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Expression, characterization and its application of a novel alcohol dehydrogenase of *Saccharomyces cerevisiae* by *Escherichia coli*

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Abstract

A NAD^+ -dependent alcohol dehydrogenase (ADH1) from *Saccharomyces cerevisiae* was expressed in *Escherichia coli* system and purified. The yeast gene encoding the alcohol dehydrogenase (*ADH1*) was cloned into the pET28a vector for expression in *E. coli* (BL21) and several conditions for optimum production of the recombinant enzyme were screened. After production of insoluble alcohol dehydrogenase in *E. coli*, purification by affinity chelating chromatography was carried out under denaturing conditions using the 6xHis tag fused to the enzyme and followed by protein refolding. The activity and stability of the recombinant enzyme ADH1 was determined and the parameter of enzyme was kinetically characterized. The purified ADH1 gave a single band on SDS-PAGE and the molecular mass was 37kDa. The results demonstrated *in vitro* activity of the recombinant enzyme similar to that of a commercial endogenous *S. cerevisiae* enzyme and showed a minimal detection limit of 2.3×10^{-4} g/L of ethanol in 2 minutes.

Keywords: alcohol dehydrogenase, cloning, purification.

Introduction

Saccharomyces cerevisiae possesses at leaves five genes (*ADH1* to *ADH5*) that encode alcohol dehydrogenase isoenzymes involved in ethanol metabolism. The isoenzymes alcohol dehydrogenase I, III, IV and V reduce acetaldehyde to ethanol during alcoholic fermentation, while alcohol dehydrogenase II catalyse the reverse reaction. Althout *ADH* I and *ADH* II share 89% sequence similarity, their respective expression products. ADHs are broadly distributed in nature and have been found in many animals, plants and microorganisms and the ability to obtain native equivalent recombinant ADH extends its applications, such as serving as a potential biomarker in cancer diagnosis (Cheng et al., 2013), enzymatic determination of ethanol in beverages and industrial products (Maitaix and Castro, 2000).

Alcohol dehydrogenases are among the most interesting enzymes in biocatalysis, for stereoselective reduction of ketones to form chiral alcohols is one of the most useful reactions in organic synthesis. Several authors highlighted the potential of enzymes to be used as efficient, economical, and environmentally friendly biocatalysts (Said and Pietro, 2004). The aim of this work was to investigate the pH and temperature of reaction, thermal stability, over recombinant alcohol dehydrogenase production and to find a method for ethanol determination in alcoholic beverages.

Material and Methods

Cloning, production of ADH1 from *S. cerevisiae*

The complete coding sequence of yeast alcohol dehydrogenease I gene (*ADH1*) was PCR-amplified from *S. cerevisiae* genome, and sub-cloned into the *Eco*RI and *Xho*I sites of pET28a vector (Invitrogen). For protein purification, BL21(DE3)pLysS cells transformed with pET28a-*ADH1* were harvested after 3 hours of growth induction with 0.4 mM IPTG at 37°C.

Purification of ADH1 from *S. cerevisiae*

The protein was affinity-purified using Ni-NTA beads (Qiagen). Ni-NTA washing buffer consisted of 50 mM NaH_2PO_4 pH 8.8; 20 mM imidazol; 300 mM NaCl and 6 M urea, whereas the elution buffer contained 50 mM NaH_2PO_4 pH 8.8; 250 mM imidazol; 300 mM NaCl and 6 M urea. The final protein sample was better than 95% purity, as judged by SDS-PAGE of protein fractions. For refolding, the concentrated denatured protein (0.5 mg/mL) was dialysed in 4 steps of 6 hours each one, at 4°C, against the refolding buffer (10 mM Tris-HCl pH 8.5), decreasing the concentration of urea per step (4M, 2M, 1M and 0M urea).

Analytical curve of ethanol and ethanol assay on beverages

The activity of purified ADH1 was analyzed spectrophotometrically recording the increment of absorbance at 340 nm promoted by the formation of NADH during the oxidation of ethanol. Ethanol was measured in samples of alcoholic beverages using the method based on the enzymatic conversion of ethanol to acetaldehyde and NADH, through oxide-reduction reaction, using the coenzyme NAD^+ as an electron acceptor (Gattás et al., 2014).

