Available online at www.sciencedirect.com
ScienceDirect
Bioresource Technology 99 (2008) 7255–7263
BIORESOURCE TECHNOLOGY

Alcoholic chestnut fermentation in mixed culture. Compatibility criteria between *Aspergillus oryzae* and *Saccharomyces cerevisiae* strains

Miguel Anxo Murado [a], Lorenzo Pastrana [b,*], José Antonio Vázquez [a], Jesús Mirón [a], María Pilar González [a]

[a] *Grupo de Reciclado y Valorización de Materiales Residuales, Instituto de Investigacións Mariñas (CSIC), r/Eduardo Cabello, 6, Vigo 36208, Galicia, Spain*
[b] *Departamento de Bioquímica, Genética e Inmunología, Facultade de Ciencias de Ourense, Universidade de Vigo, As Lagoas s/n, Ourense 32004, Galicia, Spain*

Received 25 May 2007; received in revised form 16 December 2007; accepted 18 December 2007
Available online 4 March 2008

Abstract

The main objective of the present work consisted in the transfer to the case of the chestnut of a rice fermentative process that carried out to the Japanese traditional way to lead to an alcoholic bagasse, the *moromi*, capable of obtaining distilled. This way, selection assays of amylolitic *Aspergillus oryzae* strains and studies of compatibility between microfungi and yeast were carried out. These mixed cultivations were performed operating in batch submerged culture. Later on, using solid state system (chestnut, microfungi, yeast), a fermentative fed-batch process (*koji*, *moto*, *moromi*) was defined. By means of this approach a yield of 70% was reached in the conversion of total carbohydrates in ethanol. Also, the time required by the traditional operation was reduced in half.

Keywords: Chestnut fermentation; Yeast–fungus mixed cultures; Alcoholic fermentation

1. Introduction

Sales of some popular categories of ethnic foods such as Asiatic foods experimented a noticeably raise in the last years in occidental countries. The most established sectors in the market are Indian and Chinese foods, initially as a result of their widespread restaurant, but interest by Japanese specialities have also rise. Traditional Japanese foods included very know products such as sushi and fermented foods obtained from the fermentation of amylaceus crops like soy sauce or miso, but also traditional alcoholic beverages included the sake (rice wine) and shochu among other less recognized specialities.

Elaboration of shochu, a popular distilled alcoholic drink produced from steamed cereals involves two steps using a fungus (generally, *Aspergillus kawachii* or *Aspergillus oryzae*) and a yeast (usually, *Saccharomyces cerevisiae* or *S. sake*). In industrial *shochu* brewing, *A. kawachii* is used in the first step to make moulded cereals (koji making) due to the various extracellular enzymes such as α-amylase, glucoamylase, proteases, and peptidases for digesting materials (Fukuda et al., 2001). Additionally the fungus also produces a large amount of citric acid, which allows brewers to maintain the mashed culture acidic, preventing the contamination by harmful micro-organisms. In the second step, in mixed culture, *S. cerevisiae* is used for alcoholic fermentation (moto–moromi fermentation) (Nomachi et al., 2002). Although rice and barley are the main crops used as raw materials in shochu manufacture, sweet potato soba (buckwheat) and, in lesser extend, chestnut are also fermented (Akiko et al., 2001; Yaichi, 1988).

The main difficulties to extend the exportation and consumption of some minor traditional specialities of foods is the lack of standardization of their production, because only hedonistic criteria were used along centuries to optimized the manufacture of these foods. Nevertheless the modern production imposed the application of additional

* Corresponding author. Tel.: +34 988387062; fax: +34 988387001.
E-mail address: pastrana@uvigo.es (L. Pastrana).

0960-8524/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2007.12.053

reproducibility and economic criteria. This problem arises to some local specialities of manufactured chestnut in European countries.

In this sense, Japan is the largest world consumer of raw and elaborated chestnut as well as the biggest chestnut importer. Japan's domestic production is chilled fresh and consumed throughout the winter, especially around the New Year, being widely used in the elaboration of some shochu and other delicatessen specialities.

In Galicia (NW of Spain) – one of the most productive chestnut areas in Europe – the fruit is generally processed in local industries to obtain different confectionery high-added-value products like *marron glace*. Since these delicatessen products require high quality chestnuts (in terms of size, shape or resistance for peeling and sweet processing), only a reduced percentage of the annual chestnut crop is destined to industrial transformation.

However, in basis of their starchy nature, overproduction of chestnuts can be used in the formulation of alternative foods or in biotechnological purposes. Thus, in the last years in our lab, two realistic attempted industrial alternatives for exploitation of overproduction are being tested: firstly, the development of sweet flour destined to confectionary products for celiac people (without gluten), secondly, the production of an alcoholic beverage from distillation of fermented chestnut. From an industrial viewpoint, the optimization of a process of fermentation to obtain alcoholic beverage implicate harmonize simultaneously, the maximization of the yeast metabolism in terms of the conversion of glucose to ethanol and flavour production and the prevention of the off flavours formation. Until now we have optimized the enzymatic hydrolysis of chestnut starch in solid (chopped) and submerged (pastes) operation with an enzymatic mixture of α-amylase and glucoamylase in a single step (López et al., 2004, 2005, 2006). In submerged process (López et al., 2004) total conversion of starch to glucose can be achieved only when the enzymatic mixture included a ratio of α-amylase/glucoamylase activity of 0.35 and the enzyme–substrate ratio was the highest assayed (60 total EU/g of raw chestnut). Synergistic effects in the action of these two enzymes as well as phenomena of inhibition and thermal inactivation were described and quantified (López et al., 2006). On the other hand, solid state hydrolysis allowed obtaining highest glucose concentrated hydrolysates from chestnut than submerged process (López et al., 2005).

