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Development of a reaction system for the selective conversion of (−)-*trans*-carveol to (−)-carvone with whole cells of *Rhodococcus erythropolis* DCL14

Carla Sofia R. Tecelão, Frederik van Keulen, M. Manuela R. da Fonseca *

Centro de Engenharia Biológica e Química, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisbon, Portugal

Abstract

The present article addresses the development of a microbial reaction system for the transformation of carveol to carvone, using whole cells of *Rhodococcus erythropolis* DCL14. This strain contains a NAD-dependent carveol dehydrogenase (CDH) when grown on limonene or on cyclohexanol. When a mixture of (−)-*cis* and (−)-*trans*-carveol is supplied, only (−)-*trans*-carveol is converted. Thus, besides (−)-carvone, pure (−)-*cis*-carveol can be obtained as product.

Initial experiments were performed batchwise using an aqueous system. (−)-*Trans*-carveol conversion rate gradually decreased during successive reutilisation batches. After the third reutilisation, activity was completely lost. Cells grown on cyclohexanol showed a slightly higher activity as compared to cells grown on (+)-limonene. A production of 4.3 μmol (−)-carvone formed per mg protein was achieved. A significant improvement with respect to initial reaction rate and productivity was obtained with aqueous–organic two-phase systems. Using a 5 to 1 buffer/*iso*-octane system, a 40% increase in the initial rate and a 16-fold increase of the production was observed. A further improvement resulted from increasing the volume of solvent (1 to 1 buffer/dodecane ratio). An initial reaction rate of 26 nmol/(min∗mg protein) was observed, while production increased to 208 μmol (−)-carvone formed per mg protein. As in the single-phase system, reaction rate gradually decreased along the successive cell reutilisation batches. Addition of co-substrates for the regeneration of NAD did not prevent this decay. A simple downstream process was developed for the recovery of carvone and *cis*-carveol.

Keywords: Terpenes; Carvone; Biotransformation; *Rhodococcus erythropolis* DCL14; Cofactor regeneration; Biphasic system

1. Introduction

Terpenes are unsaturated hydrocarbons derived from isoprene units. They are widely distributed in nature and their oxygenated derivatives, commonly named terpenoids, are important flavour compounds.

The biotransformation of readily available monoterpenes, such as (+)-limonene, into more valuable terpenoids, is recognised as being of great

* Corresponding author. Tel.: +351-21-841-72-33; fax: +351-21-848-00-72.

E-mail address: mmrf@alfa.ist.utl.pt (M.M.R. da Fonseca).

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economical potential to the food and perfume industry [1,2].

The production of flavours via a biotechnological route offers a number of advantages. One important attribute of microbial biocatalysis is the ability to synthesise products that can be labelled as natural, if derived from natural substrates, and added to foods without being considered as additives [3].

However, specific problems have been encountered during the biotransformation of terpenes, namely, the chemical instability and low water solubility of substrate (terpene) and/or product (terpenoid), their cytotoxicity and the existence of multiple enzymatic pathways present in the microorganism, resulting in the formation of a mixture of products [1,2].

The present article addresses the development of a microbial reaction system for the transformation of carveol to carvone, using whole cells of *Rhodococcus erythropolis* DCL14.

This strain, recently isolated at the University of Wageningen, The Netherlands, contains a NAD-dependent carveol dehydrogenase (CDH) when grown on limonene or on cyclohexanol as carbon source [4]. When a mixture of (−)-*cis* and (−)-*trans*-carveol was supplied to the cells only (−)-*trans*-carveol conversion was observed. Thus, isomeric resolution of the mixture can be obtained, resulting in pure (−)-*cis*-carveol and (−)-carvone as products at the end of the reaction [4].

An important feature of this reaction system is the cofactor requirement. In order to achieve cofactor recycling, it will be necessary to supply oxygen and an energy source to the cells during the biotransformation process.

