Bacterial Whole Cell Biotransformations: *In Vivo* Reactions Under *In Vitro* Conditions

Carla C. C. R. de Carvalho* • M. Manuela R. da Fonseca

IBB–Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

Corresponding author: * ccarvalho@ist.utl.pt

ABSTRACT

Wild and recombinant bacterial strains are able of carrying out biocatalysis and biotransformation processes involving multi-step metabolic pathways with co-factor regeneration. Bacterial cell tolerance and adaptation to organic solvents, ionic liquids and other non-conventional conditions, have allowed the biotransformation of low water soluble substrates and the production of high valued compounds. The recent developments in single cell monitoring, namely by fluorescence microscopy and flow cytometry, have resulted in increased yields and extended operation of processes for the biotransformation of toxic compounds. In this review, the utilisation of wild and recombinant strains will be discussed as well as the recent developments in metabolic engineering of bioconversion processes. The application of cells in non-conventional media and its repercussion in process design will be reviewed and the importance of studying cell viability will be presented.

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INTRODUCTION

Biotransformation of substrates can be performed by both whole cells and isolated enzymes. Amongst the advantages of using pure enzymes are the specificity for selected reactions, simple apparatus and work-up and better tolerance for co-solvents required to solubilise low-water soluble substrates (Roberts *et al*. 2004). However, enzyme isolation and purification can be quite expensive, the addition or recycling of co-factors may be necessary and it is difficult to carry out reactions requiring more than one enzyme. Multi-enzymatic reactions are easily performed by whole cells but side reactions may occur. A cascade of reactions necessary to produce complex products is possible as well as co-factor regeneration, resulting in inexpensive processes. Nevertheless, conditions to have only the desired culture(s) in a reactor are necessary and the solvents needed to dissolve low water soluble substrates may affect or even disrupt the cellular membrane.

The natural function of enzymes inside living bacteria is to promote metabolism, responsible for cell functioning and growth. The major advantage of using whole cells is efficient co-factor regeneration mechanism(s). Furthermore, they also provide a natural environment for the enzymes, avoiding loss of activity due to conformational changes in the protein structure of the enzyme in non-conventional me-

dia. Knowledge on cell physiology is therefore of paramount importance for process optimization. Biotransformation can also be primarily an act of cellular defence against a toxic compound, as an enzymatically mediated resistance mechanism through which the compound is transformed into a non-toxic form (Cloete 2003). In this case, a balance between the minimal concentration of the toxic compound necessary to induce biotransformation and the maximal concentration, above which enzyme inhibition or cell death occurs, must be achieved.

The terms biotransformation and bioconversion have often been used interchangeably (Walker and Cox 1995), but there is a subtle difference between the two. Biotransformation should be applied to a specific modification of a defined compound to a defined product, structurally similar (Lilly 1994) whilst bioconversion refers to the catalytic activity of living organisms which means that the production of the major product from the precursor compound(s) may involve several steps.

Microbial whole cells have been used more often at an industrial scale than animal or plant cells because of their vast diversity and easy handling. Single bacterial cells may produce several enzymes, which may be produced in greater or lesser number depending on e.g. the cell type, growth conditions and stage of cell development and which may work intra- or extracellularly. A single bacterium can,

Invited Review

Table 1 Substrates converted by *R. erythropolis* strains (based on de Carvalho and da Fonseca 2005a).

Substrates used by whole cells of *Rhodococcus erythropolis*						
Alkanes	**Alkanols**	**Amides**	**Aromatics**	**Halogenated**	**Lipids**	**Terpenes**
Propane	Methanol	Naproxen amide	Benzene	Fluorophenol	Cholesterol	D-Limonene
n-Pentane	Ethanol	Ketoprofen amide	Indene	Haloalkanes	Phytosterol	Limonene-1,2-
n-Hexane	Propanol		Naphthalene	Dichloropropane		epoxide
Cyclohexane	Butanol		Toluene	1-Chlorobutane		(-)-Carveol
n-Heptane	Pentanol		*o*-xylene			(-)-Carvone
n-Octane	Cyclohexanol		*m*-xylene			
Iso-octane	Dodecanol		*p*-xylene			
n-Nonane						
n-Undecane						
n-Dodecane						
n-Tetradecane						
n-Hexadecane						
Pristane						

therefore, carry out a wide array of biotransformations and use a large number of substrates (**Table 1**). During a study aimed at finding Nature's chemical diversity, more than 100 compounds belonging to more than 25 structural classes were isolated from only 6 different micro-organisms (Bode *et al*. 2002). Techniques, such as DNA recombination, have widened the array of possible whole cell catalysis, enabling the overproduction of a desired enzyme in various heterologous hosts. Combinatorial biocatalysis has allowed the creation of libraries of complex natural products or synthetic compounds (Rich *et al*. 2002). Since most of the reactions performed by micro-organisms in organic synthesis are carried out under very mild conditions, with high chemo-, regio- and stereoselectivity, there has been an increased interest in biocatalysis in the last decade to synthesise compounds that are not easily obtainable by chemical synthesis, such as precursors for the pharmaceutical industry.