Another possible strategy to obtain an alcohol beverage from chestnut consists in the action of an amylolitic fungus growing in a mixed culture with yeast. This approach, close to the traditional procedure to obtain shochu, was success assayed by us in submerged cultures with an amylaceous food waste (Siso et al., 1988; Mirón et al., 1988).

In this work, the mixed solid state fermentation of chestnut purée with *A. oryzae* and *S. cerevisiae* was studied in order to obtain an alcoholic beverage by ulterior distillation of the postincubated. Thus, in the basis of traditional shochu brewing, optimization suitable of combination of yeast and fungus strains as well as process conditions was performed.

2. Methods

2.1. Micro-organisms used and chestnut composition

The micro-organisms studied are shown in Table 1. All stock cultures were stored at 4 °C in malt media (Cultimed) supplemented with 1 g/L of yeast extract and 20 g/L of agar and re-inoculated every 2 months. The chemical composition (in g/100 g wb) of the chestnut without teguments used was as follows (López et al., 2004): water content (56.9 ± 1.0); total sugars (36.7 ± 0.8); sucrose (6.5 ± 0.1); starch (30.2 ± 0.8); glucose (traces); total nitrogen (0.46 ± 0.02); proteins (2.24 ± 0.07); total phosphorus (0.052 ± 0.002); lipids (1.70 ± 0.05); fiber (1.21 ± 0.07); ash (1.02 ± 0.03).

2.2. Experimental strategies to obtain an alcoholic postincubate from chestnut

In this work, the shochu brewing process was taken as model to obtain an alcoholic postincubated form chestnut. Thus, the experimental scheme used involved the following stages:

(1) Fermentation of chestnut with *A. oryzae* to obtain amylases postincubated called the chestnut koji (kojic).
(2) Culture of *Sacharomyces cerevisiae* on kojic to obtain an alcoholic postincubated called the chestnut moto (motoc).

Table 1
Micro-organisms used

Species	Key international[a]	Key IIM[b]
Microfungi		
Aspergillus oryzae	CBS 125–59	A1.01
A. oryzae	CBS 110–47	A1.02
A. oryzae	CBS 112–51	A1.03
A. oryzae	CBS 115–33	A1.04
A. oryzae	CBS 125–49	A1.05
A. oryzae	CBS 201–75	A1.06
A. oryzae	CBS 205–89	A1.07
A. oryzae	CBS 570–65	A1.08
A. oryzae	CBS 816–72	A1.09
Yeasts		
Saccharomyces cerevisiae	IFI 240	S1.01
S. cerevisiae	CBS 1907	S1.02
S. cerevisiae (*V. ellipsoideus*)	IFI 87	S1.03
S. cerevisiae	IFI 82	S1.04
S. cerevisiae	IFI 246	S1.05
S. cerevisiae	CECT 1319	S1.06
S. cerevisiae	CECT 1685	S1.07

[a] IFI: Industrial Fermentations Institute (CSIC), Madrid. CECT: Spanish Type Culture Collection, University of Valencia. CBS: Centraalbureau voor Schimmelcultures, Baarn, Holanda.
[b] Abbreviated notation used in this work.

(3) Extension of motoc stage to enhance the alcoholic contain by addition to the motoc, a mash of fresh amounts of kojic and chestnut. This stage was called moromic.

2.3. Culture conditions

2.3.1. Preliminary microfungus cultures

The studies for the selection of the microfungi with the highest production of amylolytic enzymes were performed in submerged culture using 300 mL Erlenmeyer flasks with 100 mL of starch culture medium. The composition and treatment of the media employed has been described in detail in previous papers (González et al., 1992; Murado et al., 1993a,b, 1997), with the only change being that these were buffered with 0.05 M bi-pthalate-NaOH at a pH of 5.5. Incubations were carried out in triplicate, with orbital shaking at 200 rpm and 30 °C. At pre-established times of 22, 46 and 68 h, culture samples were taken and centrifuged at 5000*g* for 15 min; the supernatants were analysed for total amylolytic and glucoamylase activity and total sugars. Finally, the sediments were dried at 108 °C until a constant weight was obtained in order to measure the biomass values.

2.3.2. Kojic (Chestnut koji)

The process was performed in a 100 mL Erlenmeyer loaded with 8 g of pre-cooked chestnut (in cubic pieces of ~3 mm size) and a volume of aquatic medium or liquid phase sufficient to saturate the material without leaving appreciable amounts of free liquid in the system. A 25% proportion (w/v) appeared to be adequate whose addition was performed dividing the 2 mL in two fractions: 1.5 mL of water or nutrient solution and 0.5 mL of a suspension of spores of *A. oryzae* in sterile water, a concentration sufficient to result in an initial population of 5×10^4 spores per gram of chestnut. The inoculum was aseptically added on to the mix of chestnut and nutrient solution, which had been sterilised in free steam for 1 h. The incubation temperature was 30 °C under static conditions with a brief manual agitation daily.

