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Pyruvate Decarboxylase Activity Assay *in situ* of Different Industrial Yeast Strains

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Summary

Cytoplasmic pyruvate decarboxylase (PDC, EC 4.1.1.1) is one of the key enzymes of yeast fermentative metabolism. PDC is the first enzyme which, under anaerobic conditions, leads to decarboxylation of pyruvate with acetaldehyde as the end product. The aim of this study is to develop a suitable method for PDC activity assay *in situ* for different industrial yeast strains. *Saccharomyces* sp. and *Debaryomyces* sp. yeast strains grew in fermentative medium with 12 % of glucose. Enzymatic assay was conducted in cell suspension treated with digitonin as permeabilisation agent, and with sodium pyruvate as a substrate, at temperature of 30 °C. Metabolites of PDC pathway were detected using gas chromatographic (GC) technique. Various parameters like type and molar concentration of the substrate, minimal effective mass fraction of digitonin, cell concentration, reaction time and effect of pyrazole (alcohol dehydrogenase inhibitor) were monitored to optimize PDC enzymatic assay *in situ*. In the concentration range of yeast cells from $1 \cdot 10^7$ to $1 \cdot 10^8$ per mL, linear correlation between the produced acetaldehyde and cell density was noticed. Only pyruvate was the specific substrate for pyruvate decarboxylase. In the presence of 0.05 M sodium pyruvate and 0.05 % digitonin, the enzymatic reaction was linear up to 20 min of the assay. During incubation, there was no formation of ethanol and, therefore, pyrazole was not necessary for the assay.

Key words: enzymatic activity *in situ*, pyruvate decarboxylase, yeasts

Introduction

Pyruvate decarboxylase (PDC, EC 4.1.1.1) is a key enzyme in alcohol fermentation that catalyses the decarboxylation of pyruvate to acetaldehyde (*1*). PDC was first detected in fermenting yeast in 1911 by Neuberg and Karzag (*2*), and several PDC genes were described in *Saccharomyces cerevisiae* (*3*). The catalytically active PDC enzyme is a tetramer composed of two dimers. The enzyme functions only with the thiamine pyrophosphate (TPP) cofactor along with the metal ion Mg^{2+}. The four subunits are identical and have a relative molecular mass of approx. 60 kDa each (*4*). Yeast PDC is activated by its substrate, *i.e.* pyruvate, but inorganic phosphate is a competitive inhibitor of the enzyme (*5–7*). However, a transient exposure to glucose will lead to an immediate reduction of the intracellular phosphate concentration. These properties of PDC indicate that pyruvate in Crabtree-positive yeasts will be metabolised preferentially *via* pyruvate decarboxylase during exposure to the excess of glucose (*8*).

Along with ethanol and carbon dioxide, fermenting yeasts produce a variety of fusel alcohols and esters that make an important contribution to the flavour of different alcoholic beverages. It is well known that in fermentation processes, the quality of products is affected by

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physiological conditions of yeasts. Many researchers have studied various methods available for prediction and control of the physiological state of cells. Among these methods, intracellular PDC activity can be used not only as an indicator of viability, but also as the marker of fermentation abilities of industrial yeast strains.

In recent years, quantitative procedures for the specific enzyme activity measurement in individual cells have been proved to be powerful methods, especially when applied *in situ*. Therefore, quantitative detection of acetaldehyde, a product of PDC reaction, can also be used for PDC activity assay in whole yeast cells. However, PDC is also capable of catalysing acetoin synthesis from acetaldehyde and pyruvate. There is also a known fact that some of the yeast strains can produce acetate from accumulated acetaldehyde. Therefore, in the case of PDC reactions *in situ*, control of all products of metabolic pathways in which PDC can participate is necessary (*9*).

The aim of this research is to develop a suitable method of yeast pyruvate decarboxylase activity assay *in situ* and to compare enzyme activity of industrial yeasts at different physiological states.

Materials and Methods

Strains, media and culture conditions

Different industrial yeast strains were used (Table 1). The yeast cells had previously been stored on wort agar slants under the standard laboratory conditions. Static fermentations were carried out in 100 mL of minimal medium containing (in g/L): $(NH_4)_2SO_4$ 3, KH_2PO_4 1, $MgSO_4 \cdot 7H_2O$ 0.5, $CaCO_3$ 3, yeast extract 0.5 and glucose 120 in 300-mL Erlenmayer flasks fitted with fermentation locks containing paraffin oil. Fermentation temperature was 25 °C, except for top- and bottom-fermenting yeasts with temperature of 20 and 10 °C, respectively. The number of yeast cells in the samples was determined in triplicate by direct examination under the microscope using Thoma chamber.

