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Cell adaptation to solvent, substrate and product: a successful strategy to overcome product inhibition in a bioconversion system

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Abstract Carvone has previously been found to highly inhibit its own production at concentrations above 50 mM during conversion of a diastereomeric mixture of (−)-carveol by whole cells of *Rhodococcus erythropolis*. Adaptation of the cells to the presence of increasing concentrations of carveol and carvone in *n*-dodecane prior to biotransformation proved successful in overcoming carvone inhibition. By adapting *R. erythropolis* cells for 197 h, an 8.3-fold increase in carvone production rate compared to non-adapted cells was achieved in an air-driven column reactor. After an incubation period of 268 h, a final carvone concentration of 1.03 M could be attained, together with high productivity [0.19 mg carvone h^{-1} (ml organic phase)$^{-1}$] and high yield (0.96 g carvone g carveol^{-1}).

Introduction

Rhodococcus erythropolis DCL14 cells produce several carveol dehydrogenases, each of which is dependent on a different cofactor (van der Werf et al. 1999a). When grown on limonene or cyclohexanol, the cells produce three carveol dehydrogenases and the major activity is NAD-dependent. Cell viability is therefore an issue, which makes both reactor configuration and operation conditions important. During the conversion of a diastereomeric mixture of (−)-carveol, two products are produced: (−)-carvone and isomerically resolved (−)-cis-carveol (Tecelão et al. 2001); transformation of the cis-isomer to (−)-carvone was observed only when the concentration of trans-carveol reached rather low levels (approximately 5 mM, de Carvalho et al. 2002). In both a mechanically stirred direct contact reactor and a membrane reactor, the reaction effectively stopped when the carvone concentration reached 50 mM in the organic phase (de Carvalho and da Fonseca 2002a). The same effect has also been observed at smaller scale (de Carvalho and da Fonseca 2002b). Apparently, carvone is toxic to the cells at this concentration, leading to loss of viability. In fact, cavone has both antibacterial and antifungal activity (de Carvalho and da Fonseca 2005b; Friedman et al. 2002; Jirovetz et al. 2003).

Cells were successfully adapted to higher carvone concentrations in an attempt using an air-driven 130-ml column reactor (de Carvalho and da Fonseca 2002b). During the adaptation period, biotransformation of carveol was limited to some extent by recirculating the carveol-containing organic phase through the aqueous phase in the column at a low rate. When biotransformation was promoted after an adaptation period of 20 h, a final carvone concentration in the organic phase of 94 mM was achieved and 49% of the cells were viable after 336 h. With a longer adaptation period of 136 h, carvone accumulation reached 259 mM, and 64% of the cells were still viable after 384 h of biotransformation.

A certain genetic and physiological diversity is generally found in any bacterial population. Under selective pressure, the most tolerant individuals are those that contribute more to population reproduction (Mulvey and Diamond 1991), resulting in adaptation of the population under stress. Amongst several described processes, cell adaptation can be ascribed to the following: (1) enzyme-mediated tolerance, through which the toxic compounds are transformed or metabolised to non-toxic forms (Ma et al. 1998); (2) genetic adaptation; (3) metabolic state of the microorganism, since the physiological state of the cells and the environment can lead to differences in bacterial susceptibility to biocides (Gilbert and Brown 1995); (4) membrane composition, as cells can respond to the presence of toxic compounds by adapting the composition of the cell envelope (de Carvalho et al. 2005; Pieper and Reineke 2000; Whyte et al. 1999); (5) the presence of efflux pumps that can remove the toxic compound from the cell (Kieboom et al. 1998).

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Static effectors influence a biosystem instantaneously and independently of time. Amongst these are the concentrations of substrates and products, which affect the system by limitation or inhibition (Sonnleitner 1998). When cells adapt over time to environmental conditions, dynamic adaptation is achieved. In this case adaptation could be gained through one of the mechanisms mentioned above. Interestingly, solvent tolerance mechanisms in Gram-positive bacteria are not well understood, in contrast to those in Gram-negative bacteria (Sardessai and Bhosle 2002).

