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EFFECT OF CULTURE CONDITIONS ON THE ALDEHYDE DEHYDROGENASE ACTIVITY OF *Acetobacter aceti* CYTOPLASMATIC EXTRACTS

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SUMMARY

The aldehyde dehydrogenase activity of the cytoplasmatic extracts of a *Acetobacter aceti* strain used industrially in vinegar production is investigated. Two different culture media, based on ethanol or glucose, were used to compare the strain growth and activity. The kinetic constants of the enzymatic activity have been determined for extracts from the strain grown on ethanol. Finally, a study has been made of the spontaneous enzyme de-activation, which seems to fit a first order kinetic.

INTRODUCTION

It is well known that one of the species of the *Acetobacter* genus, the *aceti* species, is the strain most commonly used for the industrial production of vinegar. However, the biochemical aspects of this species have not been as widely studied as those of other species, like beer yeasts, lactic acid bacteria and other bacteria of industrial interest. This strain performs the biological oxidation of ethanol to acetic acid by means of two enzymes associated with the cellular membrane, one an alcohol dehydrogenase (Adachi *et al.*, 1978; Adachi *et al.*, 1987) BADH (bacterium alcohol dehydrogenase) and the other an aldehyde dehydrogenase (Adachi *et al.*, 1980; Ameyama *et al.*, 1981) BDDH (bacterium aldehyde dehydrogenase), which together form the enzymatic complex responsible for acetic fermentation (Muraoka *et al.*, 1982). However, BDDH's dependent on NADP have also been found in the cytosol of *A. aceti*, which appear to be implicated in the oxidation of various

aldehydes (Muraoka *et al.*, 1980).

It should be pointed out that, in the purification processes for both the alcohol and aldehyde dehydrogenases performed in the previously mentioned studies, no uniformity is observed in respect of the carbon source employed for the growth of *Acetobacter aceti*. Thus, in various studies, an aqueous ethanol medium (Muraoka *et al.*, 1982; Muraoka *et al.*, 1980; Muraoka *et al.*, 1981a; Muraoka *et al.*, 1981b). Other authors cultured the strains on sugar media based on potato extract (Adachi *et al.*, 1978). It could, therefore, be of considerable interest to study the influence of the conditions of culture on the enzymatic activity of these strains. The choice of a suitable carbon source for the culture of *Acetobacter aceti* could encourage the catalytic activity of the enzymatic extract.

In this paper, a strain of *Acetobacter aceti* isolated from vinegars produced industrially in the Jerez area, which in principle demonstrates a high potential for oxidation, has been studied.

MATERIAL AND METHODS

Strain

Acetobacter aceti UCA1, a strain that had been isolated by our research group from vinegar industrially produced in the Jerez area, glycerinated to 80% and stored at -20°C was used as inoculum for all the fermentations carried out.

Culture medium

Two culture media of different composition were employed. YEPE (Yeast Extract, Peptone, Ethanol) and YEPD (Yeast Extract, Peptone, D-glucose), supplemented with $MgSO_4$. This because the metal ion Mg^{+2} has been observed to stimulate the enzymatic activity of the aldehyde dehydrogenases present in the cytosol of this microorganism(Muraoka *et al.*, 1980). Both types of culture medium were composed of 10 g/L of yeast extract, 20 g/L of peptone and 0.35 g/L of $MgSO_4$, being differentiated only in the source of carbon used. For this, in the YEPD medium, 20 g/L of D-glucose was added, while in the YEPE medium 60 g/L of ethanol were added, this being the maximum concentration at which efficient cellular growth is produced (Nanba *et al.*, 1984).

Culture conditions

The cultures took place in 500 mL fermenters, with a working volume of 200 mL, introduced in an rotary shaker incubator. The fermentations were performed with an agitation of 200 rpm, an aeration of 0.5 vvm and at a constant temperature of 30°C. A preinoculate was grown, with 200 μL of glycerinated culture, in 100mL of YEPD/Mg medium, from which 5 mL was re-inoculated into either the YEPD/Mg medium or the YEPE/Mg medium, according to the paricular experiment to be performed.

Determination of the biomass concentration

The growth curve of *A.aceti* was determined by spectrophotometry, using the turbidimetrical method (optical density at 580 nm) (Swings and De Ley, 1984). To express the concentration of biomass as numbers of cells per mL, a published correlation between

absorbance and total biomass for this type of culture was used (Romero *et al.*, 1994).

Obtention of the enzymatic extract

When cultures grown in the YEPD/Mg and YEPE/Mg media had reached the stationary phase, these were centrifuged for 5 minutes at 3000 g and 4°C. They were washed twice with phosphate buffer 40 mM (pH 8.0), then re-suspended in a sufficient volume of the same buffer to give a cellular concentration of 0.09 g of wet weight/mL. The suspension of cells was then passed through a high pressure homogeniser (EmulsiFlex-20000), for a single extraction cycle at 20,000 psi (Adachi *et al.*, 1978). The cellular remains (membranes, organules, and others) were eliminated by centrifugation at 100,000 g for 35 minutes at 4°C. The supernatant obtained - the enzymatic extract - was used for the determinations of enzymatic activity or was preserved at 4°C for other purposes.

