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Influence of reactor configuration on the production of carvone from carveol by whole cells of *Rhodococcus erythropolis* DCL14

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Abstract

Biocatalysis with whole cells in aqueous–organic systems is extremely attractive for conversion of substrates with low water solubility, specially if co-factor regeneration is required. However, in the latter case, cells should be able to stay viable, which makes both reactor configuration and operation conditions important.

Three reactor configuration types were tested: mechanically stirred direct contact reactor, silicone tube membrane reactor and air-driven column reactor. All reactors were operated with pulse additions of carveol. The maximum *trans*-carveol conversion (92%) and specific production rate (1.69 mg carvone/h mg prot) were obtained with the mechanically stirred reactor at, respectively, ambient temperature, in 50 mM, pH 7.0 phosphate buffer, and at 28 °C in mineral medium. The highest productivity (0.164 mg carvone/h ml org) was attained with the air-driven column reactor after the cells had been adapted to the presence of solvent, substrate and product.

Keywords: Biotransformation; (−)-Carveol; (−)-Carvone; Diastereomeric resolution; Viability; Reactor configuration

1. Introduction

Whole cells of *Rhodococcus erythropolis* DCL14 express carveol dehydrogenase (CDH), which allows them to stereoselectively carry out the oxidation of (+)-*cis* and (−)-*trans*-carveol to (+)- and (−)-carvone, respectively [1]. When a mixture of *cis*- and *trans*-(−)-carveol was supplied for biotransformation, (−)-*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 [2]). Therefore, with this system two products are obtained: (i) (−)-carvone and (ii) isomerically resolved (−)-*cis*-carveol [3]. A diastereomeric excess higher than 98% was achieved at 59% conversion of (−)-carveol [2].

The micro-organism 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. Cell viability is therefore an issue, which made operation conditions important.

Cell viability is generally defined as the ability of microbial cells to reproduce. The routine method to assess the viability of a cell population is spread plate colony counting of diluted cell suspensions. However, the time to form visible colonies may be long. Fluorescence dyes allow the rapid evaluation of cell integrity and therefore cell viability. By using a viability kit, it was possible to monitor with fluorescence microscopy, the viability loss during the biotransformations. This

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kit utilises a mixture of SYTO®9 green fluorescent nucleic acid stain, which stains all bacteria, and propidium iodide. Viable cells only allow the entrance of the SYTO®9 stain and they fluorescent green. Damaged cells allow the entrance of both stains. Propidium iodide reduces the SYTO®9 fluorescence and thus dead cells fluorescent red.

Both carveol and carvone, as most terpenes, have low solubilities in water (19 and 8.8 mM, respectively [4]). 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 [5].

In a recent article [6], the operational parameters which allow the maintenance of a high percentage of cell viability in this particular biotransformation system have been addressed. Among the solvents tested, *n*-dodecane led to the highest retention of cell activity and viability. A stable emulsion was achieved at 200 rpm with an organic:aqueous phase ratio of 1:5 and for cell concentrations corresponding to initial optical densities between 0.6 and 1.2. The aeration rate was found crucial, not only in terms of production rate, but also to maintain cell viability. Carvone was toxic to the cells, leading to cell death at concentrations higher than 50 mM, referred to the organic phase. All these results were used as a starting point for testing different reactor configurations at a slightly higher scale.

Direct contact reactors provide direct contact between the phases, high interfacial areas and the use of biological surfactants, but, often, emulsification occurs to such an extent that phase separation becomes a problem.

Alternatively, porous membrane bioreactors may be used [7]. In this kind of multiphasic reactor, the membrane acts as an interface between the organic and aqueous phases and can even be the immobilisation support. The drawback of these reactors is the difficulty of immobilisation of the liquid interface inside the membrane pores and also the control of the transmembrane pressure applied to the organic phase [8]. As an alternative to porous membranes, dense membranes can be used, such as those made of silicone. Silicone is a homogeneous polymer and the membranes are non-porous at macroscopic level [9]. Since they reject water and inorganic compounds in general, they allow organic compounds to be transferred to the aqueous phase where they are converted, but avoid the contamination of the organic phase by the aqueous medium [10].

2. Materials and methods

2.1. Strain

Rhodococcus erythropolis DCL14 was from the Division of Industrial Microbiology of the Wageningen Agricultural University, Wageningen, The Netherlands.

2.2. Growth

Cells were grown at 28 °C and 400 rpm in a 2.0 l mechanically stirred batch fermenter containing 1.5 l of medium [11], 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. Plating

Spread plating of diluted reactor samples was carried out on agar plates with a medium containing glucose, yeast extract and mineral salts. The slimy or non-slimy cell phenotype was observed after 48 h of incubation at 30 °C.

