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Production of *Rhodotorula glutinis*: a yeast that secretes α-L-arabinofuranosidase

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Abbreviations: Abf: α-arabinofuranosidase
YPD: yeast peptone glucose

Rhodotorula glutinis **is a yeast that secretes the enzyme α-L-arabinofuranosidase (E.C. 3.2.1.55) into the culture medium and thus has an interesting biotechnological potential. To determine improved culture conditions of this organism, different factors of the culture media were evaluated such as the use of peptone as nitrogen**

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source, salts composition, pH and growth temperature. Likewise, beet molasses and beet cosette were tested as industrial carbon sources to induce the production of the enzyme and how they influence the yeast growth. Based on these studies a culture medium is proposed for growth of this yeast in a continuous system. By assaying different dilution rates an average specific activity for the enzyme of 82.4 U/mg of protein was obtained.

The study of the aromas of fruits has revealed an important source of volatile compounds that may enrich the aromatic profile of juices and fermented drinks. The chemical composition of these potential aromas is formed by volatile compounds such as monoterpenes, derived from shikimate or C13 nonsoprenoids, which are linked to β-D-glucosides or diglicosides (Williams et al. 1982; Gunata et al. 1985). These compounds linked to sugars are released in two steps: initially, an α-arabinofuranosidase (Abf), an α-rhamnosidase or a β-apiosidase participate, followed closely by the action of a β-D-glucosidase (Gunata et al. 1988). Many of these aromatic compounds are naturally liberated during the fruit ripening (Cordonnier et al. 1989); however, the enzymatic activities of the plants are unable to liberate them completely leaving an important source of potential aromas in the juice (Gunata et al. 1985; Cordonnier et al. 1989). Therefore, some studies have been carried out to obtain endogenous enzymes, mainly from fungi and yeasts, which release these aromas. In general, these studies have been centred on identifying microorganisms with β-glucosidase activities and very few microorganisms have been considered that secrete the enzymes that participate in the first step of the reactions of the release of aromatic compounds (Gunata et al. 1990; Dupin et al. 1992; Gueguen et al. 1995; Lounteri et al. 1995; Charoenchai et al. 1997; Le Clinche et al. 1997, Spagna et al. 1998; Fernández et al. 2000; Manzanares et al. 2000; Spagna et al. 2000; Gallego et al. 2001; Strauss et al. 2001; Belancic et al. 2003). Generally, the glycosylated compounds are formed by a non-reductor α-L-arabinofuranoside (Gunata et al. 1990); therefore it would be interesting to use enzymes that hydrolyze this bond and allow the liberation of the volatile compound through the action of a β-glucosidase. This sequence of enzymatic activities would permit the enrichment of the aromatic profile of the product.

In grapes, the most abundant volatile compounds are monoterpenes, which can be free or linked to the disaccharides arabinofuranosilglucosides of geraniol, nerol and linalool (Williams et al. 1982; Gunata et al. 1988).

The α-L-arabinofuranosidase activity (E.C. 3.2.1.55) has been mainly described in fungus (Rombouts et al. 1988; Gunata et al. 1990; Le Clinche et al. 1997; De Ioannes et al. 2000). However, due to the physicochemical characteristics of some products, such as wine, it is difficult to maintain the enzymatic activities stable during the elaboration process. Therefore, some authors have searched for enzymes from microorganisms that participate in the must fermentation process like yeasts, and these studies have been mainly focused on the β-glucosidase activity more than on the activities responsible for hydrolysis of the disaccharide bond (Gueguen et al. 1995; Manzanares et al. 1999; Charoenchai et al. 1997; Fernández et al. 2000; Strauss et al. 2001; Belancic et al. 2003).

In light of the potential use that an Abf activity would have in the enological industry, in this work we studied the culture conditions of *R. glutinis*, a yeast that has been shown to produce Abf and are naturally present in fermentative processes. Furthermore, we evaluated the specific activity of this enzyme under continuous culture.

MATERIALS AND METHODS

Microorganism

Rhodotorula glutinis strain L-1816 was isolated from Cabernet Sauvignon grapes and was maintained on YPD medium containing 2% (w/v) glucose, 2% (w/v) peptone and 1% (w/v) yeast extract.