Due to the low aqueous solubility of substrate and product (19 and 9 mM, respectively [5]), only low dissolved concentrations of the substrate can be used in a single-phase batch system. In order to overcome this problem, an organic solvent will be introduced as substrate reservoir and product extraction phase. One very important criterion in the selection of the organic solvent is its biocompatibility. This property is often associated with the hydrophobic character of the solvent and thus its polarity [6]. A correlation between the catalytic activity of whole cells and the polarity of the solvent, expressed in terms of the Hansch parameter or log *P*, has been demonstrated by Laane et al. [7]. Ideally, the organic/aqueous system to be developed should present a low toxicity so as to maintain a high percentage of cell viability.

2. Materials and methods

2.1. Microorganism

R. erythropolis DCL14 was obtained from the Division of Industrial Microbiology, Wageningen Agricultural University, The Netherlands. The strain was stored on yeast/glucose slants at 4°C.

2.2. Growth conditions

The strain was grown in a B. Braun Biostat® MD fermenter with a working volume of 1.5 l at 28°C and 400 rpm, using a mineral medium as described by Wiegant and de Bont [8]. The fermenter was inoculated with 30 ml of a culture grown on ethanol (1 g/l) and limonene (0.42 g/l) during 48 h at 28°C. During the first stage, cells were grown on (+)-limonene as carbon source by sparging limonene saturated air through the culture broth at a flow of 200 ml/min. When the OD (600 nm) of the culture reached a value of about 5.0, ca. 1.2 l of broth were removed and fresh medium was added. After the third partial discharge, the carbon source was changed from limonene to cyclohexanol. Defined concentrations of cyclohexanol were added directly to the culture using a sterile syringe, each time GC analysis indicated complete consumption of this substrate. Growth was followed by measuring the OD (600 nm). Cells were harvested by centrifugation (4°C, 7000 rpm) and washed twice with 50 mM phosphate buffer, pH 7. The biomass was re-suspended in the same buffer and stored at −20°C.

2.3. Conversion of (−)-trans-carveol to (−)-carvone

Activity assays were carried out in 150-ml vials closed with rubber bungs that were perfurated with a small plastic tube for aeration purposes.

In preliminary runs, activity assays were performed in single aqueous phase systems. To each vial, 20 ml of 50 mM phosphate buffer (K_2HPO_4/

KH_2PO_4), pH 7.0, and a concentrated suspension of whole cells of *R. erythropolis* DCL14 (grown on (+)-limonene or cyclohexanol) were added. The vials were incubated at 200 rpm and 28°C. Experiments were started by injecting 10.4 μl of (−)-carveol (mixture of *cis* and *trans* isomers) to the reaction system. When the *trans* isomer was completely converted to carvone, a consecutive cell reutilisation was started by the addition of 10.4 μl of (−)-carveol to the biotransformation medium.

Organic/aqueous reaction systems were prepared by adding to a vial, containing 20 ml of 50 mM phosphate buffer (pH 7.0) and a concentrated cell suspension, 4 ml *iso*-octane or 20 ml *n*-dodecane containing (−)-carveol (157 mM).

All experiments were carried out, at least, in duplicate.

2.4. Analytical methods

Reactions were followed by monitoring the production of carvone. Experiments performed without the presence of an organic phase were followed by extracting 100 μl samples with 100 μl ethylacetate. The two phases were vortexed for 10 s and the ethylacetate layer was subsequently analysed by gas chromatography. Reactions carried out in the presence of an organic phase were followed by gas chromatographic analysis of the organic layer, using (−)-*cis*-carveol as internal standard. Gas chromatography was carried out on a Hewlett Packard 5890 gas chromatograph, using a 25-m HT5 capillary column (SGE). Protein was measured as described elsewhere [9].

2.5. Chemicals

Solvents used were *iso*-octane (99.5%) from Riedel-de-Haën and *n*-dodecane (99%) purchased from Aldrich Chemicals. Terpenes were (−)-carveol (97% purity, mixture of isomers: *trans* 55.2% and *cis* 44.8%), (−)-carvone (98%) and (*R*)-(+)-limonene (97% purity, 98% ee) from Aldrich Chemicals. All components of the mineral medium were analytical grade from Merck.

3. Results and discussion

3.1. Growth on (+)-limonene and on cyclohexanol

Maximum growth rate on limonene was obtained at 400 rpm by sparging limonene saturated air through the culture broth at a flow of 200 ml/min. Upon change of the carbon source from limonene to cyclohexanol, growth proceeded at a lower rate (Fig. 1). Cyclohexanol was converted readily into cyclohexanone, which in turn was then slowly assimilated. After the first period of growth on cyclohexanol (days 7 to 9), growth rate decreased and cyclohexanone accumulated in the broth medium.