Chemical reagents

Sodium pyruvate 0.005–0.5 M (Sigma, Japan) or 0.5 M glucose solutions (POCH S.A., Poland) used as substrates in PDC activity assay were sterilized by filtration (0.45 μm) and stored frozen in aliquots. Digitonin (Fluka, Italy) at mass fractions of 0–0.1 % and 0.1 mM pyrazole (Sigma, Switzerland) were used in PDC activity assay as the membrane permeabilization agent and alcohol dehydrogenase inhibitor, respectively.

Pyruvate decarboxylase activity assay

PDC activity was measured *in situ* using whole cells. Yeast cells were collected by centrifugation (10 min, 2100×*g*), washed twice and resuspended in Ringer's solution. Standardized cell suspensions in 2 mL of Ringer's solution containing from $1 \cdot 10^7$ to $3 \cdot 10^8$ cells per mL were transferred to the vials and centrifuged. Supernatants were discarded and 1 mL of 0.1 % digitonin and 1 mL of 0.05 M pyruvate solutions were added to the biomass. Then the mixtures were incubated for 20 min at 30 °C in 10-mL chromatographic vials hermetically sealed with a cap and an aluminum capsule. The reaction was stopped by incubation for 15 min in boiling water bath, and after cooling, the metabolites acetaldehyde, ethanol, acetoine and acetate were detected using gas-chromatography (GC) technique with headspace autosampler (Fig. 1). Sealed chromatographic vials with mixtures of cells and chemicals were put in headspace sampler and then heated for 30 min at 50 °C before being automatically injected into the chromatograph. For blank samples, the mixture of cells and chemicals was heated in a boiling water bath for 15 min immediately after preparing.

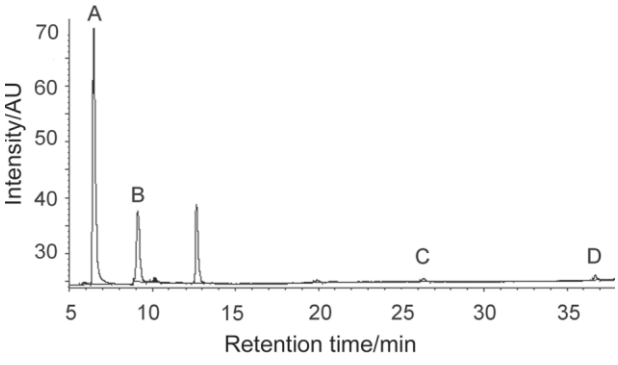

Fig. 1. Gas chromatography of PDC reaction products measured *in situ*. A – acetaldehyde, B – ethanol, C – acetoin, D – acetate

Chromatographic analysis

All samples were analyzed on 6890N gas chromatographer (Agilent Technologies, USA) equipped with flame ionization detector (FID) and 7694E headspace sampler (Agilent Technologies, USA). The GC was fitted with a 60 m×0.32 mm fused PEG capillary column INNOWax,

Table 1. Biological material and culture conditions

Yeast strain	Application	Oxygen–limited culture conditions
Saccharomyces cerevisiae Bc16a	distillery	minimal medium, 30 °C, 8 days
Saccharomyces cerevisiae TT	brewery, top-fermenting	minimal medium, 20 °C, 8 days
Saccharomyces pastorianus B4	brewery, bottom-fermenting	minimal medium, 10 °C, 8 days
Debaryomyces occidentalis Y500/5	amylolytic	minimal medium, 30 °C, 8 days
Saccharomyces cerevisiae P	bakery	minimal medium, 30 °C, 8 days

Yeast origin – International Collection LOCK 105 of the Institute of Fermentation Technology and Microbiology, Technical University of Lodz, Poland

film thickness 0.5 μm (Agilent Technologies, USA). The flow rate of carrier gas (nitrogen) was approx. 45 mL/min. The oven temperature was 40 °C and was held at this temperature for 2 min before being increased by 2 °C per min to 110 °C and then by 15 °C per min to 200 °C. The detector temperature was 250 °C. The total time of chromatographic analysis for each sample was 43 min. Parameters of the headspace sampler were the following: heating temperature 50 °C, heating time 30 min, sample pressurization time 0.13 min, loop filling time 0.15 min, loop equalization time 0.05 min, loop temperature 65 °C, and transfer line temperature 70 °C.