Growth and culture assays

The yeast inoculums for batch and continuous culture with 1 x 10^6 cells/mL were obtained by growth in YPD. Duplicate batch cultures were carried out in 250-mL flasks with 125 mL of assayed medium under mechanical shaking at 150 rpm. During the culture period growth was monitored by measuring absorbance at 475 nm every 0.5 and 1 hr, depending on the growth phase in which the culture was found. The dry cell mass concentration was determined from the optical density reading by using the following equation: dry cell mass = 0.31 OD_{475}, 0.31 being the slope of a standard curve of dry cell mass (in g/L) versus OD_{475} (r^2 = 0.9931). The specific growth rates (μ) were calculated by least-squares fitting to the linear part of

Table 1. Specific growth rates of *R. glutinis* L-1816 with different compositions of salts in the basal culture media. Means followed by different superscripted letters are significantly different ($p < 0.05$).

	Salts I Composition	Salts II Composition	Salts III Composition	Salts IV Composition
Specific growth rate (h^{-1})	0.198 ± 0.05[a]	0.131 ± 0.008[b]	0.100 ± 0.004[c]	0.097 ± 0.011[c]

Table 2. Qualitative determination of Abf activity with beet molasses and beet cosette as carbon sources. Degree of Abf activity: +++, high; ++, medium; + low.

	Abf activity	
Concentration (% w/v)	Beet molasses	Beet cosette
0.2	+++	++
0.5	++	++
1.0	++	++
2.0	+	++

the semilog growth plot. The basal culture media used contained 0.5% (w/v) yeast extract and a salts solution composed of 0.3% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) KH_2PO_4 and 0.05% (w/v) $MgSO_4$ x $7H_2O$. Modifications to the basal media are described below. Similarly, different pH and temperature conditions were evaluated.

As a way of defining an improved culture media for the growth of *R. glutinis*, several modifications to the basal media were evaluated. Growth assays of the yeast were carried out in basal media with and without peptone. Furthermore, assays were carried out with different types and concentration of salts denominated II (0.3% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4$ x $7H_2O$ and 0.005% (w/v) $FeSO_4$ x $7H_2O$), III (0.3% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4$ x $7H_2O$ and 0.001% (w/v) $MnCl_2$ x $4H_2O$) and IV (0.3% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4$ x $7H_2O$, 0.005% (w/v) $FeSO_4$ x $7H_2O$ and 0.001% (w/v) $MnCl_2$ x $4H_2O$). The salts composition of the basal culture media was denominated as salt composition I. Finally, as a means of determining the influence of the carbon source on the yeast growth, 2% (w/v) beet cosette and 2% (w/v) beet molasses were also evaluated. For this, the beet cosette was weighed, grinded and mixed with 100 mL distilled water. This mix was vigorously agitated and kept at 80°C for 20 to 30 min. Subsequently, it was filtered using a Watman N°1 filter paper for use in the preparation of the culture media.

The effect of the culture temperature on the growth of the yeast was also studied. The temperatures assayed were 20, 25, 28 and 37ºC. Furthermore, the effect of the culture media pH on the growth of *R. glutinis* was determined. The cultures were initially adjusted to pH 4.0 or 5.2. The pH was adjusted using 0.5 M citric acid.