2.4. Mechanically stirred direct contact reactor (MSDCR)

Assays were carried out in a 500 ml reactor containing 300 ml of aqueous phase (mineral medium [11] or 50 mM, pH 7.0 phosphate buffer) to which a cell suspension, 60 ml of organic phase and 50 mM of (−)-carveol (referred to the aqueous phase) were added. The reactor was mechanically stirred and operated at room temperature or at 28 °C, immersed in a water bath. The air flow was 3 ml/min, except were stated otherwise. When the concentration of *trans*-carveol was close to 5 mM, a pulse of (−)-carveol (0.49 ml) was added.

2.5. Membrane reactor (MR)

Assays were carried out in a 0.6 l reactor containing 250 ml of *n*-dodecane and an initial carveol

concentration of 81 mM, in which a silicone tube (internal diameter: 4.2 mM; wall thickness: 1.025 mM; immersed length: 62 or 112 cm) spiral was immersed. The aqueous phase (300 ml of mineral medium containing the cells) was recirculated inside the tube and aerated in a separate stirred vessel where air was injected.

2.6. Air-driven column reactor (ADCR)

Assays were carried out in a 130 ml glass column operated at room temperature and containing 70 ml of aqueous phase (mineral medium containing the cells) and 40 ml of organic phase (*n*-dodecane 50 mM in carveol).

2.6.1. Scenario 1

The organic phase was recirculated through the column using a Pharmacia LKB pump P-1 at a flow rate of 50 ml/h. Air was supplied with an air compressor (Hiblow air pump) at a flow rate of 1 ml/min.

2.6.2. Scenario 2

No external recirculation of the organic phase was induced and an air flow of 29.3 ml/min maintained the system emulsified.

2.7. Recovery and separation of products

In the case of the direct contact reactors, both phase separation and product extraction from the organic phase with methanol were carried out in a separation funnel. In the membrane reactor, product extraction was obtained by simply recirculating methanol inside the tube. The products dissolved in methanol were separated using a silica gel column and recovered by vacuum evaporation of the eluent (10% acetone in cyclohexane).

2.8. Chemicals

The terpenes used were (−)-carveol (97%) and (*R*)-(−)-carvone (98%), from Aldrich Chemicals. The organic solvent, *n*-dodecane (>99%), was purchased from Merck. Cyclohexanone (99.8%) and cyclohexanol (99%) were purchased from Aldrich Chemicals, NADH (90%) was from Sigma and glycerol (86–88%) was from Riedel-de Haën.

2.9. Analysis

At regular intervals the organic phase was sampled and subsequently analysed by gas chromatography on a Hewlett Packard 5890 gas chromatograph with a HP3394 integrator. The capillary column used was a SGE HT5, 25 m in length with an 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. The detector was at 250 °C.

2.10. 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 by a COHU RGB camera. The acquisition software was Matrox Inspector 2.1.

2.11. Error analysis

The error associated with the GC quantification of samples, injected without previous treatment, was ±6%. The errors were calculated based on the standard deviation and sample mean of seven repeated injections and are quoted for a confidence interval of 95%. Biomass concentration measurements (o.d.) 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. Mechanically stirred direct contact reactor (MSDCR)

The reactor is shown in Fig. 1. The composition of the aqueous phase is important to maintain the cells in a metabolic state which enables them to carry out the biotransformation. Thus, both the percentage of

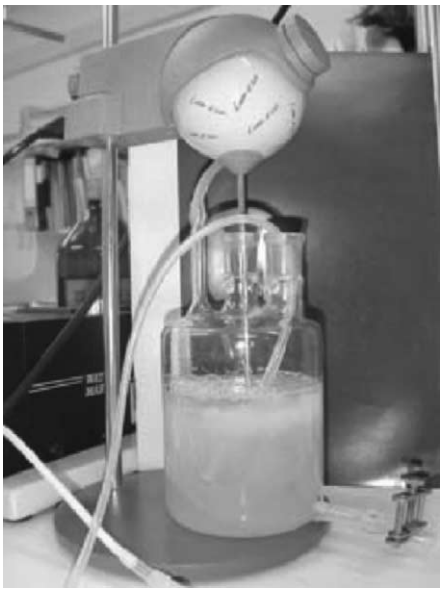

Fig. 1. Mechanically stirred direct contact reactor (MSDCR).

viability and the biotransformation activity of the cells were compared, along the time course of fed-batch runs, using a regenerative (mineral medium) and a non-regenerative medium (50 mM, pH 7.0 phosphate buffer).

