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Article

Enzymatic Hydrolysis of Cassava Starch for Production of Bioethanol with a Colombian Wild Yeast Strain

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A hidrólise enzimática do amido de mandioca para produção de xaropes de glucose foi avaliada usando alfa-amilase de *Bacillus licheniformis* e glucoamilase de *Aspergillus níger*. Também, uma mistura enzimática composta de α-amylase de *Aspergillus kawachi* e glucoamilase de *Aspergillus níger* foi testada. As condições da enzima para a hidrólise do amido foram otimizadas por um planejamento fatorial experimental ($3^3 \times 2$) usando como variáveis a concentração do substrato, a relação enzima/substrato e o tempo de reação. As condições ótimas de reação com 100 g de amido *per* L foram: α-amilase pH 5,0, 80 °C e 130,5 U g^{-1} de amido; glucoamilase pH 4,5, 70 °C e 81,5 U g^{-1} de amido. Adicionalmente, as condições ótimas da mistura enzimática foram pH 4,5, 46 °C e 16,4 U g^{-1} de amido. Finalmente, a produção de álcool usando xaropes de glucose a partir do amido hidrolisado enzimaticamente foi realizada usando uma cepa selvagem de *Candida sp* isolada do caldo de cana de açúcar, obtendo produtividades em etanol volumétrico em torno de 1,8-3,2 g $L^{-1}h^{-1}$.

Enzymatic hydrolysis of cassava starch for producing glucose syrups was evaluated using alpha-amylase from *Bacillus licheniformis* and glucoamylase from *Aspergillus niger*. Moreover, an enzyme mixture of α-amylase from *Aspergillus kawachi* and glucoamylase from *Aspergillus niger* was tested. Enzyme conditions for starch hydrolysis were optimized by a factorial experimental design ($3^3 \times 2$) using as variables substrate concentration, enzyme/substrate ratio and time reaction. Optimal enzyme reactions with 100 g of starch *per* L were: α-amylase at pH 5.0, 80 °C and enzyme dosage of 130.5 U g^{-1} of starch; and glucoamylase, pH 4.5, 70 °C and enzyme dosage of 81.5 U g^{-1} of starch. Additionally, optimal conditions for the enzymatic mixture were pH 4.5, 46 °C, and enzyme dosage of 16.4 U g^{-1} of starch. Finally, alcohol production using glucose syrups from enzymatically-hydrolyzed starch was carried out with a wild strain of *Candida sp* isolated from sugar cane juice, obtaining volumetric ethanol productivities around 1.8-3.2 g $L^{-1}h^{-1}$.

Keywords: cassava starch, enzymatic hydrolysis, amylases, bioethanol

Introduction

Increasing on energetic requirements and atmospheric contamination by combustion gases, has opened searching for new, safe, effective and more accessible energy sources. For this aim, biofuels production from different agricultural sources, such as agroindustrial by-products, vegetable materials as woods, among others, has recently started-up.[1,2] Biofuels can be produced by many different types of substrates. Among these, cassava (*Manihot esculenta* Crantz), a plant with high starch content, is considered a cheap, abundant and renewable resource for production of fermentable glucose syrups and dextrins. Moreover, it is easily produced in tropical and sub-tropical zones, mainly in Asia, South-America and South-Africa.[3]

For obtaining glucose from cassava starch, an amylose/amylopectin ratio of 18/82% is hydrolyzed by an amylolytic enzymatic complex.[4-6] These conventional processes are carried out in three steps: gelatinization, liquefaction and saccharification. Gelatinization is an important step where starch grains are heated with excess of water to increase amylopectin amorphous region and enzyme accessibility.[7-9]

On the other hand, liquefaction is carried out by amylases that hydrolyze the chemical bond α-(1-4) of starch, producing dextrin, maltose, maltotriose and maltopentoses with a dextrose equivalent (DE) below 30. This enzyme processing of starch allows a rapid reduction in the viscosity of the solution.[10,11] Amylolyltic enzymes can be obtained from different sources. Among these, we can find thermostable bacterial enzymes from *Bacillus licheniformis* or *B. amyloliquefaciens,* whose are suited for liquefaction, because this step is performed at high temperatures (80-110 °C). Finally, saccharification