Although the process is capable of progression without supplementary nutrients, we decided to compare the effects of simple wetting with water against a solution containing sources of nitrogen and phosphorous. Thus, we started with the following premise:

1: The average total content of carbohydrates in the pre-cooked chestnut used as starting material was 37% (w/w).
2: Supplementing the liquid phase with inorganic sources of nitrogen in appropriate proportions of the oxidised form ($NaNO_3$) and reduced form (NH_4Cl) as a function of the carbon source consumption (Torrado et al., 1998). Under these conditions, in an initial study we compared the results of three series of incubations: one of control with distilled water and two with supplements corresponding to theoretical consumptions of 5% and 25% (called A and B, respectively) of the total carbohydrates present in the chestnut:
   - A (5%): 9.73 g/L of $NaNO_3$, 1.03 g/L of NH_4Cl and 1.21 g/L of KH_2PO_4.
   - B (25%): 48.66 g/L of $NaNO_3$, 5.13 g/L of NH_4Cl and 6.05 g/L of KH_2PO_4.

At pre-established times, the total content of each experiment (in duplicate) was taken and briefly homogenised as a whole in 20 mL of distilled water under refrigeration in an ultra-turrax. The homogenates were then centrifuged for 15 min at 5000*g* and the supernatant used to determine the amylolytic activity and the nutrients, drying the sediments at 108 °C until a constant weight. We should point out that due the continuous generation of reducing sugars as consequence of the action of amylases this operative procedure only represent in a reproducible way the state of the system at each incubation time.

2.3.3. Motoc y moromic. Preliminary study

The first study to complete the process until obtaining moromic was performed under the following specific conditions:

1: Two liquid phases were used: water and a medium supplement (C) of nitrogen (N) and phosphorous (P) according to theoretical consumptions of 20% of the total carbohydrates (a situation close to the 3.2 assay maximum):
   - C: 31.86 g/L of $NaNO_3$, 3.32 g/L of NH_4Cl and 3.91 g/L of KH_2PO_4
2: The assays were performed in quadruplicate. All batches of kojic were produced with 10 g of pre-cooked chestnut, 2.5 mL liquid phase and an inoculum of 50,000 spores of *A. oryzae* per gram of chestnut. The motoc stage was initiated on adding 25 g of chestnut, 30 mL of liquid phase and an inoculum of 50,000 cells of *S. cervissiae* (S1.01) per gram of the total amount of chestnut (35 g). The feeding of the moromic stage implied batches of (10 g koji + 25 g chestnut + 30 mL water). From the moromic stage, the fermenting culture was stirred daily under aseptic conditions using a glass rod.
3: The analysis was performed on supernatants from centrifuged aliquots of the liquid phase.

2.3.4. Compatibility of microfungi–yeast

Cultures were carried out using the conventional submerged technique, in malt (Cultimed) medium at 30 °C with orbital shaking at 200 rpm. At 20, 40 and 70 h, the levels of ethanol present were determined together with the

biomass of each of the micro-organisms, after separating the yeast cells from the fungal mycelium using washing on a mesh of 250 μm, centrifugation and drying of the sediment at 108 °C to a constant weight.

2.3.5. Acceleration of the process and approximation to the conditions of submerged culture

In the next experimental series the following changes were introduced with respect to the preceding experiments (see results and discussion in Section 3.5):

1: The strain yeast S1.01 was substituted by S1.04 because the distillation of the postincubates of this last strain provided the best aromatic chromatographic profile (data will be presented in a further paper).
2: The liquid phase consisted simply of sterile water without the nitrogen or phosphorous supplements.
3: The incubation temperature for the motoc and moromic stages was established at 25 °C maintaining the production of kojic in the above mentioned conditions.
4: The stages for motoc and moromic were carried out in two liquid phase levels. In the lower of the set, the motoc was initiated in the normal manner adding chestnut, water and a yeast inoculum (50,000 cells per g of chestnut) in a kojic of 4 days with the aim of obtaining a system of (koji (g):chestnut (g):water (mL)): (1:2.5:3). The re-feeding during the moromic stage was performed on batches (koji:chestnut:water) of the same proportions. For the highest level of the liquid phase the proportions used were (koji:chestnut:water): (1:2.5:6).
5: In the conditions of large liquid phase, the incubation was performed in two different modes, one in an Erlenmeyer with the normal diary agitation and the other in bottles using an incubator with rollers providing a rotation of approximately two cycles per minute. By using this method we were attempting to provide a greater degree of homogeneity of the mass, avoiding the frequent agglomerations arising from the addition of kojic, without raising the levels of aeration.

2.4. Analytical methods

Total sugars (TS) were measured by the phenol–sulphuric method according to Dubois et al. (1956) using glucose as standard. Reducing sugars (RS) were analysed by the 3,5-dinitrosalycilic acid (DNS) reaction (Bernfeld, 1951) with glucose as standard. Total nitrogen was measured by the method of Havilah et al. (1977), applied to digests obtained by the classic Kjeldahl procedure. Total phosphorous was detemined by the fosfomolibdato method of Murphy and Riley (1962) according to the application of Strickland and Parsons (1968). Ethanol was analysed by ion and steric exclusion HPLC. The column was an Transgenomic ION-300 (30 cm × 0.78 cm) with a precolumn ICE-GC-801/C (Cartridge 2/pk) operated at 65 °C and used H_2SO_4 (6 mM) as mobile phase in isocratic conditions (flow = 0.4 mL/min) with a refractive index detection (Vázquez et al., 2005). Total Amylolytic and glucoamylase activity (TAA and GA, respectively) was determined in enzymatic units (EU) as described by Murado et al. (1993a). All analytical determinations were made in duplicate.

3. Results and discussion

3.1. Criteria for the selection of microorganism for koji culture

The approach proposed to obtain an alcoholic beverage from chestnut is based in shochu elaboration process that, basically, consists in a mixed culture microfungus–yeast. In this process, the fungus must provide enough amylolitic activity to obtain high amounts of reducing sugars that will be converted into ethanol by the yeast. Thus, it must be expected that the limit stage in this process could be due to the capacity of microfungus to produce amylases. For this reason a selection of a suitable *A. oryzae* is required.

