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Maintenance of cell viability in the biotransformation of (−)-carveol with whole cells of *Rhodococcus erythropolis*

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Abstract

Whole cells of *Rhodococcus erythropolis* DCL14 present carveol dehydrogenase (CDH) activity, which allows them to stereoselectively carry out the oxidation of the (+)-*cis* and (−)-*trans*-carveol to (+)- and (−)-carvone, respectively [1]. When a diastereomeric mixture of (−)-carveol was supplied for biotransformation, the (−)-*trans*-carveol was converted to (−)-carvone. When the cells grow on limonene or cyclohexanol the major activity is NAD-dependent. The relatively low water solubility of carveol and carvone was overcome through the implementation of an organic:aqueous system. The prolonged productivity of such a system depends on cell viability, since viable cells are naturally able to regenerate the co-factor. Fluorescence microscopy was used to off-line monitor cell viability during the time course of the biotransformation.

n-Dodecane was the solvent that allowed the highest retention of both cell activity and viability. The most adequate phase ratio was 1:5, at which, for ODs higher than 0.57, a stable emulsion is formed. At an OD of 0.46 only half of the solvent was emulsified. Loss of viability increased with the OD, probably due to oxygen depletion. The maximum specific production rate was obtained at an initial carveol concentration of 125 mM. At this scale (60 ml flasks) the best aeration rate was 0.01 vvm, both with regard to viability and stability of the emulsion. Carvone was found to be toxic, causing cell death at concentrations above 50 mM.

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1. Introduction

The interest for biotransformations is increasing rapidly because they allow the production of compounds with high stereo-specificity and selectivity, usually under mild conditions. Besides, the convergence of biology and chemistry, the discoveries in biodiversity and the results obtained in molecular biology and computational science are widening the range of natural and engineered biocatalysts that can be used in clean industrial products and processes [2].

Monoterpenes in plants are believed to play ecological roles mainly, serving as herbivore-feeding deterrents, antifungal defenses and attractants for pollinators [3]. In mammals, they have an important role e.g. in cellular membrane stabilisation, metabolic pathways and enzymatic regulation. Their oxidised derivatives, known as terpenoids, are used as flavours, fragrances and pharmaceuticals.

The seeds and seed oil of caraway (*Carum carvi*) have been traditionally used as source of carvone and limonene. The accumulation of these products in the fruits is a developmentally regulated process [4]. The

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fruits of annual caraway varieties generally have a lower essential oil content than the fruits of biennial varieties [5]. The use of microorganisms to produce carvone may overcome the sazonality, fruit content and other agricultural related problems.

Whole cells of *Rhodococcus erythropolis* DCL14 present carveol dehydrogenase (CDH) activity, which allows them to stereoselectively carry out the oxidation of the (+)-*cis* and (−)-*trans*-carveol to (+)- and (−)-carvone, respectively [1]. When a mixture of *cis* and (−) *trans*-carveol was supplied for biotransformation, the (−)-*trans*-carveol was converted to (−)-carvone. The transformation of the *cis* isomer was only observed when the concentration of *trans*-carveol reached rather low levels (approximately 5 mM). Therefore, with this system two products are obtained: (1) (−)-carvone and (2) isomerically resolved (−)-*cis*-carveol [6]. A diastereomeric excess higher than 98% was achieved when the conversion of (−)-carveol was 59% (data not shown).

The microorganism can produce three different CDHs, each of them dependent on a different co-factor [1]. When the cells grow on limonene or cyclohexanol the major activity is NAD-dependent. During biotransformations with whole cells, viability is therefore an issue, which makes operation conditions particularly relevant.

Cell viability may be followed by fluorescence microscopy using stains that give different colours for different cell conditions. The kit used in this work utilises a mixture of SYTO®9 green fluorescent nucleic acid stain (which stains all bacteria) and propidium iodide (which stains only bacteria with damaged membranes; since it reduces the SYTO®9 fluorescence when they are both present, cells fluorescent red).

Both carveol and carvone, as most terpenes, have low water solubility, 19 and 8.8 mM, respectively [7]. This limitation may be overcome through the implementation of an organic/aqueous phase system, in which the organic solvent acts as substrate reservoir and as product extraction phase [8].

