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Optimization of Solid-State Enzymatic Hydrolysis of Chestnut Using Mixtures of α-Amylase and Glucoamylase

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Solid-state hydrolysis of starch present in chestnut was assayed in a single step with a mixture of a thermostable α-amylase and glucoamylase at three temperatures: 17 and 30 °C, for simultaneous hydrolysis and ethanol fermentation, and 70 °C, the optimal temperature for these enzymes. Total hydrolysis was only reached at the highest temperature, leading to a more concentrated hydrolysate than in submerged hydrolysis. Mass transfer limitations and starch retrogradation appear as the main causes for the incomplete hydrolysis of chestnut starch in solid-state operation at 17 and 30 °C. Even accepting that this limitation causes a 15% reduction of the yield of the hydrolysis with respect to the submerged process or the solid process at high temperature, solid-state hydrolysis at low temperatures seems to be adequate for simultaneous solid-state hydrolysis and fermentation processes.

KEYWORDS: One-step solid-state hydrolysis; chestnut; glucoamylase; α-amylase; mass transfer limitations

INTRODUCTION

In the past years, interest in the transformation of several indigenous crops such as chestnut has been renovated because they can constitute a local alternative for the elaboration of delicatessen foods (e.g., *marron glace* and related confectionery products) and, additionally, help the conservation of marginal agricultural lands (*1*). The production of chestnut in Galicia (NW of Spain) and the North of Portugal represents ~20% of the European production. Despite this, only a minimum part of chestnuts is processed in the confectionery industry, while an important amount of the total production not useful for industrial transformation is destined to local animal feed.

One way to improve the value of chestnut overproduction as well as the bad quality fruits could be the development of new products such as a distilled spirit obtained from the postincubates of alcoholic fermentation of chestnut with yeast. Logically, the first step of this alternative requires the conversion of chestnut starch into fermentable sugars.

To obtain a suitable nutrient broth from chestnut for submerged alcoholic fermentation, we have optimized in a previous paper the simultaneous submerged liquefaction–saccharification process of chestnut purée using a mixture of a thermostable α-amylase and glucoamylase operating at 70 °C (*2*). Additionally, the existence of a synergistic effect between these two amylases was postulated to justify the rapid and total conversion of chestnut starch into glucose. Because the high viscosity of the purée limited the maximum chestnut starch concentration that can be manipulated (not higher than 225 g L^{-1}, which produces hydrolysates with ~83 g L^{-1} of total sugars, these including 68 g L^{-1} of glucose and 15 g L^{-1} of saccharose), a procedure of one-step hydrolysis in subsequent cycles was assayed to reach high concentrated glucose syrups (*2*).

Another reasonable alternative to obtain high glucose concentrated hydrolysates from chestnut could consist of performing the hydrolytic process in the solid state. This procedure is also particularly useful for simultaneous solid-state hydrolysis and fermentation processes or when hydrolysis is required as a substrate pretreatment to allow the growth of non-amylolytic or non-cellulolytic microorganisms on solid materials, thus increasing the yield of the fermentation if amylaceous or cellulosic substrates are used (*3*–*6*).

Simultaneous hydrolysis and fermentation present some operational advantages over the sequencial operation like higher simplicity, shorter time of operation, and less effect of enzymatic inhibition phenomena by the products of hydrolysis because the microorganism consumes them as they are generated. Nevertheless, some inconveniences for this procedure can be expected; the reduced amount of water in solid-state systems causes high restrictions to mass transfer, limiting the accessibility to substrates and the release of products and, consequently, affecting particularly the activity of the enzymes. Additionally, since optimal conditions of operation (mainly pH and temperature) usually differ for hydrolysis and fermentation, compromise values of the variables must be accepted. Therefore it is necessary to study the kinetics and possible limitations of the enzymatic reaction in conditions that are compatible with the fermentation process for adequate overall operation with maximum yield.