The strain A1.01 provided (data not shown) the highest amylase activity (total and glucoamylase) and the biomass production, suggesting a high potentiality to hydrolyze starch chestnut generating surplus of glucose that could be used by the yeast in mixed cultures. For this reason this strain was selected for further experiments.

3.2. The production of kojic (Chestnut koji)

In order to evaluate the suitability of the before selected *A. oryzae* strain to growth and produce amylases using chestnut as substrate (kojic) a series of cultures following the procedure described in methods section were performed. The results obtained are summarised in Fig. 1 and demonstrated the following aspects:

1: The total amylolytic activity in the system increases over a period of 6–7 days at the end of which time it tends to stabilise or diminish. Nutritional supplements favour an increase rate of production and length of the stable phase of production over the final level.
2: In general terms, the profile of the pH is the same, independently of the supplements used. Although from the initial value (7.0) here is an initial acidification phase, the minimum values are not compromised in terms of the conservation of the amylolytic activity.
3: The level of total soluble carbohydrates always shows a downward trend, while that of the reducers only descend alter first rising to a maximum (notably higher in the case of the highest nutritional supplement value) after some three days of incubation. This

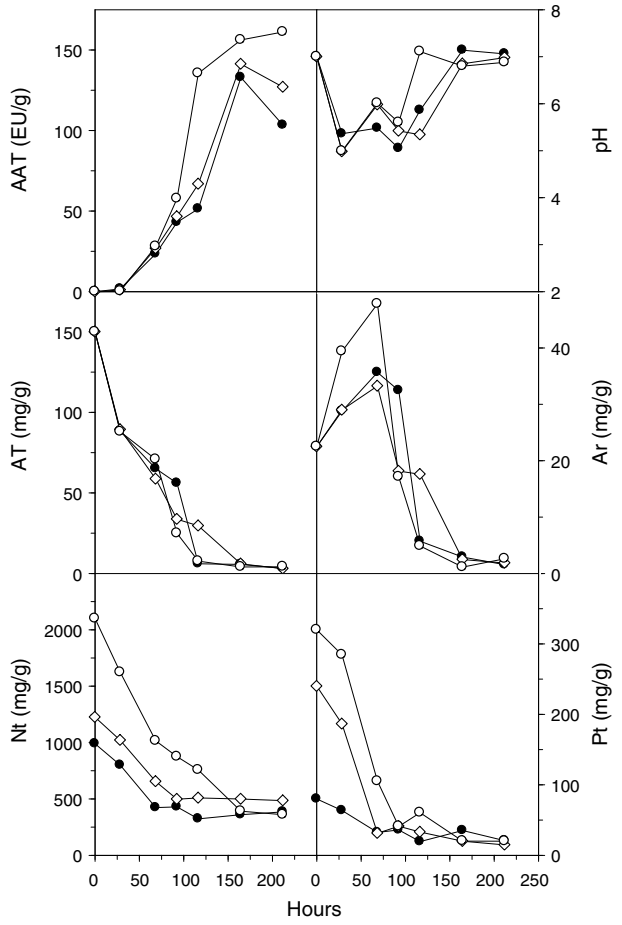

Fig. 1. Evolution of the principal variables in the system through the kojic process; TAA: total amylolytic activity; TS: total sugars; RS: reducing sugars; Nt: total nitrogen; Pt: total phosphorous. In all cases the total quantities detected in the supernatant are given (see text) in relation with the initial weight of chestnut. ●, ◇, ○: control and supplements A and B, respectively.

result is a balance between the hydrolysis as a result of the amylase action and the consumption due to the growth of the microorganism. Nitrogen and phosphorous fall to similar levels for the three cultures after 150 h of incubation, which indicates that the consumption rates are approximately proportional to the levels present, as demonstrated in the case described by Torrado et al. (1998).

Therefore, we can accept that the chestnut constitutes an adequate substrate for the growth of *A. oryzae*, with the capacity to produce a mass rich in amylases in a reasonable timeframe, homologous with koji and appropriate for studying the continuation of the process towards a product homologous with *moromi*.

3.3. Motoc and moromic. Preliminary culture

Moto and moromi are two consecutive steps in elaboration of sochu once koji stage is completed. With the aim to adapt these processes using chestnut as substrate, new experiences was performed evaluating the effect of supplementation with N and P the culture medium on the ethanol production.

The results of the study (Fig. 3) showed clearly that the supplementation with N and P had not effect in the evolution of the basic variables of the process. The maximum stoichiometric level of ethanol was estimated assuming that the carbohydrates (37%) from the chestnut are completely transformed into fermentable sugars (glucose).

Hence, when the sampling from the motoc phase includes an aliquot of the total mass in fermentation, we saw in general that the solid phase of the system contains the same proportion, in weight, of ethanol. The total result, represented by the upper lines in Fig. 3E, allows us to estimate the yield in 48% of the stoichiometric maximum. Although from this value we should discount the implied consumption of glucose in the biomass, we undoubtedly found in front of a low productivity in ethanol.

The contributions of kojic mean small increases of the amylolytic activity in the system, which tend to fall in subsequent intervals, with the exception of the first of these (4–8 days) in which we can appreciate a moderate increase coinciding with the lowest levels of ethanol. This could imply a contribution of this product to the deterioration of the enzymatic activity.