Enzymatic assays

The α-L-arabinofuranosidase activity was qualitatively determined according to Ganga and Martínez (2004). In brief, Petri dishes with culture media containing 0.17% (w/v) yeast nitrogen base (YNB, Difco); 0.5% (w/v) ammonium sulphate, 0.004% (w/v) 4-methylumbelliferyl-α-L-arabinofuranoside (Sigma) and 2% (w/v) agar-agar were used. The carbon sources assayed were beet cosette (Industria Azucarera Nacional S.A (Iansa)-Chile) and beet molasses (Iansa-Chile) at 0.2, 0.5, 1 and 2% (w/v) concentrations as indicated. The glycosidase activity was observed by the presence of UV fluorescence around the colony. To quantify the Abf activity, the culture media was centrifuged at 8000 g for 15 min at 10ºC. The supernatant was concentrated 20 times using a Minitan ultrafiltration system (Millipore Corp. Bedford, MA) with a 10,000 molecular weight cutoff polysulfone filter. The culture media without inoculum served as a control. The Abf quantification was done using 4 mM *p*-nitrophenyl-α-L-arabinofuranoside (*p*NPαAF) (Sigma) as substrate according to the methodology used by Le Clinche et al. (1997). Assays were performed by incubating a reaction mixture containing 250 µL of 1 mM of substrate in 50 mM succinate buffer, pH 5.5 and 250 µL of culture media at 40ºC for 20 min. The reaction was stopped by addition of 1 mL of 2 M Na_2CO_3, and the liberation of *p*-nitrophenol was measured spectrophotometrically at 405 nm. One unit of enzyme activity was defined as the amount of enzyme necessary for the formation of 1 µmol of *p*-nitrophenol per minute under the conditions of the assay. The protein quantification was carried out according to the equation $A_{205}/ (27 + 120(A_{280}/A_{205}))$ with readings at both 205 nm and 280 nm wavelengths (Scopes, 1974).

Production of *R. glutinis* in a continuous system

The growth of *R. glutinis* was carried out in a laboratory constructed fermentor under continuous operation. The fermentor was constructed with a 1 L glass vessel (with an internal diameter of 9.4 cm and 15.34 cm in height) submerged 14 cm in a controlled water bath. The system was agitated at 200 rpm with a magnetic stir plate (Heidolph, Germany), and fed with sterile culture media by a flow controlled peristaltic pump (Masterflex, USA). Furthermore, sterile air was injected by the use of a compressor and the flow regulated with a flowmeter (Gilmont Instruments, USA). The system was equipped with a pH probe and an acid/base flow controlled system (Cole Parmer Instruments Co, USA). Antifoam was added manually through a sterile syringe connected to the fermentor. The culture media was defined in the present study. The fermentation conditions were 1 L/min of air flow, pH 5.2 and 28ºC.

Statistical analyses

Statistical analyses were performed with Statgraphics Plus 4.0 software. Differences between treatment means were compared using the least significant differences (LSD) test with confidence level of 95%.

RESULTS AND DISCUSSION

As a way of estimating how the constituents of the culture media influence the growth of *R. glutinis* and the production of Abf, different culture assays were carried out varying the composition of our base medium as indicated above.

Effect of peptone on yeast growth

The yeast *R. glutinis* L-1816 was tested for its capacity to grow, with or without 0.5% (w/v) peptone at 25ºC, in the basal culture media supplemented with 2% (w/v) glucose as a carbon source. At 72 hrs of yeast growth, a basal value of 199 ± 0.007 h^{-1} was obtained, whilst for basal medium plus peptone the value was 0.191 ± 0.022 h^{-1}, both being statistically similar.Peptone is a broadly used component of yeast culture media because it is a rich source of nitrogen. However, our results showed that the specific growth rate is not dependent on the presence of peptone in the culture media. These suggest that the basal media used in our study contains an adequate amount of nitrogen salts that are easy assimilated by the yeast and consequently peptone would not be necessary for yeast growth, at least during the time that the assays were carried out.

Effects of the media salts composition on yeast growth

Cho et al. (2001) have shown that the use of different nitrogen sources in culture media affects yeast growth. Therefore, a study was carried out to determine the most adequate salts composition in the culture media for optimum growth of the yeast strain used in our investigation. In these experiments, we evaluated the specific growth rates of *R. glutinis* L-1816 using the basal media without peptone and varying the type and concentration of salts as is described in Material and Methods. As before, growth was followed for 72 hrs at 25ºC with 2% (w/v) glucose as a carbon source (Table 1).