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Table 1. Composition of Raw Chestnut without Teguments

component	g/100 g (wet basis)	component	g/100 g (wet basis)
water content	56.9 ± 1.0	proteins	2.24 ± 0.07
total sugars	36.7 ± 0.8	total phosphorus	0.052 ± 0.002
saccharose	6.5 ± 0.1	lipids	1.70 ± 0.05
starch	30.2 ± 0.8	fiber	1.21 ± 0.07
glucose	traces	ash	1.02 ± 0.03
total nitrogen	0.46 ± 0.02		

To design a strategy for simultaneous solid-state hydrolysis and fermentation of chestnut in order to produce an alcoholic distilled spirit, the optimization of the solid-state enzymatic conversion of chestnut starch into glucose at fermentation temperatures was studied. The same process was also developed at higher temperature, more adequate for thermostable enzymes, as a procedure to obtain more concentrated glucose syrups than in submerged hydrolysis.

MATERIALS AND METHODS

Materials: Substrate and Enzymes. The composition of the peeled raw chestnut used in this work is shown in **Table 1**. The starch content of raw chestnut was calculated from the difference between total sugars (cellulose and pentosanes are not included) and saccharose content, considering no other sugars were found in measurable amounts.

Two thermostable commercial amylases were used: α-amylase Termamyl 120 L type S and glucoamylase AMG 300 L, both purchased from Novo Nordisk A/S Industries (Bagsvaerd, Denmark).

Solid-State Hydrolysis of Chestnut. Raw chestnuts, slightly steamed to remove external teguments, were chopped and sieved to obtain five fractions with different diameters of particles, considered approximately as spheres: 4.4, 4.0, 2.7, 1.3, and 0.9 mm. After dampening with distilled water in a ratio of 0.1 mL g^{-1} of raw fruit (wet basis), chopped chestnut was heated in an autoclave for 1 h at 100 °C for simultaneous gelatinization and sterilization in view of its use as a potential substrate for fermentation.

After cooling the substrate to the temperature of the assay, a mixture of both enzymes and $CaCl_2$ (needed for the activity and stability of the α-amylase) was added as an aqueous solution prepared to provide 60 total enzymatic units (considered as the sum of the individual activities of α-amylase and glucoamylase, measured as described by Murado et al. (*7*)) and 0.053 mg of $CaCl_2$ per g^{-1} of raw chestnut (wet basis). The volume of this solution was calculated as the difference between the *added liquid phase* (*L*), indicated in each assay and defined as an independent variable in the factorial design (see below), and the water added for gelatinization. Excepting the assay of different combinations of both amylases, the ratio of α-amylase/glucoamylase applied was always 0.35:0.65, referred to 1 total enzymatic unit.

Solid-state hydrolysis was carried out in closed propylene bottles of 50 mL, loaded with the amount of moistened chestnut corresponding to 15 g of the raw fruit. Incubation was performed at controlled temperature (17, 30, and 70 °C depending on the assay) in an incubator without agitation and a humidity-controlled atmosphere by means of a saturated NaCl solution located within the chamber. Even the pH of chestnut after heating (∼6) is not the most favorable for both enzymes, specially for glucoamylase (pH ∼ 4 at 55 °C for AMG 300 L (*8*); pH ∼ 5 at 37 °C and pH ∼ 5.5 at 75 °C for Termamyl 120 L type S (*9*)); no buffer was added to avoid the increase of solute concentration that could contribute to reduce water activity and to raise the osmotic pressure.

Samples consisted of the total content of a bottle, suspended in 50 mL of NaOH 0.1 N to stop the enzymatic reaction, and homogenized with an Ultraturrax for 3 min at 9500 rpm. After sample centrifugation (12000*g*), the supernatants were recovered for analytical determinations: quantification of total and reducing sugars and identification of the soluble products of hydrolysis.