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process of partially hydrolyzed starch is carried out at lower temperatures (60-70 ºC). In this case are used enzymes such as glucoamylase (amyloglucosidase) obtained from *Aspergillus niger* or *Rhizopus sp.* These enzymes hydrolyze the chemical bonds α-(1-4) and α-(1-6), to obtain products such as maltose or D-glucose syrups with DE around 40 and 96, respectively.[1,10-12]

These amylolytic enzymes commonly are used in soluble solutions and they can be susceptible to inhibition for both reaction substrates and/or products. For this reason, identification and optimization of reaction conditions that affect enzymatic activity could improve economic and technological feasibility of this bioprocess. Among these different conditions are: temperature, pH, reaction time, enzyme concentration, viscosity, mixing rates, ionic strength, calcium concentration, etc.[11,15,16] Moreover, native starch is little soluble in water and resistant to hydrolysis at mild conditions. For this reason, it needs to carry out hydrothermal process for the gelatinization.[14] However, a high energetic requirement for this process increases economical production costs of starch hydrolysis.[8] In this work, we evaluated different amylolytic enzymes for obtaining the best conditions for hydrolysis of cassava starch, aimed to production of fermentable glucose syrups.

Experimental

Cassava starch and amylolytic enzymes

Cassava starch was obtained from a Starch Flour Plant-Manzanarez Ltda. Co. (Bucaramanga, Colombia). This starch product had a moisture content of 7 and 93% of starch. Moisture content in the cassava starch was determined by drying a starch sample to constant weight. Enzymes used in this study were the following: Liquozyme® SC DS, Spirizyme® Fuel from Novozymes, and Stargen™ 001 from Genecor International. These amylases are specialized for hydrolyzing starch from cereals such as corn and wheat.

Pre-treatment of cassava starch

Starch was dissolved in water or 0.016 mol L^{-1} sodium acetate buffer, pH 4.0. This starch solution was heated at 66 ºC [17] under mechanical shaking 390 rpm by 30 min.

Enzymatic hydrolysis of cassava starch

Starch hydrolysis was performed by two different methodologies. In the first one, it was carried out in two consecutive steps: liquefaction and saccharification. This procedure was performed in a batch reactor stirred at 390 rpm. In the liquefaction step, starch was gelatinized by thermal treatment and treated with alpha-amylase (Liquozyme® SC DS de Novozymes) for obtaining dextrins from cassava starch. Dextrins were determined as dextrose equivalent (DE) (equation 1) and reducing sugars, and reported in terms of units of enzymatic activity U (defined as 1 μmol of reducing sugar released *per* min), using the 3,5-di-nitro-salicylic acid (DNS) colorimetric method.[14] The saccharification step was carried out with glucoamylase (Spirizyme® Fuel from Novozymes) producing glucose syrups from dextrins obtained in the previous liquefaction step. Additionally, bioprocess efficiency was determined quantifying glucose concentration by spectrophotometry using a glucose oxidase method (Glucose Kit, BioSystems®, S.A).[19,20]

In the second methodology, a simultaneous liquefaction and saccharification starch hydrolysis was performed in batch mode. In this case, it was used non-gelatinized starch, adding an enzymatic mixture of α-amylase and glucoamilase (Stargen™ 001) and the same reaction conditions described previously.

$$\% \text{ DE} = \frac{\text{g reducing sugar expressed as gluose}}{\text{g dry solid weight}} \times 100 \quad (1)$$

Effect of temperature on enzymatic activity of amylases

The enzymatic activity was determined for each amylolytic enzyme at different temperatures. Liquozyme® SC DS and Spirizyme® Fuel were evaluated at 66, 70 and 80 °C, while Stargen™ 001 was determined at 30, 47 and 56 °C. Periodically, samples (1 mL) from enzyme reactions were withdrawn for determination of reducing sugars and quantification of DE.