WILD AND RECOMBINANT STRAINS

Advances in genetic and metabolic engineering have improved significantly the application potential of biotechnology, in particular, by permitting new biotransformations through the development of specific and stable biocatalysts (Liese and Filho 1999). The biocatalyst for a desired transformation may most probably be found in Nature and may be isolated from environmental samples collected in places with characteristics similar to the pretended application. Still, for the biocatalyst to be interesting it should be able to carry out the biotransformation at an industrial level. The conditions found in industry, e.g. substrate concentration, temperature, the presence of organic solvents, are seldom similar to those found in the native environment of the bacteria. Most catalysts do not present enough qualities in terms of productivity, stability and availability to be used in industrial processes (Marrs *et al*. 1999).

A few years ago, extremophiles, which include thermophiles, acidophiles, alkalophiles, psychrophiles and piezophiles, became an important source of enzymes (for reviews see Sellek and Chaudhuri 1999; Demirjian *et al*. 2001). Since wild type strains isolated from natural environments rarely produce enzymes in significant yields, it became standard to clone the genes of the desired enzymes in well-established expression hosts (e.g. *Escherichia coli*, *Pichia pastoris*, *Bacillus subtilis*). Recent developments have even allowed the screening of enzymes from gene pools collected from environmental samples. This metagenomic approach allows the cloning of DNA by polymerase chain reaction (PCR) techniques into suitable hosts without the necessity of cultivating the original strains (Hugenholtz and Pace 1996; de Long and Pace 2001; Jacobsen *et al*. 2005).

Whole cell biocatalysis has also improved due to proteomic and genomic information that have been used to identify important target genes leading to metabolic improve-

ment. Metabolic fluxes and their control *in vivo* are of paramount importance to the understanding of the metabolic behaviour of the micro-organism (Stephanopoulos 1999; Alper *et al*. 2005). McDaniel and Weiss (2005) wrote a review on the advances in synthetic biology which now allows the construction *de novo* of metabolic routes. *In silico* metabolic engineering has also been used to find novel pathways, such as the heuristic search for the *de novo* synthesis of vanillin carried out by McShan and Shah (2005).

Alcon *et al*. (2005) used *Pseudomonas putida* CECT 5279 genetically modified to study the biodesulfurisation of dibenzothiophene (DBT). The strain CECT 5279 was constructed using the genes *dszA*, *B* and *C* from *Rhodococcus erythropolis* IGTS8 (the first and best studied strain able to use DBT as the sole sulphur source) and the gene of the flavin-oxido-reductase, *hpaC*, from *E. coli*. The results obtained with this modified strain showed that there is no influence of the transport rate of the different compounds involved in the desulfurisation route across the cellular membrane on the biodesulfurisation rate, which is thus the result of the sum of several process reactions. Previous results have shown that engineered *Pseudomonas* strains, with the *dsz* cluster inserted in their chromosomes had more activity than the *R. erythropolis* wild type (Gallardo *et al*. 1997). However, in systems using *Pseudomonas*, Setti *et al*. (1995) demonstrated that the transfer of DBT from the organic phase to the aqueous phase and from the water to the cells can limit the rate of DBT metabolism. In this case, the high cell hydrophobicity of *R. erythropolis* cells allows the uptake of substrates directly from the organic phase (Monticello 2000; de Carvalho and da Fonseca 2003; Bouchez-Naitali *et al*. 2004; de Carvalho and da Fonseca 2005b). The *Rhodococcus* strains are able to access directly the hydrophobic DBT containing phases whilst the *Pseudomonas* strains can only access DBT at a much lower concentration in the water phase (Monticello 2000).

Most of the works published in recent years were aimed at finding an appropriate biotransformation system through screening of whole cells and/or DNA pools to insert the appropriate genes in a host cell that would perform the biotransformation. However, screening is made with cells that live in conditions that favour the desired enzymes (that is why there is a higher probability to find the enzymes in the first place). The original strain may possess membranes, efflux pumps, biosurfactants, etc, that may improve the overall productivity of the process and should not be disregarded at the start.

Bacillus coagulans NCIMB 9365 has two enzymes that carry out the enantioselective hydrolysis of 1,2-*O*-iso-propylidene glycerol esters to the corresponding alcohol. One enzyme is thermostable and enantioselective towards benzoate and butanoate, while the other is thermolabile and not enantioselective (Molinari *et al*. 1996). Romano *et al*. (2005) were able to produce the (*S*)-alcohol as the major enantiomer, by thermally knocking out the non-enantioselective enzyme in *B. coagulans* cells.