Analytical Methods. Total sugars (TS) were measured according to the phenol−sulfuric method of Dubois et al. (*10*) using glucose as standard. Soluble products of hydrolysis (mono-, di-, and oligosaccharides) were analyzed by reverse-phase HPLC with a refractive index detector, according to the method of Franco and Garrido (*11*). The column was a Spherisorb R ODS2 (25 cm × 0.46 cm) from Waters operated at room temperature, with water as the mobile phase in isocratic conditions, adjusting the pH of samples in the range of 6−7. Reducing sugars (RS) were determined by the 3,5-dinitrosalicylic acid (DNS) reaction (*12*) with glucose as standard. Total amylolytic activity (AAT) was measured in enzymatic units (EU) as described by Murado et al. (*7*). All analytical determinations were made in duplicate.

Table 2. Experimental Domain and Codification of the Independent Variables Analyzed by Means of a Second-Order Rotatable Factorial Design Applied to the Study of the Solid-State Enzymatic Solubilization and Hydrolysis of Chestnut[a]

coded values	natural values: *D* (mm)	natural values: *L* (mL g^{-1})
−α (−1.267)	0.9	0.165
−1	1.3	0.200
0	2.7	0.330
+1	4.0	0.460
+α (+1.267)	4.4	0.495

[a] Codification: $V_c = (V_n - V_o)/\Delta V_n$. Decodification: $V_n = V_o + (\Delta V_n V_c)$. V_n, natural value; V_c, coded value; V_o, natural value in the center of the domain; ΔV_n, increment of V_n corresponding to one unit of V_c.

Criteria for Hydrolysis Evaluation. To evaluate the progress of the enzymatic reaction, the following terms were applied (expressed as percentage): *hydrolysis* (*H*), which was defined as the ratio (expressed as mg g^{-1} of raw chestnut) between RS in the extract obtained from the solid mixture of reaction and TS (excepting cellulose, pentosanes, and saccharose) in raw chestnut including therefore both solubilization and saccharification of starch; and *solubilization* (*S*), defined as the ratio (expressed as mg g^{-1} of raw chestnut) between TS in the extract obtained from the solid mixture of reaction and TS (excepting cellulose and pentosanes) in raw chestnut. According to this definition, 100% of hydrolysis corresponds to total conversion of starch to glucose.

Statistical Methods. Kinetics assays were made in duplicate. The simultaneous effect on solid-state chestnut hydrolysis of the substrate water content, referred to as the *added liquid phase* (*L*), and the size of the particle, referred to as the *diameter* (*D*) considering spheres as an approximation, was studied by means of a second-order rotatable experimental plan with α = 1.267 and five replicates in the center of the domain, according to Akhnazarova and Kafarov (*13*) and Box et al. (*14*). As indicated above, *added liquid phase* (*L*) was defined as the sum of the water added for gelatinization and the aqueous solution of enzymes and $CaCl_2$ added after gelatinization. Percentages of *solubilization* (*S*) and *hydrolysis* (*H*) were used as dependent variables to evaluate the process. Experimental domain and coding criteria are given in **Table 2**. The significance of the coefficients of the models was calculated using Student's *t* test (α < 0.05) as the acceptance criterion. Model consistency was verified by Fisher's *F* test (α < 0.05) applied to the following mean square (*QM*) ratios:

$$\text{model/total error } (QMM/QME) \quad (F \geq F^{\text{numerator}}_{\text{denominator}})$$

$$\text{(model + lack of fitting)/model } (QM(M + LF)/QMM) \quad (F \leq F^{\text{numerator}}_{\text{denominator}})$$

$$\text{total error/experimental error } (QME/QMEe) \quad (F \leq F^{\text{numerator}}_{\text{denominator}})$$

$$\text{lack of fitting/experimental error } (QMLF/QMEe) \quad (F \leq F^{\text{numerator}}_{\text{denominator}})$$

RESULTS AND DISCUSSION

Preliminary Assay. Although mesophilic amylases are usually used in simultaneous saccharification−fermentation