Effect of pH and substrate concentration on enzymatic activity of amylases

The influence of pH on enzymatic activity was determined in a range of 3.0-6.0. Enzymatic activity was tested using substrate concentrations in the range of 100-300 g L^{-1}, determining enzyme activity and DE parameters as previously described.

Optimization of cassava hydrolysis

Once reaction conditions for enzymatic hydrolysis of cassava starch were determined, starch hydrolysis using non-diluted enzymes and high substrate concentrations

was evaluated. In this case, enzymatic hydrolysis was performed at constant pH and temperature conditions previously established for these enzymes,[8,23] similar values for these parameters to those reported in the technical sheet for these enzymes.[21,22,24] An experimental design of 3^3 using 2 measurements by run (see Table 1) was carried out for optimization of saccharification process for analyzing and validating the most important variables affecting this enzymatic process.

The experimental design for three variables, including enzyme/substrate, substrate concentration and time at three levels (low (–1), medium (0) and high (1)) (Table 1) were used for screening based on our previously study. The total number of experiments to be performed was $(n^k)r$, where n is the number of levels, k is the number of variables, and r is the number of replicates for the runs obtaining a total of $(27) \times 2 = 54$ experiments. To avoid bias, the 54 runs were performed in a totally random order. Finally, the statistical significance was determined by *P*-value, and the proportion of variance explained by the model obtained was given by the multiple coefficient of determination, R^2.

Table 1. Matrix for the experimental design of saccharification process of cassava starch using spirizyme fuel from Novozymes

Parameter	Symbol	Level		
		–1	0	1
Enzyme/Substrate × 10^3 / (U g^{-1} of starch)	X_1	1.9	3.9	5.9
Substrate / (g L^{-1})	X_2	200	300	400
time / min	X_3	15	30	45

Culture medium and yeast fermentation conditions

Inoculum was constituted by wild *Candida sp* yeast pre-cultured for 12 h at 37 ºC and under microaerophilic conditions (without shaking), using a liquid medium solution YPG, containing glucose (150 g L^{-1}), peptone and yeast extract (10 g L^{-1}). Afterwards, a fermentation medium YG containing glucose (150 g L^{-1}), yeast extract (10 g L^{-1}) was used for ethanol production.

Optimization of enzymatic saccharification process of cassava starch and ethanol production by wild yeasts strain

For these experiences, it was carried out starch hydrolysis at optimal conditions, and hydrolyzed starch containing fermentable sugars was used as carbon source for ethanol production by a native strain of *Candida sp.* Culture medium was inoculated with 0.3 g DCW L^{-1} of wild yeast, working at 0.2 mol L^{-1} sodium acetate buffer, pH 5, 35 ºC and 120 rpm of stirring, using a Shaker Max Q Mini 4450.

Ethanol production was performed by different methodologies, working with 250 g of starch *per* L as carbon source for ethanol fermentation. In the first one, starch hydrolysis and ethanol fermentation were carried out in consecutive steps, using 9.3×10^3 U g^{-1} of starch of α-amylase Liquozyme® SC DS for starch hydrolysis and 3.7×10^3 U g^{-1} of starch of the glucoamylase Spirizyme® fuel from Novozymes. In the second one, a simultaneous saccharification and fermentation (SSF) process was performed adding 39.3 U g^{-1} of starch of an enzymatic mixture of α-amylase and glucoamilase (Stargen™ 001) from Genecor. At different times (7, 20, 30 and 45 h of fermentation), samples were withdrawn from the fermentation broth. The samples were centrifuged at 10.000 g for 15 min. Supernantants were used for determination of ethanol, reducing sugars and biomass concentrations. Ethanol was determined by headspace-gas chromatography (HS-GC) using a gas chromatograph-Hewlett Packard 5890 according to Hailong *et al.*[25] Biomass concentration was determined by dry cell weight (DCW) methodology. Samples from fermentation broths were withdrawn at different times and centrifuged at 10.000 g and subsequently washed with distilled water 3 times (3×). Subsequently were dried at 105 °C until to obtain constant weight. Alternatively, dilutions from fermentation broths (in exponential phase) were made for determination of cell concentration by turbidimetry at 650 nm and correlated with in g DCW L^{-1} determinations.

