

BIOTRANSFORMATIONS OF ORGANIC ACIDS BY RHODOCOCCUS SP.

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Abstract

*Four-carbon dicarboxylic acids are important group of additives with a number of industrial applications. Tartaric acid belongs to the group of organic acids which have many applications mainly in beverages and food industry. The ability of bacteria *Rhodococcus* sp. to produce L-tartaric acid from cis-epoxysuccinic acid was investigated under various conditions. The addition of cis-epoxysuccinate to the medium was necessary to enhance the activity of key's enzyme. Pretreatment of the cells with glutaraldehyde or polyethylene imine had no significant permeabilizing effect on bacterial cells. The highest activity and stability of CEH in three repeated bioconversions by intact cells were achieved after cell's treatment by sodium deoxycholate (0.2 g/l) with a duration of 15 minutes.*

Keywords: organic acid, *Rhodococcus*, biotransformation, food additives

1. INTRODUCTION

Organic acids are widespread in the nature and may come from plant, animal and microbial sources. The demand for these acids is steadily increasing due to larger production volumes of organic acid-containing foods and materials. They have also many new applications especially in food and pharmaceutical industries. They are added to foods not only to lower the pH, but also to enhance and modify the flavor of the products, to mask undesirable aftertastes, to impart a degree of tartness and from a lot of other reasons. Although they are made by living cells, few are produced commercially. At the present time, their chemical production is replacing with biotechnological way using microorganisms or enzymes.

Maleic anhydride and maleic acid are important raw materials used in the manufacture of alkyd resins, additives, copolymers and various agriculture chemicals. With the development of biotechnology, unconventional and readily available sources of raw materials are increasingly use for the production of important substances. Maleic anhydride is good starting material for chemical and biotechnological synthesis of various organic acids, e.g. fumaric acid, malic cid, tartaric acid, succinic acid (1).

Traditionally, L(+)-tartaric acid is obtained from wine stone as a by-product during wine fermentation. This way is limited by seasonality and availability of the starting material. The increasing demand for natural form of tartaric acid has initiated the search for fermentative methods for its preparation. 5-ketogluconic acid or glucose can be utilized as a starting material for the fermentative production of tartaric acid by natural or genetically modified bacterial strains of the genera *Gluconobacter* (2). The disadvantages of saccharidic fermentation are small yields and the formation of by-products. The most effective method of L-tartaric acid production is biotransformation of cis-epoxysuccinic acid which can be easily prepared by chemical reaction from maleic anhydride. The crucial enzyme, cis-epoxysuccinic hydrolase (CEH) is produced by the bacteria of the genera *Rhizobium*, *Nocardia*, *Pseudomonas*, *Rhodococcus* or *Agrobacterium* (3). The enzyme responsible for the biotransformation must be induced by cis-epoxysuccinate salts during the cultivation of producers. Then CEH catalyses the enantioselective hydrolysis of cis-epoxysuccinate to pure natural L(+)-form of tartaric acid. The activity of enzyme can be increased by the addition of detergents and salts during the bioconversion or by genetic engineering methods (4).

The most industrially important genus of actinomycetes is *Rhodococcus*. The strains of this genus are widely used for the production of steroids, pigments, antibiotics, acrylamide and acrylic acid, terpenoids, diols, for the degradation of pollutants, transformation and synthesis a lot of intermediates

for food and pharmaceutical industries (5). These bacteria have one of the largest known genomes and are enormous source of enzymes and metabolites.

In our work we presented the possibilities of L-tartaric acid production from epoxysuccinic acid using bacteria of the genus *Rhodococcus*. The microbial production of these acids is realized in two basic steps. First, the bacterial cells are cultivated in optimal conditions given the maximal specific enzyme activity and subsequently the biomass is used in typical non-sterile biotransformation with using free or immobilized cells in high yields of acids or their salts without undesirable by-products production.

2. MATERIALS AND METHODS

2.1. Microorganisms

Bacterial strain *Rhodococcus* sp. RR1 was maintained on agar slants at 4°C. The agar consisted of yeast extract 5 g/l; glucose 10 g/l; peptone 5 g/l; agar 20 g/l and distilled water, pH 7.2.

