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# Production of phenylacetic acid from L-phenylalanine in dual reactor membrane hybrid system

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ABSTRACT

Some natural substances are impossible to produce directly using one strain of microorganisms; they have to be produced via one or more intermediates requiring the use of different production strains. After each separate production process, downstream processing of the product is necessary to prepare the substrate for another production step. In this work, two step bioproduction of phenylacetic acid from L-phenylalanine through precursor 2-phenylethanol was performed using a dual reactor membrane hybrid system. This hybrid system consisted of two bioreactors mutually interconnected with immersed capillary membrane modules used for precursor transport between the bioreactors. In the first bioreactor, 2-phenylethanol was produced by the yeast strain *Saccharomyces cerevisiae*; in the second bioreactor, 2-phenylethanol was transformed by the bacterial strain *Gluconobacter oxydans*. Extraction capability of the hybrid system was tested by a series of membrane extraction experiments. In such a system, biotransformation experiments with 2-phenylethanol bioproduction from L-phenylalanine and its biotransformation to phenylacetic acid in non-growth conditions were carried out using free cells of *Gluconobacter oxydans* and the cells immobilized in lenticular LentiKats® particles.

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## 1. Introduction

Generally, there are two ways to produce natural substances: 1. extraction from a biological source or 2. production by an enzymatic or microbial process with natural precursors. Often, the second way is much more profitable than the first one. However, microbial or enzymatic production has also some drawbacks that have to be considered: 1. product or substrate inhibition – decreasing the yield and effectivity of many biotransformation processes; 2. downstream process – obtaining the final product with high purity is often complicated. Some natural substances are impossible to produce directly using one strain of microorganisms and they have to be produced using one or more intermediates with different production strains. After each production run, downstream processing of the product has to be done to obtain pure substrate for the following biotransformation. A good example is the bioproduction of phenylacetic acid (PAA) from the amino-acid L-phenylalanine (Phe) through the precursor 2-phenylethanol (PEA) (Fig. 1). Bioconversion of PEA to PAA can be realized with bacterial strains such as *Gluconobacter oxydans* in non-growth conditions, which have an incomplete oxidative metabolism and are able to oxidize primary alcohols to the corresponding carboxylic acids [1–3]. Bioproduction of PEA from Phe is well known and it can be easily managed using ordinary yeast strains such as *Saccharomyces cerevisiae* in growth conditions [4,5]. Both, PAA and PEA, are valuable components of flavors and their natural production is interesting not only in the flavor industry but also in pharmaceutical, cosmetic and food industry [1,6]. In an ordinary bioreactor, produced PEA strongly inhibits the biomass growth and thus the biotransformation, which allows reaching the maximum PEA production of only $4\,g\,L^{-1}$ in the fermentation medium [7,8]. To increase the PEA production it is necessary to remove the product from the fermentation medium during the biotransformation, which can be done as a two-phase extraction or adsorption [9–11]. Another suitable method for PEA removal is the application of continuous membrane extraction using a capillary membrane module placed directly in the bioreactor [12,13].

*Abbreviations:* ALR, inner loop air-lift reactor; B1-3, marking of biotransformation experiments; E1-3, marking of extraction experiments; EtOH, ethyl alcohol; Glu, glucose; PAA, phenylacetic acid; PEA, 2-phenylethanol; Phe, L-phenylalanine; RES, reservoir with organic phase; STR, stirred tank reactor.

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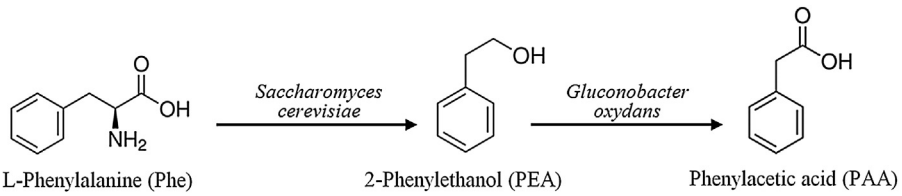


**Fig. 1.** Scheme of phenylacetic acid production from L-phenylalanine through the precursor 2-phenylethanol biocatalyzed by *Saccharomyces cerevisiae* and *Gluconobacter oxydans*.

In this paper, a unique dual reactor membrane hybrid system enabling to overcome the complicated downstream process between two production steps as well as the product (substrate) inhibition is proposed. The proposed hybrid system consists of two bioreactors each containing an immersed capillary hollow fiber membrane module, which are mutually interconnected in a loop. Each bioreactor contained a different production strain. The intermediate (PEA) produced from the substrate (Phe) in the first bioreactor in growth conditions [12], is continuously extracted by membrane extraction to an organic solvent (e.g. heptane in this work), used as an extractant and a carrier. At the same time, the intermediate is transported to the second bioreactor, where it is extracted back from the organic solvent to the fermentation medium and further converted by *Gluconobacter oxydans* in aerobic conditions to PAA. Thus, downstream processing of the intermediate is avoided and regeneration of the solvent (e.g. by distillation) is not required.