Fermentation activity

Fermentation activity of yeast populations was evaluated by quantitative determination of carbon dioxide production in 100 mL of fermentative medium.

Statistical method

Each experiment was performed in triplicate and each data was the mean value of three measurements. Standard deviations (SD) were calculated and the results were given as mean value±SD.

Results and Discussion

Optimization of PDC activity assay conditions

Effect of pyrazole as the alcohol dehydrogenase inhibitor

Pyrazole at the molar concentration of 0.1 mM had no effect on PDC activity assay *in situ*. During the enzymatic assay, there was no formation of ethanol (data not shown) and, therefore, it was not necessary to use pyrazole as effective inhibitor of alcohol dehydrogenase of yeast cells. This enzyme could be inhibited by the deficit of cytosol $NADH_2$ needed for the reaction.

Optimization of the mass fraction of digitonin

The effect of digitonin at different mass fractions (0–0.1 %) on PDC activity in whole cells was studied. Digitonin at the mass fraction of 0.05 % was optimal for membrane permeabilization (Fig. 2a). Similar results were obtained for several other enzymes (*10–13*).

Substrate specificity and pyruvate molar concentration

Pyruvate 0.005, 0.05 and 0.5 M, and 0.5 M glucose solutions were compared as the substrates for PDC activity assay. PDC activity of whole cells, measured after the incubation without substrate and in the presence of glucose, was similar, showing that only pyruvate was the specific substrate for pyruvate decarboxylase. The maximum PDC activity was obtained when pyruvate molar concentration was 0.05 M (Fig. 2b).

Yeast cell concentration

For different total cell concentrations used in the PDC assay, the linear correlation (R^2=0.9938) between the cell number and the final acetaldehyde amount was observed in the range from $1{\cdot}10^7$ to $1{\cdot}10^8$ per mL (Fig. 2c).

Incubation time

As shown in Fig. 2d, the continuous and linear increase in acetaldehyde formation was observed during the first 20 min of incubation. The standardized incubation time was set at 20 min.

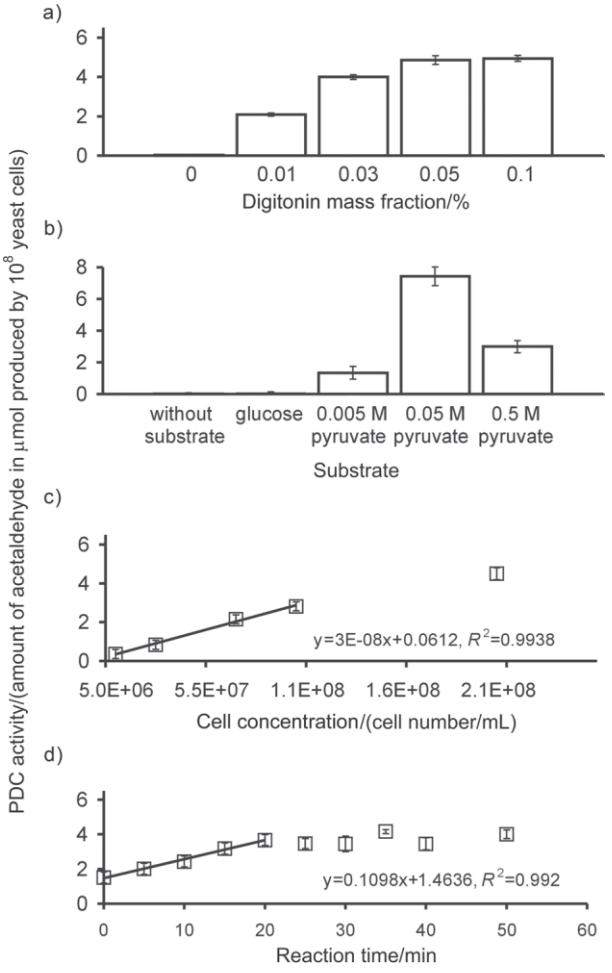

Fig. 2. Optimization of PDC *in situ* activity assay conditions for *Saccharomyces cerevisiae* Bc16a: a) digitonin mass fraction, b) substrates, c) cell concentration, d) reaction time

PDC determination in various yeast strains

Under oxygen-limited conditions, when sugars were present in excess, *Saccharomyces* sp. produced 3–4 times more acetaldehyde than *Debaryomyces* sp. (Fig. 3). Moreover, this 'non-*Saccharomyces*' yeast showed weak fermentation activity expressed in grams of excreted CO_2. In this amylolytic strain, like in other Crabtree-negative yeasts, PDC activity might influence the synthesis of cytoplasmic acetyl CoA (*14*). A significant PDC activity was observed in most yeast cells on the 3rd day of fermentation, whereas on the 8th day of anaerobic cultivation it was significantly lower. However, PDC activity of an 8-day-old population of bottom-fermenting strain *S. cerevisiae* B4 was 5 times higher than that of a 3-day-old one.

