflow dry weigh. Considering the pulp fraction retained on the skin after its removal from the fruit (residue) we obtained, in our laboratory, aproximately 100g/L of total fermentable sugars (hexoses and pentoses) in pineapple residue. Table 1 presents the results for ethanol production from sucrose, xylose and glucose in synthetic culture media and in pineapple hydrolysates containig 8% of total sugar (mainly xylose).

Table 1: Ethanol production by K. oxytoca in: LB media containing glucose, xylose and sucrose; hydrolysate; buffered pineapple juice. The effect of inocula from LB media and hydrolysate media.

Fermentation media	Maximum Ethanol (g. L^{-1})	Productivity (g. L^{-1}. h^{-1})	final pH	Viable cells inicial	Viable cells final
LB + Glucose (8%)*	35.0	1.3	6.5	10^8	10^8
LB + Xylose (8%)*	31.0	0.2	8.0	10^8	10^2
LB + Sucrose (8%)*	44.0	0.9	6.4	10^8	10^8
LB + Hydrolysate (8)* (inocula from Hydrolysate)	17.0	0.23	3.4	10^9	10^8
LB + Hydrolysate(8%)* (inocula from LB + Glucose)	26.0	0.36	3.8	10^9	10^9
Buffered pineapple juice(4%)*	13.0	0.54	5.5	---	

* total sugar concentration

Although data on ethanol production in synthetic culture media are similar to others presented in the literature (9,19), the calculated volumetric productivity around 0,2 when xylose was the fermentable sugar (Table1) is much lower than values presented by Ohta *et al.*(± 1.0) (9). High pH values at the end of fermentation process associated with high death rates (Table 1), partially explain this difference, suggesting a peculiar fermentative metabolism by *K. oxytoca* when the fermentable substrate is xylose (9). The production of acetic, lactic and succinic acids and other fermentation products as butanodiol and ethanol by *K. oxytoca* also may have contributed to a decrease of volumetric productivity by changing sugar transport systems. The same metabolic feature was not observed when xylose was the main sugar in the pineapple hydrolysate. In this case, when the inoculum was from LB medium, final pH value is lower but there is no cellular death. Although there is a high ethanol production, low volumetric productivity clearly indicate the existence of an inhibiting factor or factors present in hydrolysate responsible for a delayed ethanol production or substrate utilization. The observed in pH values and cell viability suggest that *K.oxytoca* develops distinct metabolic controls systems when fermenting similar sugars. These differences are probably related to the presence of ions originated by the hydrolysis process and/or neutralization. Despite metabolic differences, the presence of toxic derivatives as furfural, organic acids and ions in hot acid treated residues is a well known fact responsible for disturbing the fermentative process. Chemical analysis on pineapple residue hydrolysate showed the presence of 10g/L acetic acid. After 160 hours of fermentation this value increases to 12.5g/L. These data point to acetic acid as a possible candidate responsible for hydrolysate toxicity.

The search for alternative sources of nitrogen and vitamins necessary for biological fermentation processes has also received particular attention in attempts to decrease the costs of fermentative process making it an economically viable process (20). Results on Table 2 show that yeast extract (10g/l) can replace tryptone in LB medium.

Values of maximum ethanol yield and volumetric productivity from medium containning yeast autolysate instead of peptone and commercial yeast extract show that this source of vitamin and nitrogen can replace the use of tryptone and commercial yeast making the process cheaper.

Quality and volumetric proportion of inocula have been extensively studied because of their influence upon fermentative processes (20). Table 3 presents the results obtained with inocula grown on LB and on hydrolysate

medium. The influence of subculturings on solid media previous to inoculation on liquid media have also been evaluated. Althoug better ethanol yields have been obtained with 3 subculturings followed by culture on LB medium, data concerning the time spent for ethanol production show that one subculture followed by culture on LB medium is the most favorable condition (Table 3).

Table 2: Ethanol production by *K.oxytoca* in phosphate buffered LB media containing different vitamins and nitrogenous sources

Phosphate buffered LB medium with sucrose (10%) containing:	Maximum ethanol ($g.L^{-1}$)	Productivity ($g.L^{-1}.h^{-1}$)
LB(yeast extract + peptone)	46.0	0.96
Yeast Autolysate diluted to:		
1/8	37.1	0.78
1/4	43.0	0.90
1/2	33.0	0.68
Industrial yeast:		
05g/l	41.7	0.86
07g/l	36.8	0.76
10g/l	41.0	0.85
15g/l	36.0	0.75
Yeast extract (Difco):		
07g/l	43.1	0.89
10g/l	45.5	0.94

Inocula grown on hydrolysate medium were expected to present better efficiency when inoculated on similar medium. The better ethanol volumetric productivity presented by inocula grown on LB medium contradicts this expectation. More studies on the inocula influence must be performed.

Table 3. Ethanol production by *K.oxytoca* in media containig glucose or sucrose with inocula from LB and hydrolysate media.

	FERMENTATION MEDIA					
	Buffered LB + sucrose (8%)*		Buffered LB + glucose (8%)*		LB +Hydrolysate (8%)*	
INOCULA FROM:	Ethanol ($g.L^{-1}$)	Productivity ($g.L^{-1}.h^{-1}$)	Ethanol ($g.L^{-1}$)	Productivity ($g.L^{-1}.h^{-1}$)	Ethanol ($g.L^{-1}$)	Productivity ($g.L^{-1}.h^{-1}$)
LB + Glucose (2%)* 1 subculturing + LB(Glucose)	46.0	0.96	31.0	1.3	26.0	0.36
LB + Glucose (2%)* 3 subculturings + LB(Glucose)	53.0	0.60	n.d.	n.d.	n.d.	n.d.
LB + Glucose (2%)* 1subculturing + Hydrol.(8%)*	n.d.	n.d.	n.d.	n.d.	26.0	0.28

*total sugar concentration
n.d. = not determined

CONCLUSIONS

Sugar composition of pineapple waste hydrolysate favors ethanol production by *Klebsiella oxytoca*. Greater ethanol productivity values obtained with fermentation on LB medium when compared to fermentation on hydrolysate, suggest the presence of toxic derivatives in hydrolysates. The results point to good possibilities for the utilization of pineapple residue hydrolysate for the production of ethanol by recombinant *Klebsiella oxytoca*.

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RESUMO

Produção de etanol a partir de diferentes açúcares e resíduos do abacaxi por *Klebsiella oxytoca* geneticamente modificada

O Brasil está entre os cinco primeiros produtores mundiais de abacaxi. O resíduo originado da indústria de enlatados é composto pela casca e pela coroa da fruta de composição química predominantemente ligno-celulósica e hemicelulósica. Além disso parte da polpa rica em sacarose é mantida com o resíduo quando da extração da casca. A hidrólise ácida deste resíduo origina açúcares provenientes da hemicelulose principalmente pentoses e hexoses. *Klebsiella oxytoca* é uma bactéria modificada geneticamente capaz de fermentar eficientemente celobiose, pentoses e hexoses para a produção de etanol. A utilização deste microrganismo para a produção de etanol a partir de substrato barato e poluente foi o objetivo deste trabalho. Os resultados apontam grandes possibilidades de utilização do resíduo do abacaxi para a produção de etanol por *K.oxytoca* recombinante desde que haja um melhoramento genético do microrganismo ou tratamento do meio de fermentação que superem a inibição causada pelas substâncias tóxicas presentes no hidrolisado.

Palavras-chaves: *Klebsiella oxytoca*, etanol, abacaxi

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IMPROVEMENT IN ETHANOL PRODUCTION FROM SUCROSE AND SUGAR CANE BAGASSE HYDROLYSATE BY A RECOMBINANT *ESCHERICHIA COLI* AND BAKER'S YEAST

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ABSTRACT

Mild treatment of hemicellulose with dilute sulfuric acid at 120°C releases 40-45 g/L of reducing sugars (0.2g sugar/g bagasse). *Escherichia coli* KO11 is a genetically modified bacteria able of fermenting hexoses and pentoses from hydrolyzed hemicellulose. The hydrolysate was neutralized by two different alkalis and the best result was overliming with calcium hydroxide. The fermentations were carried with strains physiologically adapted to hydrolysate medium. When compared with strains from LB medium, the adapted *E.coli* presented better productivity. A second stage fermentation for ethanol production was performed by the addition of baker's yeast and sucrose (150g/L) to the hydrolyzed wine when ethanol fermentation by *E.coli* was over. After six hours of yeast fermentation the ethanol concentration accumulated in the medium was 100g/L. This two-stage fermentation can turn the bioconversion of lignocellulose to ethanol more attractive, because the increase in alcoholic concentration turning the distillation process economically advantageous.

Key words: *E.coli*, ethanol, hemicellulose

INTRODUCTION

There is a worldwide interest in renewable and alternative fuels nowadays, motivated mainly by environmental concerns. Conversion of agricultural residues to ethanol is an attractive option for utilizing all major components of biomass, besides producing an automotive fuel, is an bioremediation process (4).

Bagasse, the residue left after extraction of sucrose from sugar cane, contains about 20-30% hemicellulose, 30-50% cellulose and 10-30% lignin. Potentially, hemicellulose and cellulose could be broken down to pentoses and hexoses and fermented to ethanol (4).

Mild treatment of bagasse with dilute acid produces a mixture of monosaccharides, mainly pentoses, like D-xylose and arabinose and hexoses like glucose, galactose and mannose (6).

Recombinant *E.coli*, which harbor genes encoding ethanol-producing enzymes from

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Z.mobilis, ferments these sugars (pentoses and hexoses) with high yield and productivity of ethanol, as in synthetic broth (1,10) as in hydrolysate of different residues, such as corn cobs and hulls and wood chips (2, 3, 11).

In this study, the sugar cane hydrolysate was fermented after two different treatments, in order to remove inhibitory components. The fermentation were carried with physiologically adapted cells to hydrolysate. In order to improve the final ethanol concentration, baker's yeast and sucrose were added after hydrolysate fermentation by *E.coli* KO11, in a second step, turning the distillation process economically advantageous.

MATERIALS AND METHODS

Microorganism - *E.coli* strain KO11 is a recombinant of *E.coli* 11303 in which the genes encoding enzymes pyruvate decarboxylase (pdc) and alcohol dehydrogenase (adh) from *Z. mobilis* have been integrated into the chromosome (7). Cultures were grown in complex broth (Luria broth) containing 2% glucose (w/v) and chloranphenicol and maintained as frozen stocks (- 20°C) in 40% glycerol (v/v). Baker's yeast were maintained at 4 °C.

Culture medium - Luria broth (LB) (*5*) consisted of tryptone (10 g/L), yeast extract (*5* g/L) and NaCl (5 g/L) containing glucose 20 g/L. This broth was used to culture stocks and to prepare the inocullum.

Sugar cane bagasse hydrolysis - Hydrolysate was prepared by mixing 100g hammermilled sugar cane bagasse (45-50% humidity) with 600 mL 1% (v/v) sulfuric acid. After 24 hours at 60 °C sulfuric acid excess was extracted (about 350ml). The bagasse soaked extraction, the hydrolysate was cooled and stored a 5 °C. Prior fermentation experiments hydrolysate was neutralized and supplemented with trytone (10 g/L) and yeast extract (5 g/L).

Treatment of hydrolysate - *Neutralization I (with calcium hidroxide)* - The hydrolysate were treated with calcium hydroxide with stirring (at 80 °C) to adjust the pH to 10,0. The hydrolysate was cooled and pH was adjusted to 7,0 with concentrated sulfuric acid. Precipitants were removed by centrifugation. *Neutralization II (with calcium hydroxide and sodium hydroxide* - Calcium hydroxide was added with stirring (at 80 °C) to pH 5,5. The hydrolysate was cooled , filtered, and pH adjusted to 7,0 with sodium hydroxide (1M).

Fermentation Experiments - Inocula were grown from freshly colonies in Luria broth containing glucose 20 g/L for 6 h on rotatory shaker and inoculated to fermentation broth (10% v/v). Anaerobic conditions were set in 100 mL volumetric flasks plugged with rubber septa and 25-gauge needles to allow gas to escape and to take samples out. Fermentations were carried out at 30°C with agitation (200 rpm).

Fermentations experiments with physiologically adapted cells - Inocula were grown from freshly colonies in sugar cane bagasse hydrolysate neutralized, supplemented with tryptone (10 g/L) and yeast extract (5 g/L) for 24 hours.

Analytical Procedures - Samples were cooled, centrifuged 12100 g for 10' and analyzed for sugar and ethanol contents. Sugars and ethanol were analyzed by High Performance Liquid Chromatography (HPLC) using a refractive index monitor and integrator (Biorad). Separations were performed at 65 °C on a HPX-87H column (Biorad), at a flow rate of 0,8 mL/min. Ethanol concentration were determined by gas chromatography (Shimadzu) performed at 120°C (Chromosorb 101 column), detector at 210°C and injector at 180°C. Yield was computed on the basis of total carbohydrate was autoclaved at 120 °C for 40 minutes. After concentration and was corrected for residual sugars. The theoretical yield of ethanol from pentose and hexose sugars is 0.51 g ethanol/ g sugar.

Productivity (g de ethanol/L.h): ethanol concentration / time (h).

RESULTS AND DISCUSSION

Effect of neutralization: Figure 1 presents results from ethanol fermentation from hydrolysates neutralized by two different procedures: I and II (Materials and Methods). The difference between them was evident during the first 24 hours. In the hydrolysate neutralized I, *E.coli* KO11 produces ethanol faster than when neutralized with NaOH, and consequently, a better productivity was achieved (Table 3). Procedure I turn out to be our pattern of neutralization. Other authors utilized neutralization process I with corn cob and hulls hydrolysates (3) and *Pinus* (2). In these works they obtained 38.1 g/L of ethanol from corn cob hydrolysate containing about 75 g/L of total sugar (3), and 26 g/L of ethanol from *Pinus* hydrolysate containing 72.0 g/L of total sugar.

Table 1: Ethanol production by *E.coli* KO11 from sugar cane bagasse hydrolysate: Comparison of fermentatives parameters (see Materials and Methods) .

Procedures	Consum. Sugar (S)	Ethanol Max. (P) (g/L)	Yield (g P/g S)	Productivity (g ethanol/L.h)
Neutralization I	38.5	16.2	0.42	0.34
Neutralization II	25.0	12.0	0.48	0.16
Inocullum from LB - not adapted	22.4	10.5	0.47	0.22
Inocullum from hydrolysate - Adapted	29.5	16.8	0.56	0.70

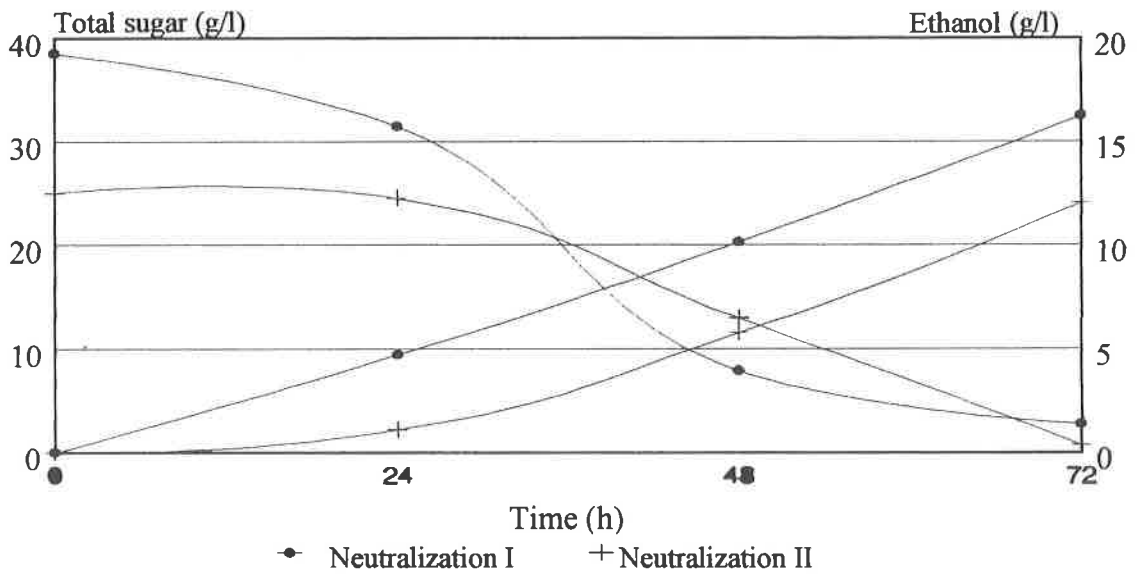

Figure 1 - Ethanol production and sugars consumption by *E.coli* KO11 from sugar cane bagasse hydrolysates submitted to different procedures of neutralization, before fermentation: with $Ca(OH)_2$ (Neutralization I) and with $Ca(OH)_2$ and NaOH (Neutralization II).