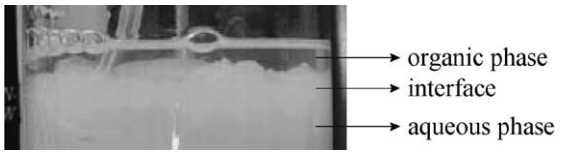

Fig. 3. View of the reaction system after cell migration towards the interface.

3.1.1. Ambient temperature, mineral medium

Fig. 2 shows the time-course of the biotransformation. After 22 h, the majority of the cells had migrated to the interface and a stable emulsion was formed (Fig. 3). This was due to the high hydrophobic character of the cells [12]. Carveol (0.49 ml) was added at 0, 24, 51 and 144 h. At 75 h, the amount of carveol added was doubled. The activity of the reaction system lasted for approximately 173 h.

Spread plating of samples revealed that initial non-slime producing cells, started to produce mucus after 24 h in the reactor. Samples taken 48 h after the start of the run produced only colonies with a slimy aspect. Cyclohexanol and cyclohexanone were able to "precipitate" the polysaccharide in aqueous solution. Thus, a biotransformation run was carried out under similar conditions but in the presence of 10 mM cyclohexanone (data not shown), to assess the potential

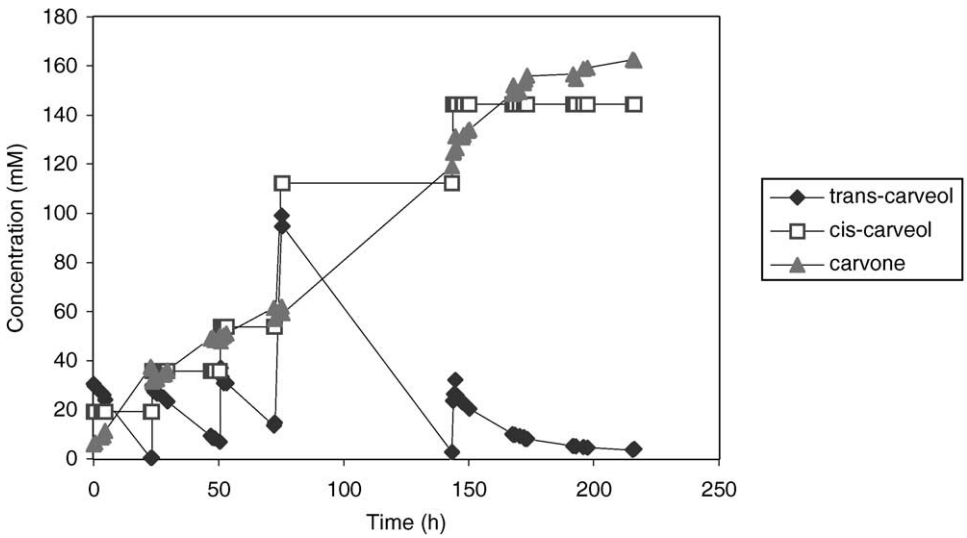

Fig. 2. Time course of the biotransformation in a fed-batch MSDCR (200 rpm; room temperature; initial o.d. 0.82, which corresponds to ca. 83 mg of protein).

benefit of polysaccharide "removal" on the substrate uptake rate. However, in this run, the concentration of carvone attained only 73 mM, compared to 162 mM in the previous case.

Although cyclohexanone could efficiently remove the polysaccharide from the aqueous phase, it acted as an anti-emulsifier, which resulted in well separated phases, even when the stirring speed was increased. Besides, cyclohexanone was consumed since it can be used by *R. erythropolis* DCL14 cells as a carbon and energy source.

In both situations, cell viability was maintained above 90% for 150 h, but a 57% decrease was observed in the presence of cyclohexanone between 144 and 216 h (data not shown). This could be the result of the accumulation of slime on the liquid surface, which hindered oxygen transfer.

Since the reaction needs co-factor regeneration, the co-substrate glycerol was tested as an energy source. Glycerol did not play the role of co-substrate (data not shown). Also, its presence led to an increase in viability loss from 78 h onwards. Respectively 9 and 29% of the cells were not viable at 18 and 93 h after glycerol addition. Compared to the situation in the absence of glycerol, the extent of viability loss for the same periods were 3 and 11%. Glycerol is widely used for cryoprotection of cells during storage [13]. It was expected that cells would, at least, not suffer from its presence.