Since survival under stress conditions takes place at the level of the single cell, methods of analysis of the single cell condition are far more valuable than methods applied on large populations of micro-organisms. While traditional agar plate count method can be useful, recent fluorescent microscopy techniques can provide more important data. The latter were used in the present study to monitor cell behaviour during both adaptation and reactor operation periods.

The aim of this work was thus the evaluation of the effect of the adaptation period, in terms of duration and conditions, on the carvone production rate and on the maximum carvone concentration attained without causing significant viability loss. Two air-driven column reactors were tested: one of 130 ml with a G4 glass sparger and the other of 65-ml with a G2 glass sparger. Each adaptation period was tested either with temperature control (set point 28°C) or without (room temperature, between 23 and 25°C).

Materials and methods

Strain

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

Growth

Cells were grown at 28°C and 400 rpm in a 2.0-l mechanically stirred batch fermenter containing 1.5 l medium (Wiegant and de Bont 1980) with limonene and cyclohexanol as carbon sources. Limonene was supplied through the air stream (de Carvalho et al. 2000). An aqueous solution containing cyclohexanol (0.2 mM) was added at a flow rate of 6.3 ml h^{-1}.

Air-driven column reactor

Assays were carried out in 130- and 65-ml glass columns operated at room temperature or thermostatically controlled at 28°C. The 130-ml bubble column, with a G4 glass sparger (pores with maximum diameter between 10 and 16 μm), contained 70-ml aqueous phase (mineral medium containing the cells) and 30-ml organic phase (*n*-dodecane 50 mM in carveol). The 65-ml column, with a G2 glass sparger (pores with maximum diameter between 40 and 100 μm), contained 30 and 8.5 ml of aqueous and organic phases, respectively. Temperature control at 28°C was achieved by recirculation of water through a jacket.

Adaptation period

1. The organic phase was recirculated through the column using a Pharmacia LKB Pump P-1 (Pharmacia, Uppsala, Sweden) at a flow rate of 50 ml h^{-1}. Air was supplied by an air compressor (Hiblow air pump; http://www.hiblow-usa.com) at a flow rate of 0.01 vvm.
2. In another strategy, which gave similar results, in a shaken flask containing 1(*n*-dodecane):12(aqueous phase), carvone and carveol were added to give 12.5 mM and 25 mM, respectively. Carveol was supplied when the concentration of trans-carveol was lower than 10% of the initial concentration. Air was injected with an air compressor (RENA Air 50; http://www.renapump.com) at a flow rate of 0.01 vvm.

Reactor operation

No external recirculation of the organic phase was induced and an air flow of 29.3 ml min^{-1} maintained the system emulsified.

Cell tolerance to carveol, carvone and *n*-dodecane

Cells suspended in growth medium (2 ml, OD~1.0) were incubated for 2 h at 28°C and 150 rpm with 0, 10, 20, 40, 80, 100 or 160 μl carveol, carvone or *n*-dodecane.

Chemicals

The terpenes used were (−)-carveol (97%) and (*R*)-(−)-carvone (98%), from Aldrich (http://www.sigmaaldrich.com). The organic solvent was *n*-dodecane (>99%) from Aldrich.

Analysis

The organic phase was sampled at regular intervals. The periodicity of sampling depended on the reaction rate. Carveol and carvone were subsequently analysed by gas chromatography (GC) on a Hewlett Packard (Los Angeles, USA) 5890 gas chromatograph with a flame ionisation detector, connected to an 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 injector, oven, and detector temperatures were set at 200°C, 120°C and 250°C, respectively.

Microscopy

Cell viability was measured by fluorescence microscopy, using a LIVE/DEAD *Bac*Light Bacterial Viability Kit from Molecular Probes (Leiden, The Netherlands), which utilises a mixture of SYTO9 green fluorescent nucleic acid stain, which stains all bacteria, and propidium iodide, which stains only bacteria with damaged membranes. Viable cells will thus fluoresce green, whilst cells with damaged membranes will appear red since, when both dyes are present, the iodide reduces the SYTO9 stain fluorescence. 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 in the Red-Green-Blue system using a COHU RGB camera. The acquisition software was Matrox Inspector 2.1 (http://www.matrox.com).