Physiologically adapted cells to bagasse hydrolysate - Table 2 shows the better performance in ethanol production by *E.coli* physiologically adapted to hydrolysate during the inocula preparation. HPLC measurements show a faster xylose consumption by physiologically adapted cells, probably caused by catabolic induction during the inocullum growth. Consequently, ethanol production was faster producing 16,8 g/L of alcohol in 24 hours, increasing yield and productivity values (Table 1).

Table 2: Sugar consumption and ethanol production *E.coli* KO11 from sugar cane bagasse hydrolysate. Inocula from: A) Luria broth (LB) - Not adapted and, B) Hydrolysate supplemented - Adapted.

A) Inocula from LB - Not adapted

Time (h)	Xylose (g/L)	Arabinose (g/L)	Total sugar (g/L)	Ethanol (g/L)
0	39.3	2.6	42.0	0.0
24	37.6	0.0	37.6	4.9
48	23.0	0.0	23.0	10.5

B) Inocula from Hydrolysate Supplemented – Adapted

Time (h)	Xylose (g/L)	Arabinose (g/L)	Total sugar (g/L)	Ethanol (g/L)
0	37.3	3.5	40.8	0,0
24	11.3	0.0	11.3	16.8
48	11.3	0.0	11.3	16.6

Two stage fermentation using baker's yeast: Ethanol concentration above 45 g/L is necessary in order to turn the distillation process economically advantageous. As *E. coli* KO11 does not tolerate ethanol concentrations above 60 g/L, baker's yeast and sucrose were added to bagasse hydrolysate after the pentose fermentation by *E. coli* KO11, looking a for an improvement in ethanol concentration in broth.

The ethanol production by *E.coli* finished in 48 hours with an alcoholic concentration of 26 g/L (Table 3). After pentoses consumption (Table 3), sucrose (150 g/L) and baker's yeast (10%) were added in and, after 6 hours, the accumulated ethanol concentration was 100 g/L, the result of bacteria and yeast production, attesting that acetic, lactic and succinic acids produced by *E.coli* KO11 (data not shown) did not interfere in ethanol production by baker's yeast.

Table 3 - Sugar cane bagasse hydrolysate fermentation by *E. coli* KO11, followed by sucrose fermentation by baker's yeast (Two step fermentations).

	Time (h)	Total Sugar (S) (g/L)	Ethanol(P) (g/L)	Yield (g P/g de S)	Productivity (g ethanol/L.h)
1st Ferm.	0	53.8	0	0	0
	24	18.7	18.5	0.53	0.77
	48	3.9	26.0	0.52	0.54
2nd Ferm.	0	150.0			
	2	N.D	45.2	0.22	0.37
	4	N.D	68.0	0.33	0.55
	6	N.D	100.4	0.49	0.80
	8	N.D	102.1	0.5	0.80

N.D. -Not determined

CONCLUSIONS

1) *E.coli* KO11 is capable of fermenting all sugars presented in sugar cane bagasse hydrolysate near the theoretical yields.

2) Neutralization using only calcium hydroxide is better than with sodium hydroxide.

3) The physiological adaptation of the cells increase the productivity values. Another advantage is the use of bagasse hydrolysate instead of synthetic broth, in the inocullum preparation.

4) The use of baker's yeast and sucrose after the fermentation by *E.coli* KO11, increase the ethanol concentration (100 g/L). This two-stage process could turn more attractive the sugar cane bagasse bioconversion by *E.coli* KO11, because it applies resources (baker's yeast and sucrose) already available in ethanol plants.

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RESUMO

Nova perspectiva na produção de etanol a partir de hidrolisado de bagaço de cana-de-açúcar e sacarose empregando *Escherichia coli* recombinante e fermento de padaria

A hidrólise da hemicelulose a 120 °C com ácido sulfúrico 1 % liberou 40-50 g/L de açúcares redutores (0,2 g de açúcar/ g de bagaço seco). *Escherichia coli* KO11, é uma bactéria geneticamente modificada capaz de fermentar hexoses e pentoses presentes nos hidrolisados de hemicelulose. Os hidrolisados foram neutralizados utilizando duas bases (NaOH e $Ca(OH)_2$) e os melhores resultados foram obtidos com a adição de hidróxido de cálcio até pH 10, e o ajuste para pH 7 com ácido sulfúrico concentrado. Foram usadas células fisiologicamente adaptadas ao hidrolisado de bagaço de cana, que, quando comparadas com as não adaptadas, apresentaram maior produtividade em etanol. Numa segunda etapa de fermentação, adicionamos, após o término da fermentação por *E.coli* KO11, fermento de padaria e sacarose (150 g/L) ao hidrolisado fermentado. Após 6 horas de fermentação pela levedura o álcool acumulado no meio de cultura foi de 100 g/L. Esta fermentação em duas etapas pode tornar mais atrativa a produção de etanol a partir de biomassa lignocelulósica por *E.coli* recombinante, uma vez que o aumento da concentração de álcool torna o processo de destilação economicamente favorável.

Palavras-chave: *E. coli*, etanol, hemicelulose

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THE ANTIBACTERIAL ACTION OF SUCCINIC ACID PRODUCED BY YEAST DURING FERMENTATION

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ABSTRACT

Benzoic acid, a yeast growth inhibitor, had shown promising results in fuel ethanol production with cell recycle, increasing ethanol yield by decreasing biomass and glycerol formation. However, the bacterial proliferation increased not only in laboratory-scale fermentation but also when benzoic acid was used in distilleries, apparently due to the reduction in succinic acid formation. The antibacterial action of succinic acid is attributed not only to the acidic effect, but mainly to the anion, in a synergetic effect with ethanol, presenting strong inhibitory action against growth of the main bacteria isolated from distilleries. An ecological function of succinic acid is suggested, since reduction of its formation causes yeast (*Saccharomyces cerevisiae*) to be less competitive in distillery environment.

Key words: succinic acid, benzoic acid, bacterial contamination, alcohol fermentation

INTRODUCTION

It is a general thought that increased ethanol yield could be achieved by minimising biomass, glycerol and succinic acid formations during fermentation (9) and such approach seems to be very attractive for industrial ethanol production. These by-products consume from 4% (9) to 10% of the metabolised substrate, depending on the environmental conditions (1). Although glycerol formation is necessary for the maintenance of the redox-balance changed by yeast growth and organic acids synthesis (9), no physiological function has been attributed to succinic acid formation at the produced amount during yeast fermentation. It has even been assumed that its formation is more probably a result of some disturbance in the cell metabolism than a necessary function of the cell under anaerobic conditions (9).

Benzoic acid, a well-known food preservative, at sublethal concentrations has a stimulatory effect upon alcoholic fermentation, increasing fermentation rates and reducing growth yields (11) and glycerol formation (5,12). Besides these effects, benzoic acid did not affect yeast viability, and its use in distilleries has been suggested (5).

In the present work, we report the use of benzoic acid on laboratory and industry-scale fermentations, with yeast reuse, in order to evaluate its applicability for fuel alcohol distilleries. However, an unexpected burst of bacterial proliferation provided a better understanding of the ecosystem of an industrial fermentation, suggesting an ecological reason for succinic acid formation.

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MATERIALS AND METHODS

Laboratory Fermentation - Fermentation was carried out in 150 mL centrifuge vials, using baker's yeast (*Saccharomyces cerevisiae*, Fleischmann) and semi-synthetic medium (pH 5.0) containing 15% sucrose, 0.25% Difco yeast extract, 0.226% diamonium citrate, 0.114% $K_2HPO_4.3H_2O$, 0.024% $MgSO_4.7H_2O$, 28 mg/L $ZnSO_4.7H_2O$ and 17 mg/L $MnSO_4.H_2O$. Prior to fermentation, 30 mL of a 33% (v/v) yeast suspension was submitted to acidic treatment (pH 2.5, with conc. H_2SO_4) for 1 hour, without (control) and with benzoic acid addition (1.2 mM) in all the four fermentation cycles. The fermentation was performed at 33 °C, imitating as much as possible the industrial procedure with fermentation time around 7 hours. Yeast was separated from the fermented medium by centrifugation (800 x *g* for 15 min.), weighed and re-utilised in a subsequent cycle. The fermented media from each cycle were analysed for ethanol by distillation and densimetry, glycerol based on periodate oxidation, residual sugar by alkaline copper oxidation (14) and succinic acid by HPLC (1). Yeast cell viability and Gram (+) bacterial contamination were estimated at the end of each fermentation cycle by optic microscopy after differential staining with methylene blue (2).

Industry Fermentation - Benzoic acid was used at the same concentration (1.2 mM), in three different distilleries, during yeast acidic treatment, once every three fermentation cycles during a week in the season of 1992 (Distillery I), 1993 (Distillery II) and 1994 (Distillery III). The fermentation time was from 7 to 9 hours, and the parameters (ethanol yield, glycerol and bacterial contamination) were measured using the standard methods (2, 14). As controls the same data were collected a week before the treatment.

Anticontaminant Action of Succinic Acid - Experiment I (Table 2) was designed to estimate the antibacterial action of succinic acid (600 mg/L, a usual concentration found in alcoholic fermentation), alone and with ethanol (6% v/v), comparing to the anticontaminant action of yeast fermentation itself (yeast at 10% v/v), against a bacteria mixture. So, 80 mL of sterilised semi-synthetic medium, the same mentioned above, but with 3% sucrose, were inoculated with 1 mL of a bacterial suspension (10^8 cell/mL) of the commonest Gram (+) bacteria isolated from Brazilian distilleries (*Acinetobacter calcoaceticus, Bacillus brevis, Bacillus coagulans, Bacillus megaterium, Bacillus subtilis, Lactobacillus coryniformis, Lactobacillus fermentum, Lactobacillus plantarum, Leuconostoc mesenteroides, Sporolactobacillus sp, Lactobacillus vaccinostercus*, from Department of Science and Agroindustrial Technology (ESALQ/USP). Sulphuric acid was used as a pH control to isolate the antibacterial action of the succinate anion. Bacterial counting was estimated by optic microscopy (14), as well as by turbidity, before and after 12 hours of incubation at 30 °C. Growth restriction was evaluated in relation to the control medium (pH 5.0).

In Experiment II (Table 3), 80 mL of sterilised semi-synthetic medium (3% sucrose, pH 4.5) without (control) and with addition of succinic acid (600 mg/L) plus ethanol (6% v/v) were inoculated with 0.25 mL of individual bacterial culture, to result in ca. 10^6cells/mL, and incubated at 30 °C for 12 hours. Bacterial counting was performed by optic microscopy and by turbidity, and the antibacterial action of succinic acid plus ethanol was estimated in relation to the control media (without succinic acid and ethanol).

All the experiments, with four replications, were completely randomised in a split plot design.

The discussed data were statistically significant at 1% level.

RESULTS AND DISCUSSION

Stimulatory effects of benzoic acid have been reported at concentrations up to 0.5 mM, while at higher concentrations (0.5 to 1 mM) it inhibits the fermentation (12, 13). As the uptake of benzoic acid depends on the proportion of its undissociated form (4), a concentration of 1.2 mM was used in the yeast acidic treatment (33% yeast suspension, pH 2.5) instead of addition to the fermentation medium, in order to potentialize its effects according to previous tests (data not shown).

The utilisation of benzoic acid in laboratory fermentation showed a significant increase in ethanol yield (Fig 1a), accompanied by a decrease in biomass (Fig 1c), glycerol (Fig 1b) and succinic acid formation (Fig 1e), without affecting yeast viability (Fig 1d), an important parameter for a process with yeast cell recycle.

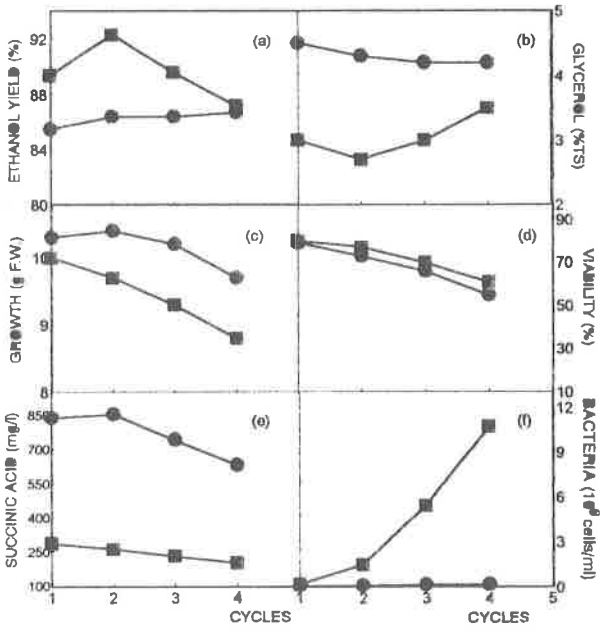

Figure 1: Effects of benzoic acid on the (a) ethanol yield (%), (b) glycerol formation (% total sugar), (c) yeast growth (g fresh weight), (d) yeast viability (%), (e) succinic acid formation (mg/L) and (f) bacterial contamination (10^6 cells/mL). Symbols: Control (●) and 1.2 mM benzoic acid (■).

An undesired side-effect, however, was the increased bacterial growth during recycling (Fig 1f). The origin of such contamination is the pressed baker's yeast used, that normally results in a bacterial counting at the level of 10^6 cells/mL during yeast recycling, even with sterilised medium. In several published works such contamination has been neglected, but when this parameter is taken into account, it helps a better understanding of factors affecting fermentation with yeast cell reuse (1).

Reduction in glycerol formation by benzoate has already been observed (5, 12) and could be explained by the connection to the redox-balance of the cell (9), since biomass and succinic acid formations were both diminished. Although succinic acid formation experienced minor changes due to temperature, pH, sulphite and yeast concentration (1), the present data show that benzoic acid brought about a drastic reduction in succinic acid production during fermentation (Fig. 1e).

High energy charge is required for the activation of pyruvate carboxylase, the key enzyme responsible for succinate formation (9), and benzoate causes a drop in energy charge, lowering ATP level (12, 13). In addition, in rat liver (not demonstrated for yeasts), benzoate sequestrates coenzyme A, inhibiting pyruvate carboxylase (6). These observations can probably explain the reduction in succinate production. It is also observed that although ethanol yield was higher in the presence of benzoic acid, there was a tendency to become lower (Fig. 1a) as bacterial contamination increased (Fig. 1f), particularly noticed after the second fermentative cycle. Same results were observed when industrial substrate (molasses and cane juice) were used (data not shown).