Results and Discussion

Enzymatic hydrolysis of cassava starch

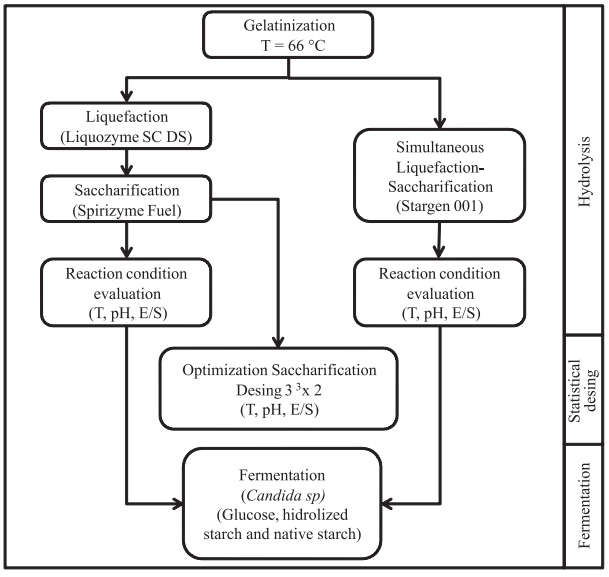

Figure 1. The schematic flow diagram of the enzymatic hydrolysis procedure.

Effect of enzyme/substrate ratio on enzymatic hydrolysis

In this study, different enzyme/substrate ratios (E/S) were tested. Higher DE values were used as criterion for selecting an enzyme for hydrolysis of cassava starch. Best results were obtained with a ratio of 130.5 U g^{-1} of starch for enzyme from Novozymes (Figure 2A) 81.6 U g^{-1} of starch for saccharification process (Figure 2B) and 16.4 U g^{-1} of starch for enzymatic mixture from Genecor (Figure 2C). The enzyme reaction conditions were the following: liquefaction and saccharification as described in pre-treatment starch, while in simultaneous liquefaction-saccharification were carried out at pH 4.0 and 37 °C. For both process we used a starch concentration of 100 g L^{-1}. Additionally, at higher E/S ratios, a both increase on released DE became and reaction rates were achieved. Nevertheless, E/S ratios reached in this study were lower than reported from providers[21,22,24] and Zhao *et al.*[23]