Image analysis

Images were analysed using Visilog 5 (Noesis; http://www.noesisvision.com). After transformation into the Hue-Intensity-Saturation colour system, automated segmentation was performed on the image intensity (analogous to a monochrome image where the cells appear with a high grey-level with respect to the background) to produce a binary image with the cells as objects. Objects smaller than 16 pixels were considered debris and discarded by erosion followed by conditional dilation. Holes in objects were filled and objects in contact with the image frame were discarded. The cell size was evaluated by its projected area. A cell was considered viable when the average number of green pixels within the area defined by the cell was larger than the average number of red pixels and, conversely, as a non viable cell.

Error analysis

The error associated with the GC quantification of samples was ±7.8%. Errors were calculated based on the standard deviation and sample mean of 15 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%.

Results

Solvent, substrate and product toxicity

When carveol conversion was carried out in a *n*-dodecane: aqueous phase system in the presence of different carvone concentrations, a decrease in carvone production rates was observed at increasing initial carvone concentrations (Table 1). Cell viability also decreased. A carvone concentration of 50 mM in the organic phase was sufficient to lower the carvone production rate to 60.9% of the value observed in the absence of carvone, but not to cause significant cell death. A concentration of 100 mM caused a 44.8% decrease in cell viability when compared to cell populations carrying out the conversion of carveol in the absence of carvone. In systems with carvone accumulation, increasing carvone concentrations apparently affect primarily the enzymatic system of the cells, and only at higher concentrations will carvone have a toxic effect on the cells sufficient to cause cell death.

The lowest cell viability values observed when *R. erythropolis* DCL14 cells were incubated for 2 h in the presence of carveol and carvone were, in each case, between 2 and 4% (v/v) (Table 2). In these cases, less than 20% of the cells were viable in the presence of carveol and nearly all cells died in the presence of carvone.

The water solubilities of carveol and carvone are rather low: 19 and 8.8 mM, respectively (Fichan et al. 1999). In the assays carried out in aqueous systems (Table 2), the terpenes were always present above the saturation concentration. Therefore, besides molecular toxicity, carveol and carvone could cause phase toxicity. Since the cells migrate towards the organic phase and are located mainly at the interface, the area of contact between the toxic agent and the aqueous phase should have a high influence on system behaviour. The increase in cell viability observed for carveol and carvone amounts higher than 4% (v/v) is, apparently, the result of a decrease in the area of contact due to coalescence of terpene droplets.

A decrease of 15.9 and 13.6% in cell viability was observed when the cells were incubated with 10 and 20 μl *n*-dodecane, respectively, as compared to the initial non-stressed population (Table 2). For volumes between 40 and 100 μl *n*-dodecane, the viability values were higher than those presented by the initial cell population. This could be the result of either of two effects: (1) changes in the permeability of the cell membrane that could interfere with entry of the fluorescence dyes into the cell; (2) the disappearance of non-viable hydrophobic cells that could have been extracted to the organic phase or could have burst. The latter is more plausible. Nevertheless, *n*-dodecane was capable of pro-

Table 1 Carvone production rate and cell viability during the conversion of carveol in the presence of carvone. Initial carvone concentrations refer to the organic phase. Cell viability determined after 24 h of biotransformation

Carvone (mM)	Carvone production rate (nmol min−1 mg protein−1)	Cell viability (%)
0	110.9	88.8
25	101.6	85.7
50	67.6	88.6
100	58.3	49.1
200	46.6	50.2

Table 2 Viability of *Rhodococcus erythropolis* cells after 2 h in the presence of different amounts of carveol, carvone and *n*-dodecane

Cell viability (%)[a]						
Amount (μl)	10	20	40	80	100	160
% (v/v)	0.5	1	2	4	5	8
Carveol	11.5	49.4	8.4	19.3	32.8	32.8
Carvone	27.4	53.5	0.0	2.7	7.8	15.9
n-Dodecane	74.4	76.4	99.9	94.2	92.0	77.5

[a]88.5% of the initial population was viable

tecting the cells from the toxic effect of both carveol and carvone in a biphasic system, leading to higher cell viability values than when the cells were in the presence of each of the terpenes alone (Tables 1, 2).