3.2. Conversion of (−)-trans-carveol to (−)-carvone in aqueous systems

Both (−)-carveol and (−)-carvone were stable when incubated at 30°C and 200 rpm in phosphate buffer for at least 24 h. Due to the low solubility of substrate and product, only a low concentration (3.27 mM) of (−)-carveol (mixture of *cis* (44.8%) and *trans* (55.2%) isomers) was introduced in the single phase reaction system. Since the aim was to develop a generally applicable reaction system for the biotransformation of terpenoids, we did not perform any experiments with the substrate present as a second phase because, in case of unstable substrates and/or products, chemical reactions would produce undesired byproducts.

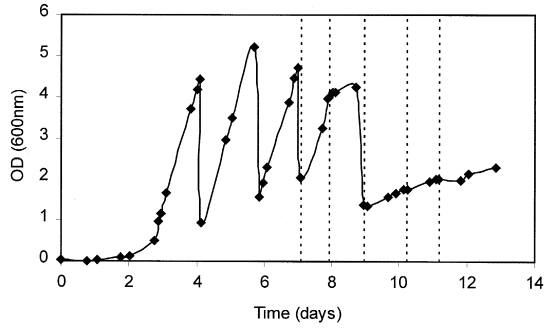

Fig. 1. Growth of *R. erythropolis* DCL14 on (+)-limonene (days 1 to 7) and cyclohexanol (days 7 to 13). Pure cyclohexanol was added (- - -) on days 7, 8 and 9 (3 ml in each addition) and on days 11 and 12 (1.5 ml). Various partial discharges were carried out during the time-course of the fermentation.

Only (−)-*trans*-carveol was converted in (−)-carvone at an initial rate of 14–16 nmol/(min∗mg protein), leaving (−)-*cis*-carveol in the reaction medium, confirming the results of van der Werf et al. [4]. Cells grown on cyclohexanol showed a slightly higher activity as compared to cells grown on limonene (Fig. 2). Once the substrate was depleted, (−)-carveol was added. (−)-*Trans*-carveol conversion rate gradually decreased during the subsequent conversions. At the third reutilisation, the cells had completely lost their bioconversion activity. In this system, a total of 4.3 μmol (−)-carvone were produced per mg protein along the three batches.

3.3. Conversion of (−)-trans-carveol to (−)-carvone in biphasic systems

In order to overcome the low solubility of substrate and product in aqueous medium, the biotransformation of (−)-carveol was attempted in aqueous/organic systems. As a first approach, a biphasic system consisting of 20 ml buffer (pH 7) and 4 ml *iso*-octane containing carveol (157 mM) was used. Both substrate and product partitioned almost completely to the organic phase. In this system, a 40% increase in the initial (−)-*trans*-carveol conversion rate was observed (Fig. 3), as compared to its aqueous counterpart. During incubation, a large interface was formed (consisting of droplets of solvent surrounded by medium). A considerable amount of cells accumulated at this interface (containing a high substrate concentration), probably originating the observed increase in the initial activity.

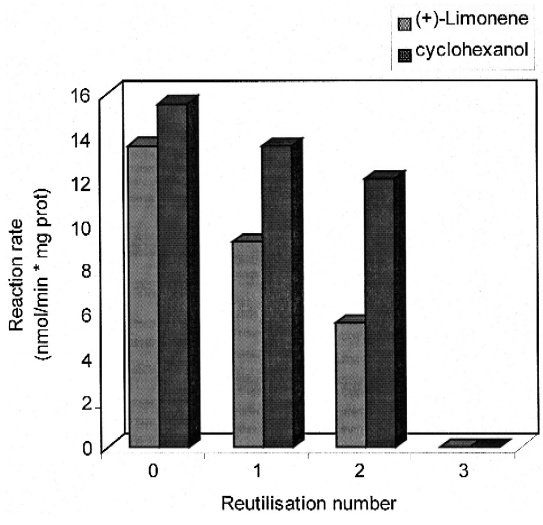

Fig. 2. Initial (−)-*trans*-carveol specific conversion activity of *R. erythropolis* DCL14 cells grown on (+)-limonene or cyclohexanol in consecutive reutilisations in single phase systems (20 ml phosphate buffer, 3.27 mM (−)-carveol, ca. 25 mg protein).