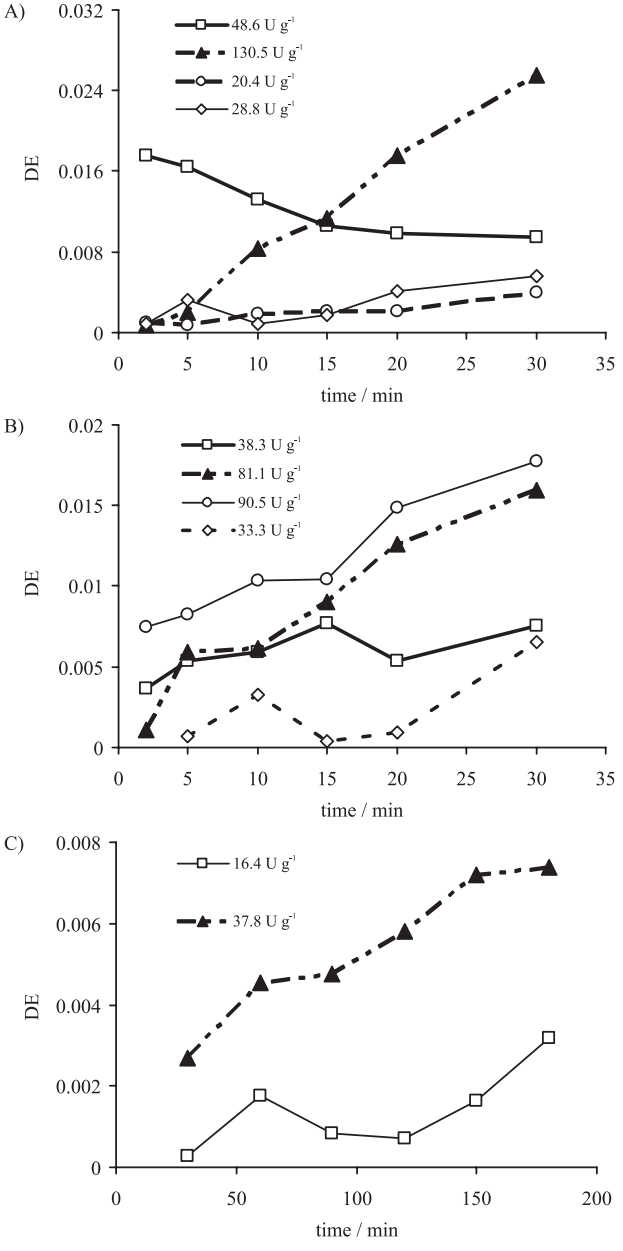

Figure 2. Dextrose equivalent production at different enzyme/substrate ratios during enzymatic hydrolysis of cassava starch.

Effect of temperature on enzymatic activity

This study was achieved at the same reaction conditions used to study effect of enzyme/substrate ratio on enzymatic hydrolysis. In Figure 3 are shown results of effect of temperature on different amylolytic enzymes hydrolyzing cassava starch. Optimal reaction temperatures varied and were dependent on type of enzyme. In general, initial hydrolysis rate was increased with temperature, obtaining optimal hydrolysis rates for Liquozyme® SC DS, Spirizyme® Fuel (amylase and glucoamylase from Novozymes) at 80 and 70 °C, respectively. These results are according to results obtained by Zhao *et al.*[23] On the other hand, we obtained an optimal temperature of 47 °C for Stargen™ 001, achieving similar data to those reported by Wang *et al.* [26] However, starch hydrolysis was decreased at high temperatures, due to thermal inactivation of amylolytic enzymes (Wu and Miao,[27] Morales *et al.*[12]).

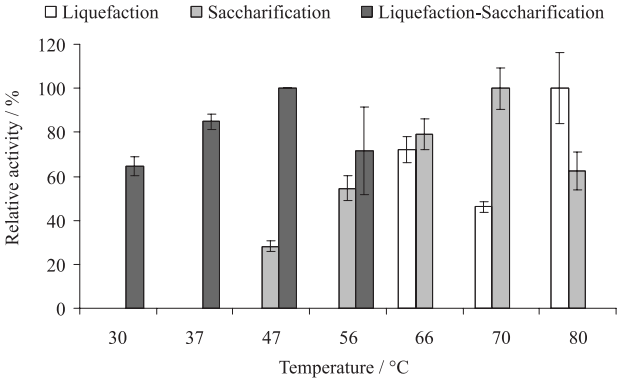

Figure 3. Effect of temperature on enzyme activity during enzymatic hydrolysis of cassava starch.

Effect of pH on enzymatic activity

In these studies, we worked in range of pH values with high catalytic activity.[13] This study was achieved at the same reaction conditions used to study effect of enzyme/substrate ratio on enzymatic hydrolysis. In Figure 4 are shown results of pH effect on enzymatic activity. In this figure we can see that optimal pH were 5.0, 4.5 and 4.0, for liquefaction, saccharification and simultaneous liquefaction and saccharification, respectively. These values were in the range to those found by Zhao *et al.*[23] for enzymes from

Novozymes, and Stargen™ 001 obtained by Wang and co-workers.[23,26]

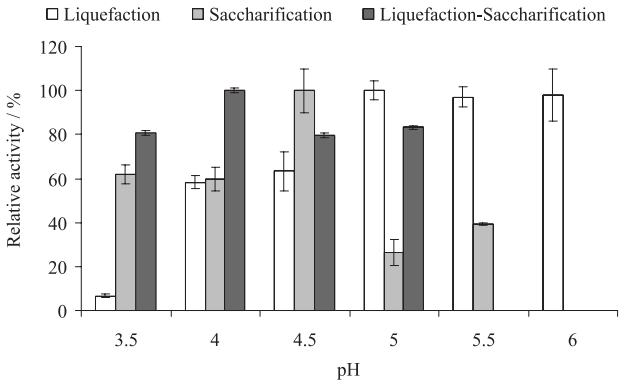

Figure 4. Effect of pH on enzyme activity during the starch hydrolytic bioprocess.

