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Comparative study of alcohol dehydrogenase activity in flor yeast extracts

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The highest activities of alcohol dehydrogenase were obtained when flor yeasts were grown on L-lactic acid as the main carbon source. The strains with the lowest average of alcohol dehydrogenase activities, grown on glucose and ethanol, make up the velum on wines during the early stages ageing. One of the strains studied (*Saccharomyces cerevisiae*, M10) may be a suitable source from which to isolate this enzyme (32 units of activity per mg protein).

Introduction

Alcohol dehydrogenase (ADH, EC 1.1.1.1), which catalyzes the oxidation of ethanol to produce acetaldehyde, is widely used in biochemical, medical and forensic science for the determination of alcohol concentrations (Bernet and Gutman, 1974). It is also used for measuring NAD^+, both directly and in cycling methods (Marko-Varga and Domínguez, 1991), and in acetaldehyde production (Lortie *et al.*, 1992). Therefore, in the last few years, new natural sources have been explored for producing this enzyme (Wills *et al.*, 1981; Bicsak *et al.*, 1982; Okuma *et al.*, 1991).

The microorganisms used in this work belong to the group known as flor yeasts because of the shape of their colonies in stationary culture. These yeasts grow spontaneously in the film phase on West Andalusian wines during biological aging. The yeasts that form this group belong to the species *Saccharomyces cerevisiae* and *Zygosaccharomyces roixii* (Barrett *et al.*, 1992). All these species are characterized by their ability to grow on very high levels of ethanol (14–15% v/v), that would be toxic for all other yeast species. It seems that yeasts that form velum have in common the capacity to develop this film as an adaptative mechanism which allows them to grow in high ethanol concentrations (Martínez *et al.*, 1995). This unusual property makes the study of the ethanol metabolism of flor yeasts very interesting.

Materials and methods

Strains

Seven different strains isolated from standard industrial ageing systems ("solera systems") for two types of Sherry wines (fino and manzanilla) were used. These strains belong to the *S. cerevisiae* (B2, B3, B16, CH15, M10 and M17) and *Z. rouxii* (R13) species.

Culture media

Three culture media of different composition were employed: YEPE (yeast extract, peptone, ethanol), YEPD (yeast extract, peptone, D-glucose) and YEPL (yeast extract, peptone, L-lactic acid). All types of culture medium were composed of 10 g yeast extract/L and 20 g peptone/L, being differentiated only in the source of carbon used, so that 20 g ethanol/L, D-glucose or L-lactic acid were added according to the particular experiment to be performed. Final pH was adjusted to 3.5.

Culture conditions

The cultures took place in 500 mL fermenters, with a working volume of 100 mL, placed on a rotary shaker incubator. The fermentations were performed aerobically with an agitation of 200 rpm and at constant temperature of 30°C. A preinoculate was grown with 50 μL of each glycerinated strain to 80% in 50 mL of YEPD medium, from which 1 mL was re-inoculated into 100 mL of YEPD, YEPE or YEPL medium according to the fermentation to be carried out.

The growth of the yeast cells was determined by spectrophotometry (optical density at 600 nm). It was observed experimentally that at $1.2 > O.D. > 2.0$ cultures were in the mid-growth phase.

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Production of the enzymatic extract

The extraction technique was modified from the original one (Schimpfessel, 1968). Once the cultures had reached the logarithmic phase, they were centrifuged for 4 minutes at 3,000 g and 0°C. The pellet obtained, containing the yeast cells, was washed with cold distilled water and re-suspended in a sufficient volume of 25 mM phosphate buffer (pH 7.4) 5 mM β-mercaptoethanol to give a cellular concentration of 0.2 g of wet weight/mL. β-Mercaptoethanol was added to prevent ADH sulfhydryl groups from oxidising (Kennedy *et al.*, 1990). The suspension of cells was then passed through a high pressure homogenizer (Emulsiflex 20.000), for a single extraction cycle at 20,000 psi. These pressure value and cellular concentration were selected because as the pressure is increased, soluble protein release increases exponentially for concentrations of yeast suspensions over the range 0.86–0.125 g of wet weight/mL (Brookman, 1974). The cellular remains (membranes, organules and others) were eliminated by centrifugation at 100,000 g for 35 minutes at 0°C. The supernatant obtained – the enzymatic extract – was used for determinations of enzymatic activity and protein concentration or was glycerinated to 30% and preserved at –20°C for other purposes.

Enzymatic activity test

Alcohol dehydrogenase activity was assayed spectrophotometrically by a modification of the Bergmeyer method (Bergmeyer *et al.*, 1974). The reaction mixture contained 2.5 mL of 0.1 M Tris/HCl buffer (pH 8.8), 200 μL of 30 mM NAD^+, 100 μL of 15 M ethanol, 25 μL of enzyme preparation, in a total volume of 3.0 mL. The enzyme reaction was carried out at 30°C in a 1 cm cuvettes, and the reaction was followed by measuring the increase in absorbance at 340 nm. One unit of enzyme activity (EAU) was defined as the amount of enzyme catalyzing the production of 1 μmol of NADH per minute under these conditions.