Air-driven column reactor

The 130-ml and 65-ml columns had different glass spargers at the bottom, resulting in air bubbles with different average size. The air bubbles were smaller in the 130-ml column due to the smaller pores of the sparger, which allowed the formation of better emulsions, with a milky appearance (Fig. 1). In the 65-ml column, the bubbles were slightly bigger and it took about 10 h longer to achieve an emulsion as stable as in the 130-ml column reactor. In the latter, a stable emulsion was observed, in general, after 22 h of operation, during which most of the cell population migrated towards the interface, as a result of *R. erythropolis* cell hydrophobicity (Fig. 1a).

During the adaptation period, the cells were in contact with slow increasing concentrations of carveol and carvone in *n*-dodecane, as described in Materials and methods. The rate of carveol conversion was kept low enough to maintain the carvone concentration below previously reported toxic concentrations.

Adaptation period

During the period of cell incubation in the presence of *n*-dodecane, carveol and carvone, cells migrated from the aqueous phase towards the organic phase. As an example, this is shown for a 136 h incubation period in Fig. 2. The previously detected strong hydrophobic character of *R. erythropolis* DCL14 cells (de Carvalho and da Fonseca 2002b, 2003) enabled the cells to be in direct contact with the organic phase containing both carveol and carvone.

The total number of cells increased with time during the incubation period (Fig. 2) at a specific growth rate of 0.005 h^{-1}. At the concentrations used during the adaptation period, *R. erythropolis* DCL14 grew considerably faster in the presence of terpenes than in *n*-dodecane: 0.066, 0.041 and 0.008 h^{-1} when carvone, carveol and *n*-dodecane, respectively, were used as sole carbon and energy sources (data not shown). The specific growth rate observed during the incubation period, lower than those observed in the presence of each terpene alone, was probably due to the higher toxicity of the system as a result of the presence of both

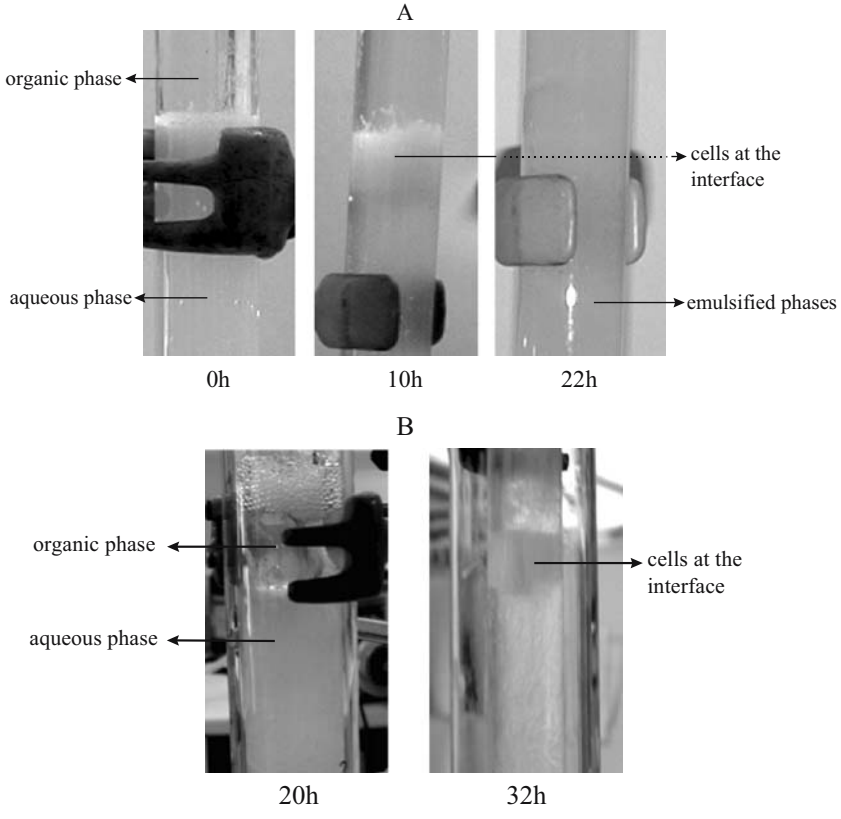

Fig. 1 Appearance of the organic and aqueous phases in a 130-ml (**a**) and in a 65-ml (**b**) column reactor over the first 32 h of operation. Images were taken 2 min after switching aeration off