3.1.2. Ambient temperature, phosphate buffer

With mineral medium as aqueous phase, cell growth was observed. Besides being a regenerative medium, the cells had a carbon and energy source available. However, since there was no increase in the production rate of carvone with time, new cells did not seem to have CDH activity. Thus, the behaviour of the cells in a non-regenerative medium was assessed.

There was no significant difference in the production rate of carvone when mineral medium or phosphate buffer were used as aqueous phase (Fig. 4).

Spread plating of samples showed that the cells in phosphate buffer remained non-mucous for a longer period. The majority of the colonies grown from cells taken from the reactor after 48 h did not form any slime. However, after 4 days in the reactor the number of mucous colonies obtained was significant. Cyclohexanone was then tested as polysaccharide precipitator.

The percentage of cell viability was lower when cyclohexanone was added (data not shown), but the carvone production rate was higher after the second addition of carveol (Fig. 4). This suggests that the transfer of substrate to the cell was easier. In fact, it was possible to observe polysaccharide "precipitation" at the surface of the reactor. This was confirmed under the microscope.

The best initial production rate was achieved in phosphate buffer without cyclohexanone (Fig. 4). However, in the subsequent carveol additions, the rate

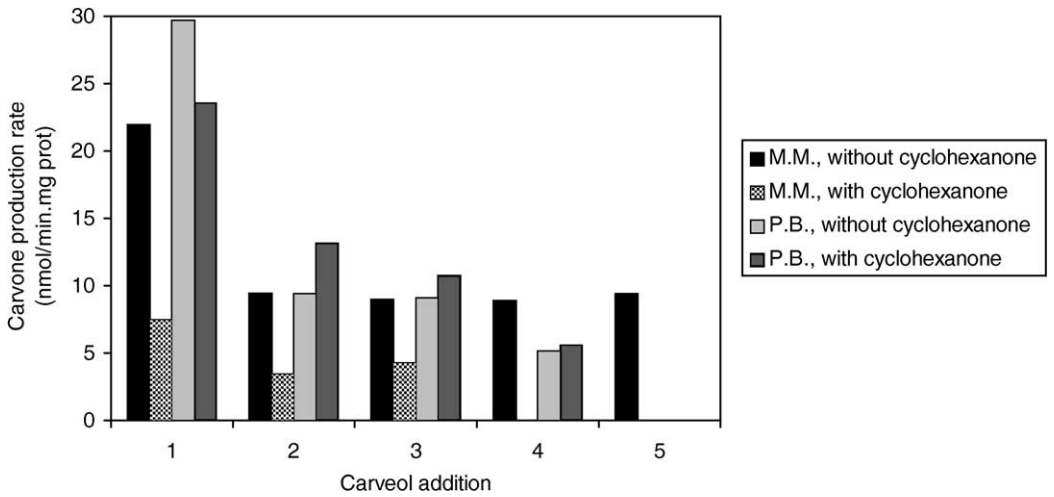

Fig. 4. Carvone production rate in the MSDCR measured after several carveol additions (cf. M&M), with mineral medium (MM) and phosphate buffer (PB) as aqueous phase, in the presence and absence of cylohexanone.

decreased, while, with mineral medium, the carvone production was almost constant between the second and fifth additions. In phosphate buffer with cyclohexanone, cells could not grow and a larger amount of cyclohexanone was used to precipitate the polysaccharide. This resulted in an increase in the production rate of carvone compared to the mineral medium counterpart, where the consumption of cyclohexanone was 30% higher (data not shown).

3.1.3. Controlled temperature, mineral medium

In the previous runs, the bioreactors were operated at room temperature with an air flow of 3 ml/min. Meanwhile, assays in 60 ml flasks had shown that the optimum temperature for the biotransformation was 28 °C [6]. Fig. 5 shows the time course of a biotransformation when the MSDCR was immersed in a water bath at 28 °C.

Particularly high carvone production rates (188 and 160 nmol/min mg prot) were obtained after the first and second additions of carveol and a carvone concentration of 76 mM was attained at 44 h. The rate decreased next to the third addition (Fig. 6). The following step (at 45 h) was to replace the organic solvent to reduce product inhibition. It was not possible to remove the whole of the *n*-dodecane volume, otherwise there would have been loss of biomass. The volume removed (47 ml) was substituted by an equal volume of fresh solvent and carveol was added to increase the concentration to its previous level (Fig. 5). This procedure only led to a slight rate improvement, which seems to indicate that the inhibition on the cells by a relatively high carvone concentration could be irreversible.

Interestingly, cell viability was maintained above 94% for the first 72 h, both in the bulk and at the interface (data not shown), indicating that the contact with carvone was not long enough to kill the cells. From 140 h onwards, the percentage of viability decrease at the interface was more than 40%.