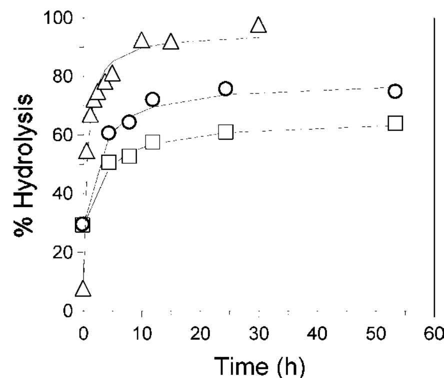

Figure 1. Kinetics of the one-step solid-state hydrolysis of chestnut with a mixture of α-amylase and glucoamylase at three temperatures: (□) 17, (○) 30, and (△) 70 °C. Diameter of particle (*D*), 1.3 mm; liquid phase added (*L*), 0.400 mL g^{-1}.

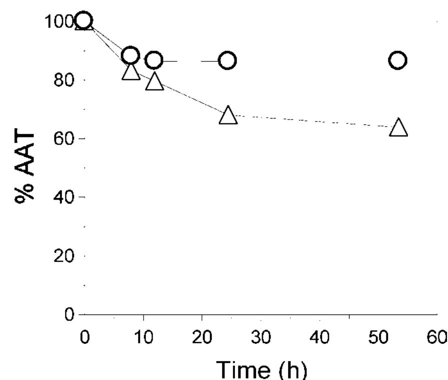

Figure 2. Residual total amylolytic activity (AAT), expressed as percentages referred to the initial total amylolytic activity (60 EU g^{-1}), during solid-state enzymatic hydrolysis of chestnut at two temperatures: (△) 17 and (○) 30 °C. Diameter of particle (*D*), 1.3 mm; liquid phase added (*L*), 0.400 mL g^{-1}.

processes, the thermophilic enzymes present some advantages even if the process is performed at moderate temperatures compatible with microbial growth. In fact, these enzymes often show higher stability in unfavorable environments such as the presence of organic solvents like ethanol (*15*, *16*). For this reason, it is reasonable to suppose that thermostable amylases are a good catalytic choice for simultaneous solid-state hydrolysis−alcoholic fermentation of chestnut.

To evaluate the hydrolytic capacity of a mixture of thermostable α-amylase and glucoamylase to hydrolyze chestnut in the solid state as a substrate treatment for simultaneous solid-state hydrolysis and fermentation processes and as a procedure to obtain concentrated glucose syrups, a series of kinetics of solid-state hydrolysis was carried out at three temperatures: 17 and 30 °C (common temperatures for alcoholic fermentation of beverages) and 70 °C. The hydrolysis was performed in a single step with a mixture of both amylases as described in a previous paper (*2*), fixing the total enzyme concentration and the ratio of both enzymes in those values that lead to total hydrolysis of starch by means of the submerged process with chestnut liquid purée at 70 °C (60 EU g^{-1} of raw chestnut; ratio of α-amylase/glucoamylase enzymatic units, 0.35:0.65). The specific variables of the solid-state operation (water addition and diameter of the particle) were fixed taking into account the need to avoid the appearance of nonretained water. Their values were 0.400 mL g^{-1} and 1.3 mm, respectively.

The results depicted in **Figure 1** show that total hydrolysis was only achieved in the series incubated at 70 °C. Extraction of sugars in a single step with a volume of water corresponding to 1.4 mL g^{-1} of raw chestnut yielded a syrup containing 133.3 g L^{-1} of total sugars and 110.5 g L^{-1} of glucose (the difference between them is due to the saccharose present in chestnut). Although the yield of extraction was only ∼50%, this mode of operation increased by 1.5 times the glucose concentration of the hydrolysate with regard to the one cycle submerged hydrolysis (83 g L^{-1} of TS from a 225 g L^{-1} chestnut purée) described in a previous work (*2*). Optimization of the extraction step must be performed to improve the total yield of the process.

Contrarily, when the process was performed at temperatures compatible with simultaneous fermentation (17 and 30 °C), no total hydrolysis was reached. The usual loss of activity of thermophilic enzymes at temperatures below 40 °C (*17*) could be proposed to explain the incomplete reaction. In effect, when the enzymatic activity of the mixture of the enzymes added (corresponding to 60 EU g^{-1}, measured at 40 °C and pH 5 as described by Murado et al. (*7*)) was analyzed at 70, 30, and 17 °C at the same pH, the following values were obtained: 108, 34, and 14 EU g^{-1}, respectively.

