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journal homepage: www.elsevier.com/locate/emtCoupled oxidation–reduction of butanol–hexanal by resting *Rhodococcus erythropolis* NCIMB 13064 cells in liquid and gas phasesP. Marchand¹, E. Rosenfeld¹, S. Lamare, T. Maugard, B. Erable, I. Goubet^{*,1}¹ Université de La Rochelle, Institut Littoral Environnement et Sociétés, LIENSS - UMR 6250, Avenue Michel Crépeau, 17042 La Rochelle cedex 1, France

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ABSTRACT

Rhodococcus erythropolis is a promising Gram-positive bacterium capable of numerous bioconversions including those involving alcohol dehydrogenases (ADHs). In this work, we compared and optimized the redox biocatalytic performances of 1-butanol-grown *R. erythropolis* NCIMB 13064 cells in aqueous and in non-conventional gas phase using the 1-butanol–hexanal oxidation–reduction as model reaction. Oxidation of 1-butanol to butanal is tightly coupled to the reduction of hexanal to 1-hexanol at the level of a nicotinoprotein–ADH-like enzyme. Cell viability is dispensable for reaction. In aqueous batch conditions, fresh and lyophilized cells are efficient redox catalysts (oxidation–reduction rate = $765 \mu\text{mol min}^{-1} \text{ g cell dry mass}^{-1}$) being also reactive towards benzyl alcohol, (S)-2-pentanol, and geraniol as reductants. However, butanol–hexanal oxidation–reduction is strongly limited by product accumulation and by hexanal toxicity that is a major factor influencing cell behavior and performance. Reaction rate is maximal at 40°C –pH 7.0 in aqueous phase and at 60°C –pH 7.0–9.0 in gas phase. Importantly, lyophilized cells also showed to be promising redox catalysts in the gas phase (at least $65 \mu\text{mol min}^{-1} \text{ g cell dry mass}^{-1}$). The system is notably stable for several days at moderate thermodynamic activities of hexanal (0.06–0.12), 1-butanol (0.12) and water (0.7).

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1. Introduction

Isolated enzymes or whole cells can be advantageous catalysts. Indeed, they perform specific and selective reactions under mild conditions and many of them are quite easily prepared. However, they can be limited by a low tolerance to organic solvent, high temperature or extreme pH and often by a low space-time yield. Among bacteria, *Rhodococcus erythropolis* strains appear as promising biocatalysts. Whole cells or isolated enzymes of this bacterium are able to carry out a very large set of bioconversions with some of them being of great environmental and biotechnological interest (for review: [1]). This ability can be related to the diversity of metabolic pathways of this microorganism [2,3] and to its resistance to solvent and hydrophobic compounds [2,4,5]. *R. erythropolis* cells have a hydrophobic surface protecting them against hydrosoluble solvents and favoring uptake of low water soluble compounds and adhesion to water/oil interfaces [6,7]. Solvent-grown cells can produce surfactants (such as trehalose mycolates and anionic trehalose tetraesters) that decrease the interfacial tension between

phases [8]. They can also aggregate to protect the population from hydrophobic solvents [9].

R. erythropolis strains (DSM 1069, DCL14, PR4) express many alcohol dehydrogenases (ADHs) [1,3]. Thus, whole cells or isolated primary and secondary ADHs may carry out a very large set of redox bioconversions, including production of flavors or synthons. Interestingly, Schenkels and Duine [10] showed that benzyl alcohol-grown *R. erythropolis* DSM 1069 can produce one nicotinoprotein(np)–ADH as the main alcohol-oxidizing enzyme in the dissimilation of many, but not all, alcohols. This enzyme, analogous to other ADH of nocardioforms and actinomycetes, is likely the main ADH produced during *R. erythropolis* DSM 1069 growth on 1-butanol. The purified enzyme exhibited a broad substrate specificity and contained tightly bound NAD as cofactor. However, high quantities of purified np–ADH were required for practical applications in different types of conversion, namely asymmetric reduction, kinetic resolution, and racemization [11].

The constraints of using ADHs which are (i) enzyme level, (ii) low substrate water solubility, (iii) instability at high (co-)substrate concentration, (iv) inhibition phenomena, and (v) requirement for cofactor regeneration, can be overcome, at least partially, by two strategies. One strategy exploits the intrinsic performances of *R. erythropolis* cells [6,7] while the other uses whole cells enriched in np–ADH-like enzymes as redox biocatalysts. An additional

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strategy (also employed in the present work) may be the use of non-conventional solid/gas biocatalysis. Indeed, this technology presents distinct advantages over liquid systems [12], and was found to improve stability of several microbial ADHs [13–15]. Moreover, we recently showed that lyophilized cells of *R. erythropolis* NCIMB 13064 were promising dehalogenating biocatalysts in solid/gas phase [16,17].

The aim of this work was to compare and optimize the redox biocatalytic performances of *R. erythropolis* NCIMB 13064 cells in aqueous and in non-conventional gas phase. 1-Butanol-grown *R. erythropolis* NCIMB 13064 cells were used as a redox biocatalyst due to their high np-ADH-like activity (see Section 3). 1-Butanol and hexanal were chosen as model substrates notably due to their low water solubility and their toxicity to bacterial cells, as many substrates of interest in agrochemical and pharmaceutical, and also due to their high volatility (well suited for solid/gas experiments). The biocatalytic potential of NCIMB 13064 fresh or lyophilized resting cells was assessed in aqueous phase using a simple batch system. The effects of substrate concentration, pH and temperature on cell viability, activity and stability of the biocatalyst were compared in both liquid and solid/gas phases. Redox performances of NCIMB 13064 cells grown under np-ADH inductive conditions are finally discussed.