When the efficiency of the biotransformation is very low in the host cells or the number of side reactions affects the process, cloning and heterologous expression of the genes necessary for the biotransformation becomes necessary. In a study to use soluble cytochrome P450 monooxygenase systems CYP105D1 and CYP107B1 from *Actinomycetes*, involved in the biotransformation of some xenobiotics, bacterial cells of the genus *Streptomyces* were chosen because: i) they have a high-GC (guanine-cytosine) content in the two genes and ii) a high probability that endogenous redox systems would work in this genus as shown by the genome sequence of *S. coelicolor* A3(II) (Ueno *et al*. 2005). Expression of these two genes in *S. lividans* TK24 showed that CYP107B1 is capable of using the endogenous electron transfer partner from the host but CYP105D1 could not, requiring a redox partner from closely related strains. The authors ascribed one of the reasons to differences in the heme-iron binding motif between the two P450 enzymes.

Speight and co-workers (2004) generated a target library containing cytochrome P450 randomized at active-site amino acids Y96 and F98 using a whole cell system to perform the hydroxylation of diphenylmethane. As pointed out by the authors, this random approach is faster than rational site-directed mutagenesis, providing information about new activities in shorter time and in a format closer to preparative scale reactions.

Genetic recombination also permits the study of enzyme systems from pathogen micro-organisms in benign hosts. The recombinant *E. coli* B834 (DE3) pDB5, expressing a Baeyer-Villiger monooxygenase from *Mycobacterium tuberculosis* H37Rv, a class 3 pathogen, performed regioselective oxidations of fused bicyclic ketones (Snajdrova *et al*. 2006). The use of living cells provides a way to recycle the cofactors (NADH and NADPH) required by this type of monooxxygenases (Seelbach *et al*. 1996). *E. coli* expressing two monooxygenases from *Brevibacterium* was used to carry out Baeyer-Villiger type biotransformations of mesomeric ketones to the corresponding lactones (Mihovilovic *et al*. 2003) and of bicyclic ketones with a cyclobutanone structural motif to which the two enzymes showed significantly different stereoselectivity (Mihovilovic and Kapitán 2004).

BIOCATALYSIS MEDIA

A significant number of the substrates and products with potential to be produced at an industrial level (e.g. aliphatic, aromatic and heterocyclic compounds) are lipophilic, presenting low solubility in aqueous systems. Several techniques have been developed to increase the solubility of these compounds such as the addition of solubilisers, emulsifiers, co-solvents or the use of biphasic systems. In these cases, the bacterial cell may help to protect the enzyme(s) from the unnatural environment necessary to increase the solubility of the substrates, as long as cell viability is maintained.

Organic phases

In the 1930s, Ernest Aleksander Sym, a Polish scientist, published several papers concerning the action of porcine pancreatic lipase in organic media (Sym 1930, 1936). However, as pointed out by Halling and Kvittingen (1999), his work was forgotten because it appeared before the dogma that enzymes are active only in aqueous surroundings.

The toxic effect of organic solvents to micro-organisms arrives from their easy entrance in biological membranes (Sikkema *et al*. 1995; Ramos *et al*. 2002). The toxicity is directly related to the logarithm of the partition coefficient of the organic compound between octanol and water (log P value). According to Laane *et al*. (1987), solvents with log P values higher than 4 are generally biocompatible, while those with values lower than 2.0 are not. Those with log P values between 2 and 4 have an unpredictable biocompati-

bility. Biocatalysis with whole cells would be, apparently, only possible with hydrophobic solvents, presenting low toxicity to cells. However, in recent years solvent-tolerant strains have been successfully used in systems with toxic, polar solvents. Tolerant bacteria are able to surmount the effects of organic solvents due to several adaptive mechanisms such as modifications in the cell membrane, *cis-trans* isomerisation of the membrane fatty acids, changes in cell hydrophobicity and action of efflux pumps (for a review on the mechanisms of solvent tolerance see Sardessai and Bhosle 2002; for a review on the technical problems resulting from interactions between cells and hydrophobic compounds see Léon *et al*. 1998 and Angelova and Schmauder 1999).