2. Materials and methods

2.1. Strain

R. erythropolis DCL14 was delivered by the Division of Industrial Microbiology of the Wageningen Agricultural University, Wageningen, The Netherlands.

2.2. Growth

Cells were grown at 28 °C in a 2.0 l batch tank fermenter containing 1.5 l of medium [9], at a stirring speed of 400 rpm, with limonene supplied through the air stream. An aqueous solution containing cyclohexanol (0.2 mM) was added at a flow rate of 6.3 ml/h.

2.3. Reactions

Activity assays were carried out, at least in duplicate, in cylindrical 60 ml flasks closed with rubber bungs, perforated by a plastic tube for aeration (unless stated otherwise), incubated at 28 °C and 200 rpm, on a rotary shaker with an amplitude of 12.5 mm.

2.3.1. Single aqueous system

To 20 ml of mineral medium [9] containing the cells (initial OD 0.9), 0.5 mmol of carveol were added. Reactions were followed by monitoring carvone accumulation in the aqueous phase.

2.3.2. Single organic system

Cells were allowed to adapt to the presence of *n*-dodecane for 24 h in a biphasic system without carveol at 28 °C and 200 rpm. After phase separation, mineral medium (20 ml, initial OD 0.9) and the emulsified interface were centrifuged in a Labofuge 200, Heraeus Sepatech centrifuge for 10 min at 5000 rpm. The liquid phase was removed and the pellet was suspended in 20 ml of *n*-dodecane. Assays were started by the addition of carveol. Reactions were followed by monitoring carvone accumulation in the organic phase.

2.3.3. Biphasic systems

The system consisted of 20 ml of mineral medium, 4 or 20 ml of organic solvent and 50 mM of (−)-carveol. Biotransformation was started by adding a concentrated suspension of whole cells of *R. erythropolis* DCL14. Reactions were followed by monitoring carvone accumulation in the organic phase. In consecutive assays for solvent selection (−)-carveol was added to a concentration of 50 mM, every 24 h. A Hiblow air pump was used when the flasks were aerated with compressed air. Air passed through a silicone tube with

a Terumo's Neolus needle (21G × 2", 0.8 mm × 50 mm) at the end, which was immersed in the aqueous phase. Reactions were followed by monitoring carvone accumulation in the organic phase.

2.4. Biomass and protein concentration

The absorbance of several samples with different cell concentrations was measured in a Merck-Hitashi spectrophotometer at 600 nm. To 1 ml of each sample contained in a test tube, 1 ml of an aqueous solution of NaOH 0.1 N was added. The tubes were closed with tin foil, boiled for 5 min, cooled with ice and treated according to the Lowry method [10]. The absorbance of each sample was read at 750 nm. The protein concentration in each sample was determined using a calibration curve "concentration of bovine serum albumine versus absorbance at 750 nm". A correlation between absorbance at 600 nm (OD) and protein concentration was thus obtained.

2.5. Chemicals

The terpenes used were (−)-carveol (97%) and (*R*)-(−)-carvone (98%), from Aldrich Chemicals. The organic solvents tested in biphasic systems were *n*-hexane (>95%), cyclohexane (>99.5%) and *n*-dodecane (>99%) purchased from Merck, *n*-hexadecane (99%) from Sigma, ethyl butyrate from Aldrich (99%) and iso-octane (>99.5%) from Riedel-de Haën.

2.6. Analysis

2.6.1. Single organic and biphasic systems

At regular intervals the organic phase was sampled. The periodicity of sampling depended on reaction rate. The carveol and carvone were subsequently analysed by gas chromatography on a Hewlett Packard 5890 gas chromatograph with a FID detector, connected to a HP3394 integrator. The capillary column was a SGE HT5, 25 m in length and with internal and external diameters of 0.22 and 0.33 mm, respectively. The oven temperature was 120 °C and that of the injector 200 °C.

2.6.2. Single aqueous system

Carveol and carvone present in samples, taken at regular intervals from the reaction system, were extracted with ethyl acetate (1:1), which was subsequently analysed by gas chromatography (GC) as described above.

2.7. Microscopy

Cell viability was measured by fluorescence microscopy, using a LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit from Molecular Probes. The microscope was an Olympus CX40, with an Olympus U-RFL-T burner and an U-MWB mirror cube unit (excitation filter: BP450-480; barrier filter:BA515). Images were captured using a COHU RGB camera. The acquisition software was Matrox Inspector 2.1.