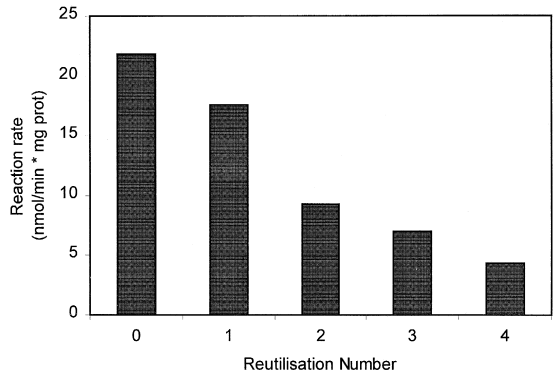

Fig. 3. Initial (−)-*trans*-carveol specific conversion activity (as calculated using the amount of biomass initially added) of *R. erythropolis* DCL14 cells (grown on cyclohexanol) in a biphasic system consisting of 20 ml phosphate buffer containing cells (ca. 25 mg protein) and 4 ml *iso*-octane containing 157 mM (−)-carveol. The organic phase was replaced by a fresh one after complete conversion of (−)-*trans*-carveol in each reutilisation.

Once (−)-*trans*-carveol was completely converted to carvone, the two phases were separated and 4 ml *iso*-octane containing carveol (157 mM) were added to the aqueous phase containing the biomass. Renewal of the organic phase was preferred over addition of (−)-carveol to the previously used solvent to avoid substrate inhibition due to accumulation of (−)-*cis*-carveol. Biomass was tentatively recovered from the interface by centrifugation. However, a large part of the biomass that had accumulated at the interface remained at the aqueous/organic interface. This might be an indication that cell lysis occurred, probably due to the prolonged contact with the moderately biocompatible *iso*-octane phase. In previous work with *iso*-octane [9], retainment of initial enzymatic activity of whole cells and subsequent decrease of this activity were observed. Thus, in the subsequent cell reutilisations, activity (calculated using the amount of biomass initially added) gradually decreased due to loss of biomass during the recovery of the aqueous phase

and probably also because of cell disintegration. In the fifth reutilisation, only 20% of the initial activity was found (Fig. 3). Even so, the total specific production obtained with this biphasic system increased 16-fold as compared to its single-phase counterpart.

To overcome the problem of loss of biomass during recovery of the aqueous phase, we decided to use a larger volume (20 ml) of organic phase, so as to facilitate phase separation. Also, from this point onwards, a more biocompatible solvent, *n*-dodecane (log $P = 6.6$) was used as substrate reservoir. After depletion of the substrate, the majority of the organic phase was removed, and the interface, consisting of an emulsion, was left in the reaction vials. Then, dodecane (containing carveol) was added in the amount that had been removed, thus keeping the phase ratio and the amount of biomass present constant.

An increase of 70% in the initial (−)-*trans*-carveol conversion rate was observed, as compared to the aqueous phase system. During consecutive biomass reutilisations, activity gradually decreased to 50% and 33% of the initial activity at the second and third batch, respectively (Fig. 4).

As compared to the previous systems, a significant gain in the amount of (−)-carvone produced per mg protein was obtained (Fig. 5).