Result and Discussion

In order to obtain a large amount of functional and stable ADH1 enzyme from *S. cerevisiae*, the recombinant enzyme gene was expressed in *E. coli* (BL21), as Figure 1.

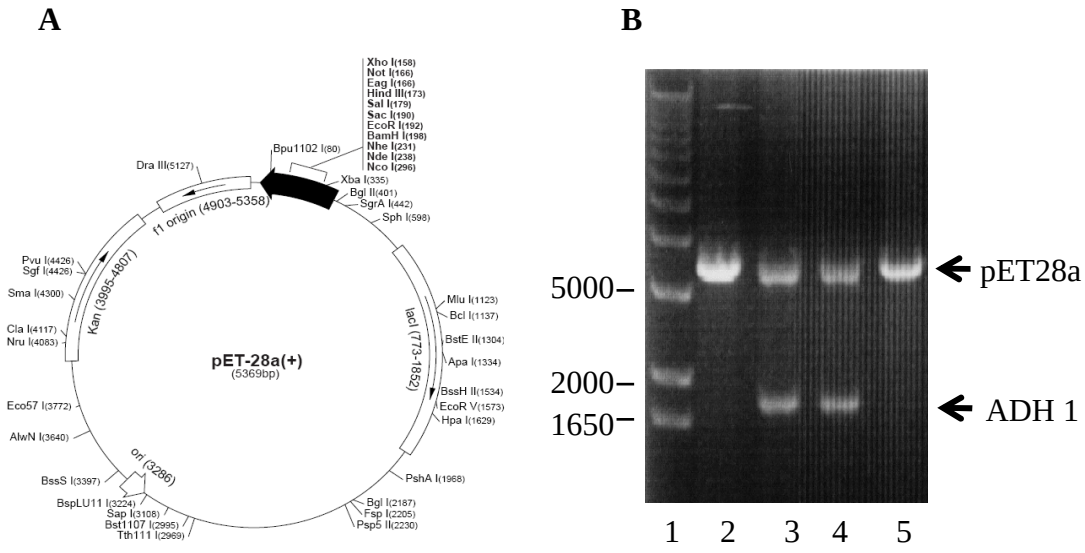

Fig . 1. Cloning of alcohol dehydrogenase of *Saccharomyces cerevisiae* by *E coli* (A) and after transformation of the ligation product in *E. coli* strain, four clones obtained were subjected to cloning checking (B).

The protein produced was purified in a unique step of affinity chromatography, as described in the methodology. The Figure 2 shows the SDS-PAGE of the chromatographic purification fractions, which various fractions containing ADH1 with better than 95% of purity can be seen.

A **B**

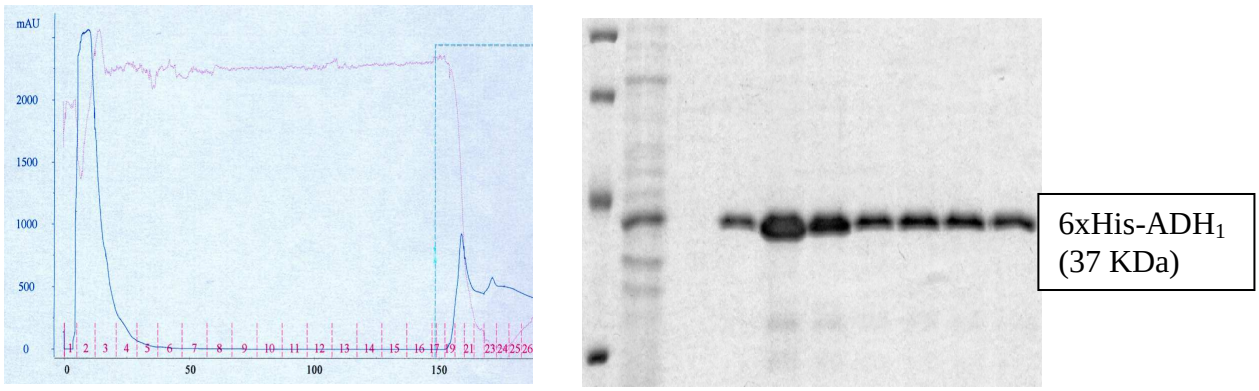

Fig 2. Purifation of ADH_1 from *E. coli* BL21 (A) and SDS-PAGE of ADH_1 (B)