Strains such as *R. erythropolis* are quite hydrophobic and cling (**Fig. 1**) or even pass to the organic phase (de Carvalho and da Fonseca 2003) while maintaining high viability and activity (de Carvalho and da Fonseca 2002a). When strain DCL14 was grown on alkanes, alkanols and terpenes to study the effect of carbon and energy sources on the production rate of (-)-carvone from (-)-carveol, it was found that all tested carbon sources caused a dose-dependent increase in the degree of saturation of the fatty acids of the cellular membrane (de Carvalho *et al*. 2005a). The exceptions were observed with short-chain alcohols, such as methanol and ethanol, which caused a decrease in the saturation degree of the fatty acids. The differences in fatty acid composition caused changes in cell hydrophobicity which affected substrate uptake and thus carvone production rate. The type of reactor used also influenced both carvone production rate and production yield (de Carvalho and da Fonseca 2002b), the best results being achieved in a air-driven column reactor which allowed a gentle mixing between phases (de Carvalho *et al*. 2005b).

Bacterial cells require, in general, more water than isolated enzymes and two phase systems have been used much more extensively than single organic systems. In this case, the reaction equilibrium can be shifted towards the product, biocatalyst and product recovery can be enhanced by phase separation and product inhibition can be reduced by extraction to the organic phase (Cabral *et al*. 1997).

During the production of limonene-1,2-diol from limonene-1,2-epoxide, which occurs with simultaneous resolution of the *trans*-limonene-1,2-epoxide diastereomer, the presence of an organic phase allowed high epoxide concentrations and the protection of substrate from chemical hydrolysis (de Carvalho *et al*. 2000). In a 500 mL fed-batch mechanically stirred reactor, the diol was produced at 197.2 g per g of protein and a yield of 98.2 and 67.9% were obtained for the *trans*-epoxide and the diol, respectively. Since the diol was more soluble in water than in the organic phase and the *R. erythropolis* cells carrying out the biotransformation remained at the organic-phase interface due to their high hydrophobicity, recirculation of the aqueous phase through an external loop containing a RP-18 column resulted in product separation *in situ*.

Fed-batch and a lower incubation temperature were used by Lee *et al*. (1998) to overcome product inhibition in the decarboxylation of ferulic acid to vinylguaiacol by *Bacillus pumilus*, in a two-phase system. A 0.1 M phosphate buffer: *n*-hexane system, at a phase ratio 1:1, gave the best results. In the biotransformation of (-)-carveol to (-)-carvone, the product was lethal to *R. erythropolis* cells above a concentration of 50 mM. However, a successful adaptation strategy, consisting of placing the cells in contact with increasing concentrations of carveol and carvone in *n*-dodecane, prior to the biotransformation, was developed (de Carvalho *et al*. 2005b). By adapting the cells for 197 h, carvone production rate increased 8.3-fold compared to non-adapted cells whilst an adaptation period of 268 h allowed a final carvone concentration of 1.03 M, thus significantly overcoming carvone toxicity.

In some cases, a small quantity of solvent, acting as co-solvent may be enough to enhance the productivity of a biotransformation. A final dimethylsulfoxide concentration
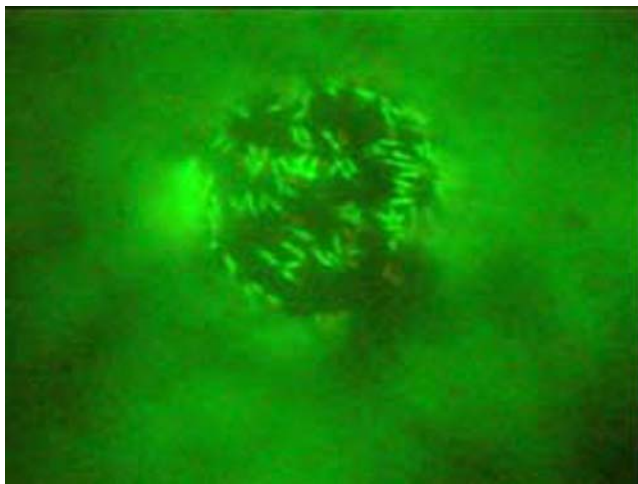
Fig. 1 *R. erythropolis* cells on the surface of a *n*-dodecane droplet in a *n*-dodecane:aqueous phase system, observed under fluorescent light. Horizontal and vertical field widths equal 0.08 and 0.06 mm, respectively.

of 2% (v/v) was sufficient to solubilise cyanohydrins used as substrate of *R. erythropolis* NCIMB11540, resulting in α-hydroxy carboxylic acids (Osprian *et al.* 2003). A concentration of 2% (v/v) of dimethylformamide was also sufficient to improve steroid hydroxylations with the fungi *Botryodiplodia malorum* and *Colletotrichum lini* (Romano *et al.* 2006).