In distilleries, identical effects could be demonstrated: increased ethanol yield and decreased glycerol formation, but bacterial contamination reached levels that renders the industrial process impracticable (Table 1).

In Distilleries II and III, a significant increase in ethanol yield and reduction in glycerol formation were demonstrated, in spite of a raise in bacterial contamination, but in Distillery I

bacterial growth was so amplified that the benefits in ethanol yield were not evident. Furthermore, in all distilleries studied, the increased ethanol yield did not compensate for the additional costs with antibiotics in order to control bacterial proliferation. As the industrial practice is performed with high levels of bacterial contamination, the use of benzoic acid in distilleries can be regarded as impractical at the present time.

Table 1. Effects of benzoic acid (1.2 mM) added during the yeast acidic treatment once every three fermentation cycles in three distilleries, on ethanol yield (%), glycerol formation (% total sugar) and bacterial contamination (10^6 cells/mL).

Distillery	Treatm.	Ethanol yield (%)	Glycerol formation (%TS)	Bacteria (10^6cells/ mL)
I	Control	90.6	3.05	66
	Benzoic	90.8	2.78	295
II	Control	87.7	3.63	12
	Benzoic	90.2	3.00	38
III	Control	90.8	4.08	8
	Benzoic	92.7	2.89	48

Such observations pointed to a possible antibacterial function for succinic acid. It is the main organic acid produced by *Saccharomyces cerevisiae* fermenting semi-synthetic, cane juice or molasses media (1).

The antagonism that yeasts exert against bacteria has been documented but only in few occasions metabolites other than ethanol has shown antibacterial activity (3 and literature therein). Succinic and lactic acids were identified as *Acidiphilium* growth inhibitory substances isolated from yeast extract (7). It has been pointed out the addition of fumaric acid to table wines for the control of malo-lactic fermentation (10). In the same work fumaric acid was demonstrated to be more efficient than succinic acid in suppressing growth of *Leuconostoc oenos*. Wines produced by cryotolerant strains of *Saccharomyces cerevisiae* presented more titratable acidity, glycerol, succinic and malic acids compared to normal strains, and were more stable with respect to malo-lactic fermentation.(4).

Isolating the effect of pH, the anticontaminant action exerted by succinic acid is due to the anion in synergistic effect with ethanol (Table 2). The antibacterial action demonstrated by alcoholic fermentation can be attributed not only to the rapid utilisation of nutrients and a drop in pH value by yeast, but additionally to succinic acid in the presence of ethanol. Table 3 shows the strong inhibitory effect of succinic acid and ethanol on growth of several bacteria isolated from distilleries. Although not shown, data of turbidity gave the same results.

Table 2. Antibacterial action of yeast fermentation, succinic acid, ethanol and succinic acid plus ethanol on a bacteria mixture. Bacterial counting (10^6 cells/mL) and pH were measured before and after 12 hours incubation at 30 °C. Growth restriction (%) was calculated in relation to the control medium (0%).

Media	Initial		Final (12 Hours)		Growth Restriction (%)
	pH	10^6 cells/ mL	pH	10^6 cells/ mL	
Control	5.00	4	3.11	185	0
Yeast fermentation	5.00	4	3.67	3	98
pH control (sulphuric acid)	3.54	4	2.93	107	42
Succinic acid (600 mg/L)	3.54	4	3.11	69	63
Ethanol (6% v/v)	3.54	4	3.27	51	72
Succinic acid + ethanol	3.54	4	3.56	7	96

Table 3. Antibacterial action of succinic acid (600 mg/L) plus ethanol (6%) on different bacteria in semi-synthetic medium (pH 4.5) incubated at 30 °C for 12 hours. Control medium (pH 4.5) without succinic acid and ethanol.

Bacteria	Control		Succin. Acid + Ethanol		Growth Restriction (%)
	pH	10^6 cells/mL	pH	10^6 cells/mL	
Acinetobacter calcoaceticus	4.40	22	4.44	7	68
Bacillus brevis	3.09	346	3.46	145	58
Bacillus coagulans	4.40	4	4.44	<0.1	>97
Bacillus megaterium	4.42	14	4.43	<0.1	>99
Bacillus subtilis	4.40	7	4.42	<0.1	>98
Lactobacillus coryniformis	3.60	40	3.86	16	60
Lactobacillus fermentum	3.16	362	3.55	78	78
Lactobacillus plantarum	3.16	193	3.67	40	79
Leuconostoc mesenteroides	3.53	38	4.24	5	87
Sporolactobacillus sp	3.76	80	4.25	26	68
Lactobacillus vaccinostercus	3.62	44	4.41	<0.1	>99

This is the first report relating succinic acid formation by yeast to bacterial growth in laboratory and industrial scale fermentations. The data provide a better understanding of the ecosystem during fermentations with yeast recycle, and although there has been no evidence for a physiological reason for succinic acid formation, it renders the yeast more competitive in an industrial fermentation environment, specially in fuel ethanol distilleries, where contamination with *Lactobacillus sp.* is very frequent. So, at least for fuel ethanol production, succinic acid formation should be regarded as a new parameter for yeast selection, since succinic acid probably will be a cheaper and natural way to help the control of bacterial contamination.

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RESUMO

Ação antibacteriana do ácido succínico formado pela levedura durante a fermentação

O uso do ácido benzóico, um inibidor de crescimento de leveduras, mostrou resultados promissores na produção de álcool combustível com reciclos de células, aumentando a eficiência em etanol e diminuindo a formação de biomassa e de glicerol. Entretanto, foi observado um aumento da proliferação bacteriana quando do emprego do ácido benzóico, não apenas em escala de laboratório, como também em destilarias, aparentemente devido à redução na formação de ácido succínico. A ação antibacteriana do ácido succínico é atribuída não apenas ao efeito da acidez, mas principalmente ao ânion, num efeito sinérgico com o etanol, apresentando forte ação inibitória contra o crescimento das principais bactérias isoladas das destilarias. Uma função ecológica para o ácido succínico é apresentada, pois uma redução na sua formação torna a levedura (*Saccharomyces cerevisiae*) tornar-se menos competitiva no ambiente de uma fermentação industrial.

Palavras-chave: ácido succínico, ácido benzóico, atividade antibacteriana, fermentação alcoólica

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ON THE MANOMETRIC METHOD FOR ESTIMATING THE ANAEROBIC MINERALIZATION IN AQUATIC ECOSYSTEMS: KINETIC AND METHODOLOGICAL ASPECTS.

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ABSTRACT

This paper discusses the kinetic and methodological aspects of gas formation due to the anaerobic mineralization of organic matter in aquatic ecosystems. Solution of carbohydrates (glucose and sucrose) were prepared in laboratory using water from the Monjolinho reservoir (22° 00' S and 47° 51' W). The rate of gas formation, temperature, concentrations of dissolved oxygen and of total carbohydrates were measured. The volume of gas formed was proportional to the initial concentration of sucrose. Gas formation started when the amount of dissolved oxygen was below 0.2 mg/L. In parallel to the gas formation, the content of glucose decreased and the processes were synchronized.

Key words: fermentation, aquatic ecosystems, kinetics, gases

INTRODUCTION

In aquatic systems, the anaerobic processes involved in the cycling of detritus generate various gases as the final products of mineralization. These include carbon dioxide, hydrogen sulfide, nitrogen, hydrogen, mercaptans, methane, etc. The production of any of these gases depends upon several biotic and abiotic factors. Among the biotic ones the most relevant are the qualitative composition of the population and the number of bacterioplankton. The temperature, the contents of nutrients, the redox potential, the pH and the chemical composition of the detritus are the main abiotic factors, which are responsible for the conditioning of the decomposition processes (6, 11, 12). Depending upon the type and concentration of gas, these fermentation products may generate significant changes in the water quality, with the risk of restricting the potential use of the hydric resources and the survival of several species. In order to elaborate on the appropriate procedures for studying the anaerobic mineralization of organic matter in aquatic systems, the kinetics and methodological aspects associated with the manometric method for estimating gas formation are discussed in this paper.

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MATERIALS AND METHODS

Eleven flasks of 4.1 L were prepared with water from the Monjolinho reservoir (22° 00' S and 47° 51' W), previously filtered through glass wool. In a set of 6 flasks, glucose was added to the final concentration of 1000 mg/L (Flasks 1 to 6). Manometers (burettes) were placed in 5 of these flasks while the sixth one was used in the determination of temperature, dissolved oxygen content (DO) and carbohydrates concentration. In the second set (Flasks 7 to 10), distinct amounts of sucrose were added to the final concentrations of 0, 24.4, 122 and 1219 mg/L. The reactors were mounted according to the procedures suggested by Sorokin and Kadota (10). In this method, the flasks containing the solutions were closed with "lids" with already-installed burettes (100 mL). The Flask no. 11 was employed for determination of the temperature of the second set of flasks. In the laboratory, the flasks were kept in the dark, with the evolution of gas and temperature being monitored daily. Gas evolution was measured through the shift in the water columns in the burettes. The gas volumes were then corrected for the ambient conditions of 20 °C and 1 atm. Periodically, the DO content was estimated by potentiometry, and the total content of carbohydrates was estimated using the colorimetric method (4).

RESULTS AND DISCUSSION

Fig. 1 shows that the kinetics of gas formation comprises three steps. This can be noted in spite of the relatively large changes observed especially in flasks 2 and 4. The first step is thought to correspond to the adaptation stage, of the organisms. In this stage, the processes for formation and consumption of gases are assumed to be equivalent, or the processes for the biological uptake prevail, or else solubilization of the gases occurs. The second step in the kinetics was characterized by the predominance of the gas formation processes, due to the anaerobic mineralization of glucose, over the processes involved in the biological assimilation or solubilization of the gases. The third step started at 138th, 56th, 141th, 189th and 187th day for flasks 1 to 5, respectively. In this step, the rate of gas consumption (solubilization + biological uptake) was greater than gas release, which caused a decrease in the amount of accumulated gas.

By comparing the results in Fig. 1, one may note that the largest changes appeared in the volume of gas formed (74.1; 30.3; 87.4; 140.0 and 67.2 mL, Flasks 1 to 5, respectively). The initial stages of intense gas assimilation lasted, for all flasks, up to the 7th day of experiments. The volume of assimilated gas ranged from 0.76 (Flask 1) to 2.37 mL (Flask 5). For Flask 4, however, the assimilation was higher, 15.3 mL. Because the water in these experiments was not previously treated to restrict the growth of microorganisms, it may be assumed that the dispersion in the results is caused by growth of distinct microorganisms, with selection of communities. These results point to the need of intense homogenization of the samples. In addition, the samples must be handled in a way to avoid contamination and/or the preferred development of some processes to the expense of the other ones throughout the experiments. On the other hand, even with very stringent precautions, growth of different communities may occur naturally in non-treated samples. Therefore, its is suggested that at least 2 bottles are used in estimating the amount of gas formed by the manometric method. With regard to the changes in the amount of gas formed, especially after the 35th day, its is possible that such changes may reflect the substitution of r-strategists species by k-strategists species.

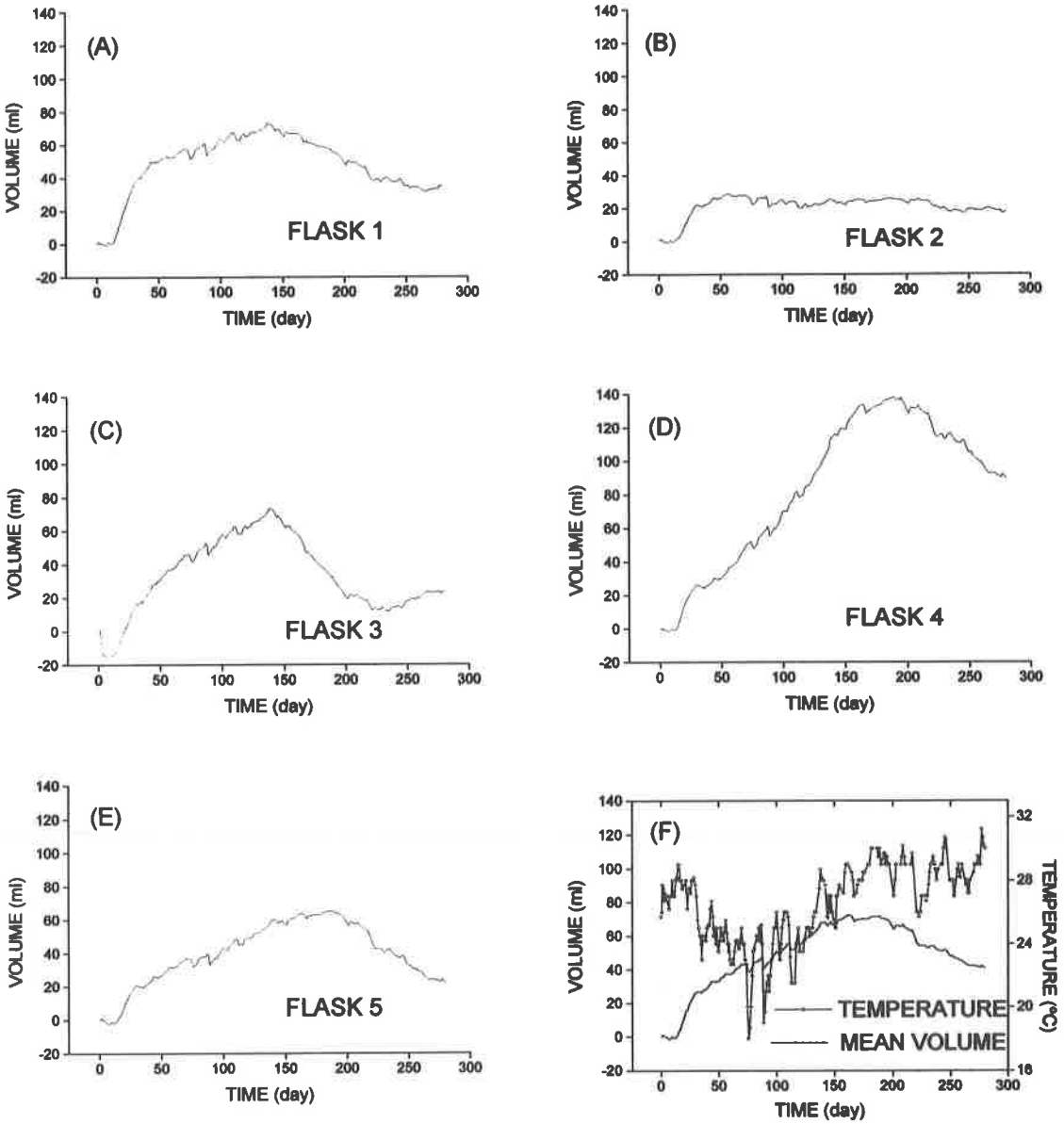

Figure 1: Time changes of the formed gas volumes and of temperature, during the anaerobic mineralization of the glucose (initial concentration: 1,0 g/l).

As shown in Fig. 1F, the maximum quantity of accumulated gas (72.4 mL) is consistent with the volume predicted by the relationship between maximum volume of formed gases and the initial amount of sucrose, derived from the results of the first set of flasks (Fig. 2A). This indicates that the maximum value of gas formation may be used as a parameter for comparing mineralization processes. The discontinuities occurring during gas accumulation suggest that, as far as kinetics is concerned, there was a succession of processes in the gas formation, with predominance of the simultaneous and consecutive reactions kinetics over those parallel reactions. Various gases are released by the organisms which act in the decomposition process of the organic matter. Under anaerobic conditions, the main gases formed are carbon dioxide, which arises from the denitrification processes and from reduction of sulfates, methane, formed through methanogenesis and molecular nitrogen, which also arises from denitrification (1). Experiments of anaerobic decay of organic matter in sediment samples indicate the following approximate composition for the gases formed: CH_4 = 85,2%, N_2 = 7,5% and CO_2 = 7,1% (7).