The salts composition I (0.3% (w/v) of $(NH_4)_2SO_4$; 0.1% (w/v) of KH_2PO_4 and 0.05% (w/v) of $MgSO_4$ x $7H_2O$) is an adaptation of that described for *Rhodotorula flava* by Uesaka et al. (1978) and our results show that it also is enough to support the growth of *R. glutinis* L-1816, being superior to the other salts compositions evaluated. The addition of Fe or Mn in the salts composition II, III or IV seems to not have important effects on yeast growth. The growth rate values showed by *R. glutinis* L-1816 in the presence of these minerals are lower than those obtained using salt composition I, which does not possess any of these metals. Likewise, it can be observed that the increase of KH_2PO_4 and $MgSO_4$ x $7H_20$ concentration could have a negative effect on yeast growth.

Study of industrial carbon sources for *R. glutinis* L-1816 growth and Abf production

The industrial carbon sources such as beet molasses and beet cosette have been used in the production of this yeast (Roche et al. 1994; Luonteri et al. 1995; De Ioannes et al. 2000). Likewise, beet cosette has been studied as a cheap substrate source in Chile for some enzyme-producing microorganisms, especially fungi (Illanes et al. 1992; Chamy et al. 1994). Considering this information, we studied the effect of these industrial carbon sources on the growth of the yeast and the production of Abf. For this, the growth and Abf activity of *R. glutinis* L-1816 were evaluated in basal media at 25ºC and an initial pH of the culture medium of 5.2 (see below). The media was supplemented with 2% (w/v) of the carbon sources as is indicated. The specific growth rates were determined from 72 hrs cultures showing that beet cosette support the lowest specific growth rate (μ = 0.151 ± 0.012 h^{-1}) in comparison to glucose (μ = 0.205 ± 0.010 h^{-1}) and beet molasses (μ = 0.219 ± 0.007 h^{-1}), where these latter carbon sources did not show significant differences in this growth parameter (p < 0.05). Rubio et al. (2002) described that *R. glutinis* has a high invertase activity that would allow the use of media with a high sugar content such as beet molasses. On the other hand, beet cosette presents cellulose and hemicellulose content with only 3.7 g/L of reducing sugars (Illanes and Schaffeld, 1983). This fact could explain the low growth rate observed when the yeast is cultivated with beet cosette, since there would be little availability of easily assimilated carbon substrates when compared with the other two carbon sources assayed.

Because the production of Abf in microorganisms is dependent on the substrate present in the culture media (De Ioannes et al. 2000) and considering the eventual applicability of beet molasses or beet cosette as industrial carbon sources for yeast growth, we study the effect of these two substrates on the production of Abf in qualitative assays. For this, *R. glutinis* L-1816 was grown in petri dishes with different concentrations of beet molasses or beet cosette (Table 2). Our results show that an increase in beet molasses concentration has a negative effect on the Abf activity. This observation could be a consequence of an increase in the sucrose content present in beet molasses whichcan have an inhibitory effect on the production of Abf. For practical considerations, the greatest enzyme secretion was obtained at a concentration of 0.2% (w/v) of beet molasses. Also, beet molasses has approximately 62% (w/v) of carbohydrates and other residues that could induce the production of Abf by the yeast.

Table 3. Effects of initial pH of the culture media on the growth of *R. glutinis* L-1816. Means followed by different superscripted letters are significantly different (p < 0.05)

pH	Specific growth rate (h^{-1})
4.0	0.156 ± 0.001[a]
5.2	0.197 ± 0.130[b]

On the other hand, the effect shown by the beet cosette on the production of Abf by *R. glutinis* L-1816 is independent of its concentration in the culture media. Illanes and Schaffeld (1983) by a hydrolysis experiment of this substrate indicated that its chemical composition includes 58.5% (w/v) of crude fiber. In our case, a hydrolysis was not carried out prior to its incorporation into the culture media, however, part of these components may have passed into solution and it is therefore possible that some induce the production of Abf. De Ioannes et al. (2000) showed that for the case of *Penicillium purpurognum*, beet cosette is a good inducer for the production of this enzyme.