Enzymatic activity test

The activity of the enzymatic extracts which had been obtained was determined by spectrophotometry (Muraoka *et al.*, 1980; Muaoka *et al.*, 1981a; Muraoka *et al.*, 1981b). The reaction mixture contained 2.5 mL of glycine-NaOH buffer 40 mM (pH 9.0), 100 µL of $MgSO_4$ 0.3 M, 100 µL of acetaldehyde 0.2 M, 0.2 µl of potassium ferrocyanide 80 mM, 100 µl of enzymatic extract and water up to a final volume of 3.0 mL. The reaction was started at ambient temperature (approximately 25°C), by means of the addition of the enzymatic extract. The reduction of the absorbance at 420 nm was monitored with a UV-V spectrophotometer.

An enzymatic activity unit (EAU) of aldehyde dehydrogenase was defined as the quantity of enzyme which catalyses the reduction of 1 µmol of ferrocyanide per minute, under the reaction medium conditions previously specified.

RESULTS AND DISCUSSION

Growth curves of *Acetobacter aceti* on glucose and on ethanol

A study was made of the biomass growth in cultures inoculated on glucose and on ethanol as the carbon source, under identical conditions. As it was to be expected, the growth on ethanol causes an expansion of the lag phase of some 12 hours, compared with the growth on glucose, as a result of the typical antiseptic effect of ethanol.

The final microbial mass measured in the YEPD/Mg medium is 2.5 times greater than that obtained in the YEPE/Mg medium (Fig. 1). It appears therefore that, from the point of view of production of biomass, it is more interesting to grow *A. aceti* on glucose than on ethanol.

Aldehyde dehydrogenase activity of the enzymatic extracts

To study the influence of the culture medium on the enzymatic activity, the aldehyde dehydrogenase activity of two enzymatic extracts of *Acetobacter aceti* UCA1 strain grown on glucose (BDDH from YEPD/Mg) and on ethanol (BDDH from YEPE/Mg) were compared.

For this, the rate of reduction of the anion ferrocyanide was monitored, which provides a quantitative indication of the amount of acetaldehyde oxidised. Illustrated in Fig. 2 are the curves of the variation in the absorbance over time, for the enzymatic extracts obtained from the YEPD/Mg and YEPE/Mg culture media at 24 hours after extraction. As can be seen, the concentration of substrate falls much faster in the case of the extract derived from the microorganism grown using ethanol as the carbon source.

From the shape of the curves of absorbance, it can be deduced that, at least in the initial moments, a zero order kinetic is followed; this normally corresponds to high values of substrate concentration in the Michaelis-Menten kinetic model. By measuring the initial slopes of the two curves obtained, and taking into account the stochiometric relationship between the ferrocyanide reduced and the acetaldehyde oxidised (1:1), the initial rates of oxidation shown by the different enzymatic extracts can be compared. The results show that the initial rate of oxidation is 246 10^{-6} mol/L'min for the extract from YEPE/Mg, and 32 10^{-6} mol/L'min for that from YEPD/Mg. Based on these data, activities of the crude extracts of 7.26 EAU/mL and 0.96 EAU/mL, can be respectively determined. Therefore, the biological oxidation of acetaldehyde is 7.56 times more efficient with enzymatic extract of cells grown on ethanol, compared with on glucose. Consequently, for the production of cytoplasmatic aldehyde hydrogenases (BDDH), it may turn out that the culture of strains in media with ethanol as the carbon source is in fact very interesting, even though the concentration of cells obtained may be less, owing to the greater activity of these extracts.

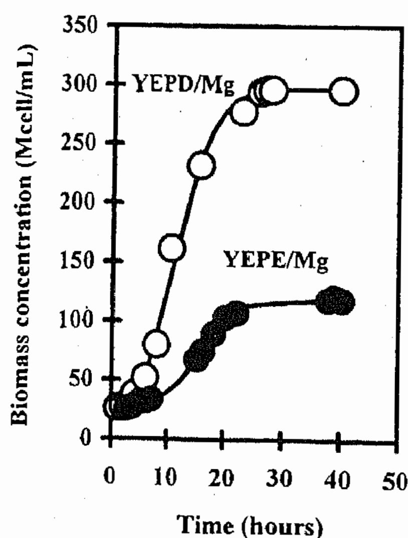

Fig. 1. Growth curves of *Acetobacter aceti* UCA1 on different culture media.