2. Materials and methods

2.1. Strain and growth conditions

The *R. erythropolis* NCIMB 13064 strain was provided by the National Collection of Industrial and Marine Bacteria (Aberdeen, Scotland). This polymorphic strain produced rough and smooth colonies [18]. The latter were further used since they were dispersed easily in aqueous media. Bacteria were routinely stored on nutrient agar plates for 1 month. The liquid minimum medium used for growth [19] had the following composition (g l^{-1}): 0.85 NaNO_3 , 0.56 KH_2PO_4 , 0.86 Na_2HPO_4 , 0.17 K_2SO_4 , 0.37 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.007 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 2.5 ml of a trace element solution consisting of (g l^{-1}): 2.32 $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.78 $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.56 H_3BO_3 , 1.0 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.39 $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 0.42 $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.66 KI , 0.1 EDTA , 0.4 $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0004 $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$. Culture medium was adjusted to pH 7.0 and was sterilized by autoclaving for 15 min at 121 °C. Benzyl alcohol or 1-butanol were filter-sterilized on 0.22 μm PTFE membrane and added at a final concentration of 0.5% (v/v) (54.6 mM) after cooling the culture medium to room temperature. Cells were pre-cultivated at 28 °C (160 rpm) for 96 h in 250 ml Erlenmeyer flasks containing 50 ml of culture medium and next cultivated for 48 h in 1 l Erlenmeyer flasks containing 200 ml of medium. Growth was followed by measuring the optical density at 690 nm ($\text{OD}_{690\text{nm}}$). Aliquots were diluted appropriately with cell-free culture medium to obtain a $\text{OD}_{690\text{nm}}$ below 0.5 units. Cultures were started using an initial $\text{OD}_{690\text{nm}}$ of 0.05 units. Cells were systematically harvested in mid-phase exponential growth when $\text{OD}_{690\text{nm}}$ reached 2.0 units and residual 1-butanol was about 25 mM.

2.2. Chemicals

1-Butanol (99.7%), geraniol (98%), 5,5'-dithio-bis(2-nitrobenzoate) (98%), CuCl_2 (98%), 1,10-phenanthroline monohydrate (98%), pyrazole (98%) and 4-nitroso-*N,N*-dimethylaniline (NDMA) (97%) were from Sigma–Aldrich, methanol (99.9%), ethanol (99.7%) from Carlo Erba, 1-hexanol (99%), butanal (98%), hexanal (98%), ($\pm$)-1-phenylethanol (98%), 2-propanol (99%), *R*-2-pentanol (98%), acetophenone (99%), 2-pentanone (99%), ethyl isobutyrate (98%) from Fluka, *S*-2-pentanol (98%) from Acros, HgCl_2 (98%) from Labosi and benzyl alcohol (99%) and KCN (98%) from Lancaster.

2.3. Biocatalysis in liquid media

The harvested cells were centrifuged (8000g, 7 min), washed twice, and resuspended at about 20.0 $\text{OD}_{690\text{nm}}$ units in cold (+4 °C) 50 mM potassium phosphate buffer (pH 7.0). A small volume of suspension was then added and mixed into the reactor, previously incubated for 30 min at the appropriate temperature. The final $\text{OD}_{690\text{nm}}$ was measured and the reactor was additionally incubated for 10 min before starting the reaction.

In preliminary experiments, each reactor consisted of 60 ml cell suspension in a 60 ml bottle closed by Teflon-lined screw plastic caps. In order to limit the air–liquid contact area and the hexanal evaporation in the headspace, we next used smaller reactors consisting of 10 ml cell suspension in a 10 ml bottle. Unless otherwise specified, cell suspension was incubated at 40 °C with continuous magnetic stirring (1100 rpm). Reactions were started after injection of the substrates and rapid mixing

with a syringe. The substrate concentrations used (0.02 M) were below the solubility limits at 25 °C [1-butanol = 1.23 M, 1-hexanol = 0.059 M [20], butanal = 0.55 M [21], hexanal = 0.056 M [22]]. Samples were periodically withdrawn with needles after two rapid mixings with a syringe. Cells were removed by centrifugation and the supernatants were directly analyzed by gas chromatography. Results were expressed as IU/g cell dry mass $^{-1}$ with 1 IU being equal to 1 μmol of 1-hexanol formed per minute.

When distinct pH values were tested, buffer strength was increased by using 100 mM phosphate buffers.

2.4. Lyophilization

Fresh washed cells were resuspended at about 20.0 $\text{OD}_{690\text{nm}}$ units (8 g cell dry mass l^{-1}) in 50 mM Tris–HCl buffer adjusted at the appropriate pH. Cell suspension (15 ml per Petri dish) was frozen at –24 °C during 8 days. Cells were lyophilized for 10 h at –30 °C and for 24 h at –20 °C and stored hermetically at –24 °C for 1–3 days before use.

2.5. Preparation of cell extracts

Fresh washed cells were resuspended in 50 mM phosphate buffer (pH 7.0) at a 1:10 cell dry mass:buffer volume ratio. Cell suspension was sonicated four times for 1 min at 50 W and 20 kHz (Vibra-cell 72434, Bioblock Scientific, France) while cooling in melting ice. After centrifugation 30 min at 17,000g, the supernatant was used as cell-free crude extract.

2.6. Protein determinations

Protein concentrations were determined by using a Quantipro BCA Assay Kit (Sigma) and desalted BSA as a standard.

2.7. Enzyme assays

Nicotinoprotein(np)-ADH activity was measured at 40 °C by following the reduction rate of 4-nitroso-*N,N*-dimethylaniline (NDMA) at 440 nm with 1-butanol, ethanol, methanol or benzyl alcohol as electron donor. The reaction mixture, containing phosphate buffer 50 mM pH 7 and cell extract (0.3–0.5 mg proteins ml^{-1}), was incubated for 30 s before addition of NDMA (28 μM). The reaction was started by the addition of 0.5 mM alcohol. Reaction rate was obtained from the reduction rate of NDMA in presence of alcohol minus the blank (no alcohol). The molar absorption coefficient for NDMA was 35400 $\text{M}^{-1}\text{cm}^{-1}$ at 440 nm [23].

2.8. Reaction in the solid/gas reactor

The solid/gas biofilter used has been previously described by Lamare and Legoy [24,25]. Briefly, the reactor is composed of a 9 cm long glass tube in which *R. erythropolis* cells (100 mg of lyophilizate containing $\approx 8\%$ of salts) were packed between two layers of glass wool. Gaseous substrates were continuously fed through the biofilter by flowing nitrogen, as carrier gas, through flasks containing the liquid substrates. The thermodynamic activity of each compound *x* in the reactor was $A_x = \text{PP}_x / \text{PP}_x^{\text{sat}}$, with PP_x , the partial pressure of *x* in the gas entering the biofilter, and PP_x^{sat} the saturation vapor pressure (atm) of pure *x* at the working temperature.

The vapor phase leaving the biofilter was sampled using a 250 μl loop on a six-ways valve (Valco) maintained at 190 °C. Samples were automatically injected in the split injector of a gas chromatograph. Calibration of 1-butanol, hexanal, butanal and 1-hexanol, was carried out by programming a range of their partial pressures in the bioreactor packed with glass wool and by analyzing the vapors leaving the reactor. Operating parameters (temperature, pressure, and substrates and water thermodynamic activities) were monitored online.