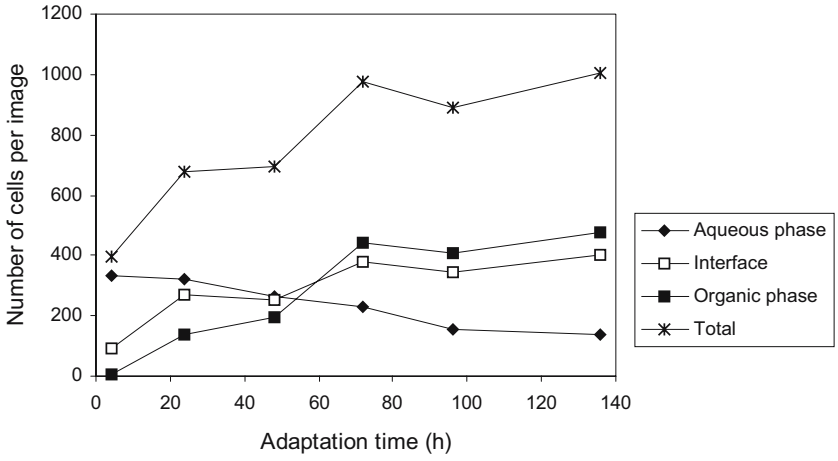

Fig. 2 Average number of cells observed per image during an incubation period of 136 h in the aqueous and organic phases and at the interface. Images were captured with fluorescence microscopy techniques. Total refers to the cumulative number of cells observed at the three sampling locations

carveol and carvone. Probably, only the most tolerant individuals were able to replicate. Nevertheless, the results clearly indicate that the cell population was growing during the incubation and thus slowing adapting to the compounds present.

The number of viable cells observed per image, using fluorescence microscopy, decreased considerably in the aqueous phase with incubation time up to 268 h (Fig. 3). Since the number of non-viable cells did not increase to the same extent, the number of cells that migrated towards the organic phase thus increased with the duration of the incubation period. This might be ascribed to a further augmentation of cell hydrophobicity.

Medium composition

The effect of the composition of the aqueous phase was assessed to evaluate the biotransformation abilities of growing cells as compared to resting cells. The adaptation period and subsequent biotransformation in the air-driven column reactor was thus carried out using phosphate buffer (pH 7.0, 50 mM) and mineral medium (elemental mineral medium as described by Wiegant and de Bont 1980).

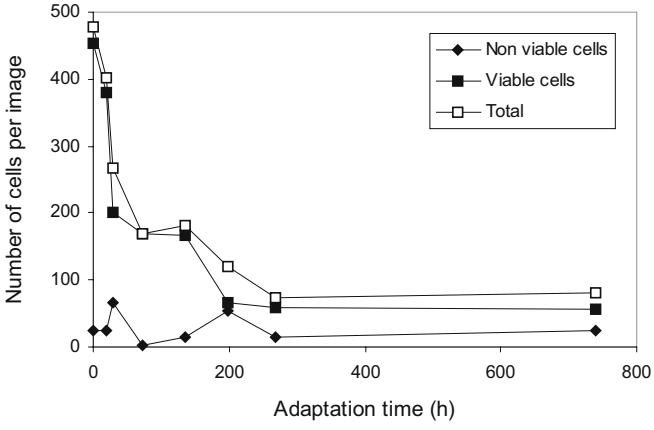

Fig. 3 Number of cells observed per image in the aqueous phase at the end of the adaptation periods. Images observed by fluorescence microscopy

Using mineral medium, the maximum carvone production rate obtained was 45.7 nmol min^{-1} (mg protein)$^{-1}$, whilst in phosphate buffer the rate attained was only 26.4 nmol min^{-1} (mg protein)$^{-1}$, after an incubation of 50 h (data not shown). Both the maximum carvone production rate and the maximum carvone concentration in phosphate buffer were ca. 60% of the values observed in mineral medium. Moreover, at 1,652.5 h, when the cells in the column with phosphate buffer were centrifuged and suspended in mineral medium, the carvone production rate was twice the value observed during the last 1,000 h in phosphate buffer (data not shown).