Protein concentration

Protein concentration of each sample was assessed by Lowry technique modified by Peterson (Peterson, 1977).

Statistics

Four different fermentations of the same strain on each different culture medium, with two replications of each enzymatic activity measurement were done. The data reported in graphics and tables are referred to the average of all the measurements and the limit of confidence of them for a probability of 95% is also included.

Results and discussion

Influence of carbon source on alcohol dehydrogenase activity

From the cytoplasmatic extracts obtained from the different fermentations that were carried out, it was possible to study the influence of the carbon source on their ADH activity. The results obtained are given in Table 1. As can be seen, the highest levels of alcohol dehydrogenase activity were obtained when the strains were grown in YEPL medium. As far as the growth on D-glucose or ethanol as carbon sources is concerned, differences between several strains were observed. So far for M17, R13 and B2 strains, the ADH activities were approximately the same, regardless of whether YEPD or YEPE was used as the culture medium; while for the rest of the strains, except for B3, higher activities were measured when the strains grew on glucose than on ethanol.

Table 1 Effect of the carbon source on alcohol dehydrogenase activity (EAU/mL) for the different stains of *Saccharomyces*

Strain	*D-Glucose*	*Ethanol*	*L-Lactic acid*
B2	7.4 ± 1.6	7.7 ± 2.1	19.5 ± 2.6
B16	10.0 ± 1.4	7.3 ± 2.4	17.6 ± 2.0
CH15	11.8 ± 1.8	8.2 ± 1.3	17.9 ± 1.7
B3	9.2 ± 1.5	11.7 ± 1.8	12.0 ± 1.8
M17	10.8 ± 1.9	11.3 ± 2.1	13.0 ± 2.9
R13	13.4 ± 2.1	13.0 ± 3.0	19.0 ± 3.4
M10	26.8 ± 2.9	19.1 ± 2.4	31.6 ± 0.6

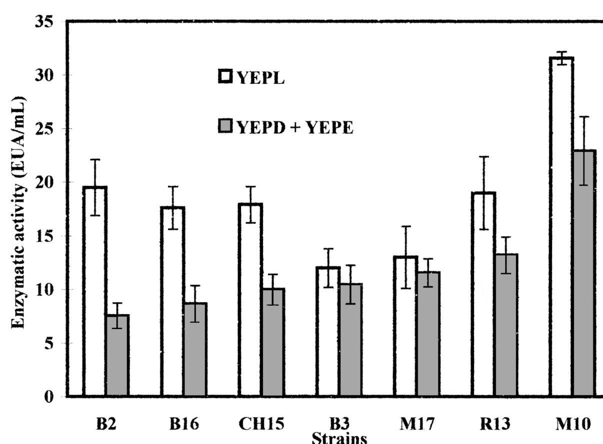

Figure 1 Average of the alcohol dehydrogenase activities of the strains extracts grown on D-glucose and on ethanol, together with ADH activity of the yeast extracts grown on L-lactic acid.

Further, it can be seen (Fig. 1) that the average of ADH activities of the strains grown on D-glucose and on ethanol allowed their classification into three different groups. Thus, strains B2, B16, and CH15 were classified as strains of low activity, B3 nd M17 as strains of medium activity, and R13 and M10 as strains of high ADH activity.

Relationship between alcohol dehydrogenase enzymatic activity and the physiological characteristics of the flor velum yeast

With the aim of relating the different results of the flor velum yeasts with regard to their ADH activities, the values of data reported in the literature concerning the ethanol consumption and acetaldehyde production, during the biological aging of pure cultures of each strain was assembled (Table 2). As might be expected, the highest levels of ethanol consumption and acetaldehyde production were recorded for R13 and M10 strain, which have been classified as strains producing high activity; while the lowest values relate to the strains of low activity.

It can also be seen that the strains taking the most time to colonise the culture surface are those belonging to the group producing high ADH activity, while strains belonging to the group producing low ADH activity make up the velum earlier.

On the other hand, it can also be observed in Table 2 that the strains that suffer the greatest percentage of inhibition in their growth, when cultivated in media with ethanol as the only carbon source, are those belonging to the group of low ADH activity; while the cellular development of flor yeasts belonging to the group of high ADH activity is affected to a lesser extent by the alcoholic substrate. This result could be explained by taking as a basis the fact that strains with high ADH activity are better adapted to the alcoholic media than the rest of the strains. That is why they would take more time to form the velum when moving to the medium surface. The strains of low ADH activity will make up the surface velum earlier as an adaptative mechanism because they can stand only low levels of ethanol.