Even though differences among the kinetics processes for gas accumulation were identified (see Fig. 1), it is important to stress that: i) the results for the first 35 days were very similar, which could be fitted with a sigmoidal kinetics model. The parameters obtained, i.e. the coefficients of formation and final volume, were similar for the various flasks, suggesting that the method may also be applied in fairly rapid incubation processes; ii) the mean rates of the gas formation $(Vol_{MAX}\text{-}Vol_{MIN})/(T_{MAX}\text{-}T_{MIN})$ were in the same range (0.57, 0.62, 0.54, 0.76, 0.36 and 0.45 mL/day for Flasks 1 to 5, respectively), indicating the possible application of these rates when employing the manometric method.

Another characteristic worth to mention refers to the 3rd step of the process, in which the consumption rates became predominant, thus causing a decrease in the volume of gas formed. Since such processes derive from biological immobilization (assimilation) and solubilization of the gases, the use of low pressure U-tube manometers is suggested for this type of incubation. The assimilation and solubilization would then be attenuated and after each measurement the flasks would undergo a depressurization process. For manometers with only one water column, as those employed in this study, a valve is recommended to be included in the reactor to permit the depressurization of the formed gases at regular intervals.

Fig. 1F shows that the average temperature in the flasks throughout the whole experiment was 26.3 °C, with a mean standard deviation of 2.5°C. The minimum observed temperature was 18.0 °C, and the maximum amplitude variation was 13.2 °C. Considering the prolonged duration of the experiment (280 days), one may say that there was no definite tend in the temperature change, either for heating or cooling. The temperatures actually were in a range (18.0 to 31.2 °C) that is usual for aquatic systems. However, due to the temperature dependence of the metabolic rates because of the possible favoring or inhibition of growth of some species (5,6), the temperature control is another factor to be considered in the attempts to minimize dispersion in the results.

Considering the gas formation involving various metabolic processes, the biological variety of the microorganisms and the chemical properties of the organic resources, it may be assumed that the experimental precautions discussed here should be sufficient for comparing the microbiological activity and also for obtaining the kinetic parameters that are representative of the aquatic systems. These precautions include: preparation of samples under aseptic conditions, homogenization of the samples, use of numerous bottles, temperature control and depressurization of the reactors at regular intervals.

Upon comparing the results, one observes that the manometric measurements pointed to the largest gas formation in Flask 7 (with no

addition of sucrose), even though 100 mg of sucrose were added to Flask 8 (24.4 mg/L). These results indicate that the manometric method employed here was not sensitive for monitoring the mineralization in media with low contents of organic matter, as in most water samples. However, upon comparing the maximum volume of gas formed for the flasks containing sucrose, it is noted that the amount in Flask 8 was similar to the ones in Flasks 9 and 10. (Fig. 2A). This indicates that the method is appropriate for comparing the cycling processes under anaerobic conditions, i.e. with high concentration of organic matter, as in the incubated samples containing sediments and detritus from aquatic plants.

From Fig. 2B, it is possible to assume that up to the 70th day, the changes in the gas volume for flasks 1 to 5 followed, in average, a two-step pattern. The initial step was characterized by the predominance of assimilation processes, with an average of 1.76 mL of assimilated volume (Fig. 2B). The second stage, which lasted until the end of the experiments (70 days), was characterized by gas formation rather than consumption processes. It was also noted that from the beginning of the second stage (13th day) up to ≈ 28th day there was an intense gas formation, which ocurred in a discontinuous way afterwards.

The analysis of gas formation in the five flasks containing the same initial concentration of glucose indicates that the method did not yield reproducible results after the 35th day. Nevertheless, the mean results described satisfactorily the global processes of gas formation. The appearance of discontinuities during gas accumulation suggests that a number of gas formation processes may have occurred. There was predominance, therefore, of the kinetics of simultaneous and consecutive reactions over the parallel reactions. In this case, the formation of various gases follows specific kinetics during decomposition of organic matter. Each kinetics has its own intermediate products and conditioning factors (6, 8,12).

Fig. 2C shows that in the first 70 days, the mean temperature was 25.8 °C, with a standard deviation of 1.72 °C. The maximum temperature was 29 °C and the variation amplitude was 6.3 °C. The temperature was in the range from 22.7 to 29,0 °C, which is similar to the are observed in the Monjolinho reservoir (3,9). Therefore, one may assume that the temperature was not a limiting factor for the development of the processes. However, because metabolic rates are affected by temperature, one may expect that the gas generation and assimilation rates were influenced (2, 7).

A large uptake of dissolved oxygen was observed in the absorption period for the organisms, as may be seen by comparing Fig. 2B and D. During this period, which lasted 14 days, i.e. until the dissolved oxygen concentration decreased to 0.2 mg/L, the formation rates did not surpass the assimilation rates. Therefore, the content of dissolved oxygen was another factor responsible for the inhibition of gas formation, as demonstrated by the intense release of gases, mainly through metabolic pathways that are characteristic of anaerobic processes. Under anaerobic conditions, CO_2 is the main gas formed. Since this product is dissociated in water and may be consumed as carbonates, there was no increase in the volume of gases. From the 14th day on, the process of gas accumulation has effectively started, after the beginning of the anoxic conditions (DO ≈ 0.2 mg/L). In parallel to these processes, a relatively slow decay in the glucose content ($t_{1/2}$ = 96.8 days) was observed up to ≈ the 20th day, as seen in Fig. 2E. From the 20th to the 27th day, the decrease in the glucose content was very fast ($t_{1/2}$ = 9.7 days). This decay has probably been reflected in a marked gas formation which started on the 14th day and reached a steady state between the 28th and the 35th day.

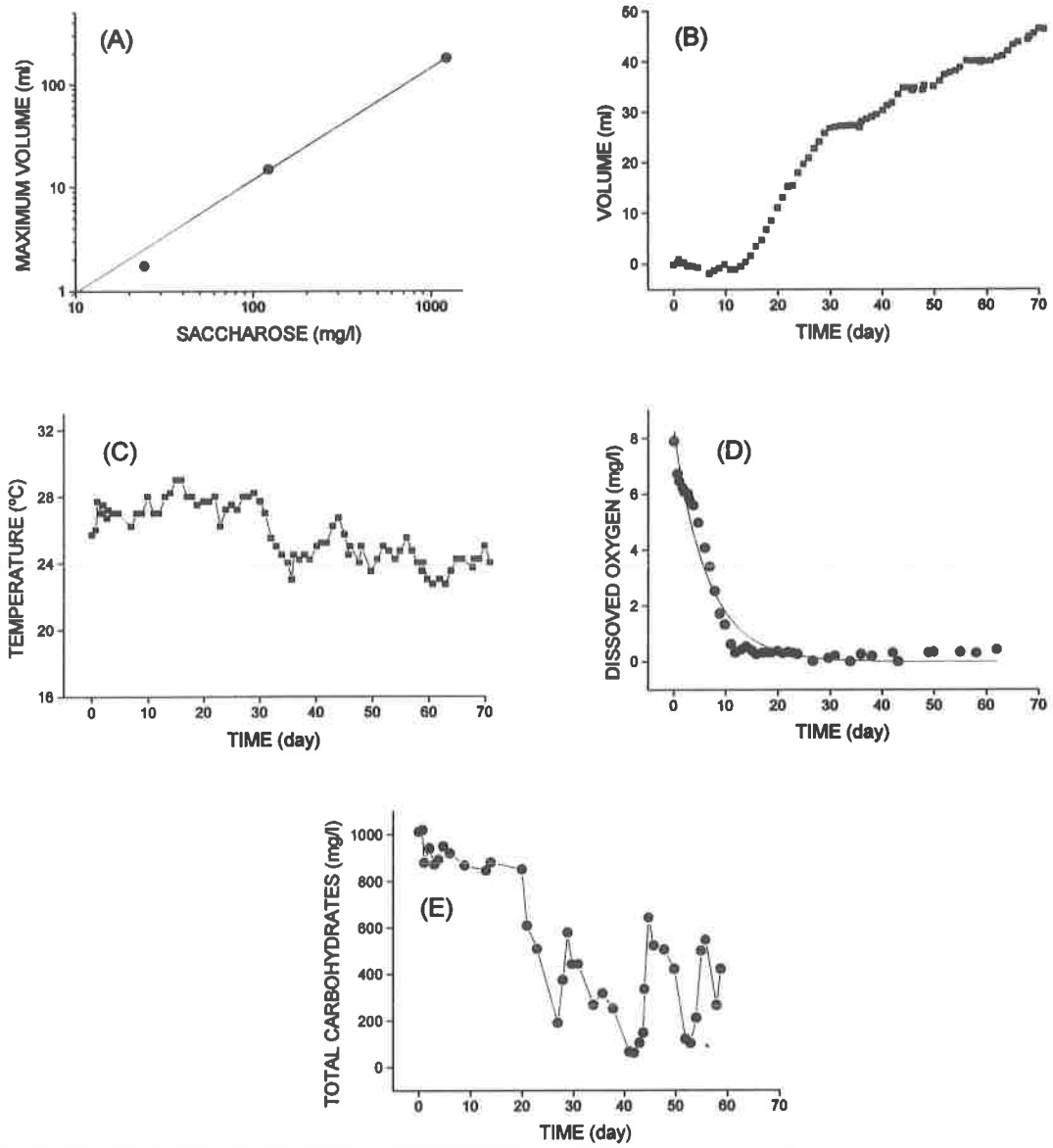

Figure 2: Relationship between the initial concentrations of sucrose and the maximum volumes of formed gases (A). Time evolution of: volume of gases (B), temperature (C), dissolved oxygen content (D) and total carbohydrates concentration (E), during the mineralization of the glucose (initial concentration: 1.0 g/L).

The increase in the glucose content observed between the 27 and 30th, 40 and 45th and 52 and 55th days was probably due to the succession of the population of microorganisms, and/or the formation of excretion products. When these results are compared to those of Fig. 2B and E, a coincidence between the appearance of the glucose peaks (carbohydrates) and the gas accumulation stages is noted. In the interval where the carbohydrate content was decreased, there was gas formation. On the other hand, when the carbohydrates content was

increased, there was no gas formation and/or accumulation. These results support the hypothesis mentioned before that the gas formation processes are mainly governed by consecutive mechanisms. This issue may be further discussed by monitoring the composition of the gas formed.

The following conclusions can be obtained: 1) Results were reproducible for the first 40 days, after which a selection of the communities can occur. 2) The microbiological activity and the gas formation among aquatic ecosystems can be compared using the manometric method. For optimizing data collection, the following experimental procedures must be adopted: experiments must be prepared under aseptic conditions, samples should be homogenized, the number of bottles must be increased, temperature must be controlled and reactors must be depressurized at regular intervals. 3) The volume of the formed gas was proportional to the initial concentration of the added source of organic matter (sucrose). 4) The gas formation processes became effective when oxygen was eliminated and were developed in a discontinuous way. 5) The rate of decay for the carbohydrates under anaerobic conditions was approximately 10 times the rate estimated when oxygen was present. 6) Under anaerobic conditions there was synchronism between the gas formation processes and the decrease of the carbohydrates content.

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RESUMO

Método Manométrico para Estimativa da Mineralização Anaeróbia em Ecossistemas Aquáticos: Aspectos Cinéticos e Metodológicos

Neste estudo discutem-se aspectos cinéticos e metodológicos relacionados com a formação de gases, decorrentes da mineralização anaeróbia de matéria orgânica em ecossistemas aquáticos. Em laboratório foram montados frascos contendo soluções de carboidratos (glicose ou sacarose). Tais soluções foram preparadas com amostras de água da represa do Monjolinho (22° 00' S e 47° 51' W). Foram determinadas as taxas de formação de gases, as temperaturas, os teores de oxigênio dissolvido e de carboidratos totais. Verificou-se que houve proporcionalidade entre concentração inicial de sacarose e volume de gases formados. A formação de gases ocorreu a partir da época em que os teores de oxigênio dissolvido encontravam-se abaixo de 0.2 mg/L. Em paralelo à formação dos gases, intensos decaimentos dos teores de glicose foram observados e tais processos apresentaram-se sincronizados.

Palavras-chave: fermentação, ecossistemas aquáticos, cinética, gases

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ESTIMATION OF THE NUMBER OF DENITRIFYING BACTERIA AND MAXIMUM RATE OF DENITRIFICATION IN ANOXIC REACTOR

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ABSTRACT

Denitrifying bacteria were enumerated (MPN) in bench scale reactor fed with synthetic sewage and nitrate, operated under controled condictions. In control conditions (without nitrate), the number of denitrifying bacteria was about $8.00x10^7$/ gVSS. At the concentrations of 10 and 50mgN-NO_3^-/L, the average was $6.00x10^7$ and $1.62x10^9$/GvSS, respectively. The maximum denitrifying rates, estimated by quantification of N_2O using the acetylene method (inhibition of reduction of N_2O to N_2), were 0.063gN/gVSS.day (10mgN-NO_3^-/L) and 0.044gN/gVSS.day (50mg N-NO_3^-/L).

Key words: denitrification, denitrifying bacteria, denitrification rate

INTRODUCTION

Denitrification has become a very important stage in wastewater treatment systems. It is an efficient method for removing nitrogen sources (mainly nitrate), contributing to mitigation of euthrophication of water bodies that receive treated effluents. In this process, NO_3^- is biologically reduced to gaseous nitrogen oxides (NO, N_2O) and, eventually to N_2 (11) under anaerobic conditions or low dissolved oxygen concentrations. This is a bacterial process, but recently, the participation of fungus which catalize the denitrification process has also been observed (10). Compared to other inorganic biotransformation processes denitrification is associated to a great diversity fo microorganisms. However, studies on the ecology of denitrifying microorganisms are scarce (11).

Most of the studies have applications in agricultural productivity. Nitrogen loss in fertilizers applied to the soil reaches 20 to 30% because of denitrification (3). For treatment of urban and other types of wastewater associated with nitrification and denitrification, different carbon sources, different types of reactors and operation conditions have been used. Denitrifying bacteria, besides using natural carbon compouds, may decompose aromatic and anthropogenic compounds (4), non-ionic surfactants (2) and chlorine-based solvents (1).

This study is a preliminary contribution to the knowledge on denitrification including estimation of the denitrifying bacteria number, isolation of bacteria groups responsible for the process and the kinetic behavior of these bacteria in anoxic reactors.

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MATERIALS AND METHODS

The experimental tests were performed with samples from an anoxic reactor fed with synthetic sewage and nitrate, at the Laboratory of Anaerobic Processes of Hydraulic and Sanitary Engineering Department of São Carlos Engineering School - USP. The bench scale reactor, made of translucent acrilic, comprised two 9,5 L volume chambers, kept at controlled temperature (28 ± 1^0 C). The chambers were operated as completely mixed reactors and were continuously fed. Chamber 1 (C1) worked as control (without nitrate) and chamber 2 (C2) received nitrate at concentrations of 10, 30 and 50 mgN-NO_3^-/L. The operational parameters were: hydraulic retention time (θh) of 12h, flow rate(Q) of 0.8 L/h and volumetric organic loading rate of 1 kg DQO/m^3.day.