In fact, in a simplistic approximation formulating the rate of descend of this activity, v_{TAA}, as the sum of both a first order kinetic term (proportionality to the present activity) and another of the second order (proportionality to the product of the amylase and ethanol concentrations)

$$v_{TAA} = k_1[TAA] + k_2[TAA][Ethanol] \quad (1)$$

An acceptable adjustment between the observed and expected rates (data not shown: $r = 0.928$) was obtained. Although the model represents a simplification that undoubtedly ignores other factors, it also constitutes a proof in favour of the hypothesis relative to the role of ethanol.

Among the possible reasons that provoke these low ethanol conversions could be: the low availability of reducing sugars, the high reducing sugars utilization for biomass production and the low yeast development. The first hypothesis seems true considering the low amylolytic activity in the moromi culture in comparison to the koji culture. However, the second is not valid since the reduced counts in the microscopic examination of the postincubates suggest scarce yeast growth (data not shown). The problem of the low yeast development, motivated by the possible low tolerance from this to the microfungi, it was already described by Kodama (1970), who described that a peculiarity of the strains of *S. cerevisiae* used as "sake yeasts" is precisely its capacity to develop in the presence of *A. oryzae*.

In addition, a low concentrations of volatile molecules commonly associated to the ethanol production could be

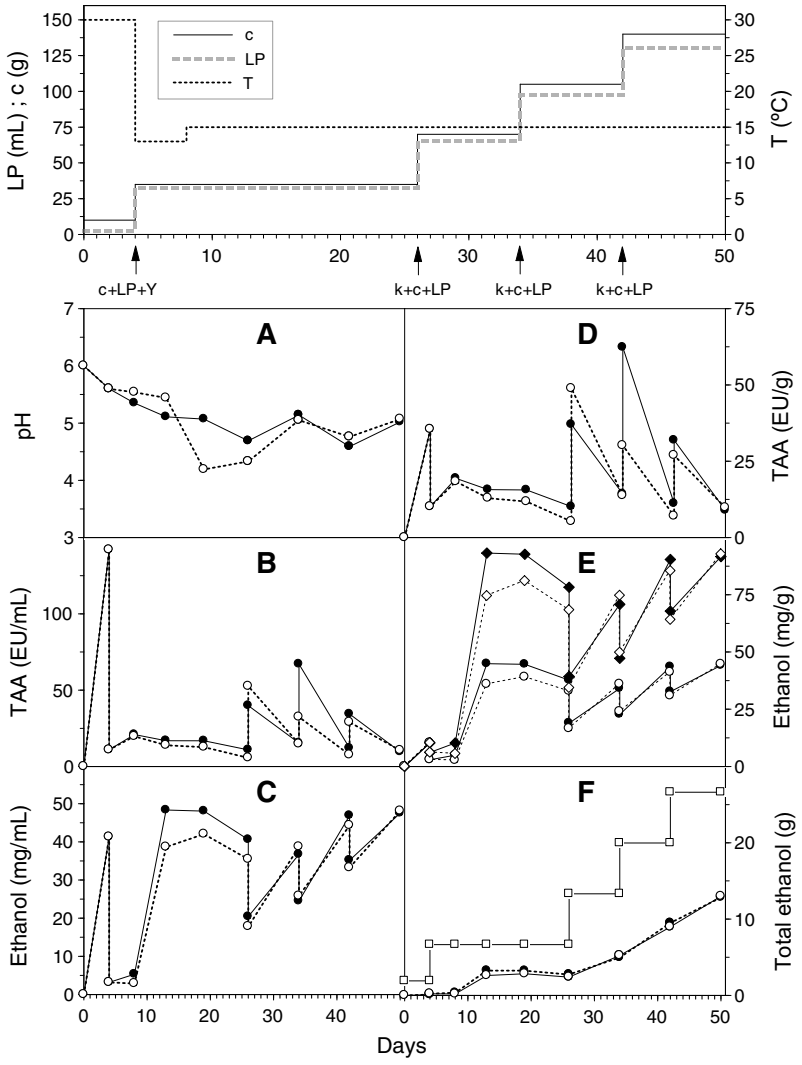

Fig. 2. Evolution of the principal variables of the fermentative system (●: control, ○: supplemented medium) throughout the kojic - motoc - moromic phases. LP: liquid phase; Y: yeast; c: chestnut; k: kojic. In **B** and **C**, the values for amylase and ethanol are referred to the concentrations detected in the liquid phase. **D**, **E** and **F**, represent the efficiency for the production of amylases and ethanol with respect to the accumulated sum of chestnut added to the system. In **E**, the upper lines (♦: control; ◇: supplemented media) correspond to the content of ethanol in the whole of the liquid and solid phases. In **F** the total levels of ethanol in the system (overall values) are compared with the stoichiometric maximum (□). The rest of the keys are as in the Fig. 1.

expected. Consequently the flavour quality of further distillates could be reduced.

3.4. Yeast–microfungus compatibility

In the bibliography relating to the traditional process (Kodama, 1970), the compatibility between the two principal microbial entities present is always described in terms of the tolerance of the yeast to the mould and not the reverse. In addition, the scarce content of reducing sugars in the chestnut necessarily forces to use of the high amylolytic capability of the microfungi A1.01. Under these conditions, the compatibility was examined following the mixed cultures development of each one of the yeasts in Table 1 (typified by mid or high fermentation capacity and mid to high tolerance of ethanol) in presence of *A. oryzae* A1.01.