Nevertheless, the above explanation is only partial because it must be expected that the reduction of the enzymatic activity at 17 and 30 °C led to a slow but continuous reaction. However, fitting the experimental values of hydrolysis to hyperbolic curves with acceptable regression coefficients (0.994 and 0.990), two asymptotes of 64.7 and 77.9 were obtained for 17 and 30 °C, respectively, suggesting that hydrolysis stopped in these conditions. Consequently, additional mechanisms including enzymatic deactivation, inadequate operational conditions (i.e., physical restrictions), or inhibition phenomena are proposed to explain the incomplete reaction of the enzymes. These possible causes are next ascertained.

Enzymatic Stability. It is reasonable to suppose that the prolongation of the time of reaction over 24 h could cause an important decrease of the enzymatic activity even working at 17 or 30 °C, increasing, therefore, the negative effect of the low amylolytic activity at these temperatures. To test this possibility, the stability of the mixture of the enzymes in the previous assay was studied at the two lowest temperatures. Although the results depicted in **Figure 2** show a slight loss of activity after the time of incubation, it does not seem to be strong enough to stop the hydrolysis before total conversion of starch.

The high stability of the mixture of enzymes at 30 °C (close to 90% of the initial activity after 55 h of incubation at this temperature) is noticeable in comparison with the enzymes incubated at 17 °C. Because hydrophobic interactions are favored when temperature increases until thermal denaturalization occurs, this behavior must be a consequence of the main role of this kind of interactions in thermal stabilization of enzymes (*18*, *19*).

Optimization of the Physical Operational Conditions. The main operational variables of the solid-state process affecting hydrolysis of chestnut in this system are *volume of liquid phase added* (*L*) and *diameter of particle* (*D*). They are closely related to mass transfer processes (i.e., substrate accessibility by the enzymes and product diffusion) and, consequently, to the extension of the reaction.

To analyze the effect of these variables on the solid-state hydrolysis of chestnut with the intention of investigating the reasons for the uncompleted reaction and, if possible, to find the optimal situation that would lead to total hydrolysis, a second-order rotatable two level factorial design was performed. The incubation was carried out at 30 °C for 24 h (time enough

Table 3. Experimental Results and Analysis of Variance of the Calculated Model 1 Describing the Effect of the Diameter of the Particle (*D*) and the Liquid Phase Added (*L*) on the Percentage of Solubilization (*S*) of Chestnut by Means of a One-Step Solid-State Enzymatic Process with a Mixture 0.35:0.65 of α-Amylase/Glucoamylase at 30 °C and 60 EU g^{-1} of Raw Chestnut

Codified Values		$\%S_{experimental}$	$\%S_{calculated}$
D	*L*		
1	1	81.2	80.2
1	-1	78.1	78.2
-1	1	86.8	85.5
-1	-1	83.5	83.5
1.267	0	78.7	79.6
-1.267	0	85.0	86.3
0	1.267	80.8	82.5
0	-1.267	80.3	80.0
0	0	82.0	82.9
0	0	83.7	82.9
0	0	84.0	82.9
0	0	83.5	82.9
0	0	82.6	82.9

Coefficients of the model[a]		*t*
i.t.	83.045*	226.474
D	-2.635*	8.550
L	0.991*	3.215
DL	-0.054	0.130
D^2	-0.228	0.627
L^2	-1.059*	2.904

Significance analysis of the coefficients[b]

Experimental error variance = 0.685

t (α<0.05; *FD*=4) = 2.776

	SS^c	FD^d	QM^e
Model (*M*)	62.93	3	20.98
Error (*E*)	10.94	9	1.22
Exp. Error (*Ee*)	2.74	4	0.68
Lack of fitting (*LF*)	8.20	5	1.64
Total	73.87	12	6.16