2.8. Error analysis

The error associated with the GC quantification of samples, injected without previous treatment, was ±6%. The errors were calculated based on the S.D. and sample mean of seven repeated injections and are quoted for a confidence interval of 95%. Biomass concentration measurements (OD) had an associated error of ±8% based on the standard deviation and sample mean of eight repeated samples, quoted for a confidence interval of 95%. The error associated with the image analysis was ±7% based on the standard deviation and sample mean of 12 repeated images taken from the same sample, quoted for a confidence interval of 95%.

3. Results and discussion

3.1. Solvent selection

The low aqueous solubility of both substrate and product was overcome by the use of an organic phase as substrate reservoir. For this purpose several solvents were tested, namely: ethyl butyrate, cyclohexane, *n*-hexane, iso-octane and *n*-dodecane, with log *P*-values of 1.85, 3.2, 3.5, 4.5 and 6.6, respectively. The best activity retention of the biocatalyst was obtained in the presence of *n*-dodecane (Fig. 1). According to its log *P*-value of 1.85, ethyl butyrate did not behave as expected, allowing a fairly good activity during the first 24 h. However, the production was close to nil during the second day.

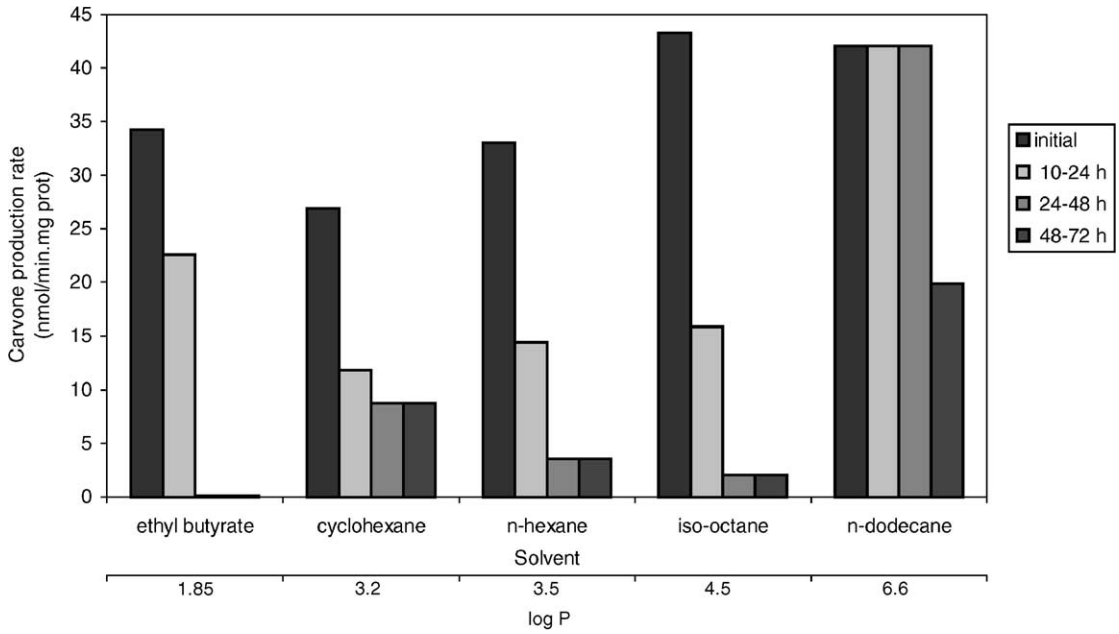

Fig. 1. Carvone production rate obtained with whole cells of *R. erythropolis* in biphasic systems during sets of consecutive batches, each with a different organic solvent (28 °C and 200 rpm; organic:aqueous ratio 1:1; 50 mM initial carveol concentration referred to the aqueous phase; 250 μl of cell suspension, containing ca. 7.4 mg of protein).

n-Dodecane was also the best performer in terms of percentage of cell viability (Fig. 2). With *n*-dodecane the viability percentage was approximately constant for 96 h, in contrast with what happened in the single phase system and in the remaining biphasic systems. This can be ascribed to the use of *n*-dodecane for maintenance purposes and/or to cell growth sustained by *n*-dodecane. Cell growth was observed on each of the tested alkanes from 24 h onwards. In fact, *R. erythropolis* can use cyclohexane, iso-octane, *n*-dodecane and also alcohols, phthalates and *cis*-carveol as carbon source. However, new cells grown on these compounds do not apparently present CDH activity (data not shown). In our biphasic systems containing *n*-dodecane, since the aqueous phase is a rich mineral medium, growth can take place if oxygen is available.