The following culture medium for L-tartaric acid production were used: **M1** was made up of (per l) 3.0 g beef extract, 10.0 g bacteriological peptone, 3.0 g NaCl, 3.0 g KH₂PO₄, 2.0 g MgSO₄·7H₂O, 0.01 g FeSO₄·7H₂O, 0.03 g CaCl₂·2H₂O, 0.025 g MnSO₄·H₂O. **M2** was made up of (per l) 5.0 g beef extract, 5.0 g bacteriological peptone, 3.0 g NaCl, 3.0 g KH₂PO₄, 2.0 g MgSO₄·7H₂O, 0.01 g FeSO₄·7H₂O, 0.03 g CaCl₂·2H₂O, 0.02 g MnSO₄·H₂O. **M3** was identical to M2 except that beef extract and bacteriological peptone was replaced by 10 g/l yeasts extract. **M4** was identical to M2 except that beef extract and bacteriological peptone was replaced by 10 g/l beef extract. **M5** was identical to M2 except that beef extract was replaced by 10 g/l yeasts extract and concentration of bacteriological peptone was 3.0 g/l. The pH was adjusted with NaOH to 7.0 in all media. The medium (100 ml) was placed into 500 ml flasks on the shaker and 1% (w/v) sodium cis-epoxysuccinate (pH=7.0) was added to the culture medium after sterilisation. The cultivation was carried out at 30°C and 200 rpm, using a rotary shaker, for 24 h. After cultivation, cells were collected by centrifugation, washed with physiological solution (0.85% NaCl) and used for bioconversion of cis-epoxysuccinic acid to L-tartaric acid.

2.2. Biotransformation of cis-epoxysuccinic acid to L-tartaric acid

After the cultivation, the cells were centrifugated at 6000g for 15 min and pellets were washed and resuspended in 0.6 M sodium cis-epoxysuccinate (pH 7.0) with 0.02 % (w/v) sodium deoxycholate. Bioconversions were carried out at 30°C with constant stirring and the pH was maintained at 7.0 with NaOH or HCl. One unit of the enzyme activity was defined as the amount of enzyme that generated 1 µmol tartaric acid per 1 hour under the above conditions.

2.3. Optimalization of reaction conditions

Effect of detergents

Effect of detergents on the activity of cis-epoxysuccinate hydrolase in the intact cells was studied. Three detergents - glutaraldehyde, polyethylene imine and sodium deoxycholate were tested in various concentrations according to the following procedures:

After the cultivation, 50 ml of the cells were centrifugated (6000g, 15min), washed with 25 ml physiological solution (0.85% NaCl) and biomass after the treatment by detergents was used for bioconversion of cis-epoxysuccinic acid to tartaric acid.

A: Control (biomass without pretreatment by detergents). The bioconversion was carried out according Chapter 2.2.

B: Effect of glutaraldehyde: The biomass was resuspended in 10 ml of 1% solution of glutaraldehyde and intensively mixed for 1 minute. After this time 10 ml of physiological solution was added and centrifugated (6000 rpm, 15 min). The biomass was subsequently resuspended in substrate solution and used for bioconversion (Chapter 2.2).

C: Effect of polyethylene imine: The biomass was resuspended in 30 ml of 0.02% solution of polyethylene imine and 10 ml of phosphate buffer (0.1M, pH 7.0). After one hour 10 ml of physiological solution was added and the mixture was centrifugated (6000 rpm, 15 min). The biomass was subsequently resuspended in substrate solution and used for bioconversion (Chapter 2.2).

D: Effect of both, polyethylene imine and glutaraldehyde: First the biomass was processed by protocol C. After centrifugation and washing with physiological solution the biomass was affected by glutaraldehyde according the protocol B. At the end the cells were used for bioconversion of substrate (Chapter 2.2).

2.4. Evaluation of sodium deoxycholate time exposure and its concentration for cis-epoxysuccinate hydrolase activity

Biomass after cultivation (40 ml) was centrifugated (6000g, 15 min), washed with physiological solution and again centrifugated. The biomass was resuspended in 20 ml of 0.6 M sodium cis-epoxysuccinate solution with the addition of various concentration of sodium deoxycholate in the range 0.005 – 0.02% (w/v). The time of detergent's exposure was 15, 30 and 60 minutes. After this time the biomass was diluted by phosphate buffer (0.1M, pH 7.0, 80 ml), centrifugated (6000g, 15 min) and resuspended in 20 ml of 0.6 M substrate solution. The bioconversion was carried out by protocol in the capture 2.2. At 90-100% degree of bioconversion, the cells were separated by centrifugation (6000g, 20 min), washed with phosphate buffer (40 ml, 0.1 M, pH 8.0) and then used for a new bioconversion of 0.6 M cis-epoxysuccinate (20 ml). The cells were used in three repeated bioconversions.