The total flow passing through the biofilter was set at 500 $\mu\text{mol min}^{-1}$ giving a volumetric flow of 12.3 ml min^{-1} . 1-Butanol, hexanal and water thermodynamic activities were fixed at 0.12 (1.4 $\mu\text{mol min}^{-1}$), 0.06 (1.9 $\mu\text{mol min}^{-1}$) and 0.7 (24.8 $\mu\text{mol min}^{-1}$), respectively. A few hours of substrate feeding were required for the proper rehydration of the dried cells and for reaching a steady state. We checked that inhibition by the products did not occur and that specific initial rates measured at steady states were not modified when total flow varied from 300 to 1000 $\mu\text{mol min}^{-1}$. 1 IU corresponds to 1 μmol of 1-hexanol or butanal formed per minute.

2.9. GC analysis

Samples were analyzed by gas chromatography using a Hewlett-Packard 5890 gas chromatograph with a flame ionization detector connected to a 3396 integrator. The column used was a OV 1701 fused silica capillary column (25 m $\times$ 0.25 mm i.d. $\times$ 0.25 μm film thickness; Chrompack, France). The injector and detector were kept at 220 and 250 °C, respectively. The column temperature was held at 60 °C for 5 min, then programmed to increase at 15 °C min^{-1} to 160 °C. Ethyl isobutyrate was used as internal standard. Retention time were 2.48 min for butanal, 3.70 min for

ethyl isobutyrate, 3.22 min for 11-butanol, 4.53 min for hexanal and 5.51 min for 11-hexanol.

2.10. Cell viability

Viability of fresh or lyophilized cells was assessed by counting the number of colonies grown on Petri dishes containing nutrient agar after 48 h incubation at 30 °C. Serial dilutions in sterile saline water (NaCl 9 g l⁻¹) were made to obtain 30–300 colonies per Petri dish.

2.11. Cell dry mass

Cell dry mass was determined by filtering 25–50 ml of culture medium through membrane filters (pore size 0.22 µm). Filters were rapidly rinsed three times with 1 ml of distilled water, and desiccated at 105 °C until reaching a constant weight (48 h).

2.12. Microscopy

Lyophilized cells were gently suspended in 50 mM sodium cacodylate buffer (pH 7). The fixation step was performed using solutions of glutaraldehyde 2% and osmic acid 2%. Successive baths of acetone (50%, 70%, 90% and 100%) and hexamethyldisilazane (50% with acetone and 100%) were used for the dehydration steps. The preparations were examined using Philips Quanta 200 ESEM/FEG microscope with an Everhart Thornley-BSE detector.

2.13. Measurement of oxygen uptake

Oxygen uptake by washed cell suspensions was measured at 40 °C in a Hansatech oxygen electrode unit (type DW1) as described by Rosenfeld et al. [26]. The calibration of the electrode was carried out assuming the dissolved oxygen concentration in air-saturated phosphate buffer to be 195 µM at 40 °C.

3. Results

3.1. 1-Butanol–hexanal oxidation–reduction catalyzed by resting *R. erythropolis* cells in aqueous phase (at 40 °C)

The biocatalytic properties of 1-butanol-grown *R. erythropolis* NCIMB 13064 cells were first determined in simple aqueous conditions (phosphate buffer 50 mM pH 7.0, 40 °C). Each substrate was used at a considerable concentration (20 mM) but below the solubility limit in water at 25 °C. In the blank, 1-butanol is not converted at detectable levels by fresh resting cells (Fig. 1A). Blank experiments showed that hexanal, and to a lesser extent butanol, can evaporate at significant levels (e.g. 1 mM h⁻¹) in the presence as well as absence of *R. erythropolis* cells (Fig. 1A). We used batch reactors minimizing this phenomenon (see Section 2).

Under similar conditions, oxygraphy experiments showed that the cells are able to respire actively the 1-butanol added (about 30 µM O₂ min⁻¹. OD_{690 nm}⁻¹ ≡ 70 µM O₂ min⁻¹ mg cell dry mass⁻¹). This catabolizing activity is low compared to the initial 1-butanol concentration (20 mM).

When co-added at equimolar amounts both 1-butanol (reductant) and hexanal (oxidant) are converted in butanal and 1-hexanol, respectively (Fig. 1B). We found that equimolar quantities of 1-hexanol and butanal are formed. Interestingly, initial specific reaction rate is high despite the simple conditions used. A mean value of 765 ± 140 IU g cell dry mass⁻¹ was obtained from 10 independent experiments performed at final OD_{690 nm} ranging from 0.2 to 0.8. Butyric and hexanoic acids are not produced during the reaction and total substrate and product concentration is almost constant even though a minor part of hexanal evaporates (Fig. 1B). Under similar conditions, respiration becomes null (data not shown). Taken together, these results indicate that oxidation of 1-butanol into butanal is tightly coupled to the cofactor regenerating reduction of hexanal into 1-hexanol, and that this oxido-reductive reaction is mainly uncoupled from other cellular oxido-reductive and carbon-consuming reactions.