Since CDH is NADH-dependent, 1 mM of NADH was supplied to the system at 71 h. Under these conditions, the carvone production rate was constant during a further period of 70 h (Figs. 5 and 6).

In conclusion, the productivity obtained in the MSDCR at the optimum biotransformation temperature is very high (Fig. 6), but rapidly leads to cell intoxication by carvone. Future work should address practical means for in situ product separation to enable prolonged operation. Exogenous NADH was apparently used by the whole cells and allowed to extend in time the activity of the system. This is, however, an expensive solution.

3.2. Membrane reactor (MR)

The aqueous phase, aerated in a separate vessel, was recirculated inside the silicone tube which was

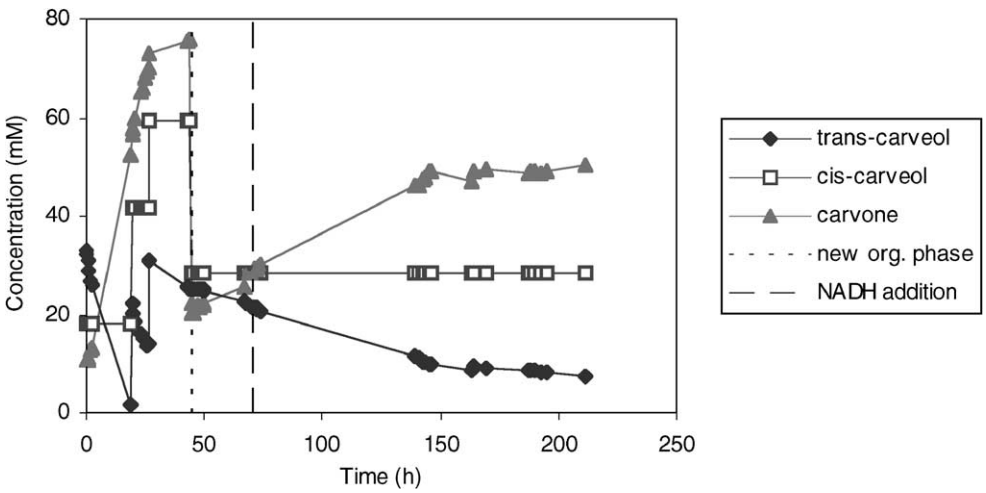

Fig. 5. Production of carvone by *R. erythropolis* cells in a fed-batch MSDCR (200 rpm; 28 °C; air flow 3.6 ml/min; initial o.d. 0.94 which corresponds to ca. 89.8 mg of protein).

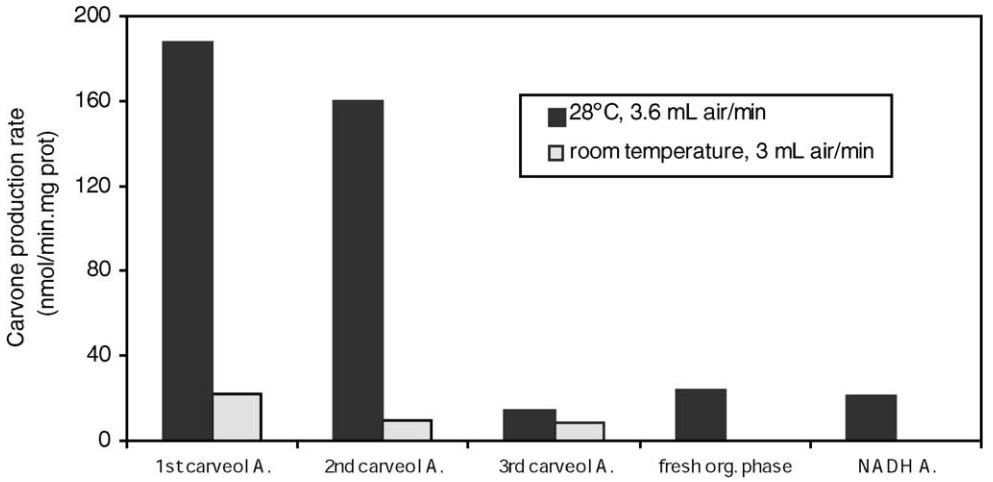

Fig. 6. Carvone production rate in the MSDCR with and without temperature control (A.: addition; conditions as referred in Figs. 2 and 5).

immersed like a coil, in the organic phase containing the substrate and the accumulated product (Fig. 7). With the organic phase circulating in the tube, there would have been solvent leakage to the aqueous phase.