3.2. Phase ratio

3.2.1. Rhodococcus erythropolis

DCL14 cells are very hydrophobic. This characteristic leads to extensive cell accumulation at the interface [11]. At an organic:aqueous phase of 1:1 and an agitation of 200 rpm hardly any emulsion is formed and cell migration is not complete. With a ratio of 1:5, the whole solvent volume is emulsified. Once the stirring is stopped cells accumulate at the interface and the aqueous phase becomes almost cell free. With a ratio of 2:5, only half of the solvent is emulsified and thus a lower interfacial area is available. At an organic:aqueous ratio of 1:5 a good contact between the cells and the substrate is achieved. This ratio resulted in a 2.6 fold increase in reaction rate as compared to that obtained with a 1:1 ratio (Table 1).

In single phase systems, either aqueous or organic, the carvone production rate was low (Table 1). *n*-Dodecane may increase the permeabilization of the cell membrane, facilitating the entrance of carveol and leading to higher activities in biphasic systems [12]. In the absence of a macroscopic aqueous phase, the initial carvone production rate was only 8.1 nmol/min mg protein, probably due to enzyme conformational changes.

Cell viability was measured, after 24 h, in these systems (Fig. 3). The organic single phase system was

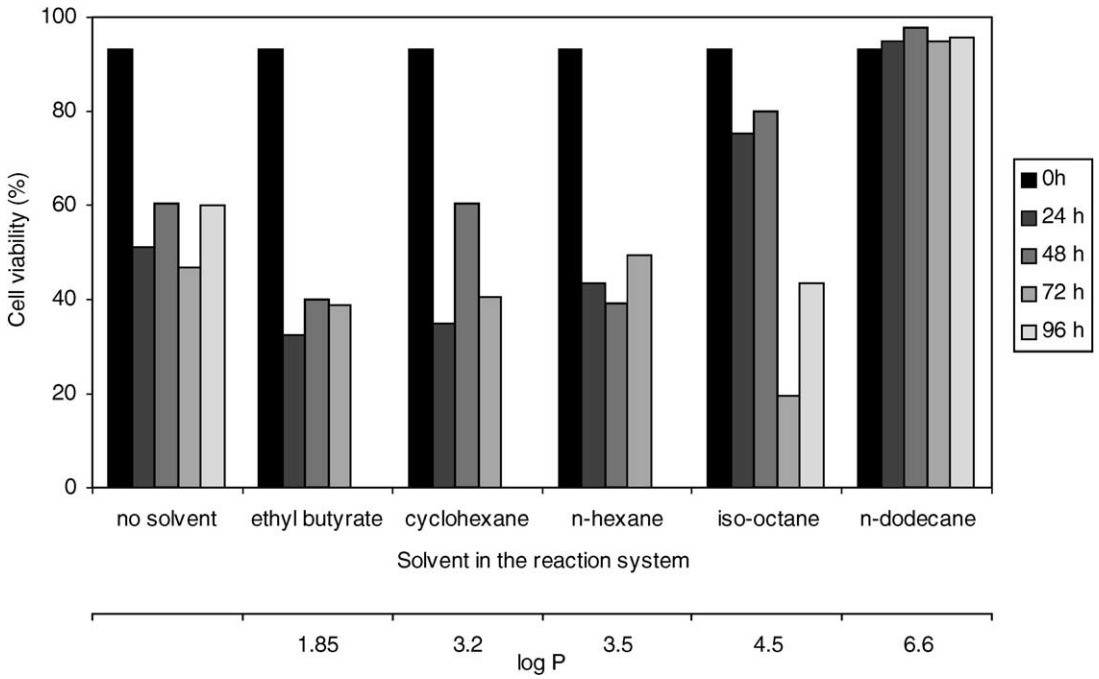

Fig. 2. Percentage of viable cells along the time course of the biotransformations carried out with different solvents (28 °C and 200 rpm; organic:aqueous ratio 1:1; carveol initial concentration 50 mM referred to the aqueous phase; 250 μl of cell suspension, containg ca. 7.4 mg of protein) as compared to the single aqueous system.

able to maintain a higher percentage of cell viability than the single aqueous system. This beneficial presence of *n*-dodecane can be ascribed either to a protective effect of the solvent or to cell growth. Since cell viability was maintained above 70% in the biphasic systems, the low activities obtained at 1:1 and 2:5 ratios (Table 1) were, most probably, due to a limitation on the supply of substrate to the cells.