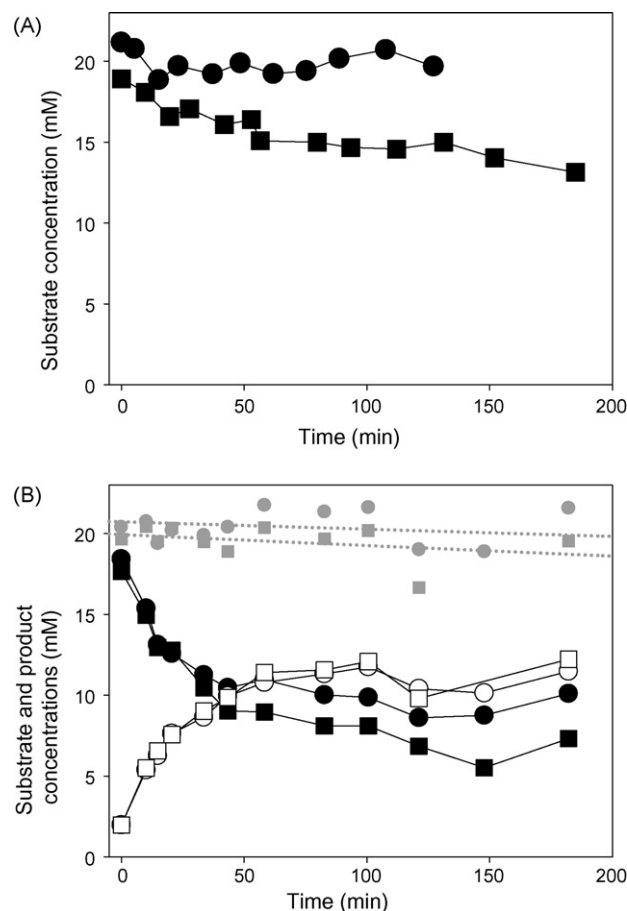


Fig. 1. Coupled oxidation–reduction of 1-butanol–hexanal by 1-butanol-grown *Rhodococcus erythropolis* NCIMB 13064 cells in aqueous batch conditions (40 °C, pH 7.0). (A) 1-Butanol (●) or hexanal (■) were added separately to the fresh cell suspension (OD_{690 nm} = 0.78). (B) 1-Butanol (●) and hexanal (■) were added together to the cell suspension (OD_{690 nm} = 0.78) allowing the production of butanal (○) and 1-hexanol (□). Total concentrations of C4- (●) and C6- (■) compounds are shown together with linear regressions (dotted lines). C4- and C6-acids were not produced at detectable levels. Data are means of at least two GC determinations.

Fig. 1B also shows that saturation-like curves of all substrates and products reach simultaneously a plateau. At this stage, we could not identify the factor (thermodynamic equilibrium, loss of viability or inactivation of the biocatalyst) limiting the reaction.

3.2. Parameters controlling the butanol–hexanal oxidation–reduction (at 40 °C)

We observed that viability of *R. erythropolis* cells rapidly decreases during the reaction in aqueous phase (Fig. 2) whereas oxidation–reduction reaction still efficiently proceeds (Fig. 1B). Viability is thus dispensable for the reaction. The sole hexanal is sufficient to observe a detrimental effect on cell viability. 1-Butanol has only partial toxic effects (at 40 °C).

To identify the factor limiting the reaction, we tested several initial substrate concentration (varying from 5 to 40 mM) and catalyst concentration (varying from 0.5 to 2.5 OD_{690 nm} units). Under all the conditions examined, we found that reaction stops when the product versus substrate ratio ([butanal][hexanol]/[butanol][hexanol]) surrounds 1.9 ± 0.25 (Fig. 3A). This suggests that reaction stops when the thermodynamic equilibrium is reached. Accordingly, no reaction occurs if substrates and products are added at time zero at a ratio of 1.9 (Fig. 3B).

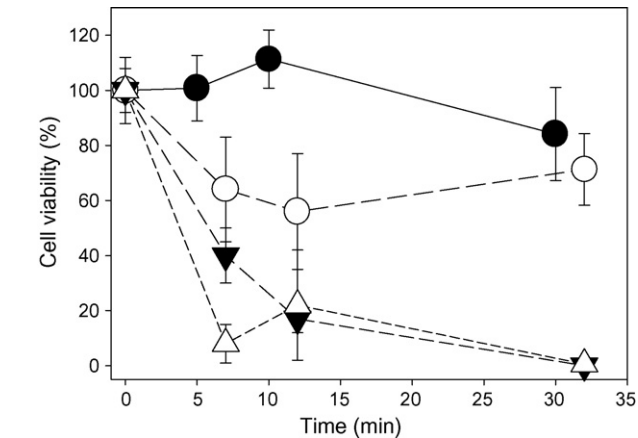


Fig. 2. Effect of 1-butanol and hexanal on cell viability (40 °C, pH 7.0). Fresh cells (final $OD_{690\text{nm}} = 0.3\text{--}0.5$) were incubated in batch reactors in the phosphate buffer (●, control) with 20 mM 1-butanol (○), 20 mM hexanal (▼), or both (△). Data are means ($\pm$ S.D.) of six or eight colony counts obtained from two independent experiments.

Initial reaction rate is linearly dependent on cell concentration at low $OD_{690\text{nm}}$ (data not shown). Cell densities below 1 $OD_{690\text{nm}}$ unit were thus used for further determinations. In additional experiments (also not shown), we checked that 1-butanol

Table 1
Relative oxidation–reduction activities of 1-butanol- and benzyl alcohol-grown *R. erythropolis* NCIMB 13064 cells

	Substrate for biocatalysis	
	1-Butanol	Benzyl alcohol
1-Butanol-grown cells	100	47
Benzyl alcohol-grown cells	46 ^(*)	32 ^(*)

Fresh resting cells were used at final $OD_{690\text{nm}}$ of 0.5. Hexanal was used as electron acceptor and 1-butanol:hexanal oxidation–reduction rate (32 UI/g cell dry mass^{–1}) was used as 100% reference. Data are means of measurements obtained from four (or two^(*)) independent experiments.

and hexanal concentrations (20 mM) are not limiting for reaction rate. The equimolar ratio 1-butanol (20 mM):hexanal (20 mM) was found to constitute a good compromise between reaction rate (being optimal at this ratio) and conversion level (about 50% for both substrates, Fig. 1B).

3.3. Substrate specificity of whole cells and extracts

ADH activity of the strain NCIMB13064 has not yet been studied. Since 1-butanol and benzyl alcohol were found to be efficient growth substrates to induce the np-ADH in the DSM 1069 strain [10], we compared the oxidation–reduction activities of butanol- and benzyl alcohol-grown NCIMB13064 cells using either butanol or benzyl alcohol as reductant (Table 1). We found that the best biocatalytic activity is obtained with butanol-grown cells and 1-butanol as reductant. This combination (also well suited for solid/gas experiments) was therefore used to further precise the properties and the nature of the ADH(s) produced by the strain NCIMB 13064. As shown in Table 2, 1-butanol is the best substrate for 1-butanol-grown *R. erythropolis* NCIMB 13064 cells but ethanol, (S)-2-pentanol, benzyl alcohol, and geraniol are also converted at interesting rates when hexanal is used as co-substrate. Conversely, methanol, 1-phenylethanol, acetophenone, and 2-pentanone are poorly converted. These results resemble those observed by Schenkels and Duine [10] for the np-ADH produced by *R. erythropolis* DSM 1069 grown on benzyl alcohol as carbon source. However, contrary to DSM 1069 cells, we found that 1-butanol (rather than benzyl alcohol) is the best substrate for NCIMB 13064 1-butanol grown cells.

One property of the np-ADH enzyme is that substrate specificity for the aldehydes reflects those of the corresponding alcohols [10].