Denitrifying bacteria were enumerated by the Most Probable Number (MPN) technique originally developed for soil samples and modified by Tiedje (11). This technique was adapted for liquid samples in this work. Five tubes with replicate sample volumes with decimal dilutions were incubated under anaerobic conditions, during seven days at 28 ± 1^0 C in selective media: generic nutrient media (Nutrient Broth of "Difco") and $NaNO_3$ 5mM. The number of denitrifying bacteria was expressed per gram of volatile suspended solids (VSS) (9). From the highest dilution tube presenting a positive result (absence of nitrate and production of N_2O), one type of denitrifying bacteria was isolated and kept in solid and liquid generic nutrient media with $NaNO_3$ 5mM. This purified culture was photographed by scanning electron microscopy after treatment with hexamethyldisilazane (HMDS) (8).

The kinetic study of the process was accomplished based on the quantification of N_2O as the final product of the denitrifying bacteria activity, using the acetylene blockage to the enzymatic reduction of the N_2O to N_2 (13). The samples were kept in 400 ml "reaction flasks", being 250 ml of reactive material (synthetic sewage, sludge and nitrate in the same proportions of the bench scale reactor chambers) and 150ml of gaseous phase. After flushing N_2 into the flasks, they were sealed, and 10% of the gasous phase was substituted by acetylene under the partial pressure of 10 Kpa. The flasks were incubated in the dark, in shaker at controlled temperature. The accumulated production of N_2O in the headspace was quantified by gaseous chromatography with thermal conductivity detector, column "Porapak Q" (80-100 mesh) with 2m of length and ¼ of one inch of internal diameter, for oven temperature at 40^0 C and H_2 as carrier gas. The N_2O concentration in the reaction flasks was calculated according to Tiedje (11). From the mathematical adjustment of the experimental data of the N_2O accumulated production as function of time, N_2O production maximum rates (5) were estimated. The specific denitrifying activity was estimated by dividing the maximum N_2O production rate by the VSS concentration in the flasks.

RESULTS

The number of denitrifying bacteria under control condition (C1) ranged from $1.49.10^7$ to $1.19.10^8$/g VSS, with an average of $8.00.10^7$/g VSS. In reactor C2 with 10 mg N-NO_3^-/L, the number ranged from $1.12.10^7$ to $1.08.10^9$/g VSS, with average value of $6.00.10^8$/gVSS. Still in C2 with 50 mg N-NO_3^-/L, the number ranged from $7.36.10^7$ to $3.18.10^9$ /gVSS.

For 10 mg N-NO_3^-/L, the maximum N_2O production rate of N_2O of 0.063gN/g VSS.day was achieved (Fig.1), while for 50 mg N-NO_3^-/L this rate decreased to 0.044gN/g VSS.day (Fig.2).

Fig. 3 shows denitrifying bacteria, thin rods with 0.5 to 1.0μm lenght, isolated from the chamber fed with nitrate, photographed by scanning electron microscopy with magnification of 5000 times. Identification of these bacteria is not available yet. A preliminary study showed that these bacteria do not grow in specific media for *Pseudomonas* (Agar Cetrimide of "Merck").

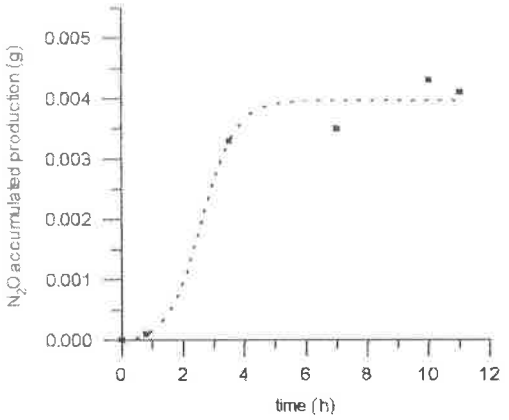

Figure 1: N_2O accumulated production as function of time (10mg $N\text{-}NO_3^-$/ L).

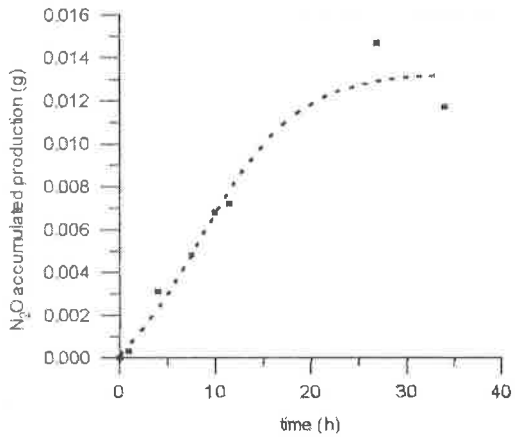

Figure 2: N_2O accumulated production as function of time (50 mg $N\text{-}NO_3^-$/ L).

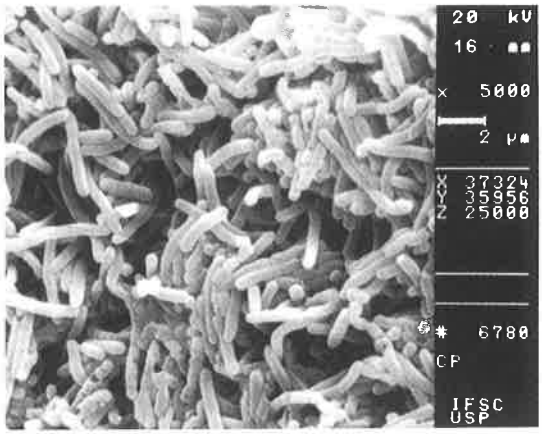

Figure 3: Denitrifying bacteria isolated from the chamber fed with nitrate. (X 5000)

DISCUSSION

In spite of the similiarity of test conditions, the number of denitrifying bacteria per gram of VSS was not the same in the three reactors studied. Under control (C1), the number ranged between 10^7 to 10^8 /g VSS. In reactor (C2), fed with 10mg and 50 mg N - NO_3^- / L, this number ranged between 10^7 to 10^9. The number of denitrifiers increased in the reactors fed with nitrate. However, the variation in the same test conditions suggests that these results are not conclusive, mainly when the two studied nitrate concentrations are compared. However, these results indicate considerable potential for denitrification occurrence. Even in control condition the number of denitrifying bacteria is high, possibly due to the biochemical diversity of these individuals, constituting a physiologically well succeeded group in nature. The majority has heterotrophic nutrition. Some utilize one-carbon compounds, others grow autotrophically using H_2, CO_2 or sulfur reduced compounds. One of bacterial groups is photosynthetic. Thus, in a reactor with diversified microbiota different kinds of denitrifying bacteria can occur, some with varied metabolism.

Very few references on the quantication of denitrifying bacteria are available. Some authors found 10^5 to 10^{10} bacteria/g (dry weight) in the freshwater sediment (6). Fig. 1 shows that the complete reduction of 10 mg $N\text{-}NO_3^-$/L to N_2O occurred after approximate-ly 10 hours. For 50 mg

$N–NO_3^-/L$, it occurred after 25 hours.

The maximum rates of nitrogen production, calculated from the adjustment of the data experimentally obtained through a sigmoidal curve model (5) were 0.063 gN/g VSS.day (10 mg $N-NO_3^-/L$) and 0.044 g N/g VSS.day (50 mg $N-NO_3^-$ /L). Reactors treating domestic sewage showed values of denitrifying rates varying between 0.03 to 0.11 gN/gVSS. day (7).

The estimated number of denitrifying bacteria and the correspondent denitrification rates obtained in this work show that the anoxic reactor assayed exhibited a considerable potencial for denitrification, when compared to the data available in the literature.

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RESUMO

Estimativa do número de bactérias desnitrificantes e da velocidade máxima de desnitrificação em reator anóxico

Foi estimado o número de bactérias desnitrificantes (NMP) em reator de bancada alimentado com esgoto sintético e nitrato, sob condições controladas. Na condição de controle tal estimativa apresentou valor médio de $8.00.10^7$ indivíduos/gSSV, com 10 e 50 mgN-NO_3^-/L a média foi de $6.00.10^7$ e $1.62.10^9$ indivíduos/gSSV, respectivamente. As velocidades máximas de desnitrificação, estimadas a partir da quantificação do N_2O empregando o método do bloqueio pelo acetileno à redução do N_2O para N_2, foram de 0.063 gN/gSSV.dia (10 mg N-NO_3^-/L) e de 0.044 g N/gSSV.dia (50 mg N- NO_3^-/L).

Palavras-chave: desnitrificação, bactérias desnitrificantes, velocidade de desnitrificação

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OXIDATIVE DISSOLUTION OF RESEARCH-GRADE MINERALS BY *THIOBACILLUS FERROOXIDANS* AND *THIOBACILLUS THIOOXIDANS*

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ABSTRACT

Solution- and solid-phase changes associated with galena (PbS) and sphalerite (ZnS) oxidation by *T. ferrooxidans* and *T. thiooxidans*, were determined. In experiments with galena, anglesite ($PbSO_4$) was detected as a solid-phase product in biotic and abiotic experiments. In *T. ferrooxidans* cultures supplemented with $FeSO_4$, jarosite [$MFe_3(SO_4)_2(OH)_6$] was also detected as a new solid phase product, whereas S^0 was not detected in the residues. In sphalerite experiments, minor amounts of S^0 accumulated in $FeSO_4$-amended sphalerite media with or without *T. ferrooxidans* or *T. thiooxidans*. Jarosite was only detected in *T. ferrooxidans* culture with $FeSO_4$. By comparison with *T. thiooxidans*, *T. ferrooxidans* was more efficient in the oxidation of galena and sphalerite.

Key words: *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans*, mineral sulfides

INTRODUCTION

The oxidation of galena (PbS) in sulfide concentrates by *Thiobacillus ferrooxidans* has been demonstrated in previous studies (7, 4). Evidence for direct bacterial oxidation of PbS is ambiguous because the concentrates have contained several other sulfide minerals which could potentially support bacterial growth and produce sulfuric acid and dissolved iron. Anglesite is the major mineral product in the residue. It has been suggested that S^0 could also be a product of galena oxidation in accordance with the following chemical reactions:

$$PbS + H_2SO_4 + 0.5O_2 \rightarrow PbSO_4 + H_2O + S^0 \quad (1)$$
$$PbS + Fe_2(SO_4)_3 \rightarrow PbSO_4 + 2FeSO_4 + S^0 \quad (2)$$

Biological oxidation of Zn-sulfides has also been previously investigated in samples of sulfide concentrates or high-grade ores (1, 3, 5, 6). Sphalerite (zinc blend; ZnS) is the most common Zn-sulfide in such materials. It has also been established that *T. ferrooxidans* can directly oxidize Zn-sulfides, but Zn solubilization is greatly enhanced by Fe^{3+}-mediated dissolution where *T. ferrooxidans* re-oxidizes Fe^{2+}. Direct oxidation is sometimes difficult to distinguish from Fe^{3+}-mediated oxidation because of the occurrence of Fe in at least minor or trace amounts in Zn-sulfides and in leach solutions. Under chemical (abiotic) leaching conditions, the surface layers of sphalerite grains become Zn-deficient as acid leaching progresses and may become enveloped

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with S^0. These surface alterations cause the leaching to become increasingly controlled by solid-state diffusion. Analytical evidence for S^0 accumulation has yet to be documented in biological leaching reactions.

The purpose of the present work was to investigate solid- and solution-phase transformations associated with the oxidation of galena and sphalerite as the sole mineral substrates by acidophilic, Fe- and S-oxidizing (*T. ferrooxidans*) and S-oxidizing (*T. thiooxidans*) bacteria. *T. ferrooxidans* and *T. thiooxidans* were used with and without additional Fe and S^0, respectively, in an effort to differentiate between indirect (active redox cycling of Fe) and direct (no re-oxidation of Fe) biological oxidation of these non-ferrous sulfide minerals.

MATERIALS AND METHODS

Mineral Samples - Galena and sphalerite were obtained from Ward's Natural Science Establishment (Rochester, NY). The samples were ground in a disc mill and sieved to 99% < 0.5 mm. No mineral impurities were detected in the galena sample by x-ray diffraction (XRD) analysis. The ZnS sample was composed of sphalerite (cubic) but also contained minor amounts of isochemical wurtzite (hexagonal).

Mineral Oxidation Experiments - *T. ferrooxidans*-I35 (8) and *T. thiooxidans*-FG01 (2) were initially adapted to grow with galena and sphalerite by successively replacing ferrous sulfate (for *T. ferrooxidans*) or sulfur (for *T. thiooxidans*) in a mineral salts solution which contained 0.4 g each of $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$ and K_2HPO_4 per liter of distilled water. The medium was acidified with H_2SO_4 to pH 2.0 and amended with either galena or sphalerite (2.5% w/v), which were equilibrated for 48 h in the mineral salts solution to allow for the stabilization to pH 2. The flasks were sterilized by autoclaving (30 min, 120°C) followed by inoculation (5% vol/vol) with *T. ferrooxidans* or *T. thiooxidans*. The experiments were carried out in 100 ml cultures in 250-ml shake flasks which were incubated at 150 rev/min and at 22± 2°C. In some experiments 30 mM $FeSO_4 \cdot 7H_2O$ or 10 g elemental S/liter was used as a supplementary energy source.

Analytical Methodology - Samples were aseptically withdrawn from the flasks at intervals for measurement of pH and redox potential (a Pt-electrode against an $Ag^0/AgCl$ reference), and for the titrimetric determination with $K_2Cr_2O_7$ of Fe^{2+} and total soluble Fe, after its reduction with Sn(II)-chloride; the concentration of Fe^{3+} was calculated as the difference between total soluble Fe and Fe^{2+}. Suspended solids were recovered by centrifugation (5000·g for 10 min), and the supernatants were preserved in 1 M HCl for subsequent analysis of Pb and Zn by atomic absorption spectrometry. The solid residues were washed with distilled water, air dried and gently ground with an agate mortar for XRD analysis. Powdered specimens were analyzed with CuKα radiation and a wide-range goniometer equipped with a diffracted-beam monochromator and a Θ compensating slit. Step scans were conducted from 10 to 70°2Θ in 0.05 °2Θ increments using a 4 sec step time (7). Relative peak heights were used as indicators of changes in the abundance of minerals in solid residues.

RESULTS AND DISCUSSION

Galena Experiments - The oxidation of galena by *T. ferrooxidans* and *T. thiooxidans* in unsupplemented media showed no net change in acidity, although there were transient pH changes during the experiment (Fig. 1A). In contrast, the pH in the abiotic control increased to around 4.0. The redox potential increased to around 500 mV in inoculated *T. ferrooxidans* cultures (Fig. 1B). The increase in redox potential was relatively slow in the *T. thiooxidans* culture. The abiotic control remained at around 300 mV. These results indicate that galena acted as a strong reductant in the leaching system.

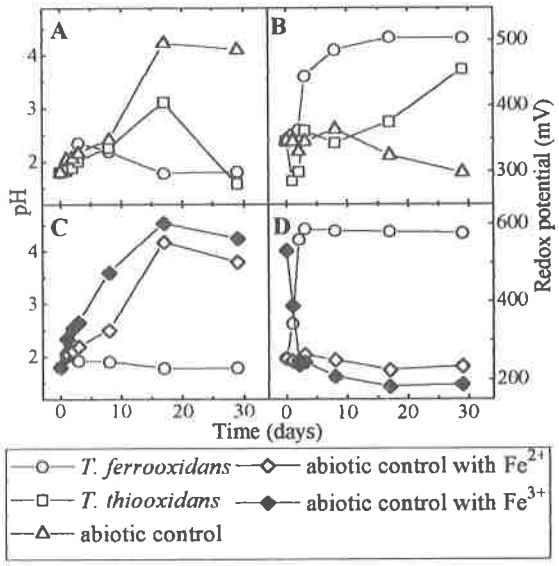

Figure 1. Changes in pH and redox potential in galena experiments in absence (A and B) or in presence (C and D) of supplemental Fe^{2+} or Fe^{3+}.