The amount of solvent in the system may affect i) the mass transfer rate of the substrate and product from and to the organic phase and ii) cell integrity due to phase toxicity as the interfacial area increases. Prichanont and co-workers (1998) showed how the specific epoxidation rate of allyl phenyl ether to phenyl glycidyl ether by *Mycobacterium* M156 is influenced by the volume fraction of the aqueous phase in two phase systems. As the volume fraction decreased, the phase toxicity caused by the solvent became, apparently, an influential factor reducing the specific epoxidation rate. On the other hand, over 95% of *Mycobacterium* sp. NRRL B-3805 cells remained viable for more than 150 h in pure bis(2-ethylhexyl) phthalate whilst in a biphasic system less then 20% remained viable (de Carvalho *et al.* 2004). Since these cells present a tendency to accumulate at the organic:aqueous interface, they should be subject to mechanical stresses resulting from droplet coalescence/division and collision in biphasic systems.

By joining the solubility capabilities of solvents with the protective effect of immobilisation supports, it is possible to carry out biotransformations with whole cells, but the immobilisation matrix and the mixing conditions must be carefully chosen to avoid problems related to mass limitation and low yields (Fernandes *et al.* 1998). The cleavage of β-sitosterol to 4-androstene-1,17-dione, a multi-step

pathway, by *Mycobacterium* sp. NRRL B-3805 in bis(2-ethylhexyl) phthalate was possible with the cells immobilised on Celite, but the activity was strongly affected by the amount of water adsorbed on the support (Fernandes *et al.* 1998). When the same cells were tested using chrysotile, a low-cost and readily available inorganic material, immobilised resting cells performed better than free cells and the system could be used continuously for up to one month (Wendhausen *et al.* 2005).
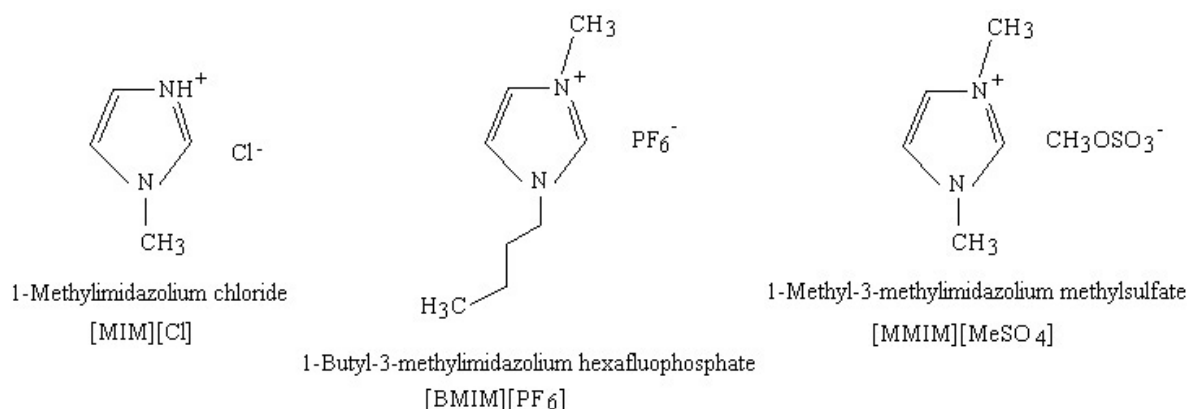
Immobilisation of cells has the advantage of permitting the reuse of cells which is particularly important when the cells produce enzymes at low concentration or grow at slow rates under conditions that induce the enzyme(s) required. However, the stability of the enzymes during repeated use may depend on the substrate type: nitrile hydratase of *R. equi* A4 was most stable while transforming (*R*,*S*)-3-hydroxy-2-methylenebutanenitrile but activity decreased during repeated bioconversion of benzonitrile (Kubáč *et al.* 2006).

Ionic liquids

Ionic liquids are non-aqueous, not flammable, thermally stable solvents containing an organic cation and an organic or inorganic anion (**Fig. 2**). By changing the proportion between the amount of the cation and anion, their polarity, hydrophobicity and solvent miscibility may be fine tuned. The first report of an ionic liquid was done by Walden in 1914, who reported the synthesis of ethylammonium nitrate (Gordon 2001). However, the first application in biocatalysis was only reported in 2000 by Cull *et al.* These authors compared the biotransformation of 1,3-dicyanobenzene in water-toluene and in water-[BMIM][PF$_6$] systems by *Rhodococcus* R312 cells.

In the presence of the ionic liquid, the initial rate of 3-cyanobenzamide production was lower than in the system with toluene but the specific activity of the cells was an order of magnitude greater than in water-toluene, which led the authors to the conclusion that the bioconversion is limited by substrate mass transfer rather than by the activity of the cells.