2.5. Preparation of crude enzyme extract

After the cultivation, the induced cells were harvested by centrifugation (7000 g, 15 min, 4°C). The sediment was subsequently resuspended in 20 ml of 0.1M phosphate buffer (pH 7.0). The cell suspension was disrupted in a continual cell disruptor (CF Range, Constant Systems, Ltd., Daventry, UK) in three cycles at 4°C and 20 kPSI. The cell debris was removed by centrifugation (60 000 g, 30 min, 4°C). All fractions (intact cells, crude enzyme extract after disruption, soluble enzyme fraction and also insoluble fraction of cell debris) were used for biotransformations of 0.2M sodium cis-epoxysuccinate (Chapter 2.2.).

Analytical methods

For the determination of dry weight, the cells were washed twice with distilled water. The biomass was separated by centrifugation after each cycle and the dry weight was determined by incubating the biomass at 105°C for 3 h.

Carboxylic acids were determined by isotachopheresis using a column-coupling isotachophoretic analyser (model EA102, Villa Labeco, Slovak Republic). The following electrolytes were used in analyses: Leading: 10 mM HCl, β -alanine, 0.1% methyl-hydroxyethylcellulose; Terminating: 5 mM acetic acid. The compounds were also analysed by HPLC (Agilent Technologies 1260 Infinity, USA) on an 8 μ m, 250x8 mm column with a Polymer H⁺ form (WATREX, Czech Republic). The temperature in the column was 50°C, RI detector at 40°C. As the mobile phase, 1,4 mM H₂SO₄ with flow rate 0.7 ml/min was used.

3. RESULTS AND DISCUSSION

3.1. Effect of different carbon and nitrogen sources

Carbon and nitrogen sources are the most important elements at the period of the growing and metabolic processes of the microbial strains (6). The biomass production and enzyme activity of CEH in *Rhodococcus* sp. were associated with the changes in different sources of C and N. We tested 5 different media with various composition of beef extract and bacterial peptone and yeast extract (Fig.1.). The results demonstrated that all carbon and nitrogen sources accelerated the cells growing and epoxide hydrolase production. The inducer of CEH was sodium cis-epoxysuccinate (1% w/v). The

cells cultivated in the medium without inducer had no CEH activity. The highest biomass concentration was observed when 10 g/l of yeast extract and 3 g/l peptone were used in the medium. Unfortunately, the enzyme activity in these cells was the lowest (136.4 U/mg). The maximum epoxide hydrolase activity (273.8 U/mg) was obtained after 24 hours cultivation in the M2 medium. This medium contained beef extract and bacteriological peptone in the ratio 5:5. The results confirmed high sensitivity of bacterial cells to composition of fermentation medium and also to CEH activity.

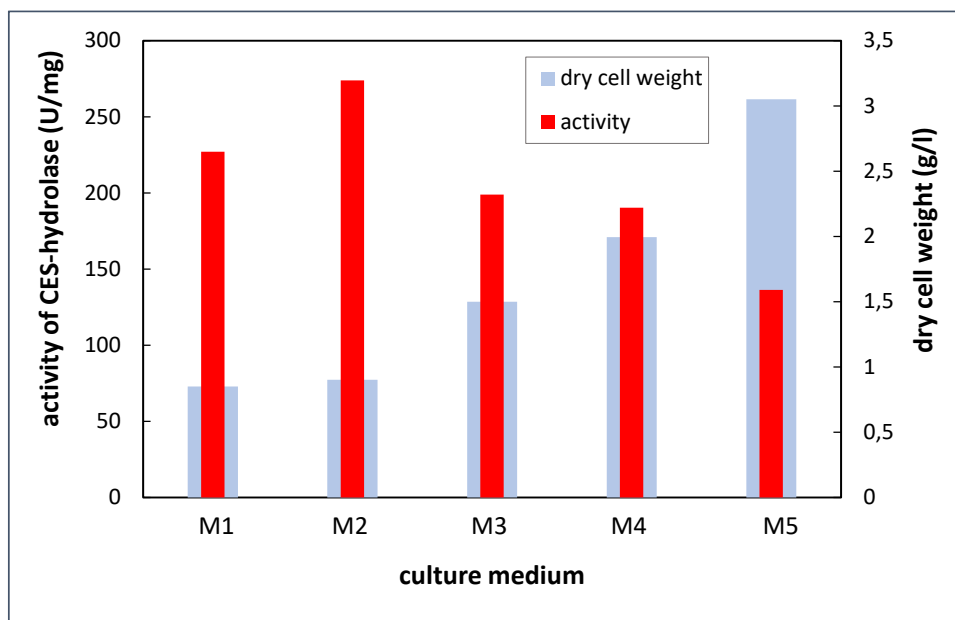


Fig. 1. Effect of culture medium composition on growth and activity CEH in *Rhodococcus* sp. RR1