Effects of temperature and initial pH of the culture media on yeast growth

The growth temperature conditions described for *R. glutinis* have been 22ºC (Cho et al. 2001) and 30ºC (Buzzini and Martin, 2000; Rubio et al. 2002). As a way of defining an adequate temperature for the growth of the yeast under our conditions, assays were carried out using culture temperatures of: 20, 25, 28 and 37ºC. In these assays, we estimated the specific growth rates of *R. glutinis* L-1816 at the temperatures indicated using the basal media without peptone. As before, yeast growth was monitored for 72 hrs with 2% (w/v) glucose as a carbon source. Our results suggest that 28ºC is the optimum growth temperature, with a specific growth rate value of 0.198 ± 0.006 h^{-1}, although previous reports have obtained yeast growth at 37ºC (Kurtzman and Feel, 1999).

On the other hand, we have carried out assays in Petri dishes of several native isolates to obtain yeasts with Abf activity (Ganga and Martínez, 2004). As a continuation of these studies, we found that *R. glutinis* L-1816 presents this activity at an acid pH (data not shown). As a way of evaluating the effects of the acid culture media on the growth of this yeast strain, assays were carried out in culture media with an initial pH of 4.0, 5.2 and 7.0. Cho et al. (2001) showed that this yeast showed an important biomass production at an initial pH of between 4.0 and 7.0. Our results are shown in Table 3 and indicate that at an initial pH of 5.2, in unbuffered media, the yeast growth rate is greater. Under our culture conditions and pH 7.0, only a residual growth was observed.

The study of pH evolution in unbuffered *R. glutinis* L-1816 72 hrs cultures showed an increase of this parameter reaching a value of 7.0, when basal media without peptone at 28ºC and beet molasses as carbon source was used. Therefore, we analyzed the effects on the yeast growth of maintaining the pH stable during the whole growth period using phosphate citrate pH 5.2 as buffer. Our results showed that there are no statistical differences in the specific growth rate of the yeast using media at a constant pH of 5.2 or media without pH control but with an initial pH of 5.2. Furthermore, the biomass concentration values at the end of the growth period showed differences of less than 6%. Since growth of this yeast is affected by neutral pHs and these values are observed in our studies in batch cultures, it is therefore important to control this parameter in continuous cultures.

Considering the assays above, we proposed that the best growth conditions for Abf production by *R. glutinis* L-1618 are 0.2% (w/v) beet molasses, 0.3% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4$ x $7H_2O$, 0.5% (w/v) yeast extract, pH 5.2 and 28ºC.

Production of α-arabinofuranosidase in a continuous system

Using the culture conditions described in the previous paragraphs, the growth of *R. glutinis* L-1816 was carried out in a continuous culture system for80 hrs to produce Abf. Two dilution rates were assayed as is shown in Table 4. Under these conditions it was possible to obtain a positive correlation between dilution rate and Abf activity, where the latter increased almost five times. Furthermore, an increase in the production of total proteins is also observed, which allowed the specific activity of the enzyme under study be maintained constant.

Uesaka et al. (1978) by growing *R. flava* in a medium with purified beet arabinan as carbon source obtained an α-arabinofuranosidase activity of 3.6 mU/mL, a lower value than that obtained in our study at the different flow velocities assayed. On the other hand, the specific activity described by these authors was 0.26 U/mg. In our case, the specific activity obtained for the *R. glutinis* strain L-1816, did not show significant differences between the flows velocities assayed, obtaining an average value of 82.4 U/mg, approximately 23 times greater than that described by Uesaka et al. (1978). This is mainly because of the low

Table 4. α-L-arabinofuranosidase production in a continuous culture reactor at different flow velocities. Means within the same column followed by different superscripted letters are significantly different ($p < 0.05$).

D (h^{-1})	Enzyme activity (mU/mL)	Total proteins (µg/mL)	Specific activity (mU/µg)	Enzyme activity (mU/g dry weight)
0.064	29.238 ± 2.617[a]	0.349 ± 0.089[a]	83.775 ± 28.885[a]	16.565 ± 2.806[a]
0.178	145.571 ± 13.957[b]	1.798 ± 0.810[b]	80.951 ± 44.231[a]	105.640 ± 3.4046[b]

amount of proteins secreted by the yeast, which could correspond to the enzyme under study and therefore our growth conditions for this yeast permit a greater increase in the Abf production.

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