Fig. 8 shows carveol consumption and carvone production during three runs carried out in the membrane reactor with an immersed tube length of 62 cm. The organic solvent was kept from one run to the next while the aqueous phase, including the biomass, was changed between runs.

In the first run, the air was off during the first 80 h and on thereafter. Without aeration cell viability decreased to only 11% after 75 h, although cells recovered when air was turned on (Fig. 9). In the second run, the aqueous phase was aerated from the beginning. The result was a 3-fold increase in the amount of carvone produced. Furthermore, cell viability was maintained above 48% during 216 h. In the third run, which was carried out with an aeration rate similar to the second, carveol consumption decreased sharply while the carvone concentration was approaching 50 mM. Still, 61% of the cells were viable at the end of the run. In all runs, the o.d. considerably increased between 50 and 75 h and the majority of the colonies, obtained on agar plates from samples taken from the bioreactor after 24 h, produced mucus. This obviously enhanced cell retention inside the tube and biofilm adhesion was observed, which limited the transfer of carveol and carvone. The same set-up was attempted with a longer immersed section of the tube (112 cm) and an air flow of 18 ml/min.

Fig. 7. Membrane reactor (MR).

In this situation, the initial carvone production rate (16 nmol/min mg prot) was higher than in the previous runs and 7.8 mmol of carvone were produced after 150 h (data not shown). Meanwhile, a thick pink biofilm was already visible inside the tube at 48 h. Carvone production rate decreased as carvone concentration approached 50 mM and did not increase further after another pulse of carveol, although cell viability was higher than 70% after 172 h (data not shown).

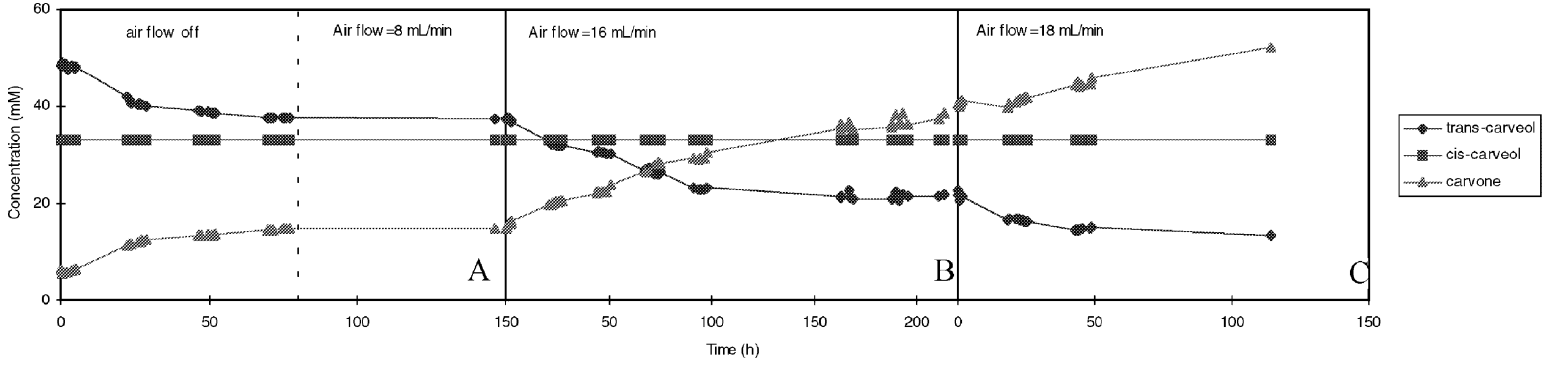

Fig. 8. Time course of three runs in the MR (62 cm of silicone tube immersed in the organic phase: (A) initial o.d. 1.96 corresponding to ca. 140.5 mg of protein; (B) initial o.d. 1.77 corresponding to ca. 132.1 mg of protein; (C) initial o.d. 1.51 corresponding to ca. 120.2 mg of protein).