Table 2
Oxidation–reduction rates with various substrates (40 °C, pH 7.0)

Substrate	Initial reaction rate (%)
With hexanal as electron acceptor	
Methanol	0.3 ± 0.3
Ethanol	47.9 ± 4.8
2-Propanol	7.7 ± 0.8
1-Butanol	100.0 ± 4.8
S-2-Pentanol	19.4 ± 6.3
R-2-Pentanol	1.1 ± 0.15
Benzyl alcohol	46.9 ± 21
1-Phenyl ethanol	0.6 ± 0.15
Geraniol	79.8 ± 3
With 1-butanol as electron donor	
2-Pentanone	0.2 ± 0.1
Hexanal	100.0 ± 11
Acetophenone	0.0
With butanal as electron acceptor	
1-Hexanal	47.0 ± 23

Fresh resting cells were used at final $OD_{690\text{nm}}$ of 0.2–0.5. 1-butanol:hexanal oxidation–reduction rate (32 UI/g cell dry mass^{–1}) was used as 100% reference. Data are means ($\pm$ mean deviation) of at least two independent experiments.

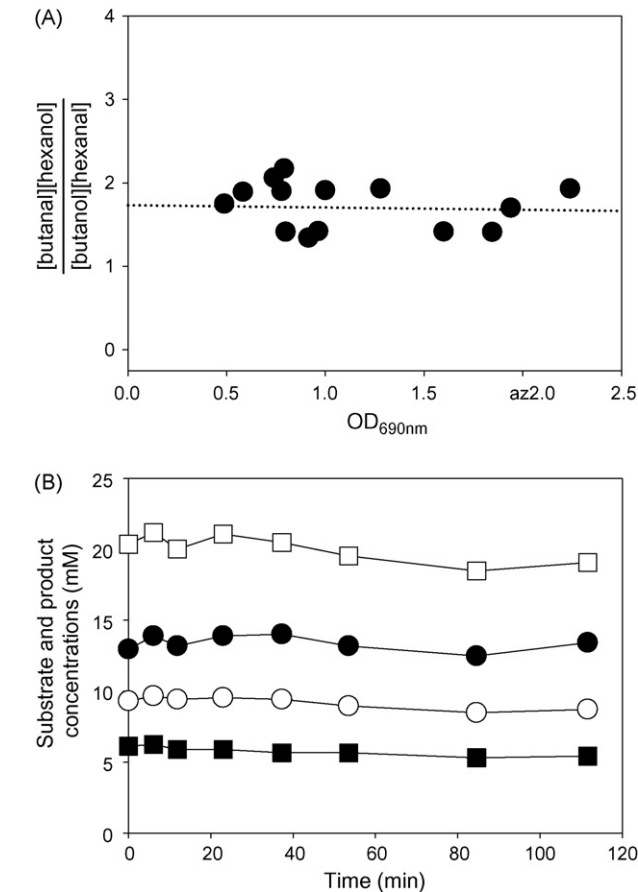


Fig. 3. Apparent equilibrium of 1-butanol:hexanal oxidation–reductions catalyzed by *R. erythropolis* cells (40 °C, pH 7.0). (A) Ratios of products:substrates, obtained after reaction stopped, were measured at various cell concentrations and equimolar initial substrate concentrations ranging from 5 to 40 mM. (B) A mixture of 1-butanol (●), hexanal (■), butanal (○), and 1-hexanal (□) miming a K_{eq} of 1.9 was directly added to the fresh cell suspension ($OD_{690\text{nm}} = 0.422$). Data are means of at least two GC determinations.

Table 3

Nicotinoprotein (np)–alcohol dehydrogenase activities exhibited by extracts of 1-butanol-grown *R. erythropolis* NCIMB 13064 cells

Substrate	Specific np-ADH activity (nmol NDMA reduced min ⁻¹ protein mg ⁻¹)
Methanol	0
Ethanol	8.4 ^a
1-Butanol	9.5 ± 0.3
Benzyl alcohol	6.7 ± 0.6

Data are means (± mean deviation) of four measurements obtained from two duplicate cell extracts.

^a Mean of three measurements obtained from one cell extract.

Accordingly, high oxidation–reduction rate is observed in aqueous phase when the reverse couple 1-hexanol (20 mM)–butanal (20 mM) is used (Table 2).

Substrate specificity of cells extracts is logically comparable to that of whole cells. Cell extracts are able to reduce the artificial electron acceptor NDMA when 1-butanol, benzyl alcohol, and ethanol, are used as electron donors (Table 3). Methanol does not reduce NDMA. This also argues for the involvement of a np-ADH-like enzyme and excludes the possible involvement of a NDMA-dependent methanol dehydrogenase (MNO).

We examined the effect of different inhibitors on the np-ADH activity of cell extracts. KCN and HgCl₂ completely inhibit the np-ADH activity with 1-butanol as substrate (Table 4). Similar results have already been reported for the np-ADH of *Amycolatopsis methanolica* [27,28]. Nevertheless, we found a weak (rather than

Table 4

Inhibition of the np-ADH activity of cell extracts of *R. erythropolis* NCIMB 13064

Inhibitor	Concentration (mM)	Remaining activity (%)
KCN	2	0
HgCl ₂	1	0
CuCl ₂	1	88
1,10-Phenanthroline	1	0
5,5'-Dithio-bis(2-nitrobenzoate)	1	71
Pyrazole	1	99

a strong) inhibition of the np-ADH by CuCl₂. The np-ADH activity of cell extracts is not inhibited by pyrazole and 5,5'-dithio-bis(2-nitrobenzoate) (Table 4) whereas those compounds (together with CuCl₂) are strong inhibitors of the carbonyl reductase of *R. erythropolis* DSM 743 [29].

3.4. Influence of pH and temperature

Butanol–hexanal oxidation–reduction is highly dependent on the pH of the potassium phosphate (100 mM) buffer (Fig. 4A). Reaction rate is maximum at pH 7.0. While such result could be expected for a purified enzyme, it was quite surprising for whole cells.