Significance analysis of the model[f]

F(QMM/QME)=	17.25	F_9^3 (α<0.05)=	3.86
F(QM(M+LF)/QMM)=	0.42	F_3^8 (α<0.05)=	8.85
F (QME/QMEe)=	1.78	F_4^9 (α<0.05)=	6.00
F (QMLF/QMEe)=	2.40	F_4^5 (α<0.05)=	6.26
$r^{2\,g}$ =	0.852		
$r^2_{adj}{}^g$ =	0.802	***SIGNIFICATIVE***	

[a] Coefficients for the terms of the model (*i.t.*, independent term; *D*, diameter of the particle (mm); *L*, added liquid phase (mL g^{-1}); *, significative coefficients). [b] Coefficients significance was calculated using Student's *t* test (α < 0.05). [c] Sum of squares. [d] Freedom degrees. [e] Mean squares (*QM*=*SS*/*FD*). [f] Models consistency was verified by Fisher's *F* test (α < 0.05) as indicated in Materials and Methods. [g] Regression coefficients (*adj*: adjusted).

to reach the asymptote), fixing the rest of the conditions as before. In this case, solubilization was also measured to investigate if there is a limiting step of the total reaction (solubilization + saccharification) in this system.

Tables 3 and **4** summarize the responses for solubilization and hydrolysis along with the predicted responses. The model equations fitted by regression analysis are given by

$$\%S = 83.0 - 2.6D + 1.0L - 1.1L^2 \quad (1)$$

$$\%H = 79.4 - 3.2D + 1.2L - 1.3L^2 \quad (2)$$

In both cases, the model terms *D*, *L*, and L^2 were found to be significant according to the Student *t*-test (α < 0.05); meanwhile D^2 and the interaction term between *D* and *L* (*DL*) were found to be nonsignificant in both equations (**Tables 3** and **4**). The analysis of variance (ANOVA) applied simultaneously to the four mean square ratios indicated in the materials and methods section considering α < 0.05 confirmed the consistency of both models 1 and 2.

These empirical models showed acceptable fittings and predicted satisfactory maximum responses inside the experimental domain assayed (86.6 and 83.8% for solubilization and hydrolysis) for the same combination of the independent variables in both cases (*particle diameter* = 0.9 mm, *added liquid phase* = 0.390 mL g^{-1}). The respective response surfaces (**Figure 3**) demonstrated the inability of the system to reach total solubilization and hydrolysis inside the experimental domain assayed in these conditions of operation. A more detailed analysis of the terms of models 1 and 2 allows one to reach some conclusions that can be related to the incomplete reaction of the enzymes.

The coincidence between models 1 and 2 indicates the same effect of both variables on solubilization and hydrolysis and suggests that total hydrolysis of chestnut starch in the solid state depends mainly on the solubilization of sugars. Because total solubilization was not reached inside the experimental domain, and considering that α-amylase is mainly responsible for solubilization, diffusional restrictions for this enzyme to access to the substrate located inside the particles of chestnut could be a cause for incomplete hydrolysis at low temperatures. In fact, in low-porosity materials such as chestnut, degradation processes take place mainly at the surface of the particles in such a way that the substrate in the deeper regions of the solid remains inaccessible (*20*, *21*). Additionally, the fact that total hydrolysis was reached operating at 70 °C sustains this idea because diffusivity increases as temperature augments.

This idea is also supported by the role of *D* and *L* in the system. *Diameter*, which shows the strongest influence on the process (higher coefficients in both models), only appears as a

Table 4. Experimental Results and Analysis of Variance of the Calculated Model 2 Describing the Effect of the Diameter of the Particle (*D*) and the Liquid Phase Added (*L*) on the Percentage of Hydrolysis (*H*) of Chestnut by Means of a One-Step Solid-State Enzymatic Process with a Mixture 0.35:0.65 of α-Amylase/Glucoamylase at 30 °C and 60 EU g^{-1} of Raw Chestnut