The results (Fig. 3) revealed, indeed, important differences in the tolerances of the yeasts. S1.01 showed the most deficient development, with the microfungi reaching the highest value of biomass. It also suggests that the other yeasts exercise some form of control on the system, modifying the conditions in those that the development of the microfungi are limited. If this "yeast regulation" is accepted as the primary criteria for selection, the strains S1.01, S1.06 and S1.07 should be discarded. In addition, the incapacity of the yeast for such regulation seems to correlate with the tendency for the pH to rise in the culture.

As shown in Fig. 4, we can recognise that the entity responsible for the production of ethanol is basically the yeast (there is an acceptable positive linear correlation between the level of ethanol and the biomass of yeast; negative and less acceptable for the fungi). This does not imply, however, that *A. oryzae* does not produce ethanol,

nor that the situation in which we see the maximum relationship between the yeast/mould coincides with the maximum ethanol production. If all ethanol were attributed to the activity of the yeast, we would have to accept that the culture S1.01, even though it is the lowest producer in absolute terms, because it would be the most productive in terms of the relationship ethanol/yeast. On the contrary, if the ordinate in the origin (Fig. 4) is interpreted literally, we would have to accept that the ethanol produced in these conditions is a result of the fungii activity. The probable real situation is an intermediate between these two extremes, and the most appropriate selection is to consider the relationship between the ethanol produced and the biomass.

This criterion would favour those systems in which less carbohydrate is consumed during the generation of the biomass and would suggest (Fig. 4) the order: S1.03 > S1.02 > S1.04 = S1.05.

3.5. Acceleration of the process and approximation to the conditions of submerged culture

In order to improve the ethanol production, S1.01 was substituted by S1.04 due to that the distillation from the postincubates provided the best aromatic chromatographic profile (data will be presented in a further paper). Also, the increase of the liquid phase in the system could be a favourable factor upon increase of the bioconversion sugar to ethanol. In the first place, it would take to reduce the ethanol concentration and, consequently, would eliminate it toxic effect in the liquid phase (without affecting the total extensive production). In second place, because the microfungus are, in general, more appropriate micro-organisms for development in solid state culture, thus the increase of liquid phase proportion should favour to the yeast.

On the other hand, and with the purpose to accelerate this fermentative process, we decided to elevate the incubation temperature in the motoc and moromic phases. This temperature was maintained inside of an interval: (a) favourable to the yeast, (b) sufficiently far from the temperature that maximizes the growth rate of both micro-organisms and (c) with low risk of eventual loss of aroma.

The results (Fig. 5) confirmed the expected tendencies and the efficiency of ethanol production according to the chestnut load, without any evidence of effects attributable to a lack of nutritional supplements. Therefore:

(1) The level of ethanol equivalent to that of the experiment described in Section 3.3 was achieved in less time, and the process did not present such a pronounced stabilisation. This way, the fermentation was completed in half the time required for the traditional method.
(2) Throughout the motoc and moromic phases, the pH remained stable in the three series and with appropriate values for the conservation of the amylolytic

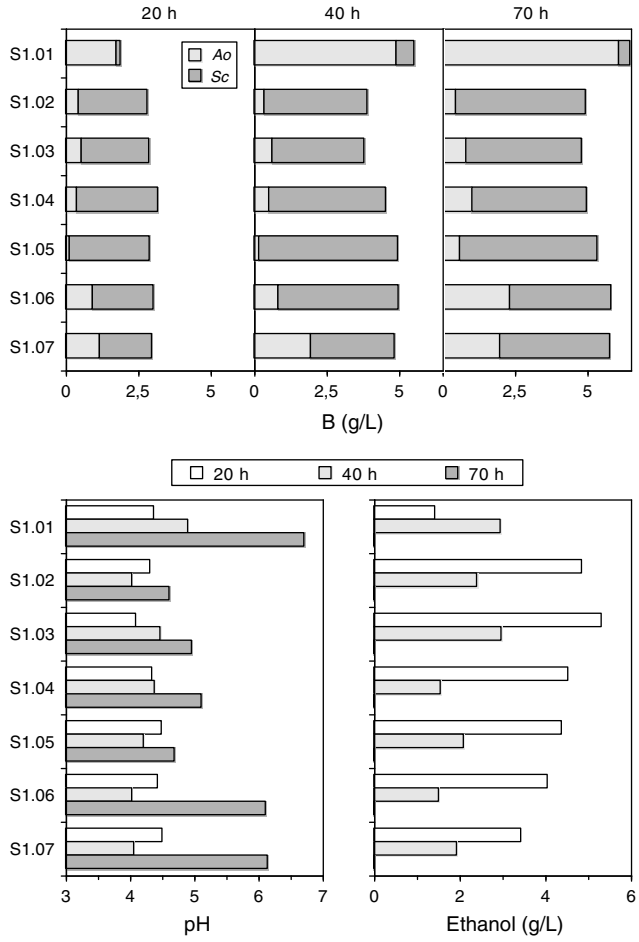

Fig. 3. Tolerance of the 7 strains of *S. cerevisiae* (*Sc*) referenced in Table 1 to the strain A1.01 of *A. oryzae* (*Ao*), as per the results for the corresponding mixed cultures at three incubation times. B: biomass.