In the absence of hexanal, cell viability is maintained at high levels in the pH range 5.0–9.0 (Fig. 4B). In this range, viable cells are probably able to maintain the intracellular pH near neutrality. In the presence of hexanal, cells rapidly lose their viability (Fig. 2) and they are thus unable to maintain the intracellular pH homeostasis. As a result, reaction rate is strongly influenced by the external pH and

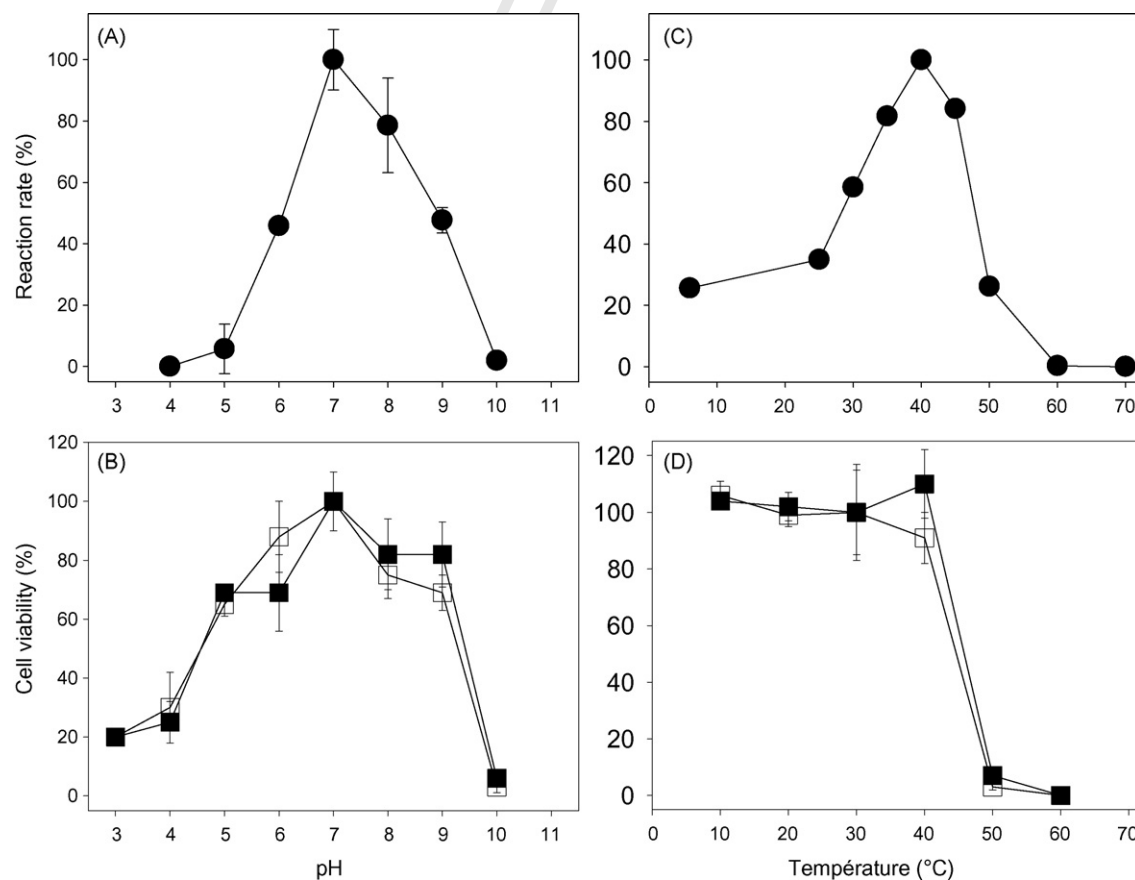


Fig. 4. Effect of pH and temperature on oxidation–reduction rate and cell viability. (A and C) Initial rates of 1-butanol (20 mM):hexanal (20 mM) oxidation–reductions were measured using fresh cell suspensions at final OD_{690 nm} surrounding 0.3. Maximal rate (100%) corresponded to 765 IU/g cell dry mass⁻¹. pH was not significantly modified during reactions. Data are means (±S.D.) obtained from two independent experiments. (B and D) Cells (final OD_{690 nm} = 0.2–0.5) were incubated 10 min in batch reactors with 20 mM butanol. Data (% mean ±S.D.) were obtained from four colony counts using 10⁻⁴ (■) or 10⁻⁵ (□) dilutions.

is maximum likely near the optimum pH value (7.0) of the np-ADH enzyme.

Reaction rate is maximum at 40 °C and become null at 60 °C (Fig. 4C). Control experiments performed in the absence of hexanal (Fig. 4D) revealed that viability is unaffected between 4 and 40 °C and becomes rapidly null at 50–60 °C. The sole thermal treatment can therefore be sufficient for the loss of the cellular enzymatic activity.

3.5. 1-Butanol–hexanal oxidation–reduction catalyzed by lyophilized *R. erythropolis* cells in a solid/gas reactor (at 40 °C)

Lyophilization of *R. erythropolis* cells is a prerequisite for reaction in solid/gas conditions (see Section 2). We observed that freezing (8 days) at –24 °C and lyophilization do not affect significantly cell viability and, more importantly, the biocatalytic properties in aqueous phase (data not shown). These lyophilized cells (Fig. 5) were used for solid/gas biocatalysis. Using a water thermodynamic of 0.7 activity, 1-butanol vapors were converted by the cells only when hexanal vapors were co-added. Similar to aqueous phase, butyric and hexanoic acids were not produced (data not shown) suggesting that reaction was an oxido-reductive conversion strictly uncoupled from potential interfering reactions. For practical reasons, 1-butanol and hexanal thermodynamic activities were systematically fixed at 0.12 (1.4 $\mu\text{mol min}^{-1}$) and 0.06 (1.9 $\mu\text{mol min}^{-1}$), respectively.

3.6. Influence of water activity, pH and temperature in solid/gas phase

At 40 °C, 1-butanol–hexanal oxidation–reduction rates are strongly improved when lyophilized cells are sufficiently hydrated

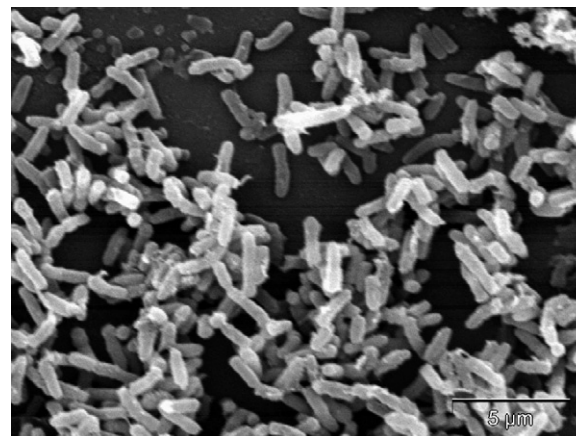


Fig. 5. Scanning electron microscopy of lyophilized *R. erythropolis* NCIMB 13064 cells.