The results indicate that a regenerative medium is required for adapting a population, suggesting that adaptation results from growth of the most apt individuals.

Duration of the adaptation period

The carvone production rate increased with increasing adaptation periods up to a maximum in both reactors tested, at 28°C and at room temperature (23–25°C). A 3-fold increase in carvone production rate was observed in the 65 ml column reactor, maintained at 28°C, when the cells were allowed to adapt for 72 h, as compared to the situation with no adaptation (Fig. 4). However, increasing the adaptation period to 268 h resulted in an increase in the production rate of only 15.5%. When the air-driven column reactor was operated at room temperature, carvone production was faster for longer adaptation periods, achieving an 8.3-fold increase when the cells were adapted for 197 h, as compared to their non-adapted counterparts. After an incubation period of 268 h, the production of carvone was slower than after an adaptation period of 197 h.

The same type of response was observed in the 130 ml column reactor: a 18.7-fold increase was obtained when the cells were adapted for 136 h as compared to operation with non-adapted cells; after an adaptation period of 740 h, the carvone production rate was similar to that obtained with cells adapted for 50 h (Fig. 4a). When the biotransformation was carried out with non-adapted cells, the values obtained with this column maintained at 28°C were the same (0.1% difference) as those obtained with the column working at

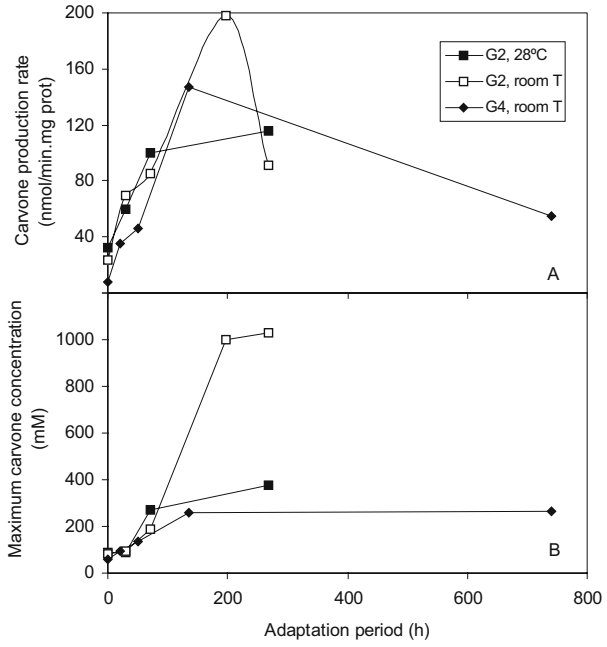

Fig. 4 Influence of the adaptation period on **a** the carvone production rate and **b** the maximum carvone concentration attained in air-driven column reactors with G2 or G4 glass spargers, operated at 28°C or at room temperature (23–25°C)

room temperature (data not shown). For an adaptation period of 740 h, the maximum carvone production rate was 1.9-fold higher in the column operated at room temperature. The final carvone concentrations achieved in the organic phase, after a total operation time of 1,550 h, were 267.1 and 407.2 mM at room temperature and at 28°C, respectively.

The maximum concentration of carvone attained in each reactor was also dependent on the adaptation period. Using the 65 ml column with a G2 glass sparger, and cells adapted for 268 h, a concentration of 375.3 mM and an impressive 1,030.9 mM were obtained after 167 h of reactor operation at 28°C and at room temperature, respectively (Fig. 4b). In the 130 ml column with a G4 glass sparger, a concentration of 267.1 mM was attained after a total time of operation of 1,550 h after adapting the cells for 740 h. However, a concentration of 259.0 mM of carvone was obtained after a total time of 341 h with an adaptation period of 136 h.

During the incubation periods, viability losses were around 10%, except for the cells used in the 65-ml column reactor, which presented a viability loss of 20% (data not shown) even though cells were adapted in a similar way. The different values of viability observed suggest that distinct batches of cells may present slightly different responses to adaptation. Nevertheless, the cells used in the various tests in each reactor were from the same batch and thus the comparisons are valid.