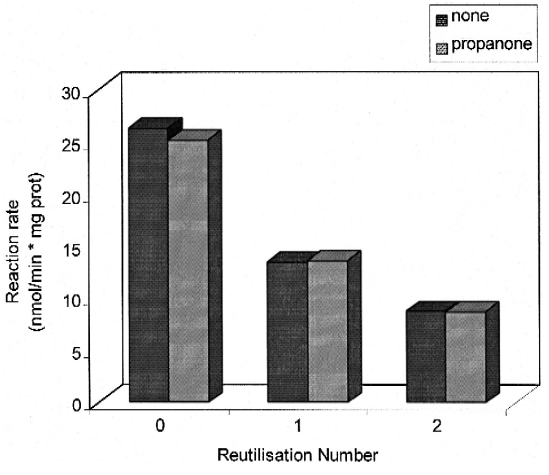

Fig. 4. Initial (−)-*trans*-carveol specific conversion activity in the presence and absence of propanone as cosubstrate, of cells (grown on cyclohexanol) in a biphasic system consisting of 20 ml phosphate buffer containing cells (ca. 25 mg protein) and 20 ml *n*-dodecane containing 157 mM (−)-carveol. The organic phase was renewed after complete conversion of (−)-*trans*-carveol.

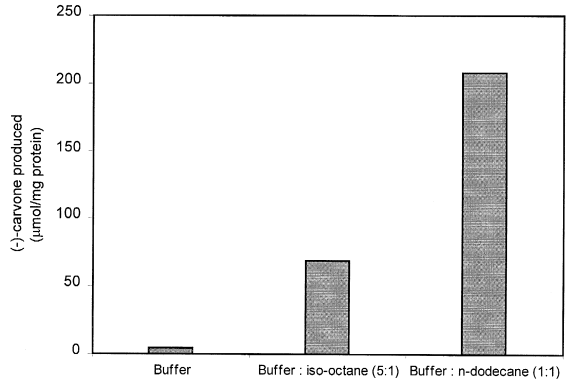

Fig. 5. Mass of (−)-carvone produced per mg protein in different biotransformation systems.

During the transformation of (−)-*trans*-carveol, NAD^+ is used as co-factor. Thus, in terms of cell re-utilisation, conditions for NAD^+ regeneration should be implemented, namely, supply of oxygen and an energy source. If viable cells are used, the strain might be able to use carveol for maintenance, since this strain is able to grow on carveol [4]. However, conversion of carveol to carvone occurred in an equimolar amount, i.e. no detectable amount of carvone was metabolised. Thus, the observed decrease in activity might be due to insufficient cofactor regeneration. Cofactor regeneration in whole cells can, in theory, be achieved by supplying cosubstrates that are catabolised. Since the cells have a high activity in ADH, aldehydes or ketones are appropriate cosubstrates. However, use of propanone as cosubstrate did not lead to the retention of biomass activity (Fig. 4). Other cosubstrates, namely glutamic acid, propanal and butanal, were tested without success (data not shown).

3.4. Purification

Once complete depletion of (−)-*trans*-carveol was observed by GC, the various reaction mixtures from activity assays carried out in biphasic systems containing *n*-dodecane as the second phase were gathered in a separation funnel. Following the separation of the aqueous phase (containing the cell suspension) from the organic layer, unreacted *cis*-carveol and carvone were tentatively extracted from the *n*-dodecane into methanol. Several extractions were per-

formed, each one with a proportion of 100 ml *n*-dodecane to 50 ml methanol, until less than 10% of either substrate and product were present in the dodecane layer. The pooled methanol fractions were concentrated by evaporating the methanol on a film evaporator and the residue was dissolved in a solution of 10% acetone in cyclohexane. This solution was then applied onto a 100-ml column containing silica gel (particle size ranging from 0.2 to 0.5 mm) and complete separation of carvone and carveol was achieved using 10% acetone in cyclohexane as eluent.

4. Conclusions

A model reaction for the biotransformation of (−)-carveol (mixture of *cis* and *trans* isomers) to (−)-carvone with whole cells of *R. erythroplis* DCL14, was implemented.

Aqueous–organic systems were developed, in order to overcome the low water solubility of both substrate and product. Using an organic phase as reservoir, it was possible to considerably enhance the initial conversion rate of (−)-*trans*-carveol and the amount transformed per unit of protein. The addition of cosubstrates did not prevent the gradual decrease of the reaction rate along with the reutilisation of whole cells.

Since *R. erythropolis* DCL14 cells only convert (−)-*trans*-carveol, two products are obtained with this bioreaction system: (i) (−)-carvone from the transformation of (−)-*trans* carveol and (ii) isomerically resolved (−)-carveol. A simple downstream process was developed to separate and recover both (−)-carvone and (−)-*cis*-carveol.

Acknowledgements

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