The first biotransformation requiring cofactor regeneration in which ionic liquids were used as substrate reservoir and product extracting phase was the asymmetric reduction of 4-chloroacetophenone to (*R*)-1-(4-chlorophenyl)ethanol with *Lactobacillus kefir* (Pfruender *et al.* 2004). The ionic liquids [BMIM][PF$_6$], [BMIM][Tf$_2$N] and [OMA][Tf$_2$N] were also found biocompatible to *E. coli* and *S. cerevisiae* cells (Pfruender *et al.* 2006). The lactic acid producing bacterium *L. delbruekii* subsp. *lactis* NRIC 1683 can even grow in the presence of imidazolium-based ionic liquids, but the bacterial activity decreases with an increase in the alkyl chain length of the imidazolium (Matsumoto *et al.* 2004). Nevertheless, further research is imperative to design ionic liquids to improve biocatalytic reactions and to develop sound processes with industrial application.

1-Methylimidazolium chloride
[MIM][Cl]

1-Butyl-3-methylimidazolium hexafluophosphate
[BMIM][PF$_6$]

1-Methyl-3-methylimidazolium methylsulfate
[MMIM][MeSO$_4$]

Fig. 2 Examples of ionic liquids used in biotransformations.

CELL MONITORING

In a biotransformation system, particularly if co-factor regeneration is required, it is not only important to monitor both substrate and product concentration but it is of paramount importance to monitor the status of the cells. In the presence of organic solvents, ionic liquids, toxic substrates and products, the cells may use adaptive mechanisms that require energy or even loose viability, thus decreasing the productivity of the system. In recent years, technical advances have provided several tools that allow better insights of the state of the cells, such as fluorescence microscopy and flow cytometry.

Cell viability is usually determined by the plate count method, which is time consuming since it needs a relatively long time for colony development. Furthermore, no information is given about the cells that are unable to form colonies. Any technique enabling measurements at the individual level provides more useful information than traditional biochemical assays, which study large populations of cells (Davey and Kell 1996).

The metabolic condition of individual cells can be rapidly assessed by fluorescence microscopy. Presently, there are fluorescent dyes which allow the assessment of e.g. membrane integrity, enzyme activity, electron transport, membrane potential and intracellular pH (Breewer and Abee 2000). By combining fluorescence microscopy with image analysis, it is possible to carry out quantitative analyses and to obtain information about the status and morphology of each cell, which is not possible by growing cells on agar plates (**Fig. 3**). Laser microscopy techniques, such as confocal laser scanning microscopy (CLSM) and the new infrared multiphoton laser scanning microscopy (NIR-LSM), are highly sensitive and permit the detection of biomolecules within the cells (for a review on the application of laser scanning microscopy to biological systems see Halbhuber and König 2003).

Protein activity and localization in bacterial cells have also improved by the use of green fluorescence protein (GFP) cloning. *In vivo* protein-protein interactions can be measured by fluorescence resonant energy transfer (FRET) while protein diffusion coefficients and protein mobility can be, respectively, measured and assessed by fluorescence recovery after photobleaching (FRAP) using GFP and its spectral variants (for a review on the applications of GFP in fluorescence-imaging techniques see Meyer and Dworkin 2007). However, metabolic activity studies are hampered because during the long-term measurements required, fluorescent compounds suffer photochemical decomposition or photobleaching. Scanning electrochemical microscopy (SECM) allows the study of electron transfer reactions inside living cells, giving information about the respiratory activity, cell viability and other redox processes. The redox activity of individual cells of *Rhodobacter sphaeroides* (purple bacteria), which contain a membrane-bound reaction centre protein with bound redox co-factors, was studied by Cai *et al*. (2002). Longobardi *et al*. (2006) used membrane fragments and liposomes of the same strain to study the electron transfer to the co-factors of the photosynthetic centre protein.

Fluorescence techniques can be useful during the development of biotransformation systems to follow bacterial growth (Iwabuchi *et al*. 2002), the transport and extrusion of compounds (Xu *et al*. 2003), the toxic effect of substrates and products (de Carvalho and da Fonseca 2002a), the effect of the organic solvents in biphasic systems (de Carvalho and da Fonseca 2002a; de Carvalho *et al*. 2003; de Carvalho and da Fonseca 2004a, 2004b) and of reactor conditions (de Carvalho and da Fonseca 2002b).

Flow cytometry is a relatively new and powerful technique for studying the integrity of the cellular membrane and membrane polarisation of individual cells within a population. Flow cytometry uses principles of light scattering, light excitation and emission of fluorochromes using generally a laser as a light source. As cells pass through the sensing region, a light scatter pulse is generated and detected both in the forward (FALS, *Forward Angle Lights Scatter*) and side (RALS, *Right Angle Light Scatter* or SSC, *Side Scatter*) angle. The FALS signal is an indication of the cell size whilst the RALS signal is influenced by the refractive index and the internal cell structure. The specific fluorescent stains added to the cells, to bond at specific intracellular binding sites, are excited to higher energy states as the cells intercept the light source and scatter light. The energy of the fluorescent dye is then released as a photon of light with a specific wavelength and the technique is powerful enough to allow the rapid throughput of thousands of individual cells per second, providing real-time and statistically significant information. The intracellular environment, even within living cells, may thus be assessed.