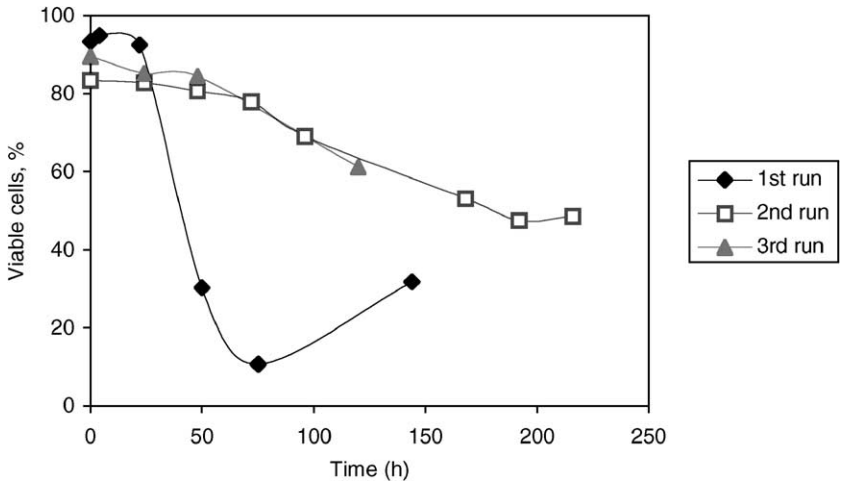

Fig. 9. Cell viability during the three runs carried out in the MR (conditions as in Fig. 8).

In the MR, product recovery was facilitated because no emulsion was formed and the extraction of both products from the *n*-dodecane phase was achieved by recirculating methanol through the silicone rubber tubing. This procedure enabled the reduction of the volume of methanol to 25% of that used in the extraction from the MSDCR. A corresponding gain in product concentration was thus obtained.

The set-up and operation conditions used in the MR enabled the maintenance of a high percentage of viability during prolonged operation times, e.g. aeration in the vessel could supply enough oxygen for the cells to endure circulation inside the silicone tube. However, this had the disadvantage of promoting cell growth, resulting in biofilm adhesion to the inner tube surface. Both viability percentage and film thickness actually increased when the immersed length was changed from 62 to 112 cm. In fact, with the longer immersed tube, a high initial productivity was obtained only during the first 24 h while biofouling was negligible. Thus, this set-up should be avoided, at least for hydrophobic, slime producing strains.

3.3. Air-driven column reactor (ADCR)

In both the MSDCR and the MR carvone production practically stopped when the carvone concentration reached 50 mM. Thus, an attempt to adapt the cells was performed. A 130 ml glass column was tested as an air-driven direct contact bioreactor. *n*-Dodecane containing carveol was recirculated through the column containing the aqueous phase at 50 ml/h. At this circulation rate, the contact time between the cells and the substrate is low enough so that the biotransformation takes place to a small extent only.

In the first run, recirculation was stopped after 20 h, the whole organic volume was allowed to enter the column and air was injected at the bottom of the column at 29 ml/min (0.3 vvm). A good emulsion was formed and the whole of the cell population had migrated towards the organic phase after 10 h. The aqueous phase became completely cell free (Fig. 10).

The cells were able to carry out the biotransformation for at least 310 h. At this time carvone production reached 94 mM (data not shown). This suggests that if cells are allowed to stay in contact with solvent, substrate and slow increasing concentrations of carvone, they do not lose viability as quickly as when the carvone concentration is high when the reaction starts, even for a short period (cf. MSDCR under *controlled temperature*). In fact, at 336 h, 49% of the cells in the *n*-dodecane were viable (data not shown). Somehow, cells in *n*-dodecane were protected from the toxic effect of carvone.

As a further attempt to adapt the cells to the presence of solvent, substrate and product, an assay was carried out in which *n*-dodecane containing the substrate was

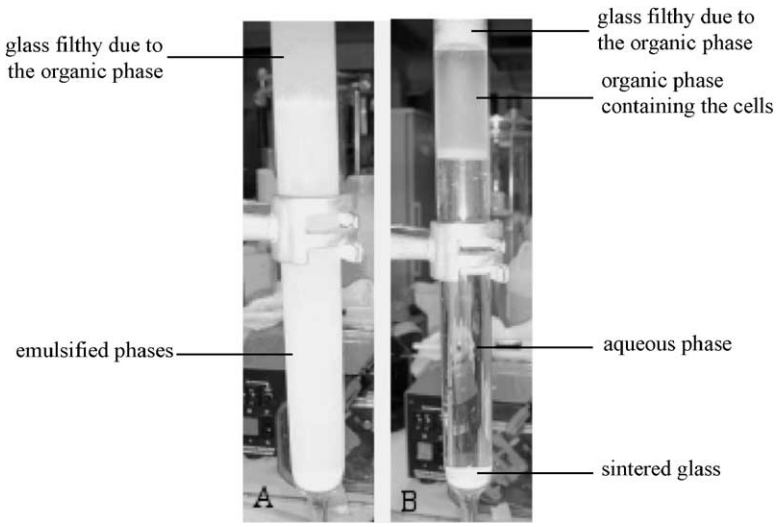

Fig. 10. ADCR: (A) with the air flow on, the emulsification was complete; (B) 5 min after an air-driven agitation period of 10 h, phase separation and cell location in the solvent phase could be observed.

recirculated for 136 h. This long adaptation period was actually a successful strategy, allowing a carvone accumulation up to 259 mM (Fig. 11), thus, overcoming the product inhibition problems previously encountered. Plus, during at least the first 59 h, the production rate was extremely high (147 nmol/min mg prot). Once again practically all cells had migrated to the organic phase and 64% were viable after 384 h (data not shown).