As S^0 was not identified in the solid residues, the transient pH increase in the *T. thiooxidans* culture suggested the formation of an uncharacterized S-intermediate through acid-consuming steps before final oxidation to sulfate. In the corresponding abiotic control, the oxidation to sulfate apparently did not occur to the same extent because the pH did not decrease toward the end of the time course.

The pH changes were again minor when galena was inoculated with *T. ferrooxidans* and supplemented with 30 mM ferrous sulfate (Fig. 1C). In the corresponding control flasks amended with 30 mM Fe^{2+} or Fe^{3+}, the pH values increased to around 4.0, again indicating incomplete oxidation of the S-entity of PbS to sulfate. Oxidation of Fe^{2+} by *T. ferrooxidans* caused an increase in the redox potential to 600 mV. The redox potential remained unchanged in abiotic control containing 30 mM iron added as Fe^{2+}. In the abiotic control experiment with 30 mM Fe^{3+} addition, the initial redox potential of 530 mV decreased rapidly to 200 mV as a result of the redox reaction with PbS (Fig. 1D). The low redox potential reached in the control galena supplemented with 30 mM Fe^{3+} confirmed that galena acted as a reductant and thereby completely depleted Fe^{3+} from solution.

No mineral impurities were detected in the galena sample (Fig. 2A) and anglesite ($PbSO_4$) was the only new solid phase detected by XRD analysis of leach residues in the experiments with galena as the sole energy source for *T. thiooxidans* and *T. ferrooxidans* (Fig. 2C).

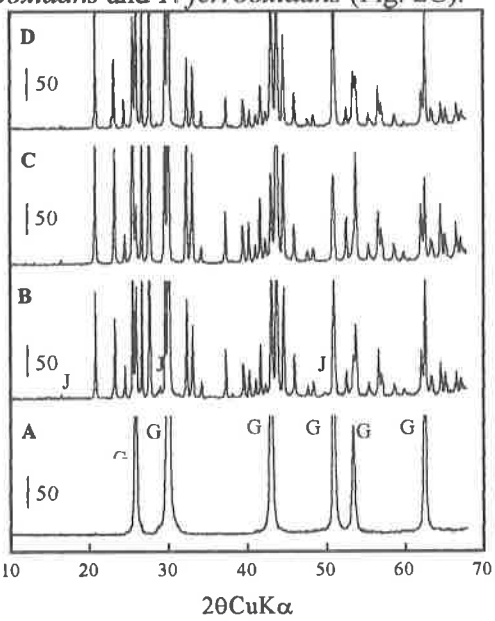

Figure 2. X-ray diffractograms of pure galena (A) and residues after 8 days in *T. ferrooxidans* cultures with Fe^{2+} (B) and 29 days without Fe^{2+} (C) and 29 days in abiotic control with Fe^{3+} (D). **G** and **J** stand for galena and jarosite. All the other peaks are anglesite. Bars indicate intensity of counts.

Additionally, jarosite was detected within 8 days from solids generated by *T. ferrooxidans* cultures grown with 30 mM ferrous sulfate (Fig. 2B) and it was not detected in any of the abiotic controls which were initially amended with Fe^{2+} or Fe^{3+} (Fig. 2D). Anglesite is a relatively insoluble compound (log K_{s0} = -7.85). Dissolved Pb concentrations in culture supernatants were in the range of 1.1-5.0 mg Pb^{2+}/liter throughout the time course in all experiments. Anglesite formation and concurrent galena dissolution were more pronounced in *T. ferrooxidans* experiments as compared with *T. thiooxidans* cultures or the abiotic controls. The relative peak intensities have indicated that the

biological leaching of galena declined toward the end of the incubation (data not shown).

This trend suggests that anglesite, and jarosite in Fe-amended cultures, may precipitate on the surface of galena particles so that further oxidation becomes diffusion limited. Elemental S was not detected in solids from any of the galena leaching experiments sampled at 0, 8, 17 and 29 days.

In view of the solid-phase products found in this work, the rate limitation in bacterial leaching systems for galena may be due to the formation of mineral surface coatings (anglesite, jarosite) that hinder the diffusion of reactants and products and thereby slow the rates of some abiotic or biological oxidation steps involving O_2 and Fe^{3+} as the available electron acceptors.

Sphalerite Experiments. The oxidation of sphalerite by *T. ferrooxidans* produced a small decrease (< 0.3 units) in pH, whereas the pH was constant in the chemical leaching system (Fig. 3A). In the presence of Fe^{2+}, the pH of the abiotic control increased slowly, while remaining more or less constant in the inoculated system; a more dramatic increase in pH was noted in the abiotic control that received Fe^{3+} (Fig. 3C). The redox potential levels were higher for the *T. ferrooxidans* cultures as compared to abiotic controls (Figs. 3B and 3D), in keeping with the presence of more oxidized S and Fe species in the bacterial cultures. *T. thiooxidans* produced similar pH and redox potential changes in unsupplemented media (Figs. 3A and 3B). In *T. thiooxidans* cultures supplemented with S^0, the final pH decreased to pH 1.1 at the end of incubation (Fig. 3C). In the corresponding abiotic control, supplemental S^0 did not enhance acid production. In the abiotic control supplemented with Fe^{3+}, the redox potencial decrease rapidly, as a result of reduction of Fe^{3+} to Fe^{2+} (Fig. 3D).

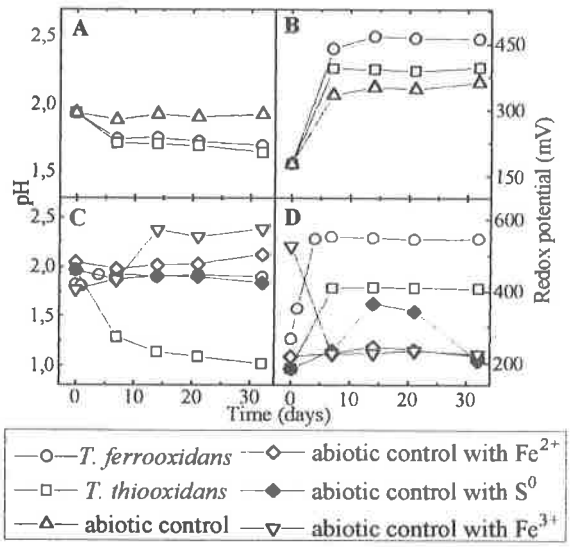

Figure 3. Changes in pH and redox potential in sphalerite experiments in absence (A and B) or in presence (C and D) of supplemental Fe^{2+} Fe^{3+} or S^0.

The release of Zn was enhanced when *T. ferrooxidans* cultures were amended with ferrous sulfate (Figs. 4A and B). This enhancement was presumably due to the Fe^{3+}-mediated oxidation of ZnS, involving re-oxidation of Fe^{2+} by *T. ferrooxidans* in addition to the direct oxidation mechanism.

The addition of Fe^{3+} in the form of sterile spent media slightly facilitated ZnS dissolution, but the effect was temporary due to the limited pool of Fe^{3+} and the absence of a biological mechanism to replenish Fe^{3+}. In *T. thiooxidans* cultures the rate and extent of Zn release were uninfluenced by S^0 addition (Figs. 4A and B), and Zn dissolution was about 25% of that achieved in *T. ferrooxidans*-inoculated experiments.

In unsupplemented *T. ferrooxidans* and *T. thiooxidans* cultures and in the respective abiotic controls, incubation for up to 32 days did not change the XRD pattern of sphalerite, and S^0 was not detected in the solid residues (data not shown).

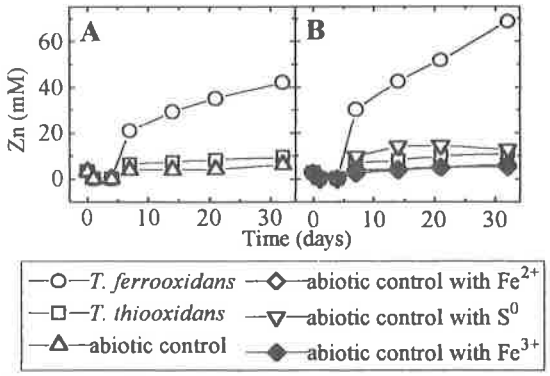

Figure 4. Changes in dissolved Zn in sphalerite experiments in absence (A) or in presence (B) of supplemental Fe^{2+}, Fe^{3+} or S^0.

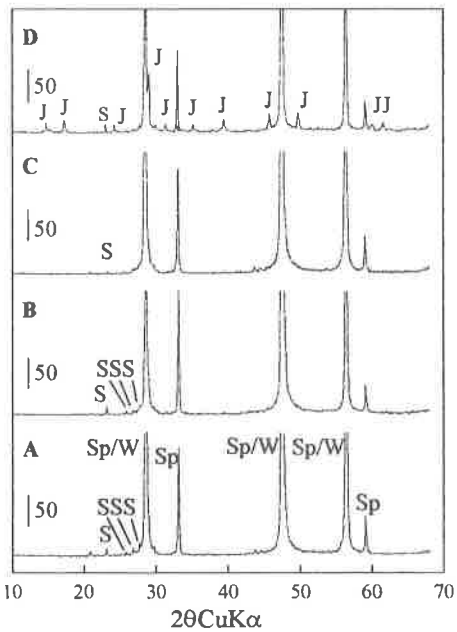

Figure 5. X-ray diffractograms of sphalerite residues after 32 days in abiotic controls with Fe^{2+} (A) or Fe^{3+} (B), and in *T. thiooxidans* culture with S^0 (C), and in *T. ferrooxidans* culture with Fe^{2+} (D). **J, Sp, S** and **W**, stand for jarosite, sphalerite, sulfur and wurtzite, respectively. Bars indicate intensity of counts.

In Fe^{2+} and Fe^{3+} supplemented abiotic controls, S^0 was present in samples leached for 32 days, suggesting a gradual accumulation over time (Figs. 5A and B). By contrast, elemental sulfur was almost depleted, in inoculated cultures supplemented with S^0 and Fe^{2+}, respectively (Figs. 5C and D), consistent with bacterial oxidation of S^0, mainly in *T. thiooxidans* culture. Jarosite, as expected was found in *T. ferrooxidans* (Fig. 5D).

The initial oxidation of ZnS to S^0 is acid-consuming and is balanced by acid-production due to S^0 oxidation. In the direct oxidation pathway, S^0 may not accumulate to detectable levels because of its concurrent oxidation.

Actual experimental results confirmed that the oxidation of ZnS by thiobacilli in unsupplemented media did not accumulate S^0. Supplemental S^0 was also consumed in the presence of *T. thiooxidans* indicating rapid depletion as an intermediate phase in ZnS oxidation. By contrast, the indirect, Fe^{3+}-mediated mechanism of ZnS oxidation in *T. ferrooxidans* cultures was coupled with S^0 accumulation. The formation of S^0 was also detected in abiotic controls that were initially supplemented with ferrous sulfate or with spent *T. ferrooxidans* culture filtrate. In Fe-amended experiments, Fe^{2+} oxidation by *T. ferrooxidans* enhanced Zn dissolution, and jarosite formation occurred exclusively in the presence of *T. ferrooxidans*. In the absence of *T. ferrooxidans*, Fe^{3+} was neither generated nor replenished, redox potential remained low, and dissolution of ZnS was relatively insignificant when compared to the inoculated system.

With these particular bacterial strains, *T. ferrooxidans* was more efficient than *T. thiooxidans* in the oxidation of galena and sphalerite.

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RESUMO

Dissolução oxidativa de sulfetos minerais por *Thiobacillus ferrooxidans* e *Thiobacillus thiooxidans*

O objetivo desse trabalho foi determinar as alterações das fases sólida e líquida de minerais sulfetados de referência, associadas com a atividade oxidativa das espécies *T. ferrooxidans* e *T. thiooxidans* nesses minerais. Os minerais sulfetados escolhidos para esse estudo foram galena (PbS) e esfalerita (ZnS). Nos ensaios com galena, anglesita ($PbSO_4$) foi detectada como um produto da fase sólida tanto nos controles como nos frascos inoculados. Nas culturas com *T. ferrooxidans* contendo $FeSO_4$ como fonte adicional de energia, jarosita [$MFe_3(SO_4)_2(OH)_6$] foi também detectada, enquanto que S^0 não foi encontrado em nenhum dos resíduos. Nos ensaios com esfalerita, pouco S^0 acumulou nos testes contendo $FeSO_4$ ou S^0 com a presença do *T. ferrooxidans* ou *T. thiooxidans*, respectivmente. Jarosita foi somente detectada nas culturas de *T. ferrooxidans* com $FeSO_4$. Os resultados revelaram que o *T. ferrooxidans* foi mais eficiente que o *T. thiooxidans* na oxidação da galena e esfalerita.

Palavras-chave: *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans*, mineral sulfides

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SPECIFIC PRODUCTIVITY AND RHEOLOGICAL CHARACTERIZATION OF BATCH AND FED-BATCH CEPHALOSPORIN C PRODUCTION BIOPROCESS

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ABSTRACT

Studies on cephalosporin C production with *Cephalosporium acremonium* ATCC 48272 in batch and fed-batch fermentations were performed utilizing starting medium containing glucose as carbon source. After its depletion a medium with sucrose as carbon source was added. In the fed-batch experiments the effect of initial glucose and sucrose concentrations on productivity was examined and rheological characterization was performed. The results obtained in the fed-batch experiments showed that the highest glucose/sucrose ratio led to higher cephalosporin C specific productivity. However, this productivity was lower than that of batch experiments. Concerning rheology, the broth presented Newtonian and pseudoplastic behavior depending on the fermentation time both for the batch and fed-batch process.

Key words: broth rheology, fungal morphology, specific productivity, fed-batch fermentation, cephalosporin C production

INTRODUCTION

Cephalosporin C, CPC, is a secondary metabolite obtained industrially with high production mutant strains of *Cephalosporium acremonium*. The study of its production is important as it is used for obtaining of semi-synthetic cephalosporins, which possess high chemotherapeutic value (11). The presence of two sources of carbon in the medium is required: glucose and sucrose. They are consumed in two different consecutive phases: cell growth and antibiotic production due to the diauxic effect presented by the microorganism (2,4,10).

Demain and Kennel (4) observed that the enzymatic complex responsible for the formation of cephalosporin C is inhibited in the presence of glucose and ammonium salts in the beginning of the bioprocess. Concurrently they observed increasing activity of this enzymatic complex after glucose exhaustion in the medium when consumption of less metabolizable sugars, like sucrose, takes place. Demain *et al.* (5) concluded that the initial glucose /sucrose concentrations of 27/36 used in the batch fermentation medium in bioprocesses

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carried out in a shaker results in higher productivity.

In aerated tanks culture broth presents non-newtonian characteristics. As filamentous growth prevails in the majority of the processes, the long, thin and branched hyphae create a tridimensional net structure which results in a very viscous broth. The effect of the broth flow properties on the reactor performance is well known, but few systematic studies on rheological properties of fermentation broths have been published and the use of rheometry in such processes is limited. This is due to the difficulties encountered in assessing rheological properties of non-homogeneous broth and consequent uncertainty in interpretation of results. The techniques usually employed allow measu-rements over a very narrow shear rate range, making it difficult to separate different types of non-newtonian broths.

Relations among biomass concentration, broth rheological properties, and other operational parameters, need to be studied (3).