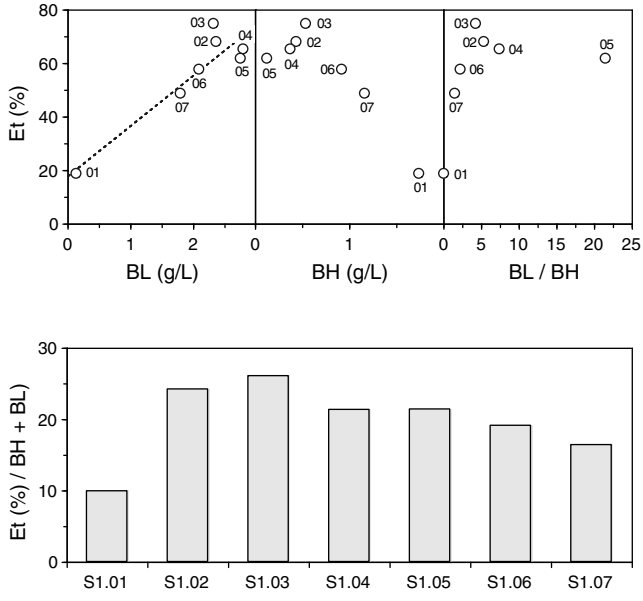

Fig. 4. Relationship between the principal process variables in mixed cultures at 20 hours. BL: yeast biomass; BH: microfungi biomass; Et (%): ethanol produced as a proportion of the stoichiometric maximum.

activity. If we accept the correlation suggested by the compatibility experiment this could indicate a certain efficacy of the "regulation by the yeast" (compare Fig. 2 with Fig. 5).

(3) The concentration of reducing sugars in the liquid phase descended rapidly from values approximating to 20 g/L at the beginning of the motoc phase, until reaching a stable level of ~5 g/L for the rest of the process. Although, as previously mentioned, this level may not be a limiting factor for growth it can hardly be in doubt that the maintenance of higher concentrations would benefit the accumulation of ethanol.

(4) As expected, although the level of ethanol in the liquid phase was below 23% in the diluted cultures, the efficiency of its production with respect to the total load of chestnuts added actually increased 50% over the control. In accordance with the criteria of quantification established in Section 3.3, the control culture (concentrated) provides a conversion equivalent to 48% of the stoichiometric maximum, while the diluted cultures reached almost 70% in both methods of mixing.

(5) In spite of the increased homogeneity using rotary movement, its results were not significantly different from those achieved by the daily stirring with a glass rod. Given that the rotation seems preferable in principle and the risk of promoting an excessive aeration does not seem to apply, this agitation procedure would constitute the best option suited to the definitive scale.

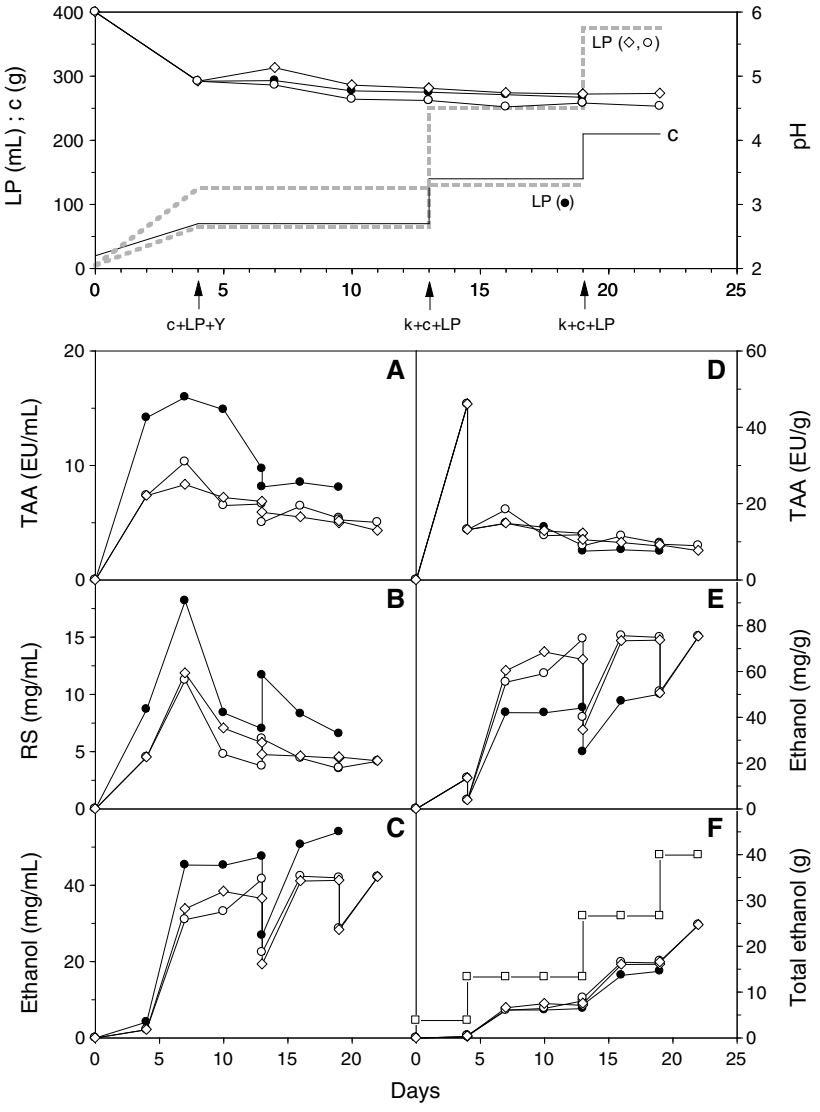

Fig. 5. Evolution of the principal variables for the fermentative system, performing the motoc and moromic stages at 25 °C, with the normal proportions in the liquid phase (●), with double liquid phase and daily stirring (○), and with double liquid phase and continuous low speed rotation (◇). TAA: total amylolytic activity; RS: reducing sugars; LP: liquid phase; c: chestnut; Y: yeast; k: kojic. In **A**, **B** and **C**, the values for amylase, reducing sugars and ethanol are referred to the concentrations detected in the liquid phase. **D**, **E** and **F**, represent the efficiency for the production of amylases and ethanol with respect to the accumulated sum of chestnut added to the system. In **F** the total levels of ethanol in the system (overall values) are compared with the stoichiometric maximum (□).