The 130-ml column reactor, with a G4 glass sparger, operated with cells adapted for 136 h produced the highest quantity of carvone, 1,560 mg, and also high carveol conversion rates (Table 3). Nevertheless, the highest productivity, 0.19 mg carvone h^{-1} (ml organic phase)$^{-1}$, and yield, 0.96 g carvone (g carveol)$^{-1}$, were attained with the 65-ml reactor with a G2 glass sparger when cells adapted for 268 h were used as biocatalyst. For the same adaptation period, slightly better results were achieved using the 65-ml column reactor at room temperature than at 28°C.

Discussion

Carvone was found to inhibit its own production by *R. erythropolis* DCL14 whole cells after reaching a concentration of 50 mM in mechanically stirred direct contact and membrane reactors, even though cell viability was high (de Carvalho and da Fonseca 2002a). In organic:aqueous phase systems, the thickness of the interface decreased with increasing initial carvone concentrations, and cell viability decreased by one-half after 24 h when the initial carvone concentration was higher than 50 mM (de Carvalho and da Fonseca 2002b). When carveol biotransformation was carried out in biphasic systems in the presence of carvone, an initial carvone concentration of 50 mM was sufficient to decrease carvone production, but not to cause significant cell death, indicating that the enzymatic system of the cells was affected before other more vital systems of the cell. However, when the cells were incubated with terpenes in the absence of a protective organic phase, such as *n*-dodecane, cell viability decreased considerably in the presence of 40–80 mM carvone.

Exposure to a toxic substance has been found to promote cell adaptation to the conditions to which they are exposed

Table 3 Performance of the air-driven column reactors

	G2, 28°C				G2, room temperature					G4, room temperature				
	0 h	29 h	72 h	268 h	0 h	29 h	72 h	197 h	268 h	0 h	20 h	50 h	136 h	740 h
Carveol added (mg)	239.50	302.73	277.32	516.82	239.50	302.73	277.80	469.40	661.00	431.10	521.31	1,291.38	2,300.00	1,224.71
Carvone produced (mg)	103.68	110.45	264.62	479.24	107.42	116.58	121.65	387.92	636.57	225.21	426.20	685.88	1,560.00	961.97
Productivity [mg carvone h^{-1} (ml organic phase)$^{-1}$]	0.10	0.08	0.01	0.11	0.12	0.08	0.07	0.17	0.19	0.02	0.04	0.03	0.16	0.04
Yield [g carvone (g carveol)$^{-1}$]	0.43	0.36	0.95	0.93	0.45	0.39	0.44	0.83	0.96	0.52	0.82	0.53	0.68	0.79

by favouring individuals that better tolerate the stress (Genoni et al. 2001; Mulvey and Diamond 1991; Montague et al. 2001). Cells that grow slowly or have entered the stationary phase acquire general tolerance to stress, the slowing of growth being seen as the inducing signal that leads to acquisition of stress tolerance (Booth 2002). Furthermore, the slowing of growth induces separate, but overlapping pathways that lead to tolerance to diverse types of stress (cells can become simultaneously tolerant to acid, heat, alkali, etc).

Strain DCL14 is naturally quite tolerant to both water-miscible and immiscible organic solvents (de Carvalho et al. 2004). In the presence of low log *P* solvents, *R. erythropolis* cells aggregate due to the hydrophobic character of the cellular membrane, thus protecting the cell population against toxic hydrophilic compounds (de Carvalho and da Fonseca 2004a; de Carvalho et al. 2004). When the cells were allowed to adapt to the organic solvent used as substrate reservoir in biphasic biotransformation systems, carvone production rates increased, even when solvents like *n*-hexane, which was expected to be toxic according to its log *P* value, were used (de Carvalho and da Fonseca 2004b). Image analysis of fluorescence microscopy observations, and principal components analysis, indicated that loss of viability occurs after incorporation of molecules of solvent into the cellular membrane, leading to alterations in the cell shape. The presence of non-viable cells with the same shape as the initial cells suggested that some cells in contact with toxic solvents lost viability before they could adapt to the presence of the solvent. This supports the theory that adaptation of a population derives from the ability of an organism and its progeny to remain viable under conditions that would kill or inhibit other members of the same population.