Multi-parameter flow cytometry has been used to assess the physiological state of cells after exposure to a toxic compound (Amanullah *et al*. 2002a), to help defining the best substrate feeding strategy (Amanullah *et al*. 2002b), to study the effect of starvation periods and substrate pulses to continuous cultures (Hewitt *et al*. 1999; da Silva *et al*. 2005), to monitor the proportions and population dynamics in mixed cultures (Müller *et al*. 2002), to distinguish between "viable" and "viable but non-culturable cells" (Nebe-von-Caron *et al*. 2000) and to develop and optimize bio-

A

B
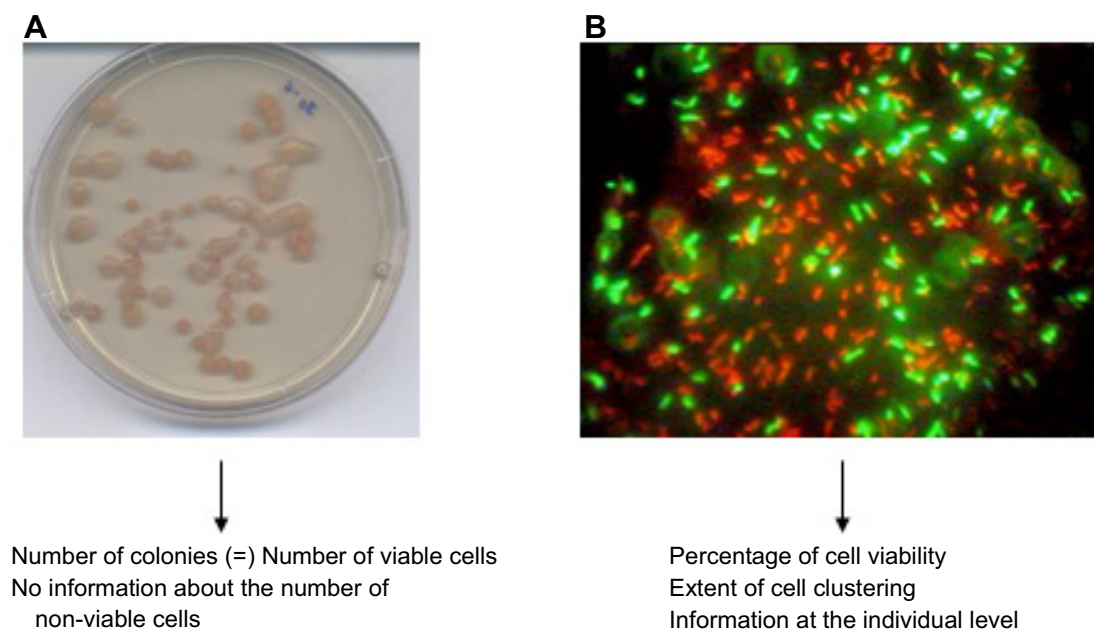
Fig. 3 Viability assessment: by colony counting on an agar plate (A) and fluorescence microscopy (B). Entrance of specific fluorescence dyes in the cells depends on their state, thus distinguishing viable from non-viable cells.

Number of colonies (=) Number of viable cells
No information about the number of
    non-viable cells

Percentage of cell viability
Extent of cell clustering
Information at the individual level

processes by evaluating cell viability and the evolution of cell populations in reactors (Cánovas *et al.* 2007).

Several other techniques have been used to monitor cell viability and number. Bučko and co-workers (2005) followed the content of viable biomass encapsulated in SA-CS/PMCG capsules and CPG beads by measuring the ATP with a luminometer. The majority of the bioluminescence assays use bacterial luciferase with an oxidoreductase and the applications include the determination of coenzymes (e.g. ATP, NADP, FAD, FMN) the metabolism of compounds such as glucose, ethanol, oxygen and pyruvate and the detection of reporter genes or enzymes by coupling their assays to the bacterial luminescence reaction (Meighen 1991; Alcon *et al.* 2005).

Bundy *et al.* (2001) studied the toxicity of heterocyclic and hydrocarbon compounds towards *P. fluorescens* by assessing the inhibition of light production of a strain construct that expresses gene *lux* constitutively. The biotransformation ability was then determined by the amount of light produced by *P. fluorescens* HK44 (pUTK21), which only produces light in the presence of salicylate, a metabolic intermediate.