The enzymatic activity assay of the ADH1 protein was carried out as described by Racker (Racker, 1955), which is based on the oxidation of the ethanol by the enzyme, with the concomitant reduction of NAD+. The formation of NADH was determined by the absorbance at 340 nm for minute and the result is showed in the Figure 3. The enzymatic activity of ADH1 is proportional to the increase of the enzyme concentration used in the assay and not important differences on the absorbance between 1 and 90 minutes of the time reaction. Similar assay was done with the purified enzyme kept at 4°C and – 20°C, for until 120 days and the enzyme ADH1 lost the activity when it was kept at 4°C after 3 days. However, the enzymatic activity was constant until 120 days, when the enzyme was kept at - 20°C (data not showed). The method of ethanol quantification using the purified ADH1 showed sensitivity in the minimal ethanol concentration assayed 5 mM (2.3 x 10^{-4} g/L), after 2 minutes of reaction (Figure 3).

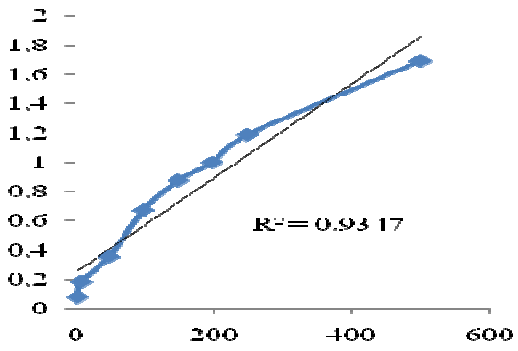

Fig. 3. Analytical curve of ethanol.

The quantification of ethanol in different alcoholic beverages was also performed using the same protocol presented, with the use of 0.5 U of purified ADH1, the samples were diluted 2000 times and it was observed a maximum deviation in the ethanol concentration of only 0.4% when compared with the specifications of the producer (Table1).

Sample	Manufacturer's (vol. %)	Found (vol. %)
Brama Beer	4,8	4,8 +/- 0,2
Antartica Beer	4,6	4,6 +/- 0,3
Whisky J&L	40	39,5 +/- 0,4
Ypióca (sugarcane brandy	39	41 +/- 0,3

Table 1. Assay of ethanol in samples of alcoholic beverages**.**

Conclusions

In the present study, the recombinant enzyme alcohol dehydrogenase 1 (ADH1) from *S. cerevisiae* was produce in bacteria and purified under denaturized conditions. After refolded treatment, it was possible to produce 30 U of enzyme using 1 L of bacterial culture, which one enzymatic unit (U) was defined as the amount of enzyme converting 1.0 µmole of ethanol to acetaldehyde per minute at pH 8.8, 25° C. This enzyme showed a minimal detection limit of 2.3×10^{-4} g/L of ethanol in 2 minutes and the ethanol determination in different sample presented a maximum deviation of only 2 %. In addition, the ADH1 was stable up to 120 days when stocked at -20ºC. All the features presented by the recombinant alcohol dehydrogenase produced using the methods determined herein enable the use of this enzyme in a kit to be low-cost, fast and accurate for assaying ethanol concentration.

References

CHENG F, HU T, NA Y, HUANG J, XU Y (2013) Purification and enzymatic characterization of alcohol dehydrogenase from *Arabidopsis thaliana*. Protein Expres Purif. 90:74-77.

MATAIX E, CASTRO MDL (2000) Simultaneous determination of ethanol and glycerol in wines by a flow injection-pervaporation approach with in parallel photometric and fluorimetric detection. Talanta 51: 489-496.

SAID S, PIETRO RCLR (2004) Enzimas como agentes biotecnológicos. Ribeirão Preto: Editora Legis Summa.

GATTÁS EAL, RIBEIRO MHL, PERES MFS (2014) Improvement of the alcohol dehydrogenase from baker's yeast using polymers and salts for alcohol determination in beverages. J Food Agr Env 12: 80-83.

RACKER E (1955) Alcohol dehydrogenase from baker's yeast. In: Colowich, S.P., Kaplan N.O (Eds.). Methods in enzymology 1: 500-503.