at water activities up to 0.4 (Fig. 6A). An optimum value of 0.7 was obtained. The *R. erythropolis* cells used were also washed and suspended at different pH prior lyophilization. Fig. 6B showed that acidic pretreatments, even performed in aqueous phase, strongly affect reaction rates in gas phase. Activity decreases by 40% at pH 6.0 and become null at pH 4.0. In contrast, activity is unaffected between 7.0 and 9.0. Surprisingly, the optimum pH range for oxidation–reduction reaction in solid/gas phase (Fig. 6B) is much broader than that observed in aqueous phase (Fig. 4A). This discrepancy might be explained by the rapid detrimental effect of 20 mM hexanal on cell viability (and thus on pH homeostasis) in aqueous phase. Since no hexanal was used when cells were prepared for lyophilization, the cells remained up to 60% viable in a pH range

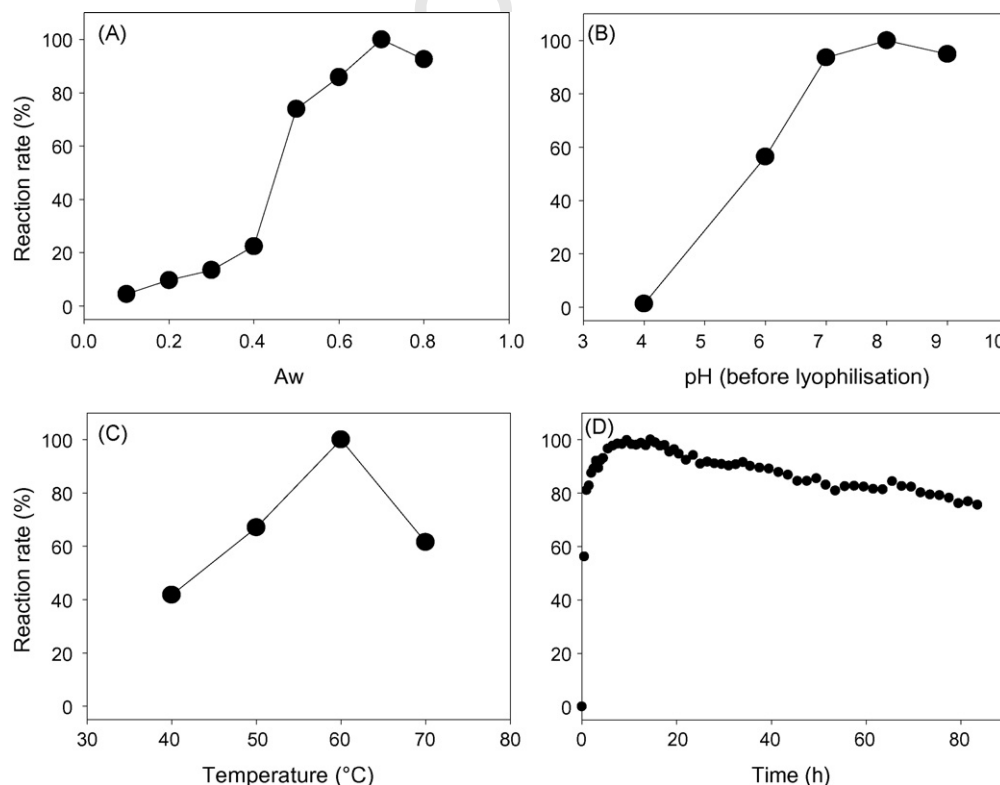


Fig. 6. Influence of water activity (a_w), pH, temperature, and time, on 1-butanol:hexanal oxidation–reduction catalyzed by lyophilized *R. erythropolis* cells in continuous gas phase. Reactions were carried out with 100 mg of lyophilizate as described in Section 2. Data (means of five GC determinations) were obtained from single typical experiments. (A) Measured at 40 °C, pH 9.0. (B) Measured at 40 °C, $a_w = 0.7$. (C) Measured at pH 9.0, $a_w = 0.7$. (D) Measured at pH 9.0, $a_w = 0.7$, 60 °C. The rates of 100% corresponded to 13 IU/g cell dry mass $^{-1}$ and 32 IU/g cell dry mass $^{-1}$ for (A and B) and (C and D), respectively.

from 5.0 to 9.0 (Fig. 4B) and could therefore maintain their intracellular pH probably near neutrality until freezing and lyophilization.

The optimal temperature (60 °C) for 1-butanol–hexanal oxidation–reduction reaction in solid/gas phase strongly differs (Fig. 6C) from that observed (40 °C) in aqueous phase (Fig. 4C). Similar trends have already been observed with isolated enzymes [12].

3.7. Stability and relative activity of the biocatalyst in solid/gas phase (at 60 °C)

Cell viability is rapidly and entirely lost in the solid/gas reactor at 40–60 °C (data not shown) confirming that reaction proceeded independently from viability. Stability and activity of the biocatalyst was finally evaluated under optimal conditions (pH 9.0 prior lyophilization, $a_w = 0.7$, $T = 60$ °C). 80% of the initial activity was maintained after 80 h of conversion in the continuous solid/gas reactor (Fig. 6D), whereas a 90% decrease was observed after 2 h of reaction in aqueous phase. A first reason for this enormous gain of stability is the limited water thermodynamic activity (0.7). Indeed it has already been shown that thermostability of enzyme is increased by a reduced water activity and also a limited hydration [30]. A second is that cells were exposed to lower toxic levels of hexanal, even if a quite high flow of hexanal ($1.9 \mu\text{mol min}^{-1}$) was used compared to that of 1-butanol ($1.4 \mu\text{mol min}^{-1}$). Using the same lyophilizate, we found that specific reaction rate is lower in gas phase ($32 \text{ IU g cell dry mass}^{-1}$) compared to aqueous phase (730 IU g^{-1} at 40 °C). In additional experiments (not shown), we observed that the 1-butanol thermodynamic activity used (0.12) is near the optimal value for reaction rate. In contrast, a two-fold increase of the hexanal thermodynamic activity (from 0.06 to 0.12) leads to a two-fold increase in reaction rate (65 IU g^{-1}). In our conditions, the low hexanal partial pressure used thus limits biocatalytic activity but probably improves stability of the biocatalyst.

4. Discussion

Whole cells have already been used for alcohols, aldehydes and ketones syntheses. The cheap recycling of cofactor can be achieved either by addition of a co-substrate or by cell metabolism. Redox biocatalytic properties of resting and/or lyophilized *R. erythropolis* cells have not been thoroughly studied yet [1] contrary to *R. ruber* DSM 44541 cells [31–33]. In this work, we used fresh resting or lyophilized NCIMB 13064 cells grown on 1-butanol to favor np-ADH induction [10]. We showed that these cells are able to efficiently perform the coupled oxidation–reduction of 1-butanol–hexanal and other couples of interest.