3.3. Substrate concentration

In aqueous systems, with initial carveol concentrations of 15 and 25 mM, respectively below and above saturation, the carvone production rate obtained was 0.9 and 2.5 nmol/min mg protein.

In 1:5 biphasic systems the highest activity (124.1 nmol/min mg protein) was achieved at an initial

Table 1
Effect of the organic:aqueous ratio on the reaction rate (28 °C and 200 rpm; 250 μl of cell suspension, containing ca. 7.4 mg of protein; *n*-dodecane as organic solvent; initial amount of carveol 0.99 mmol, except in the aqueous phase system to which 0.50 mmol of carveol were added)

Organic:aqueous	Emulsion	Carvone production rate (nmol/min mg protein)
1:0	–	8.1
0:1	–	2.5
1:1	Almost none; cell migration not complete	42.1
1:5	Whole solvent volume emulsified	110.9
2:5	Half of the solvent emulsified	12.5

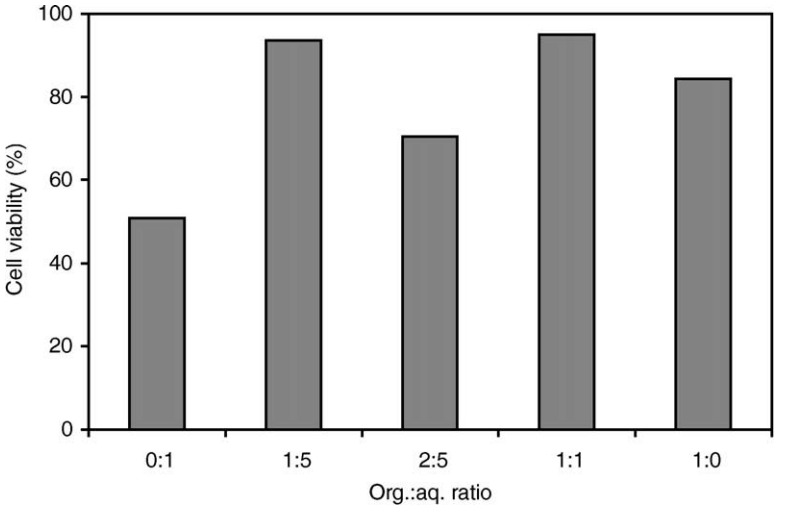

Fig. 3. Percentage of viable cells after 24 h in systems with different organic:aqueous ratios (conditions as in Table 1). The initial cell viability was 97%.

carveol concentration of 125 mM, referred to the organic phase (Fig. 4). Above this concentration, the production rate decreased, indicating a toxic effect of carveol on the cells.

3.4. Cell concentration and aeration

The cells, besides being the biocatalyst, play also an emulsifying role. Under our operation conditions