Codified Values			
D	*L*	$\%S_{experimental}$	$\%S_{calculated}$
1	1	81.2	80.2
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0	1.267	80.8	82.5
0	-1.267	80.3	80.0
0	0	82.0	82.9
0	0	83.7	82.9
0	0	84.0	82.9
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t (*α<0.05; FD=4*)= 2.776

	SS[c]	FD[d]	QM[e]
Model (*M*)	62.93	3	20.98
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Total	73.87	12	6.16

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first-order negative term, indicating a linear effect on the response, which increases as the diameter size diminishes. This suggests that smaller particles than those assayed could lead to higher degrees of hydrolysis, supporting the idea of limitations to access to the core of the particles. However, this possibility implies the disadvantages of a more complicated pretreatment of the solid substrate and a more difficult operation because of the change of the rheology to a more viscous matrix after humectation and gelatinization.

Liquid phase (*L*) influences starch accessibility by the enzymes and inhibition phenomena affecting substrate and product concentration and diffusion. This variable presents in both models a negative second-order term, which implies the existence of a maximum that, in this case, corresponds to a value of *L* situated inside the experimental domain. Considering that this maximum does not reach 100% in any of the models, it means that total solubilization and hydrolysis of starch in this system does not depend on this variable. Taking into account that high liquid phases are beneficial to reduce inhibition and favor the diffusional processes but low water contents are also favorable by increasing the stability of enzymes, optimal *L* must be a compromise value between them, being close to the higher value assayed since the loss of amylolytic activity along the time of incubation did not seem to be critical, as reflected in **Figure 2**.

Effect of the α-Amylase/Glucoamylase Relationship. The optimal combination of both amylases for the submerged operation (*2*) applied in the above experiments is not necessarily the same for the solid-state operation. Possible inhibition phenomena and the unsuitable pH of chestnut for these enzymes, as indicated before, can affect in a different way the activity of the α-amylase and the glucoamylase. If this happens, the mixture must be enriched in the more affected enzyme to compensate these negative effects.

To investigate this possibility, four different combinations of both amylases were assayed at 30 °C in the conditions of humectation and diameter of particle that lead to the maximum response of hydrolysis according to model 2 (diameter, 0.9 mm; added liquid phase, 0.390 mL g^{-1}).

The results (**Figure 4**) showed that the optimal ratio for submerged hydrolysis of chestnut starch is also the best combination in the solid state, although it is unable to reach total conversion of starch in this mode of operation. This suggests two explanations: both enzymes suffer in a similar way the negative conditions defined by the operation in the solid state, and/or they are not much affected.

Considering that glucoamylase is the enzyme less favored by the pH of the medium (*8*, *9*, *22*, *23*) and that the optimal ratio of both amylases did not change with regard to the reaction in pH 4.75 buffered chestnut liquid purée (*2*), it seems that pH

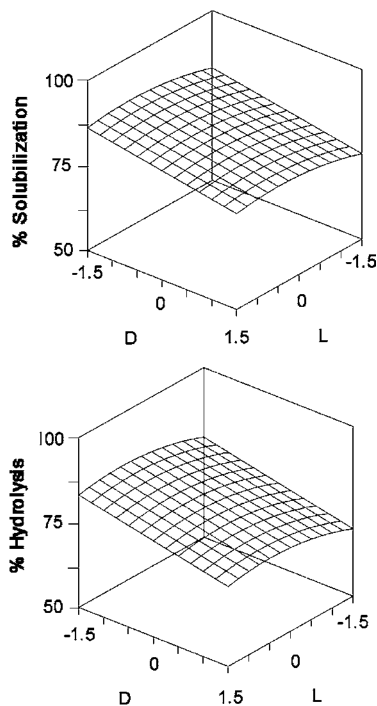

Figure 3. Response surfaces showing the effect of the diameter (*D*) and the liquid phase added (*L*) on the one-step solid-state enzymatic solubilization and hydrolysis of chestnut with a mixture of 0.35:0.65 α-amylase/glucoamylase and 60 EU g^{-1} of raw chestnut at 30 °C, according to models 1 and 2 (**Tables 3** and **4**).