3.2. Effect of detergents

Detergents are known to enhance the rates of bioconversion of cis-epoxysuccinate to tartrate. They increase the permeability of cells membrane thus the yields of product can be increased. The effect of detergents pretreatment on the enzyme activity of CEH in the intact cells *Rhodococcus* sp. RR1 is shown in Fig.2. We tested two detergents (glutaraldehyde and polyethylene imine) at various concentrations. They are used as cross-linking agents during immobilisation of cells or enzymes. After pretreatment with detergents the cells were washed and used for bioconversion of 1M cis-epoxysuccinate to L-tartrate with 0.02 % (w/v) sodium deoxycholate (Chapter 2.2). The positive effect of sodium deoxycholate on the enzyme activity during bioconversion was studied previously (7). The specific activity of intact cells without pretreatment with detergents was found to be 204.6 U/mg. A slight increasing of specific enzyme activity was observed when polyethylene imine as detergent for biomass pretreatment was used. The specific activity after one hour of enzymatic reaction was 218.0 U/mg. The CEH activity in the presence of glutaraldehyde (B) decreased around 50% compared to the control (A). As shown on Fig.2., the pretreatment of the cells with cross-linking agents had no significant permeabilising effect on the bacterial cells. Our findings indicate that the pretreatment of the cells before biotransformation of substrate to product is not necessary and in the next experiments we skipped this operation.

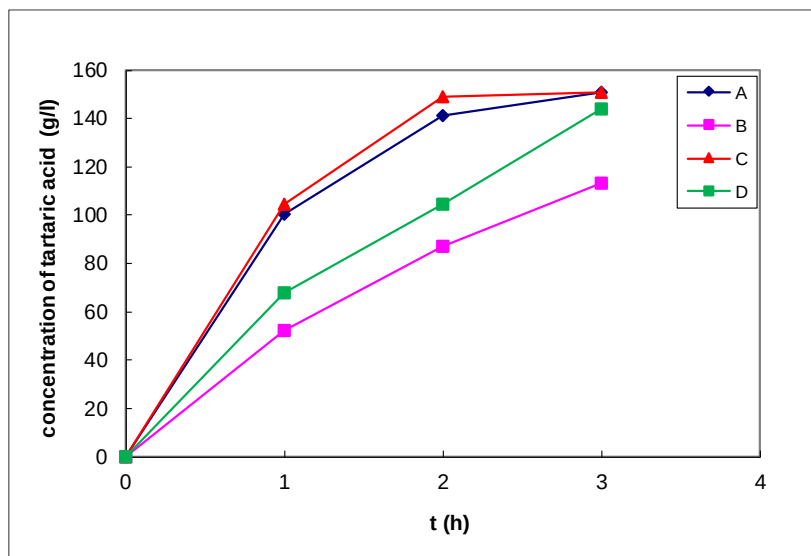


Fig.2. Effect of detergents on the tartaric acid production.

(A-control, B-glutaraldehyde, C-polyethylene imine, D- polyethylene imine and glutaraldehyde)

3.3. Effect of sodium deoxycholate

The addition of sodium deoxycholate to the reaction mixture rapidly increased the specific activity of CEH in the intact cells (Rosenberg, 1999). The different concentration of sodium deoxycholate and various time of its exposure on the cells during three repeated biotransformations were investigated in this experiment.

We tested three different concentrations of deoxycholate (0.1; 0.15; 0.2 g/l) with a duration of action of 15, 30 and 60 minutes. After the detergent treatment, the cells were separated from the reaction mixture by centrifugation and used to the first bioconversion. The goal was to find a optimal conditions for permeabilisation of the intact cells *Rhodococcus* sp. RR1 with very good stability and enzyme activity in repeated biotransformations. A details of procedure are described in Chapter 2.4.