Mid-infrared spectroscopy was used by Dadd *et al.* (2000) to study the kinetic of nitrile bicatalysis by *R. rhodochrous* LL100-21 cells. The React IR spectrophotometer is based on a Fourier transformed infrared technology (FTIR) and carries out real-time analyses of the identity and quantity of species present in a chemical reaction or bioprocess. Since the absorption spectrum of the compounds is taken in the mid-infrared region, a unique fingerprint of the reaction is created. The system is calibrated by generating reaction profiles of the concentration of the compounds involved as function of time.

INDUSTRIAL APPLICATIONS

The production of acrylamide from acrylonitrile by a nitrile hydrolase from *R. rhodochrous* J1 is the best known example of an industrial application of biocatalysis. The production by Mitsubishi Rayon reaches nowadays 30,000 t/year (Kobayashi and Shimizu 2000). The reaction is carried out in fixed-bed reactors using cells of strain J1 immobilised in polyacrylamide (Thomas *et al.* 2002). The same strain is used by Lonza Guangzhou Fine Chemicals to produce 3,400 t/year of nicotinamide (niacinamide) in stirred batch tank reactors with continuous feed of 3-cyanopyridine (Thomas *et al.* 2002).

The biotransformation of steroids at industrial scale is well established. For example, the German company Schering produces androstendione and androsta-dien-dione on a 200 m^3 scale using *Mycobacterium* strains. A successful multi-step coenzyme-dependent process is used by Kyowa Hakko Kogyo Co. in the production of oligosaccharides such as globotriose which is produced at a final concentration of 188 g L^{-1} (Koizumi *et al.* 1998). In this case, the cells are in a non-viable but metabolically active state.

The biotransformation of 2-cyanopyrazine in two-steps to 5-hydroxypyrazinecarboxylic acid, which is used in the preparation of antituberculosis drugs, by whole cells of *Agrobacterium* DSM 6336 results in a product concentration as high as 40 g L^{-1} (Wieser *et al.* 1997). To induce the activity in the cells, they are grown on 3-cyanopyridine as carbon and energy source.

In general, bioprocesses are environmentally cleaner than chemical processes and the action of enzymes and whole cells may decrease environmental pollution. The production of 1,3-propanediol from cornstarch sugar by a genetically modified *E. coli* by DuPont is a high yield process (Gavrilescu and Chisti 2005). Various carbohydrates may be used to produce 1,3-propanediol. The bioprocess performed by DuPont Tate & Lyle Bio Products consumes 40% less energy and reduces greenhouse gas emissions by 20% in comparison with the petroleum-based propanediol production process. It was estimated that the alkaline protease from *Bacillus lentus* may have reduced in more

than 60% environmental pollution associated with detergents (Gavrilescu and Chisti 2005). The hydration of adiponitrile from 5-cyanovaleramide by nitrile-hydratase of whole cells of *P. chloraphis* immobilised in calcium alginate beads carried out by DuPont is more efficient, cleaner and produces less by-products than the chemical counterpart (Zaks 2001). The re-cycling of the cells allowed a production of c.a. 3150 kg product/kg catalyst.

In the last few years, several companies have produced biopharmaceuticals using recombinant micro-organisms by biocatalytic and fermentation processes. For example, Lonza has produced purified bulk solutions of recombinant proteins and large peptides, hormones, antibodies and protein vaccines since 2004 by micro-organisms such as *E. coli*, *P. pastoris* and *Saccharomyces cerevisiae*.

Although a large number of processes, involving biotransformations of commercially interesting compounds, has been successful at laboratory scale, its application at industrial scale is still difficult. The scale up of e.g. biocatalytic oxidation processes is difficult due to the complexity of the processes and the large number of process variables to consider. The permeation of substrates across the cell membrane, the accumulation of by-products, the interactions between cells and several other behaviours can increase the unpredictability of these processes.

Nevertheless, several advantages over traditional chemical processes have been pointed out and in a near future the number of biocatalytic processes used in synthesis of high interesting compounds should significantly increase.

CONCLUSIONS

Efficient high-throughput screening and new microbiological techniques involving genetic and metabolic engineering are widening the number of biotransformations using whole bacterial cells with industrial application. Multi-step pathways and reactions requiring co-factor regeneration have more probability of being successful using whole cells than with cascades of isolated enzymes. The cells provide an *in vivo* environment to the enzymes, protecting them from *in vitro* reaction conditions that may be harsh if non conventional medium is used. The development of reactors and reaction conditions allowing high cell viability is being helped by the increased knowledge acquired by techniques that allow the study of the status of individual cells in large populations. Novel strategies can thus be developed to produce high valued products using environment-friendly biocatalytic processes.

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