Strains growth on L-lactic acid

The values of specific alcohol dehydrogenase activity (enzymatic activity by protein weight unit) of the cytoplasmatic extracts for each one of the strains grown on L-lactic acid can be found in Fig. 2. From the values obtained, it can bee seen that the strains B3 and M17 have a low specific activity, strains B2, B16, CH15 and R13 have a medium specific activity, and strain M10 has this activity in a high range.

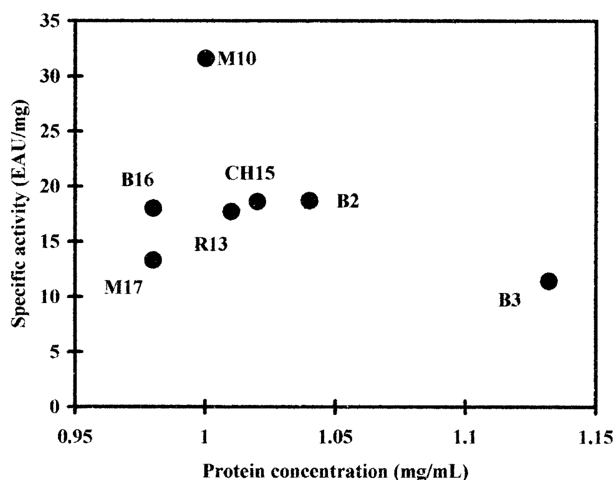

Figure 2 Specific ADH activity of flor yeast grown on L-lactic acid versus their total protein concentration. B2, B3, B16, CH15, M10 and M17: *Saccharomyces cerevisiae* and R13: *Zygoccharomyces rouxii.*

Table 2 Comparison of ADH activity with published physiological characteristics (Martínez, 1995) when grown on ethanol

Strains	*Average of activities on YEPD and YEPE (EAU/mL)*	*Time on velum formation (days)*	*Inhibitory effects of ethanol (%)*	*Ethanol comsuption %(v/v)*	*Acetaldehyde production (mg/mL)*
B2	7.5 ± 1.2	15	66.2	3.4	162
B16	8.6 ± 1.7	14	75.0	3.7	170
CH15	10.0 ± 1.4	14	71.3	3.5	144
B3	10.4 ± 1.8	19	58.3	3.3	211
M17	11.0 ± 1.3	20	68.9	–	–
R13	13.2 ± 1.7	31	50.0	4.8	375
M10	22.5 ± 3.2	28	58.2	4.7	358

Further, the graphic representation of the specific activity versus protein concentration of the extracts of each strain (Fig. 2), allows the extract quality/quantity to be assessed. Thus, the strain M10 might constitute a suitable source from which to isolate the ADH enzyme, as this will produce an enzyme of high quality, or high specific activity. The strain B3, however, could be selected with a high content of proteins, although this shows very low specific activity.

References

Barrett, J.A., Payne, R.W. and Yarrow, D. (1992). In: *Yeasts: Characteristics and Identification*, Cambridge Univ. Press, Cambridge, England.

Bergmeyer, H.V., Gacoehm, K. and Grassl, M. (1974). In: *Methods of Enzymatic Analysis*, H.V. Bergmeyer, eds. vol. 2 pp. 428–429, New York: Academic Press.

Bernet, E. and Gutman, Y. (1974). In: *Methods of Enzymatic Analysis*, H.V. Bergmeyer, eds. pp. 1499–1502, New York: Academic Press.

Bicsak, T.A., Kann, L.R. Reiter, A. and Chase, T.J. (1982). *Arch. Biochem. Biophys.* 216, 605–615.

Brookman, J.S.G. (1974). *Biotechnol. Bioeng.* 16, 371–383.

Kennedy, C.L. and Domach, M.M. (1990). *Biotechnol. Prog.* 6, 41–47.

Lortie, R., Fassovane, A., Laval, J.M. and Bourdillon, C. (1992). *Biotechnol. Bioeng.* 39, 157–163.

Martínez, P., Codón, A.C., Pérez, L. and Benítez, T. (1995) *Yeast.* 11, 1399–1411.

Marko-Varga, G. and Domínguez, E. (1991) *Trends. Anal. Chem.* 10, 290–297.

Okuma, Y., Ito, Y. and Endo, K. (1991). *J. Ferment. Bioeng.* 71, 309–312.

Peterson, G.L. (1972). *Anal. Biochem.* 83, 346–356.

Schimpfessel, L. (1968). *Biochim. Biophys. Actas.* 151, 317–329.

Wills, C., Kratofil, P., Londo, D. and Martin, T. (1981) *Arch. Biochem. Biophys.* 210, 775–785.

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