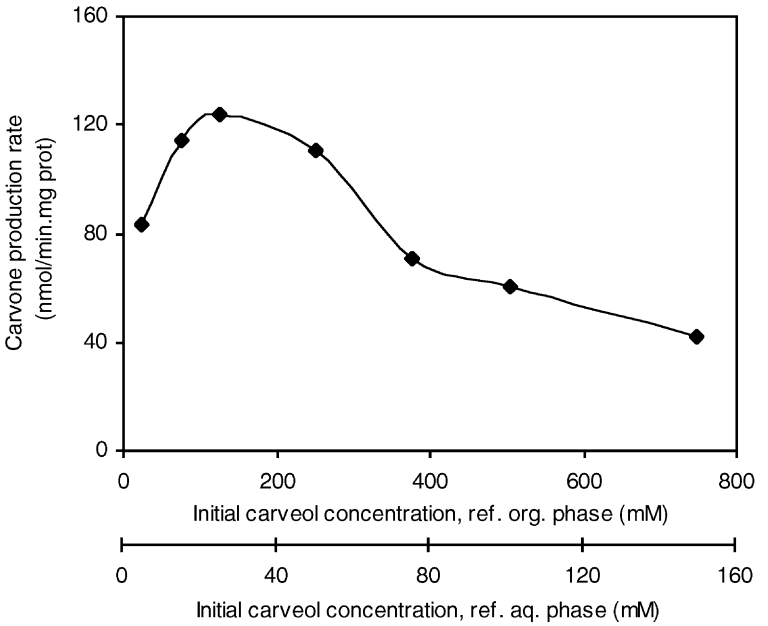

Fig. 4. Production rate of carvone in biphasic systems at different initial concentrations of carveol (28 °C and 200 rpm; organic:aqueous ratio 1:5; 500 μl of cell suspension, containing ca. 7.8 mg of protein; initial concentrations of carveol referred to the organic and aqueous phase, although in reality the aqueous phase is saturated for concentrations higher than 19 mM).

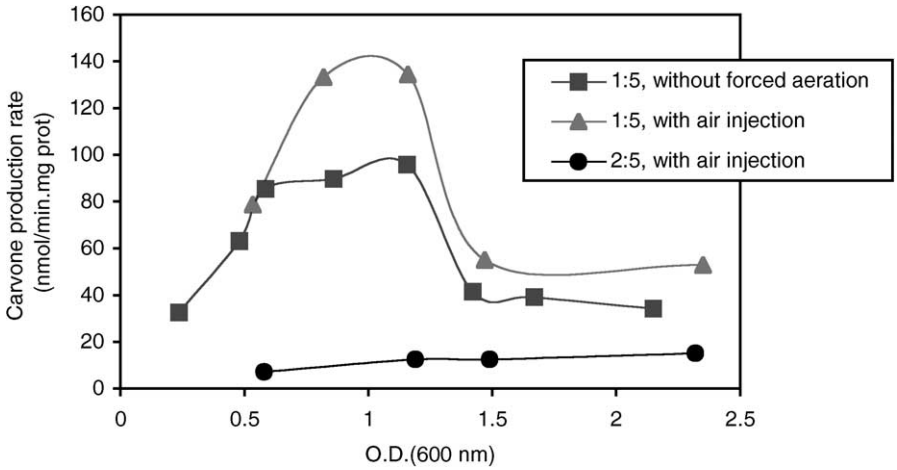

Fig. 5. Carvone production rate in biphasic systems with different initial concentrations of biomass (28 °C and 200 rpm; 50 mM carveol initial concentration, referred to the aqueous phase). In the "without aeration" situation the flasks were closed with rubber bungs perforated by a plastic tube; in the "with aeration" case, compressed air was bubbled at 0.01 vvm.

and with a 1:5 organic:aqueous ratio, the phases were completely separated at optical densities lower than 0.36 (data not shown). At an OD of 0.46 approximately 50% of the organic phase was emulsified. A stable emulsion was observed only for ODs higher than 0.57.

At a defined phase ratio, there should be an optimal biomass concentration to maintain a monolayer of cells at the interface. At higher cell concentrations, some of the cells may not have direct access to the substrate reservoir. Thus, at higher ODs, a decrease in the average specific reaction rate was expected and, indeed, obtained (Fig. 5). We also expected production to remain constant or increase due to the contribution of the cells not in direct contact with the solvent. Still, a decrease in production was observed for ODs higher than 0.86 in the absence of forced