4. Conclusions

This work showed that is possible to adapt of the traditional Japanese shochu fermentation to the conversion of the starch chestnut into ethanol studying the compatibility of microfungus–yeast in mixed cultures in relation with the concentrations in the solid state system of the amylolitic activity and ethanol. The selected fermentation procedure allowed a high conversion of total carbohydrates in ethanol in a reasonable incubation time, envisaging an easy scale-up of the process in the future.

Acknowledgements

This work was funded through FEDER Project IFD97-0020-C02-02. We wish to thank to Margarita Nogueira, Ana Durán and Araceli Menduiña for their technical assistance. Dr. J.A. Vázquez Álvarez had a postdoctoral contract (CSIC-I3P-PC 2003, financed by the European Social Fund). The English usage in the manuscript has been revised by J&J Barry Consulting, S.L. (Global Pharmaceutical Support, Vigo, Spain).

References

Bernfeld, P., 1951. Enzymes of starch degradation and synthesis. Adv. Enzymol. 12, 379–427.

Dubois, M.K., Pilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Biochem. 28, 350–356.

Fukuda, H., Kizaki, Y., Tsukihashi, T., Wakabayashi, S., 2001. *Shochu* brewing characteristics and properties of a trichothecin-resistant *shochu* yeast mutant. Biotechnol. Lett. 23, 2009–2013.

González, M.P., Siso, M.I.G., Murado, M.A., Pastrana, L.M., Montemayor, M.I., Mirón, J., 1992. Depuration and valuation of mussel-processing wastes. Characterization of amylolytic postincubates from different species grown on an effluent. Bioresource Technol. 42, 133–140.

Havilah, E.J., Wallis, D.M., Morris, R., Woolnough, J.A., 1977. A microcolorimetric method for determination of ammonia in Kjeldahl digests with a manual spectrophotometer. Labor. Prac. July, 545–547.

Kodama, K., 1970. Sake yeast. In: Rose, A.H., Harrison, J.S. (Eds.), The Yeast, vol. 3. Academic Press, London, pp. 225–282.

López, C., Torrado, A., Fuciños, P., Guerra, N., Pastrana, L., 2004. Enzymatic hydrolysis of chestnut purée: process optimisation using mixtures of alpha-amylase and glucoamylase. J. Agric. Food Chem. 52 (10), 2907–2914.

López, C., Torrado, A., Fuciños, P., Guerra, N., Pastrana, L., 2005. Optimization of solid state hydrolysis of chestnut using mixtures of alfa-amylase and glucoamylase. J. Agric. Food Chem. 53 (4), 989–995.

López, C., Torrado, A., Fuciños, P., Guerra, N., Pastrana, L., 2006. Enzymatic inhibition and thermal inactivation in the hydrolysis of chestnut purée with an amylases mixture. Enzyme Microb. Technol. 39, 252–258.

Mirón, J., Siso, M.I.G., Murado, M.A., González, M.P., 1988. Microfungus–yeast mixed cultures in the degradation of amylaceous wastes. II: an experimental design for optimization of yeast production. Biotech. Lett. (Techniques) 2 (3), 171–176.

Murado, M.A., Siso, M.I.G., González, M.P., Montemayor, M.I., Pastrana, L.M., Pintado, J., 1993a. Characterisation of microbial biomasses and amylolytic preparations obtained from mussel processing waste treatment. Bioresource Technol. 43, 117–125.

Murado, M.A., González, M.P., Pastrana, L., Siso, M.I.G., Mirón, J., Montemayor, M.I., 1993b. Enhancement of the bioproduction potential of an amylaceous effluent. Bioresource Technol. 44, 155–163.

Murado, M.A., González, M.P., Torrado, A., Pastrana, L.M., 1997. Amylase production by solid state culture of *Aspergillus oryzae* on polyurethane foams. Some mechanistic approach from an empirical model. Proc. Biochem. 32, 35–42.

Murphy, J., Riley, J.P., 1962. A modified single solution method for the determination of phosphate in natural waters. Anal. Chem. Acta 27, 31–36.

Nomachi, W., Urago, K., Oka, T., Ekino, K., Matsuda, M., Goto, M., Furukawa, K., 2002. Molecular Breeding of *Aspergillus kawachii* Overproducing Cellulase and Its Application to Brewing Barley *Shochu*. J. Biosci. Bioeng. 4, 382–387.

Siso, M.I.G., Murado, M.A., Franco, J.M., Mirón, J., González, M.P., 1988. Microfungus–yeast mixed cultures in the degradation of amylaceous wastes. I: Interactions affecting amylolytic activity. Biotechnol. Lett. 10, 431–436.

Strickland, J.D.H., Parsons, T.R., 1968. A practical handbook of sea water analysis. Bull. Fish Res. Board Can 167, 57–62.

Torrado, A., González, M.P., Murado, M.A., 1998. pH regulation in solid state culture through the initial ratio between oxidized and reduced sources of nitrogen. A model applicable to the amylase production by *Aspergillus oryzae*. Biotechnol. Tech. 12, 411–415.

Vázquez, J.A., Mirón, J., González, M.P., Murado, M.A., 2005. Bacteriocin production and pH gradient. Some mathematical models and their problems. Enzyme Microb. Technol. 37, 54–67.

Yaichi, S., 1988. Patent number: JP63185366.