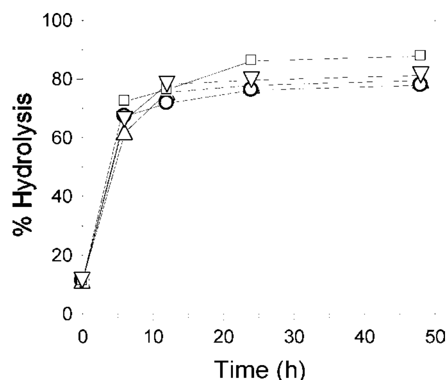

Figure 4. Kinetics of the one-step solid-state hydrolysis of chestnut at 30 °C, in the best conditions predicted by model 2 ($D = 0.9$, $L = 0.390$ mL g^{-1}), with different ratios of α-amylase/glucoamylase in the mixture of enzymes: (△) 0.75:0.25, (○) 0.5:0.5, (□) 0.35:0.65, and (▽) 0.25:0.75.

is not responsible for the incomplete hydrolysis in this case. Moreover, the measurement of the amylolytic activity of the mixture of both amylases at two values of pH (5.0, close to the optimal for both enzymes, and 6.0, the operational pH of chestnut) at the three temperatures assayed (17, 30, and 70 °C) reflected a much stronger loss of activity at pH 6.0 at 70 °C (around 46% of the initial activity in comparison with 4 and 16% at 17 and 30 °C, respectively), just for the series that was able to reach 100% of hydrolysis. These results support once again the existence of physical restrictions as the main reason for incomplete hydrolysis.

Product Inhibition. The low water content in solid-state systems can originate high local concentrations of solutes and make more intense inhibition phenomena. Despite this, product inhibition does not seem to be too strong in this case for glucoamylase, which is able to practically hydrolyze all the

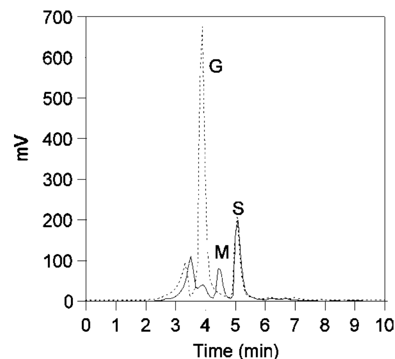

Figure 5. Profiles of the soluble sugars from chestnut (continuous line) and of the solubilized sugars after 24 h of solid-state hydrolysis of chestnut (dashed line) at 30 °C with a mixture 0.35:0.65 of α-amylase and glucoamylase and 60 EU g^{-1} of raw chestnut, in the best conditions predicted by model 2 ($D = 0.9$, $L = 0.390$ mL g^{-1}): glucose (G), maltose (M), and saccharose (S).

solubilized sugars into glucose, as shown in **Figure 5**. α-Amylase inhibition by glucose is reported to appear at concentrations even lower than those estimated in this system (~300 g L^{-1}) considering the degree of hydrolysis reached in the best situation and the water content of the solid matrix, although osmotic effects are proposed as the reason for this behavior (*24*). α-Amylase inhibition must be thus considered as a contribution to the decrease of the reaction rate, but not as the cause for the stop of the hydrolysis.

Finally, in the case of gelatinized starchy materials incubated at low temperatures, and especially for those containing a relatively high amylose/amylopectin ratio (*25*, *26*) such as chestnut (~21/79), which is between corn and cassava (*27*), retrogradation must be also taken into account as a factor increasing the resistance of starch to the enzymatic attack as a consequence either of the limitations to mass transfer in the system caused by the increase of the viscosity or of the polysaccharide structural changes that could affect the amylase affinity toward the substrate. Taking into account that the limiting step is solubilization, which is mainly due to α-amylase, this hypothesis is supported by references (*26*, *28*) and by the higher (*29*) sensitivity of this enzyme toward retrograded starch in comparison with glucoamylase.

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