Kim and Yoo (7), investigated the rheological properties of fermentation broth with *C. acremonium*, as well as the contribution of each morphological cell type on the variation of the broth apparent viscosity (μ_{ap}). They concluded that the suspension of filamentous hyphae was more viscous than that of swollen hyphae. At low shear rate the apparent viscosities of suspension broths of filamentous hyphae were very high and declined rapidly with increasing shear rate. Suspension broths of filamentous and long swollen hyphae showed more pseudoplastic behavior, while the suspension broth of short swollen hyphae approached newtonian behavior.

The procedure for broth rheology control normally done by dilution of the broth, results in a decrease in the apparent viscosity in a short time interval, therefore being , insufficient for the process control. A promising approach in rheology control may be physiological manipulation of cell growth, possibly by manipulating either the morphology of the micelial aggregates or of the surface properties of the micelium. That is, several parameters, such as nutrient concentrations and the nature of the limiting nutrient, specific growth rate and effect of the mixture quality can affect morphology and cell wall composition and hence the rheological properties of fermentation broths(8).

In this work experimental results obtained from batch and fed-batch processes, for comparative study of specific productivity and rheological characterization of the fermentation broth were presented. For the investigation of the specific productivity the initial glucose and sucrose concentrations were varied, according to the relationship proposed by Demain *et al.* (5). Rheological characterization of the fermentation broth was performed by means of a viscometer with concentric cylinders as suggested by Queiroz *et al* (9), and simultaneous assessment of cell morphology, through photomicrographs.

MATERIALS AND METHODS

Microorganism: The strain *Cephalosporium acremonium* ATCC 48272 (C-10) was utilized.

Culture Medium: In all experiments, in the germination stage, a fermentation medium was employed with pH 7.0±0,1 whose composition follows (in %): glucose (3.0); DL-metionina (0.5); oleic acid (0.15); KH_2PO_4 (0.23); K_2HPO_4 (0.58); $NH_4CH_3CO_2$ (0.88); $Fe(NH_4)_2(SO_4)_2$ $6H_2O$ (0.016); Na_2SO_4 (0.081); $MgSO_4 7H_2O$ (0.0384); $CaCl_2$ $2H_2O$ (0.008); $MnSO_4H_2O$ (0.0032); $ZnSO_4$ $7H_2O$ (0.0032); $CuSO_4$ $5H_2O$ (0.0002) and $CaCO_3$ (0.2).

For the fermentation stage, each experiment was performed with different glucose/sucrose concentration ratios, maintaining the other nutrients constant.

Fed-batch fermentation (A), with pH 7,0±0,1 was had the composition (in %): oleic acid (0.15); DL-methionine (0.5); $NH_4CH_3CO_2$ (0.88); KH_2 PO_4 (0.108); K_2HPO_4 (0.297); $Fe(NH_4)_2$ $(SO_4)_2.6H_2O$ (0.016); Na_2SO_4 (0.081); $MgSO_4$ $7H_2O$ (0.0384); $CaCl_2.2H_2O$ (0.008); $MnSO_4$ H $_2O$ (0.0032); $ZnSO_4.7H_2O$ (0.0032); $CuSO_4$ $.5H_2O$ (0.0002); $CaSO_4.2H_2O$

(0.343); glucose (8.1); sucrose (10.0) and antifoam agent.

Fed-batch fermentation (B), was identical, except for the sugars, whose concentration was (in %): glucose (2.7); sucrose (12.6).

Fed-batch fermentation (C), was the same, except for the sugars, with concentrations (in %): glucose (2.6); sucrose (18.7).

Batch fermentation (D), was also the same, except for the sugars, with concentrations (in %): glucose (2.7); sucrose (3.6).

Agar slant culture was incubated for one week at a temperature of 25°C. The germination stage and inoculum preparation took place in a shaker with a controlled temperature of 26°C, and agitation speed of 250 rpm. The germination was performed within 48 hours and the inoculum preparation within 28 hours.

The experiments were conducted in a New Brunswick 5 liters BIOFLO II bioreactor controlling pH, temperature, dissolved oxygen, agitation, and equipped with a peristaltic pump. The stirrer speed was kept between 300 and 750 rpm, and aeration maintained at 1 vvm. Dissolved oxygen was kept at 40%, pH at 6.9± 0.3 and temperature at 26°C. Feed flow rates of the medium were maintained at 0.36 mL/min, 0.24 mL/min, 0.33 mL/min for experiments A, B and C, respectively. In all experiments, a 10% v/v inocula suspension was used, with reactional volume maintained at approximately 3.8 liters.

For determination of cell mass, sugars, and the antibiotic obtained during the experiments, collected samples were centrifuged under refrigeration.

Cell concentration was determined by the weight of the dry mass in an incubator at 105°C, after 24 hours.

Concentration of sucrose was determined by the difference between total reducing sugars and glucose concentration, determined by enzimatic-colorimetric method (GOD-PAP).

CPC concentration was determined by the agar diffusion method using *Alcaligenes faecalis* ATCC 8750 as the test bacteria.

To examine cell morphology a microscope equipped with a camera was used.

For the rheological characterization of the broth a Brookfield viscometer, model LVT, with concentric cylinders was employed. Shear stress τ and shear rate γ, were calculated using equations (1) and (2), whose constants of proportionality are presented in table 1. The rheological parameters consistency index K, and flow index n, from the power law model (equation 3) were calculated by non-linear regression of the experimental values of τ and γ.

During sampling, bioreactor stirrer speeds were recorded for later estimates of broth's apparent viscosities (μ_{ap}).

$$\tau = \kappa'.T \quad (1)$$

$$\gamma = \kappa''.N \quad (2)$$

$$\tau = K.\gamma^n \quad (3)$$

Table 1: Proportionality constants k' and k", in equations (1) and (2)

	UL-adapter	21/13R	15/7R
k'	0.01068	0.0648	0.3287
k"	1.22	0.93	0.48

RESULTS AND DISCUSSION

Specific Productivity: The result of the batch experiment allowed determination of kinetic parameters of growth and substrate consumption as presented in Tables 2 and 3. These results are in agreement with those presented by other authors, i.e, the values of $Y_{X/S}$ and μ_{max} obtained in all tests are similar to those found in glucose and sucrose assimilation (1, 2, 4, 10).

The batch process (experiment D) all those used presented the largest specific productivity: 0.734 ($mg_{CPC}/(g_{cél}.h)$), utilizing the glucose/sucrose concentrations relation 27/36, the same used by Demain *et al* (5).

In the fed-batch processes, specific productivities of 0.619 ($mg_{CPC}/(g_{cell}.h)$), 0.507 ($mg_{CPC}/(g_{cell}.h)$) and 0.369 ($mg_{CPC}/(g_{cell}.h)$ were obtained with glucose/sucrose ratios of 81/100,

27/126 and 27/187 for experiments A, B and C, respectively. They show a direct relation between specific productivity reached and the glucose/sucrose concentration ratio employed for each experiment, as well as the global productivity obtained.

Table 2: Kinetic parameters of growth: μ_{max1}; μ_{max2}; $Y_{x/s1}$ e $Y_{x/s2}$. Experiments A, B e C (fed-batch), experiment D (batch).

Experiment	Fermentation time (h)	$Y_{x/s1}$	$Y_{x/s2}$	μ_{max1}	μ_{max2}	Initial glucose (g/l)	Initial sucrose (g/l)	CPC_{max} (g/l)	Specific Productivity (mg_{CPC}/g_{cell} h)
A	127	0.182	0.004	0.022	0.003	81	100	1230	0.619
B	113	0.465	0.089	0.046	0.026	27	126	824	0.507
C	96	0.600	0.427	0.059	0.003	26	187	646	0.369
D	96	0.501	0.071	0.054	0.057	27	36	1093	0.734

Table 3: Glucose, sucrose, cellular mass, CPC concentrations and specific productivity obtained through the processes.

Experiment	Fermentation time (h)	Dry mass (g/l)	Glucose (g/l)	Sucrose (g/l)	CPC (mg/l)	Specific Productivity ($mg_{CPC}/g_{cell}.h$)
A	48	13.7	36.90	16.40	144.0	0.219
A	72	14.3	11.98	25.30	348.0	0.338
A	126.5	15.7	0	7.60	1230.0	0.619
B	36	14.2	0	0.28	13.6	0.027
B	72	13.7	0	0.53	204.1	0.208
B	113	14.4	0	0.80	824.3	0.507
C	48	17.7	0.21	3.60	102.8	0.120
C	72	23.9	0.15	0.58	515.4	0.300
C	96	18.2	0.15	9.81	646.2	0.369
D	46.3	21.7	0.27	23.34	67.8	0.068
D	77.8	18.1	0	1.20	693.1	0.492
D	96	15.5	0	0.66	1093.0	0.733

Rheological Characterization: The results, presented in tables 4, 5 and figures 1 and 2, show that the calculated values of parameters n and K, indicate similar rheological behavior in both of the experiments. The broth presented newtonian and pseudoplastic behavior for distinct fermentation times. These results are in agreement with those of Gomes *et al* (6). The influence of the dilution rate on the rheological parameters under the conditions studied can be noted by the lag phases of K and μ_{ap} profiles obtained for each type of processes.

Table 4: Cell mass concentrations, bioreactor stirrer speed (N), flow behavior index (n), consistency index (K), and apparent viscosity (μ_{ap}) of experiment C.

Fermentation time (h)	Dry mass C_x(g/L)	N (rpm)	n	K (din.s^n.cm^{-2})	μ_{ap} (din.s.cm^{-2})
8.0	3.04	300	0.5570	0.1349	0.0224
11.6	4.24	300	0.4844	1.0160	0.126
24.0	5.90	325	0.3869	3.3410	0.2650
32.0	13.42	485	0.4275	5.8740	0.4390
39.0	14.64	522	0.5055	3.4140	0.3500
48.0	17.72	420	0.5328	0.8676	0.1120
62.0	18.68	394	0.4060	1.2340	0.0946
96.0	18.22	342	0.4804	0.1921	0.0219
120.0	21.44	326	0.6526	0.0921	0.0219

Table 5: Cellular mass concentrations, bioreactor stirrer speed (N), flow behavior index (n), consistency index (K) and apparent viscosity (μ_{ap}) of experiment D.

Fermentation time (h)	Dry mass C_x(g/L)	N (rpm)	n	K (din.s^n.cm^{-2})	μ_{ap} (din.s.cm^{-2})
5.1	2.72	300	0.6347	0.0623	0.0142
14.1	5.65	300	0.4195	0.2929	0.0279
17.0	4.31	300	0.4549	0.4068	0.0447
22.1	9.96	300	0.4826	0.7907	0.0972
29.0	9.04	359	0.3883	2.6951	0.2030
34.0	12.84	446	0.4027	5.5721	0.3910
38.2	16.60	605	0.5152	6.8631	0.6850
39.5	14.62	662	0.4774	9.3451	0.7440
41.0	17.96	715	0.4981	8.5181	0.7210
46.3	21.69	548	0.5914	4.8271	0.7210
52.2	20.34	452	0.5734	2.9630	0.4420
62.0	19.89	390	0.6748	0.3558	0.0875
69.0	18.70	364	0.8335	0.0509	0.0251
77.8	18.05	360	0.9065	0.0288	0.0194
96.0	15.35	300	0.6698	0.0964	0.0253

Morphology: To verify the cell morphology alteration, for each sample photomicrographs were prepared as shown in figures 3 and 4. It can be observed that the filamentous forms are the most likely to contribute to the apparent viscosity rise. This is substantiated by the observation of the cells at the end of the process, where they are predominantly encountered as artrospores. In this case, the calculated apparent viscosity decreases and almost becomes constant. The values of n tend to unity and at the same time cell concentration remains practically unchanged (tables 4 and 5).

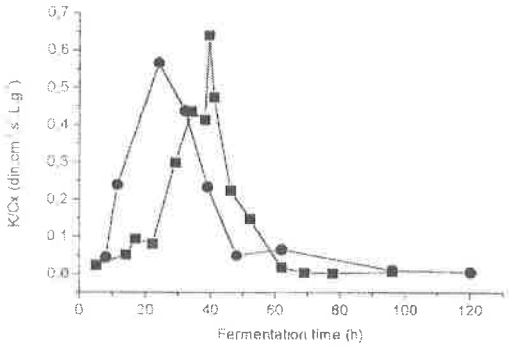

Figure 1: Parameter K/C_x as a function of time, obtained in the fermentations: (•) fed-batch and (■) batch.

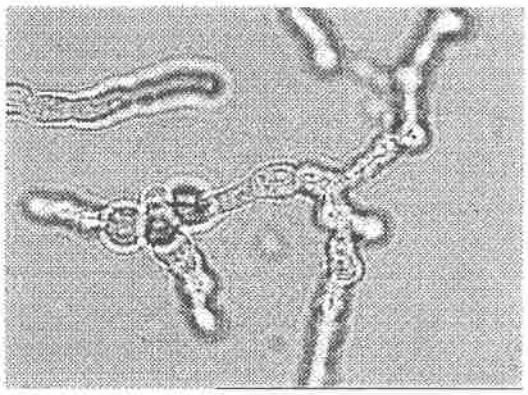

Figure 3: Photomicrographs of fungi cells: swollen hyphae filaments, occurring between 20-40 h of process (400X).

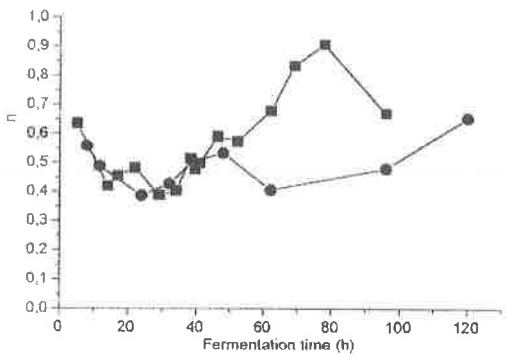

Figure 2: Parameter n as a function of time, obtained in the fermentations: (•) fed-batch and (■) batch.

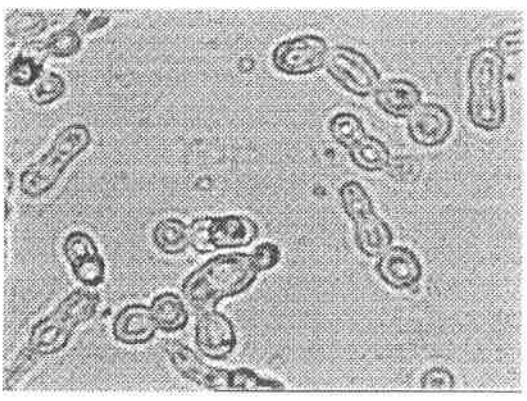

Figure 4: Photomicrographs of fungi cells: round cells occurring at the end of process after 55 h (1000X).

CONCLUSIONS

It can be concluded that the stirred tank batch process, utilizing sugar concentrations similar to those used by Demain *et al* (5), presented larger specific productivity than the results obtained in the fed-batch experiments. As for effect of the initial concentration of sugars on the medium it was observed that in the fed-batch process a direct relation was established between the glucose/sucrose ratio and specific productivity. It can also be concluded that high glucose concentrations utilized in experiment A clearly show the occurrence of catabolite repression, that resulted in a delay in the CPC formation process and consequently in the maximum production time, which was reached 30 hours later than at which maximum production time for the batch process.

From experimental determinations with the viscometer, it was concluded that the fermentative broth presented the same rheological behavior regardless of the process utilized, batch or fed-batch. That is, the broth presented both newtonian and pseudoplastic behavior at different times during the same fermentation process. A time lag occurred only in the values of K and μ_{ap} obtained in experiments C and D.