3.4. Comparison of reactor performance

In the MR, production rate became mass transfer controlled due to biofilm adhesion to the membrane. Mass transfer limitations in the MR were also suggested by the value of 73.5% achieved for carveol conversion (Table 1), indicating consumption of the *cis* isomer, which only occurs when the concentration of the *trans*-carveol is lower than 5 mM [2].

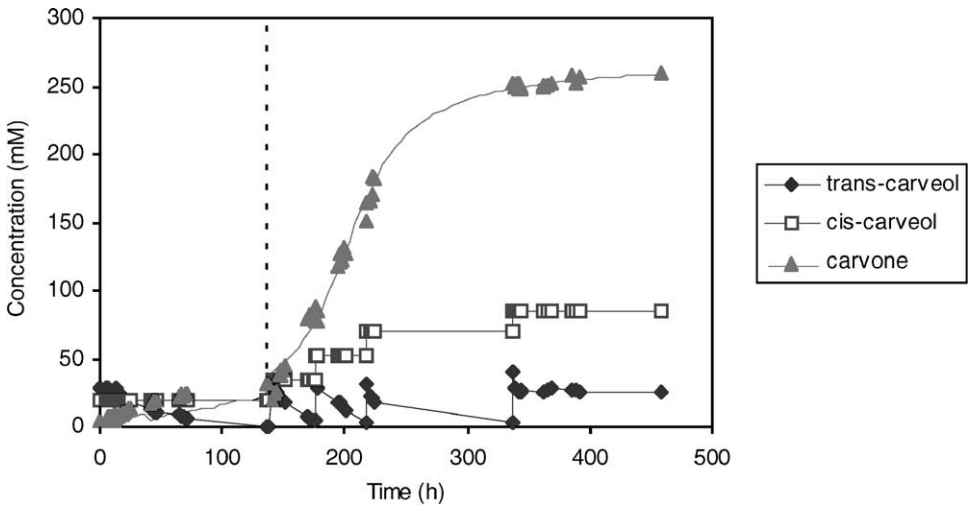

Fig. 11. Carvone production and carveol consumption in the ADCR (initial o.d. of 0.95, corresponding to ca. 21.1 mg prot).

Table 1
Comparison of reactor performance

	MSDCR			MR (112 cm tubing)	ADCR (t_{adapt} = 136 h)
	Room temperature		Thermostatised		
	Mineral medium	Phosphate buffer	Mineral medium		
Carveol added (g)	2.74	2.74	2.28	5.48	2.30
Carveol conversion (%)	68.49	59.91	64.58	73.50	64.16
trans-Carveol conversion (%)	87.07	91.79	83.54	57.20	76.83
Carvone produced (g)	1.39	1.54	1.10	2.01	1.56
Maximum production rate (mg/h mg prot)/duration (h)	0.20/22.9	0.28/20.4	1.69/19.4	0.14/20.4	1.33/58.9
Productivity ($mg_{carvona}$/h ml_{org})	0.119	0.130	0.126	0.070	0.164

The highest *trans*-carveol conversion was obtained in the MSDCR at room temperature using phosphate buffer as aqueous phase, in the absence of cyclohexanone. The maximum production rate, 1.69 mg/h mg prot, was obtained with the thermostatised MSDCR during 19 h. However, a production rate of 1.33 mg/h mg prot was attained in the ADCR during 59 h, when cells were allowed to adapt to the organic phase. The highest productivity was therefore achieved with this reactor, as well as the highest yield, 0.68 g carvone/g carveol. Cell viability was maintained at high levels also with the ADCR, probably due to mixing being gentle.

4. Conclusions

Direct contact bioreactors gave better results than membrane reactors since in the latter cell adhesion to the silicone tubing occurred, hindering the transfer of carveol from the organic to the aqueous phase.

The presence of cyclohexanone, which was able to precipitate the polysaccharide produced by the cells, did not improve the performance when 50 mM, pH 7.0 phosphate buffer was used as aqueous phase. With mineral medium, a 3.3-fold decrease in carveol conversion was observed, because cells could use carveol and/or carvone as carbon source.

The best performance in terms of productivity was obtained with the air-driven direct contact reactor, probably because mixing was more gentle and also because cells were allowed to adapt to the solvent.

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