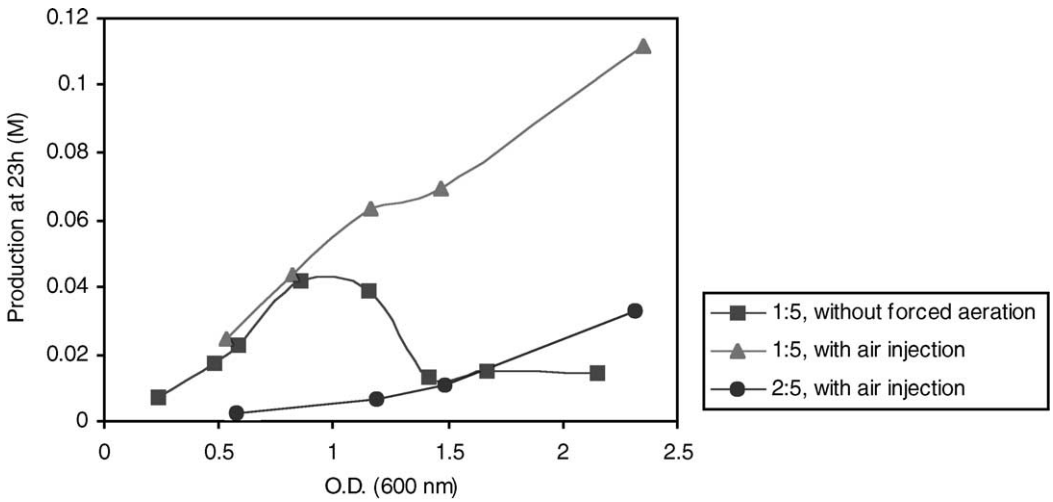

Fig. 6. Effect of the initial amount of biomass on the production of carvone at 23 h reaction time in biphasic systems (same conditions as in Fig. 5).

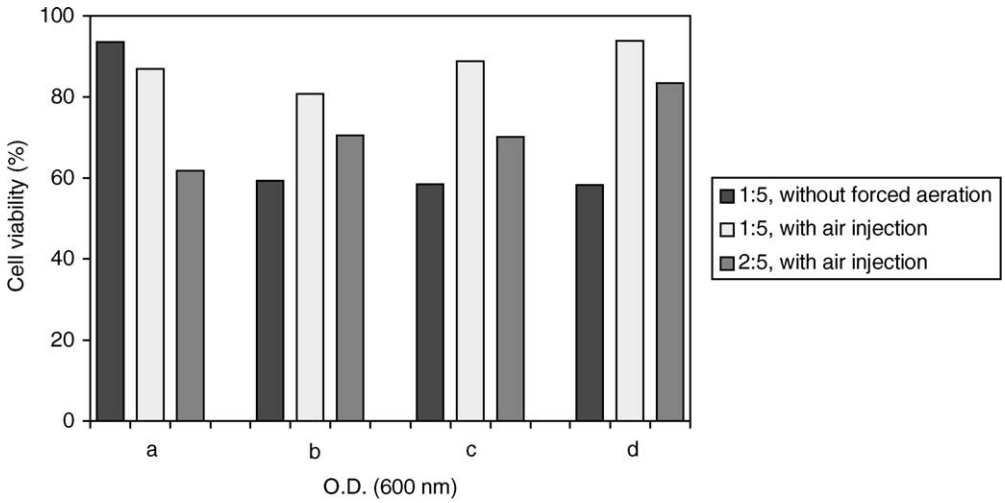

Fig. 7. Cell viability after 24 h in biphasic systems with different initial biomass concentrations (optical density range: (a) 0.53–0.58; (b) 1.07–1.19; (c) 1.42–1.49; (d) 2.15–2.35; other conditions as in Fig. 5). The initial cell viability was 93%.

aeration (Fig. 6). Viability studies were able to clarify the situation. In fact, without forced aeration, a viability loss of 40% was observed for ODs higher than 0.86 after 24 h (Fig. 7).

One of the reasons for the loss of viability in the flasks containing higher biomass concentrations was, clearly, the shortage of oxygen. When air was supplied, the production of carvone during the first 23 h of biotransformation increased 10 fold for an OD of 2.2, as compared to the situation when no air was forced to enter the flasks (Fig. 6). This was due to a higher percentage of viable cells (Fig. 7).

Production of carvone was followed in flasks where compressed air was injected (*c.f.* M.&M.) at four distinct flow rates. Fluorescence microscopy showed that the highest percentage of cell viability, after 24 h of reaction, was obtained at an air flow of 0.010 vvm (data not shown). Evaporation of carvone and water were insignificant at low flow rates. At a flow rate of 0.010 vvm small air bubbles were formed, while at