Effect of substrate concentration

In Figure 5 is shown the effect of substrate concentration on enzymatic activity at optimal pH and temperature determined in previous studies. In this figure is observed that increasing in substrate concentration produced a decrease in enzymatic activity on starch hydrolysis. This effect could be due to diffusional constraints during the enzymatic hydrolysis of starch,[16] caused mainly by increasing in viscosity by gelatinized starch. Similar observations have been reported by Baks *et al.*,[28] working with high starch concentrations (650 g of strach L^{-1}). On the other hand, it is possible that enzymatic inhibition of amylases could be produced by high substrate concentrations.

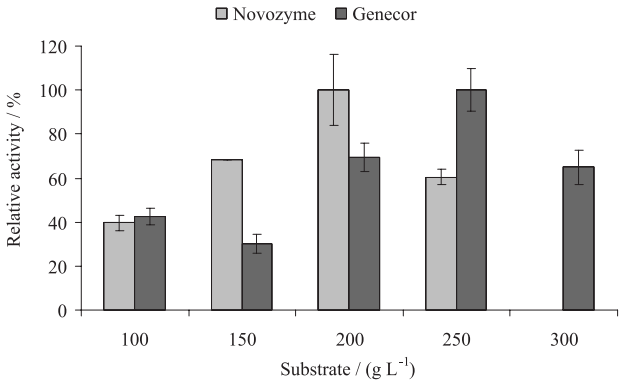

Figure 5. Effect of substrate concentration on enzyme activity.

For this reason, it was selected 200 g of starch L^{-1} of cassava starch for enzymes from Novozymes and 250 g of starch *per* L) for the enzyme from Genecor.[24,25] However, these enzymes are not saturated at these starch concentrations. On the other hand, stirring rate was kept at 390 rpm during all starch hydrolysis process, and it was not detrimental for enzyme stability,[9] allowing god mass transfer and mixing performances for enzymatic starch hydrolysis.[16]

Optimization of starch hydrolysis by factorial design

Analysis of variance (Table 1) indicates that the second-order polynomial model (equation 2) was significant and adequate to represent the actual relationship between the response (glucose released by enzymatic starch hydrolysis in units of concentration (g L^{-1})) and the significant variables, with a very small *P*-value ($p < 0.05$) and a coefficient of determination $R^2 = 0.7977$.

$$Y = 194.8 + 40.3\,X_1 + 39.0\,X_2 + 29.8\,X_3 + 5.6\,X_1^2 + 22.9\,X_1X_2 + 8.9\,X_1X_3 - 9.4\,X_2^2 + 0.86\,X_2X_3 - 12.1\,X_3^2 \quad (2)$$

where Y is glucose concentration (g L^{-1}) and X_1, X_2 and X_3 are enzyme/substrate ratio (U g^{-1} of starch), starch (g L^{-1}) and time (min), respectively.

This equation was statistically adjusted and could explain 75.6% of variability on glucose concentration which means that 24.4% is due to factors not considered in this investigation (uncertainty), with 23% of mean absolute error.

From these regression coefficients obtained in the equation 2, it is possible to determine that the most linear important independent variables for an efficient production of glucose from cassava starch are: X_1, X_2 and X_3 variables. This equation corroborates that these variables are very significant (with a very small *P*-value ($p < 0.05$); see Table 2), followed by the interaction of linear variables of X_2X_1, for finding finally other second order combinations, with minimal contribution to the response variable or indirect relation over response.