The hydrophobic character of *R. erythropolis* DCL14 results in cell migration from the aqueous towards the organic phase. Furthermore, in *n*-dodecane:aqueous emulsified systems, most of the cells were found to be located inside solvent droplets (de Carvalho and da Fonseca 2003). In the present study, the number of cells leaving the aqueous phase increased with increasing incubation times. Direct contact between the cells and the organic phase containing carveol and carvone was thus possible.

Since the number of cells in the organic phase increased with the duration of the incubation period, cell hydrophobicity must also have increased with time. Bacteria can change the fatty acid composition of the membrane only by de novo synthesis, which is strictly related to cell growth. Also, our results indicate that cells grew during the adaptation period at an average growth rate of 0.005 h^{-1}. According to Booth (2002), this slow growth can be a sign of cell adaptation.

R. erythropolis DCL14 cells may use both carveol and carvone as carbon and energy sources (van der Werf et al. 1999a,b). Additionally, they are also able to metabolise *n*-dodecane (de Carvalho and da Fonseca 2005a), although cells grown on this compound do not, apparently, exhibit CDH activity (de Carvalho and da Fonseca 2002b). Growth of strain DCL14 is also much faster on terpenes than on *n*-dodecane.

Alkanes and terpenes were shown to cause a dose-dependent increase in the degree of saturation of the fatty acids of the membrane phospholipids when used as sole carbon and energy source (de Carvalho et al. 2005). The differences in the degree of fatty acid saturation led to a considerable variation in the hydrophobic behaviour of the cells and therefore influenced the uptake rate of hydrophobic/hydrophilic compounds. Nevertheless, not only is the bacterial membrane affected by the presence of an organic solvent, in particular by the presence of terpenes, but the enzymes involved in their metabolism may also be activated (de Carvalho and da Fonseca 2004b).

In the air-driven column reactors, comparison of the results obtained with cells carrying out the bioconversion of carveol in phosphate buffer as aqueous phase and those attained with cells performing the biotransformation of carveol in mineral medium show that conditions that promote cell growth are required to achieve adaptation of the population. With adaptation periods up to 72 h, slightly better results were attained in the column with a G2 glass sparger, but for longer adaptation periods, temperature influenced the system more than aeration and mixing. Unexpectedly, columns reactors working at room temperature (23–25°C) allowed better carvone production rates than those maintained at 28°C. This could be the result of a slower cell growth and, consequently, a slower adaptation process, which seems of paramount importance in achieving high carvone production rates and concentrations. The results also indicate that, for each bubble column and reaction condition, there is a particular period of adaptation that leads to a maximum production rate. Longer periods will not, apparently, increase production rates. The importance of having an adaptation period during which growth slowly takes place can also be inferred by comparison with the results obtained in a mechanically stirred direct contact reactor. In fact, in the latter, where no cell adaptation was attempted, there was no significant difference in the production rate of carvone when mineral medium or phosphate buffer were used as aqueous phase (de Carvalho and da Fonseca 2002a).

Conclusions

The results clearly demonstrate that if *R. erythropolis* DCL14 cells are allowed to come into contact with low, increasing, concentrations of carveol and carvone in *n*-dodecane with aeration, they will be able to endure higher carvone concentrations. Furthermore, the carvone production rate and maximum carvone production depend on the length of the incubation period, maximums being achieved after 136 and 197 h for the G4 and G2 reactors, respectively. Loss of viability also decreases after longer adaptation periods. Cell growth during the incubation phase and/or during the biotransformation run, is assumed to have been responsible for the adaptation of the population and thus for the increase in carvone production. During bioconversion, cell growth is traditionally, and usually, undesired if the cells are using the substrate, product, or both as carbon

source. However, in the present case, higher activities were obtained when the cells were able to adapt/grow in the presence of both carveol and carvone. The present study provides scope to investigate the mechanism(s) involved in cell/population adaptation, in order to implement rational strategies for cell adaptation in whole cell bioconversion systems.

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