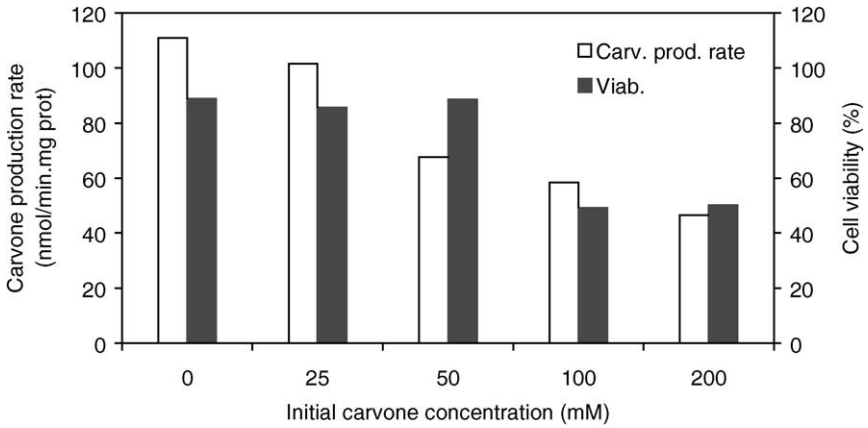

Fig. 8. Effect of the initial carvone concentration on its production rate and on cell viability after 24 h in biphasic systems (28 °C; organic:aqueous ratio 1:5; 500 μl of cell suspension, containing ca. 7.0 mg of protein; initial concentration of carveol: 250 mM, referred to the organic phase).

slightly higher aeration rates larger air bubbles were observed and these tended to coalesce rapidly.

The aeration rate is of prime importance for the maintenance of viability, but it also has an effect on the stability of the interface. At flow rates higher than 0.14 vvm, biomass was expelled from the liquid phase and a biofilm attached to the upper surface of the flask.

Since the results in systems with an organic:aqueous ratio of 1:5 indicated that part of the biomass was not in contact with the solvent containing the substrate, the ratio was increased to 2:5. However, as previously mentioned, at this ratio and under the current operation conditions, the phases are kept separated. This led to a substantial decrease in production (Fig. 6).

3.5. Carvone concentration

The carvone concentration had a remarkable effect upon the system behaviour. For instance, the thickness of the interface decreased in flasks containing increasing initial carvone concentrations (data not shown). This probably resulted from (1) a variation in interfacial tension and/or (2) cell disruption, with cell debris being visible on the surface.

A decrease in the carvone production rate was observed at increasing initial carvone concentrations (Fig. 8).

When the initial carvone concentration increased from 50–100 mM, referred to the organic phase, cell viability reduced to the half after 24 h (Fig. 8).

4. Conclusions

Since carveol and carvone have low solubilities in water and *R. erythropolis* cells are very hydrophobic, an organic:aqueous system was the best choice to carry out the biotransformation. Besides, when *n*-dodecane was used as organic phase, this solvent was able to protect the cells from the toxic effect of the substrate. In fact, using *n*-dodecane, viability and high production rates could be maintained for at least 3 days in consecutive batches of 24 h each. *R. erythropolis* cells are able to grow on a wide range of compounds, e.g. on all of the tested alkanes and on alcohols, monoterpenes and phthalates, even though cells do not show CDH activity when grown on the majority of these substrates. A solvent which would allow the cells to carry out the biotransformation, but which could not be used as carbon source, would be more adequate to clarify aspects related to co-factor regeneration. So far, such solvent could not be found. In the absence of growth the viable cell population will probably have a fairly homogeneous CDH activity. Under conditions which do not support growth, but do support co-factor regeneration, an improved correlation between activity and viability may thus be obtained. This subject is currently being addressed.

At 200 rpm, a *n*-dodecane:aqueous ratio of 1:5 led to the formation of a stable emulsion which promoted the contact between cells and substrate, resulting in a production rate 2.6 times higher than that obtained with a 1:1 phase ratio. The viability loss was only 3.6% after 24 h. The highest carvone production rate was obtained at an initial carveol concentration of 125 mM, referred to the organic phase. Further key parameters to achieve good emulsions and maintain the cell viability high were the initial amount of biomass and the aeration rate. For this purpose, an OD higher than 0.6 should be used. At a 1:5 ratio, for an OD of 2.2, cell viability was 61% higher, after 24 h, when air was injected at 0.01 vvm than when no air was forced into the system.

Carvone accumulation was found to have negative effects on the performance of the system. Maintenance of its concentration below 50 mM, by in situ removal, will be advantageous.

Acknowledgements

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