Nowadays, immobilization and encapsulation of bacteria, yeasts or enzymes into particles is more frequently applied in biotechnological production due to such benefits as enhanced stability of enzymatic activities and significantly simplified biocatalyst recovery. LentiKats® particles developed by Vorlop and Jekel [14] represent one of the best immobilization method for the entrapment of living cells and cross-linked enzymes [15] currently available. Particles are lenticular-shaped and are made of polyvinyl alcohol (PVA), the particles are ideal for different biotechnological applications as biotransformation, wastewater treatment, ethanol production, or food industry due to their high mechanical stability, non-toxicity, non-biodegradability and their adequate elasticity and density [16–19].

Our previous study [3] was focused on the production of PAA from PEA using free cells of *Gluconobacter oxydans* and cells immobilized in LentiKats® particles in an airlift reactor. The results showed that long-time PAA production (95 h) in the airlift reactor with immobilized biomass is feasible with the final PAA concentration of 7.2 g $L^{-1}$ and the average specific PAA productivity of 44 mg $h^{-1}$ $g^{-1}$. The production ability of free cells of *Gluconobacter oxydans* was from 7 to 20 times higher than that of the immobilized cells depending on the conditions; however, the production stamina of free cells was only a quarter of the stamina of immobilized cells. In addition, free cells are more sensitive to PEA concentration which has to be kept below 0.5 g $L^{-1}$ to ensure satisfactory PAA production.

In this work, PAA was produced from Phe in a dual reactor membrane hybrid system consisting of a 3 L stirred tank bioreactor for PEA production and a 15 L internal loop airlift reactor for PAA production. Extraction capability of the hybrid system was tested by a series of membrane extraction experiments. The ability of the hybrid system to transport PEA between the bioreactors with simultaneous production of PAA was also tested. Finally, in the presented hybrid system, a set of biotransformation experiments with real PEA bioproduction and its continuous biotransformation to PAA were carried out using free and immobilized cells.

## 2. Experimental

### 2.1. Chemicals

2-Phenylethanol (99%) and methyl benzoate (99%) were purchased from Sigma–Aldrich (Steinheim, Germany). Acetophenone (98%) was purchased from Merck (Hohenbrunn, Germany). Heptane (99%) and glucose (monohydrate) were from Mikrochem (Pezinok, Slovakia). Ethyl acetate was purchased from Lachema (Brno, Czech Republic). L-phenylalanine was from Evonik (Slovenská Ľupča, Slovakia). Dipotassium phosphate, monopotassium phosphate and natrium hydroxide were from Centralchem (Bratislava, Slovakia).

### 2.2. Analytical methods

Concentrations of PEA and PAA were measured by a gas chromatograph (GC) 6890N (Agilent Technologies, Santa Clara, USA) equipped with a column Agilent 19091N-113 (30 m × 0.32 mm, 0.25 μm of active layer) and a flame ionization detector. The sample, 1 μL, was injected into the column using an automatic sampler. Temperature of the injector was 285 °C. The column was operated in the temperature range starting from 150 °C with a ramp to 220 °C and a 3.2 mL $min^{-1}$ volumetric flow rate of nitrogen used as the carrying gas. The detector was operated at the temperature of 280 °C, hydrogen flow of 25 mL $min^{-1}$, and air flow of 350 mL $min^{-1}$. Each sample was analyzed twice and the arithmetic means of the measured peak areas from the gas chromatograph were used.

For each analysis of PEA and PAA, a 4% vol solution of acetophenone and methyl benzoate in ethyl acetate was used as the internal standard (in case of acetophenone analysis, acetophenone in the internal standard was substituted with PEA). For the analysis of aqueous samples from the airlift reactor, 1 mL of the sample was treated by double extraction of the sample to 2 mL of ethyl acetate (mixed for 3 min in a reciprocal shaker) after the addition of 50 μL of the internal standard and 10 μL of sulfuric acid (50% mass water solution) in a 10 mL test tube. Then, 200 μL of the ethyl acetate phase was used for the GC analysis. The addition of sulfuric acid was needed to enable the analysis of PAA dissociated in the aqueous phase at pH > 2. For the analysis of PEA in the aqueous samples from the stirred tank reactor, 1 mL of the sample was treated by extraction of the sample to 0.7 mL of heptane (mixed for 5 min in a reciprocal shaker) in a 10 mL test tube. Then, 200 μL of the heptane phase with the addition of 50 μL of the internal standard was used for the GC analysis. When biomass was present in the samples, the sample was centrifuged for 10 min at 5000 rpm and the supernatant was taken for the analysis. Balancing of components used in the analysis was performed on a single pan electronic balance to four decimal places.

Biomass (*Saccharomyces cerevisiae*) concentration in the fermentation medium was analyzed spectrophotometrically at the wavelength of 600 nm using a SPEKOL 11 spectrophotometer (Carl Zeiss, Jena, Germany). Concentrations of Phe, glucose and ethanol

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