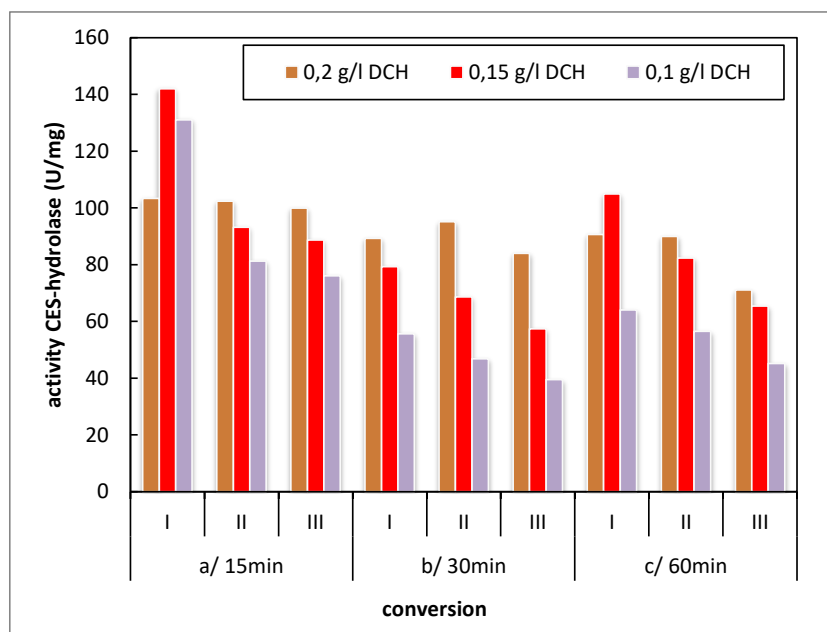


Fig. 3. Influence of sodium deoxycholate time exposure and its concentration on CEH activity in three repeated bioconversions.

As shown on Fig.3, the lowest CEH activity was observed at the lowest concentration (0.1 g/l) of sodium deoxycholate, at 15 to 60 minutes in all three consecutive bioconversions. The optimal results (the highest activity and stability of CEH) were achieved using the highest concentration of sodium deoxycholate (0.2 g/l) with a duration of 15 minutes. In the following three repeated bioconversions, the decrease in CEH enzyme activity was minimal (only 4%).

3.4. Biotransformation of *cis*-epoxysuccinate by cell free extract

Due to the intracellular localization of CEH, the enzyme activity measured with intact cells can be regarded as the apparent activity which is much lower than the real activity obtained with the cells after disruption. To examine this possibility, biomass disruption was performed in a continuous mode using a constant cell disruption system. Continuous disruption of the cells is advantageous, because the process is easily customisable for larger volumes, which is important for potential applications of enzymes in industry. The specific enzyme activity was measured in four fractions of CEH. Our results are summarised in the Table 1. The specific enzyme activity in the crude enzyme extract (42.4 U/mg) was 11-times higher compared with the specific CEH activity in the intact cells (3.74 U/mg) of bacterial strain *Rhodococcus* sp. RR1. Subsequently, we decided to separate the cell debris by ultracentrifugation and determine the enzyme activity in soluble enzyme fraction and also in particulate fraction. The enzyme activity 209 U/ml was observed in soluble fraction, which is 90 % of total enzyme activity in crude extract (233 U/ml).

Table 1. Specific CEH activity of intact cells and cells disrupted in a continual disruptor

Type of cell's preparete	specific CEH enzyme activity (U/mg of dry cells)	specific enzyme activity (U/ml of enzyme's solution)
intact cells	3.74	-
crude enzyme extract	42.4	233.0
soluble enzyme fraction	-	209.0
particulate fraction	-	26.7

The residual enzyme activity (26.78 U/ml) remained in the particulate fraction of cell debris. Our results show that in this way is possible to prepare cell free extract with high specific enzyme activity of CEH.

4. CONCLUSIONS

The bacterial strain *Rhodococcus* sp. RR1 was successfully used for biotransformation of *cis*-epoxysuccinic acid to L-form of tartaric acid. Beef extract and bacteriological peptone in the ratio 5:5 were selected as the best C and N source for studied bacterial strain to produce epoxide hydrolase, respectively. The CEH activity was 273.8 U/mg under the optimal cultural conditions. Pretreatment of the cells with cross-linking agents doesn't increase the enzyme activity of CEH. After biomass disruption 90 % of enzyme activity was found in soluble enzyme fraction. In further studies, we will stabilise enzyme by immobilisation, which can make basis for its reusability and practical application of this biotransformation for the production of L-tartaric acid.

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