From the photomicrograph obtained, it may be stated that the cell morphology forms appear predominantly as round cells at the final stage of fermentation, when the apparent viscosity presents a decrease. This shows that the cell morphology alteration of the mold also influences the broth rheology in this fermentation process.

ACKNOWLEGMENTS

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RESUMO

Produtividade específica e caracterização reológica do bioprocesso de produção de Cefalosporina C em batelada e batelada alimentada

Ensaios em batelada alimentada foram conduzidos fazendo a alimentação do meio de fermentação com os açúcares separadamente. O processo foi iniciado com meio de cultivo contendo apenas glicose. A alimentação de meio contendo sacarose foi feita após o esgotamento da glicose. Os resultados obtidos em batelada alimentada mostraram que a maior relação de concentração glicose/sacarose, proporcionou maior produtividade específica de cefalosporina C, porém menor quando comparada com os experimentos realizados em batelada. Quanto à reologia, os caldos fermentativos apresentaram comportamento newtoniano e pseudoplástico para tempos distintos de fermentação em ambos os processos.

Palavras-chave: reologia, morfologia fúngica, produtividade específica, batelada alimentada, cefalosporina C

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SELECTION OF INDUSTRIAL COMPLEX MEDIUM FOR CEPHALOSPORIN C PRODUCTION

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ABSTRACT

Six different industrial nitrogen sources were tested in order to obtain an increase of cephalosporin C titres by *Cephalosporim acremonium* in a complex medium. Production and degradation of cephalosporin C using soybean as a nitrogen source were studied in this work too. The sources of raw material tested were: casein, lactalbumin, soybean meal, corn gluten and cottonseed. Fermentation occurred in 250ml fermentor flasks at 28 °C on a rotatory shaker at 250rpm. The raw material were added in a concentracion of 4 g/L as total nitrogen. The highest cephalosporin C concentration was obtained when soybean was used as nitrogen source. The kinetic data showed than maximum cephalosporin C production was obtained when the fermentation reached 144h (0.93g/L). At the end of fermentation the cephalosporin C concentration dropped to 0.39g/L.

Key words: Cephalosporin C, *Cephalosporium acremonium,* industrial fermentation media

INTRODUCTION

The cephalosporins are included in a large group of β - lactams antibiotics produced by microorganisms that include penicillin, norcardicins and thienemycins (16).The fungus *Cephalosporium acremonium* was the first microorganism associated with the cephalosporin C production. It was first isolated from sea water near sewerage in Sardena by Giusepp and Brotzu (13).

Since this antibiotic was produced for the first in industrial scale, efforts have been made in order to increase its yield. A way of doing this includes more productive strains (4, 10,15) as well as modified compositions in culture media (6). Different raw materials have also been used as fermentation substrate in order to increase the cephalosporin C level.

Nowadays the community of researches has the C-10 strain of *C. acremonium* available to its work. This microorganism is descendent from CW-19 strain, which is a low industrial producer of cephaloporin C (12).

In this work different nitrogen sources were tested as fermentation substrata to increase the cephaloporin C production by *C. acremonium* (strain C-10). Production and degradation of cephalosporin C using soybean as a nitrogen

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source were studied in this work. All the raw material tested is for industrial use.

MATERIALS AND METHODS

Strain - *C. acremonium* strain C10 from American Type Culture (ATCC 48272). This culture was obtained in a criovial from the culture collection of Fundação Tropical de Pesquisas (Brazil).

Raw Material - (1) HY-SOY® (lot No M010412), (2) EDAMIN® S (lot No M010322), (3) N-Z AMINE® A (lot No MRP503680), (4) N-Z AMINE® As (lot NoMO10335), (5) Hydrolyze Corn Gluten (lot No 9NA04A) and (6) Hydrolyze Cottonseed (lot No 1NG27) from QUEST INTERNATIONAL SHEFFIELD PRODUCTS division were used as nitrogen sources. The sources of each raw material are: papain digest of soybean meal, enzymatic digest of lactalbumin, pancreatic digest of casein, pancreatic digest of casein, enzymatic digest of corngluten and enzymatic digest of cottonseed respectively.

Culture Conditions - 0.2 ml from frozen (-20°C) culture was thawed and used to inoculate a seed medium. The seed medium contained: 25g/L of glucose; 5g/L of yeast extract; 3g/L of beef extract; 10g/L of tryptone; 1g/L of K_2HPO_4 ; 1g/L of KH_2PO_4 and 10 ml of salts solution (sodium sulfate 15g/L; magnesium sulfate 3,2g/L; calcium chloride 0,8g/L; manganese sulfate 0,08g/L; zinc sulfate 0,6g/L and cupric sulfate 0,04g/L) The seed was propagated in 250ml Erlenmeyer flasks containing 20 ml of medium for 3 days at 28°C on a rotatory shaker at 250rpm. The medium used for cephalosporin C production was a modification of Demain *et al.* (3) (36g/L of sucrose; 27g/L of glucose; 1,5g/L of soybean oil; 1,08g/L of KH_2PO_4, 2,0g/L of calcium carbonate; 0,16g/L of ferrous sulfate; 2g/L of KH_2PO_4 and 50 ml/l of salts solution (sodium sulfate 15g/L; magnesium sulfate 3,2g/L; calcium chloride 0,8g/L; manganese sulfate 0,08g/L; zinc sulfate 0,6g/L and cupric sulfate 0,04g/L) added to 4g/L of total nitrogen to each raw material tested. The fermentation was carried out in 250 ml Erlenmeyer flasks containing 20 ml of medium and 10% of inoculum. The fermentation to select the industrial protein source lasted 96 h at 28°C on a rotatory shaker at 250rpm. Samples were harvested at 72 and 96 hours. Each sample was made in duplicate. The fermentation to investigated the production and degradation of cephalosporin C lasted 216 hours at 28^0 C on a rotatory shaker at 200 rpm. Samples were harvested from 72 thru 216 hours (every 24 hours). Each sample was made in duplicate.

Antibiotic Assay - Cephalosporin C was determined by high-performance liquid chromatography (HPLC) using Hewlett-Packard pump model 1050, Rheodyne injector; UV detector Waters model 484 at 254nm and integrator Hewlett - Packard model 3396. Operational conditions were: mobile phase 1,36% of KH_2PO_4 pH=6,0 (set with KOH)/ CH_3CN 100/3 (v/v); flow rate: 1ml/m; injection volume was 20µl; Brownlee ODS reverse phase column (250x4,6mn particle size 5µ).

Sugars Assays - Sugars were determined by high-performance liquid chromatography (HPLC) using Hewlett-Packard pump model 1050, Rheodyne injector; R.I. detector Waters model R-401 and integrator Hewlett -Packard model 3396. Operations conditions were: mobile phase 100% water; flow rate: 0,6ml/m; injection volume was 20µl; Biorad Aminex HPX - 87C column at 85 °C (300x7,8mn particle size 5µ).

Two steps were used in this test. First of all glucose and fructose were determined. Then, the enzymatic hydrolysis was made in these samples using INVERTINA® by Merck and the same sugars were analyzed again in order to determined the sucrose present in the fermentation broth.

Dry Mass - 10 ml portions of fermentation broth were centrifugated at 6000 rpm for 10 minutes. The pellet was washed with distilled water and centrifugated again. This operation was done twice. The pellet was dried in an oven (80 ±5°C) to constant weight.

RESULTS AND DISCUSSION

Selection of Protein Source - The results obtained in this test are in Table 1 and Figure 1 .

They show that the raw materials that obtained the best cephalosporin C concentration at 72 hours of fermentation were 1-HYSOY® (0.21g/L), 6-Cottonseed (0.19g/L), 2-EDAMIN® 'S (0.11g/L), 3-N-Z-AMINE® A (0.08g/L), 5-Corn gluten (0.07g/L) and 4-N-Z-AMINE® AS (the antibiotic was not detected this time). At the end of fermentation 1-HYSOY® (0.63g/L) was the raw material that produced the highest cephalosporin C titres followed by 6-Cottonseed (0.58g/L), 2-EDAMIN®'S (0.27g/L), 4-N-Z-AMINE® AS (0.24g/L), 5-Corn gluten (0.21g/L) and 3-N-Z-AMINE® A (0.20g/L).

For all substrata tested, high sucrose concentrations were detected at the end of fermentation.

The maximum dry mass was produced by Cottonseed (49.8g/L) and Corn gluten (36.9g/L).

Table 1: Results in g/L to dry mass, cephalosporin C, and sucrose for 72 and 96 hours of fermentation.

Raw material	A 72h	A 96h	B 72h	B 96h	C 96h
1	35.9	32.4	0.21	0.63	29.9
2	21.3	26.7	0.11	0.27	35.2
3	13.7	23.0	0.08	0.20	36.1
4	19.6	23.1	n.d.**	0.24	35.8
5	30.3	36.9	0.07	0.21	36.0
6	45.1	49.8	0.19	0.58	36.9

A - dry mass (g/L)
B - cephalosporin C (g/L)
C - sucrose (g/L)
** n.d. = not detect

Literature (1,2,8,9) reports that methionine stimulates cephalosporin C production. The amino acid distribution of the substrata tested in this work show that the highest methionine concentration is in N-Z-AMINE® A. The methionine concentration in the fermentation medium was 816mg/L for this raw material. However, the N-Z-AMINE® A did not obtain the best cephalosporin C titres in this test. The maximum cephalosporin C concentration was obtained when HYSOY® was used as nitrogen substrate. The concentration of this amino acid in the HYSOY® medium is of 416mg/g. The studies that mention methionine as a cephalosporin C stimulator, use this amino acid in medium composition in its free form. In this work the methionine added to the fermentation media was in a protein form.

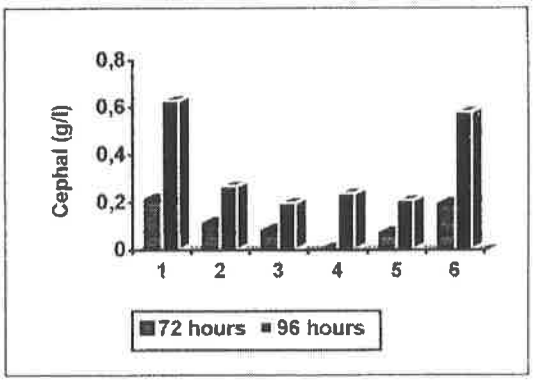

Figure 1: Cephalosporin C concentration obtained at 72 and 96 hours of fermentation using 1, 2, 3, 4 5, and 6 as nitrogen source

The high sucrose levels at the end of fermentation indicate that cephalosporin C could be increased if the fermentation time were expanded. Since this sugar is slowly assimilated by the microorganism, it is an excellent carbon source for cephalosporin C production (7).

The time expansion necessary to end up with sucrose would be useful to check if it is possible to obtain higher antibiotic concentration using protein sources that contain large methionine concentration.

The substrata that produced the highest dry mass were Cottonseed (49.8g/L) followed by Corn gluten (36.9g/L). As it is evident that there is a relationship between cephalosporin C and the dry mass produced, the insolubility of these two substrata can account for the dry cell over estimation.

Production and Degradation of Cephalosporin C- The profile of cephalosporin C production and degradation, sugars consume, dry mass produced are in Table 2 and Figure 2.

The results show that the maximum cephalosporin C production was reached at 144h of fermentation. From then on the cephalosporin C began to be degraded. The glucose exhausted in 96 hours. From the on the antibiotic production began. The sucrose exhaustion coincides with the antibiotic degradation. The dry cell raised its maximum production (35g/L) in parallel with the maximum cephalosporin C production.

Regarding the pH, it increased from 6.0 to 8.7. Probably because of the cellular death during the antibiotic production.

The maximum cephalosporin C production was obtained at 144 hours of fermentation. The concentration reached 0.93g/L. From then on (168hours) the cephalosporin C concentration started to decrease. It dropped to 0.39g/L. The fermentation time for the maximum production can change due to the raw material. Data that were not published show that when hydrolyzed casein (N-Z-AMINE ® A) is used as a substrate this fermentation time increases to 192 hours.

Table 2: Results in g/L of sucrose, glucose, frutose, dry mass and cephalosporin C obtained for different fermentation time

Time (hours)	1	2	3	4	5	6
0	0	27.0	2.0	30.0	0	6.0
72	0.01	11.7	2.2	34.9	11.7	6.5
96	0.18	<0.1	2.4	24.8	28.1	6.6
120	0.60	<0.1	0.8	26.3	32.7	7.9
144	0.93	<0.1	0.5	15.6	35.0	8.0
168	0.8	<0.1	0.5	<0.2	35.0	8.3
192	0.39	<0.1	0.9	<0.2	32.8	8.6
216	0.39	<0.1	0.9	<0.2	28.2	8.7

1 - Cephalosporin C (g/L) 4 - Sucrose (g/L)
2 - Glucose (g/L) 5 - Dry mass (g/L)
3 - Frutose (g/L) 6 - pH

The degradation of cephalosporin C in the fermentation broth has been reported (5,14). The chromatogram shows that when cephalosporin C decreased, another product was present in the fermentation broth As the concentration of this new product increased the cephalosporin C decreased. However, this new product was not recognize. Published works (14) suggest that the antibiotic loss happens through a chemical breakdown, since cephalosporin C disappears even when the sample is stored in a buffer solution. This chemical breakdown depends on temperature and pH. Some works even report (5), that the cephalosporin C degradation occurs through enzymatic hydrolysis. This extracelular enzyme is produced by the strains of *C. acremomium* itself and it is able to hydrolyze cephalosporin C to deacetylcephalosporin C. The beginning of cephalosporin C degradation happens in parallel with the carbon source exhaustion. It could be suggested that the antibiotic is probably being used as a carbon source by the microorganism.

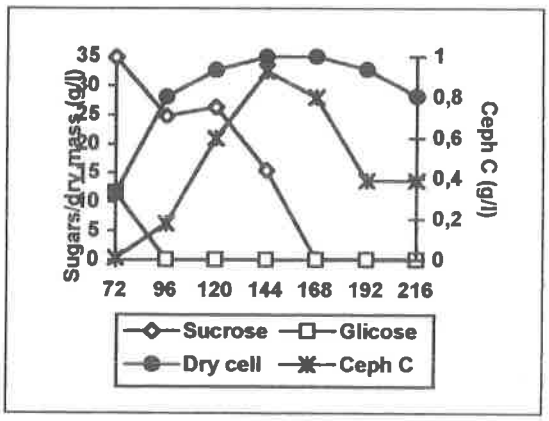

Figure 2: Profile of cephalosporin C, sucrose, glucose and dry mass concentration (g/L) during the fermentation time.

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RESUMO

Seleção de meio de cultivo industrial complexo para produção de cefalosporina C

Seis diferentes fontes de proteínas (caseína, lactoalbumina, farinha de soja, glutén de milho e semente de algodão) foram testadas na composição de meio de cultivo industrial para produção de cefalosporina C. Foi feito ainda um estudo relativo a produção e degradação da cefalosporina C quando farinha de soja é utilizada como fonte de proteína. Os ensaios foram realizados em frascos de 250 ml a 28 ^{o}C sob agitação de 250rpm. As matérias-primas foram adicionadas em concentração de 4g/L de nitrogênio total. A maior concentração de cefalosporina C foi obtida quando farinha de soja foi utilizada na composição do meio (0,63g/L). Os dados cinéticos em relação a produção e degradação de cefalosporina C quando farinha de soja foi utilizada como fonte de proteína mostraram que o pico de produção do antibiótico foi alcaçada com 144 horas de fermentação (0,93g/L). No final da fermentação (216 h) a concentração de cefalosporina C no meio de fermentação diminui para 0,39g/L.

Palavras-chaves: Cefalosporina C, *Cephalosporium acremonium*, meios de cultivo industriais.

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