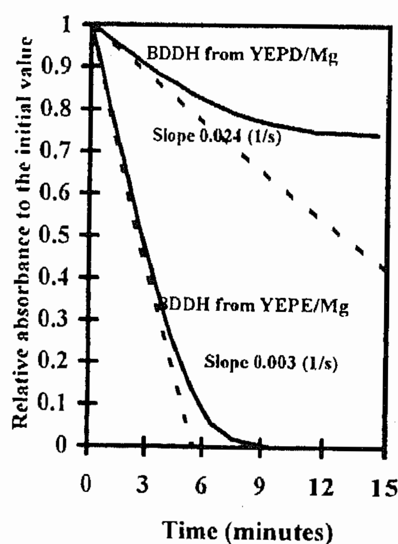

Fig. 2. Measure of the aldehyde hydrogenase enzymatic activity from *A. aceti* UCA1, grown on different culture media.

Determination of the kinetic constants of the enzymatic extract

To obtain the kinetic constants of the enzymatic extracts from the strain grown on ethanol as carbon source, different enzymatic tests of aldehyde dehydrogenase activity were carried out. These tests were done at 4 and 7 days after the extractions and for each extract five tests were done, using five different concentrations of acetaldehyde, while keeping the concentration of ferrocyanide constant. From the falling curves of absorbance obtained, the rates of enzymatic reaction were calculated and, subsequently, a regression analysis was performed adjusting the data for these rates to the Lineweaver-Burk equation (Smith, 1972)(Fig. 3). In this way, it was determined that the enzymatic extract at 4 days after extraction shows a maximum reaction rate (Vm) of 137 10^{-6} mol/L·min ($r^2 = 0.99$), while at 7 days this is reduced to 112 10^{-6} mol/L·min ($r^2 = 0.99$). Equally, the saturation constant (Km) obtained is 0.688 mmol/L, which coincides closely with the mean of those obtained by other authors for diverse purified BDDH's (Muraoka *et al.*, 1980). Values like these indicate that the cytoplasmatic extracts have a strong affinity for acetaldehyde as substrate.

De-activation kinetic of the enzymatic extract

A study of the stability of the enzymatic extracts derived from *A. aceti* grown on ethanol as the carbon source, when stored at 4°C, was carried out. The maximum rates of reaction for fresh extracts and for those at 24 hours after extraction were determined by applying the general type of the Michaelis-Menten kinetic. The results obtained, together with the maximum rates of reaction of the enzymatic extract at four and seven days after their extraction are shown in fugure 4.

In the Michaelis-Menten equation, the maximum reaction rate (Vm) can be considered as the product of the total (initial) concentration of enzyme (g/L) and the kinetic constant for product formation rate (kp) (mol/g of enzyme·min). Therefore the graphic representation of Vm versus time provides a direct measure of the evolution of the enzymatic activity of an extract.

In this case it is possible to fit the values obtained to a first order kinetic of de-activation (Lencki *et al.*, 1992). In the model considered, the active enzyme (E_a) suffers an irreversible chemical or structural change to an inactive form (E_i) at a rate (r_d) proportional to the concentration of active enzyme being kd the kinetic constant of deactivation.

From the graphical representation, a constant of de-activation of 2.24 10^{-3} s^{-1} ($r^2 = 0.97$) is obtained. These results suggest that the enzymatic extract under study is quite stable to storage at 4°C.

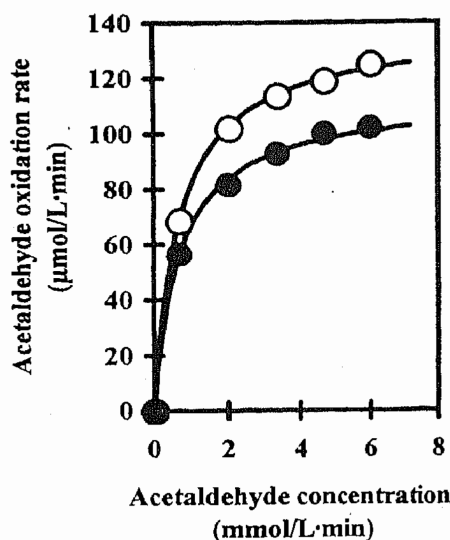

Fig. 3. Effect of the concentration of acetaldehyde on the activity of the enzymatic extract at four days (○) and at seven days (●) after its extraction.

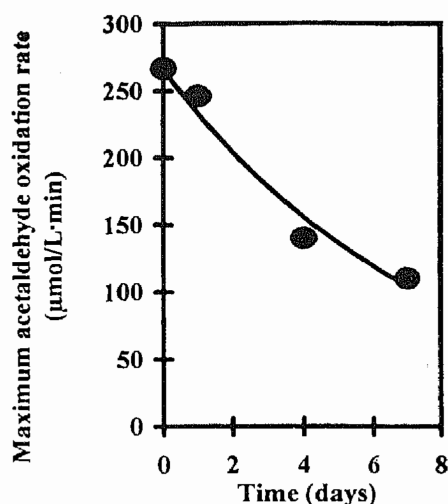

Fig. 4. De-activation of the enzymatic extract derived from *Acetobacter aceti* UCA1 grown on ethanol, as a function of time of storage at 4°C.

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