In-depth knowledge on yeast growth and metabolism is indispensable for application of yeast in any bio-

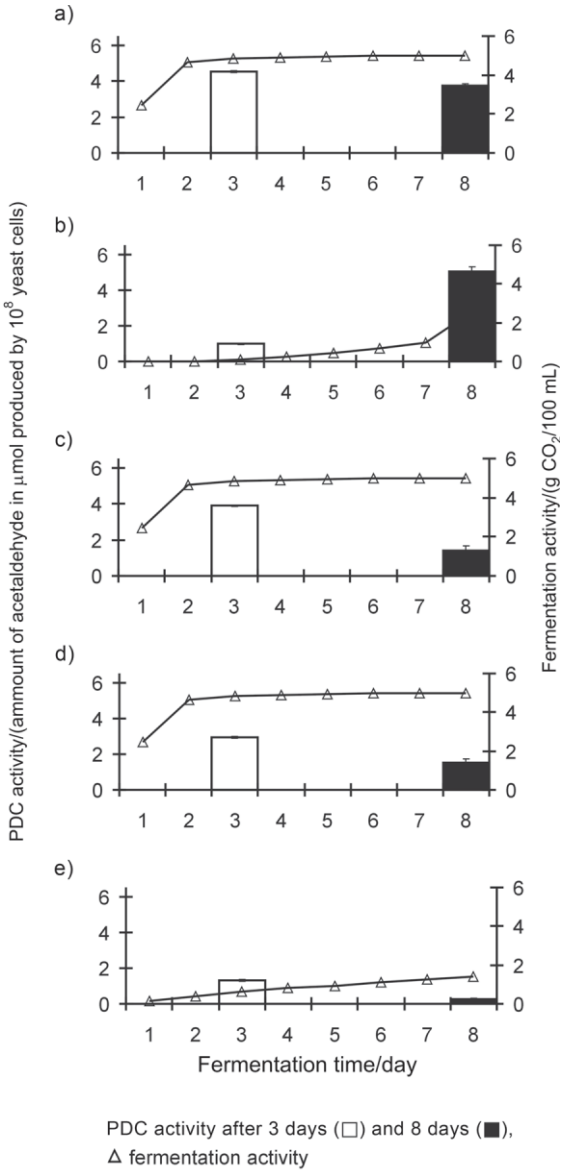

Fig. 3. PDC activity of different yeast strains during the course of fermentation: a) *Saccharomyces cerevisiae* TT – top-fermenting yeast, b) *Saccharomyces pastorianus* B4 – bottom-fermenting yeast, c) *S. cerevisiae* Bc16a – distillery yeast, d) *S. cerevisiae* P – baker's yeast, e) *Debaryomyces occidentalis* Y 500/5 – amylolytic yeast

technological process. In beverage industries, quality of the final product is affected by physiological conditions and fermentation ability of yeasts.

Pyruvate decarboxylase, the key enzyme for ethanol production, has an important physiological function in yeast metabolism (*15–17*). The proposed new PDC activity assay *in situ* with digitonin permeabilization, conducted under strictly standardized conditions, gives an opportunity to evaluate cell age and their physiological activity.

In the case of enzymatic reactions *in situ*, control of all metabolic pathways in which PDC could participate was necessary. The obtained results show that only traces of other metabolites were detected and, therefore, their presence can be neglected in PDC activity measurement.

Conclusion

Chromatographic detection of acetaldehyde, a product of PDC reaction, by GC technique has many advantages like relatively low price of chemicals, a suitable reaction speed and easiness, so it may be widely used not only for estimation of physiological state, but also for evaluation of fermentation abilities of yeast cells. Apart from ethanol and CO_2, yeasts produce many other compounds, which are commonly reffered to as by-products of fermentation, and can also be detected and measured by GC technique. Therefore, gas-chromatographic detection of acetaldehyde and other metabolites forming aroma profiles during fermentation should give an estimation of biotechnological usefulness of yeast strain in different fermentation processes.

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