Table 2. Analysis of variance for synthetic variables pertaining to response glucose concentration (g L^{-1})

Source[a]	Df	*P*-value
X_1	1	< 0.0000
X_2	1	< 0.0000
X_3	1	< 0.0000
X_1^2	1	0.5431
X_1X_2	1	< 0.0009
X_1X_3	1	0.1699
X_2^2	1	0.3048
X_2X_3	1	0.8943
X_3^2	1	0.1896

[a]X_1, X_2 and X_3 are enzyme/substrate ratio (U g^{-1} of starch), starch (g L^{-1}) and time (min), respectively.

The optimal saccharification parameters were obtained by solving the regression equation (equation 2). The optimal parameters were: 5.9 U g^{-1} of starch of spirizyme fuel, 400 g L^{-1} dry matter and 45 min for saccharification. Under optimal conditions obtained by design we reached a glucose yield of 320.7 g L^{-1}.

Ethanol production by wild yeast using hydrolyzed cassava starch as substrate

The results obtained during fermentation of hydrolyzed starch (Table 3) were favorable for both hydrolytic processes used in this study. We used two approaches for ethanol production: first one, starch hydrolysis and then yeast fermentation, and second one, simultaneous saccharification and fermentation (SSF).

Based on the obtained results the time of the overall process of ethanol production may increase in the simultaneous saccharification and fermentation. The energy savings could be attained since the SSF process was effectively performed at 35 ºC, which is a lower temperature than the optimal temperature from the action of enzymes for starch hydrolysis and then yeast fermentation (> 60 ºC).

In addition, the SSF process bring some advantages such as prevention of inhibition by substrate concentration, and that as the sugars are produced, these are fermented also avoids previous process of starch hydrolysis and the use of other process.

Table 3. Kinetic parameters of ethanol production obtained during fermentation of a wild yeast strain using different fermentable sugar as carbon sources

Fermentation substrate	qp / h^{-1}	Qp / (g L^{-1} h^{-1})	t-f / h
Hidrolized starch	64.8	3.2	20
Native starch	36.4	1.8	20
Glucose	82.5	3.6	23

qp = specific productivity; Qp = volumetric productivity; t-f = fermentation time.

We achieved ethanol production values comparable with data reported by Wang and co-workers[23] and Białas *et al.*[29] These authors used starch from crude corn and flour corn, and obtained volumetric productivities around 1.6 and 1.7 g L^{-1} h^{-1}, respectively. Other authors have used other enzymes and worked with simultaneous saccharification and fermentation of cassava starch. In these cases, maximum ethanol productivities, ranging from 0.95 to 2.1 g L^{-1} h^{-1} and using free and co-immobilized cells of *Saccharomyces diastaticus* and *Zymomonas mobilis,*[30] *Saccharomyces cerevisiae* IR2,[31] *Saccharomyces cerevisiae* CHY1011 and CHFY0901[32] have been produced. Likewise, ethanol productivities in the range of 0.24 to 0.48 g L^{-1} h^{-1}, and using Cassava pulp and a thermotolerant yeast *Candida tropicalis* BCC7755[33] and a native *Saccharomyces cerevisiae Kyokai N. 7*[34] have been obtained.

Conclusions

We worked in the saccharification of cassava starch using commercial enzymes: α-amylase Liquozyme® SC DS and glucoamylase Spirizyme® Fuel from Novozymes, and a mixture of α-amylase and glucoamylase Stargen™ 001 from Genecor obtaining high concentrations of glucose (around 300 g L^{-1}) when reaction conditions of pH, temperature and substrate/enzyme ratios were optimized.

Additionally, it was possible to save time and energy on the conversion of cassava starch to glucose by using novel enzymes like Liquozyme SC DS and spirizyme fuel from Novozyme and Stargen 001 from Genecor. Saccharification rates were improved dramatically by using these enzymes, reducing in 50% the time for both liquefaction and saccharification process compared to hydrolysis of starch obtained from other sources.

Finally, production of glucose syrups from cassava starch pre-gelatinized was not favourable with the enzyme mixture Stargen. However, simultaneous saccharification and fermentation (SSF) studies with enzyme mixture Stargen and native cassava starch is quite promising because this bioprocess increase ethanol productivity by reducing of pre-fermentation times and energy required for conventional enzymatic starch hydrolysis.

Acknowledgments

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