Substrate specificity observed for whole cells or extracts excludes the involvement of a MNO. The very low conversion observed with 1-phenyl ethanol and the lack of conversion of acetophenone shows that the enzyme involved in biocatalysis is different from the S-ADH isolated from *R. erythropolis* DSM 43297 [34]. Oxidation rate observed with benzyl alcohol is incompatible with the involvement of a long chain secondary ADH similar to that of *R. erythropolis* ATCC 4277 [35]. The ability to oxidize ethanol and 1-butanol led us to conclude that the enzyme is different from the carbonyl reductase isolated from *R. erythropolis* DSM 743 [29]. Moreover, strong inhibitors of the carbonyl reductase did not, or only slightly, reduce the 1-butanol dehydrogenase activity of *R. erythropolis* cell extracts confirming that the enzyme involved in biocatalysis is not similar to the carbonyl reductase of *R. erythropolis* DSM 743. Neither optimal pH nor substrate specificity of our catalyst correspond to that of the formaldehyde dehydrogenase produced by *R. erythropolis* DSM 1069 [36,37]. Conversely, opti-

mal pH and substrate specificity of whole *R. erythropolis* NCIMB 13064 cells or cell extracts are in agreement with those observed by Schenkels and Duine [10] for the np-ADH of *R. erythropolis* DSM 1069. Interestingly, reduction rates of NDMA with 1-butanol, benzyl alcohol and methanol are comparable to that measured by Schenkels and Duine [10] on cells extracts of *R. erythropolis* DSM 1069. Moreover, a significant sequence identity (51% of amino acids) has been shown between the np-ADH of *R. erythropolis* DSM1069 and a putative ADH from *R. erythropolis* NCIMB 13064 [10]. Taken together, these data support the hypothesis that the main enzyme produced by *R. erythropolis* NCIMB 13064 grown on 1-butanol, and that the enzyme involved in the biocatalysis, is a np-ADH similar to that already characterized by Schenkels and Duine [10].

Biocatalysis was first studied in aqueous phase. When provided as a sole substrate, 1-butanol is not converted to butanal. When hexanal is co-added the recycling of the required cofactor (likely bound NADH) is achieved in a coupled–substrate reaction probably involving only the intracellular np-ADH-like enzyme. This leads to an apparent thermodynamic equilibrium between all four components ($[\text{butanal}][\text{hexanal}]/[\text{butanol}][\text{hexanal}] = 1.9 \pm 0.25$ at 40 °C) determining the maximum achievable conversion (surrounding 55% at 20 mM initial 1-butanol and hexanal concentrations). Theoretical Gibbs free energy of the coupled reaction is $\Delta G^\circ = -3.73 \text{ kJ/mol}$ and the equilibrium constant $K_{\text{eq}} = 4.5$ at 25 °C. Using the van't Hoff equation, we calculated a value of 4.3 for K_{eq} at 40 °C. This value, calculated for an ideal system, slightly differs from that observed during biocatalysis (1.9). Indeed, activity coefficients in our complex systems could differ from 1 and explain this discrepancy.

Cell viability is clearly not required for the coupled redox reaction which is uncoupled from cell metabolism. Thus, the use of lyophilized cells (viable or not) may be of great interest for future applications. However, several pretreatments affecting viability may also affect, with time, pH homeostasis and enzymatic stability, and as a result, np-ADH activity and biocatalytic performances.

Importantly, initial reaction rates reached $765 \text{ IU g cell dry mass}^{-1}$ in aqueous phase. This value largely exceeds those reported in distinct redox biocatalytic systems. For instance, solvent-grown DCL14 cells were found to oxidize carveol into carveone in a *n*-dodecane:aqueous system at about $50 \text{ IU g cell dry mass}^{-1}$ (considering that total proteins account for 35% of the biomass, [6]). Elsewhere, no redox bioconversion rates higher than $25 \text{ IU g cell dry mass}^{-1}$ were reported for fresh or lyophilized cells of *Rhodococcus* sp. [38] or *R. ruber* DSM 44541 [31,33,39]. Hence, 1-butanol-grown *R. erythropolis* NCIMB 13064 cells appeared as efficient redox biocatalysts. Moreover, these cells are able to convert substrates of interest such as benzyl-alcohol, (*S*)-2-pentanol, 2-propanol, and geraniol (Table 2).

We clearly showed that 1-butanol-grown *R. erythropolis* NCIMB 13064 cells are also a promising redox biocatalysts in gas phase. Qualitatively, such cells might be of industrial interest for redox conversions due to the properties of the np-ADH-like enzyme (e.g. substrate specificity for the aldehydes reflecting those of the corresponding alcohols). Quantitatively, these cells also exhibit high redox performances in gas phase.

First, reaction is optimal at $a_w = 0.7$. Similar results were obtained in gas phase when lyophilized *Saccharomyces cerevisiae* cells were used for coupled oxidation–reduction of pentanol:hexanal [40], and when *R. erythropolis* cells were used for 1-chlorobutane hydrolysis [16]. Importantly, maximum specific reaction rate is quite high ($65 \text{ IU g cell dry mass}^{-1}$ at 60 °C) even lower than those obtained in aqueous phase ($765 \text{ IU g cell dry mass}^{-1}$ at 40 °C). The calculated space-time yield is $48 \text{ g 1-hexanol (or butanal)}^{-1} \text{ day}^{-1}$ per 1 g of lyophilizate. This value largely exceeds those we found previously with whole ADH-enriched *S.*

cerevisiae cells (65 °C, $a_w = 0.57$, $a_{\text{ethanol}} = 0.2$, $a_{\text{butanol}} = 0.1$, Grizon et al. [42]), and it resembles that reported for ADH-enriched *Lactobacillus brevis* extracts co-immobilised with NADP⁺ and stabilised with sucrose (50 °C, $a_w = 0.5$, $a_{\text{acetophenone}} = 0.2$, $a_{\text{isopropanol}} = 0.06$, Ferloni et al. [13]). In contrast, this space-time yield (when expressed per g of enzymes using protein-content data [6,41]) appears significantly lower than those reported for isolated ADHs of *Thermoanaerobacter* sp. and *L. brevis* co-immobilised with NADP⁺ and sucrose (60 °C, $a_w = 0.55$, $a_{\text{acetophenone}} = 0.15$, $a_{2\text{-propanol}} = 0.12$, [15]). These discrepancies might be partially explained by the positive effects of NADP⁺ and sucrose on these purified ADHs [14].

Contrary to aqueous phase, and without adding “stabilizer” such as polyols, stability of the redox reaction is maintained at high levels for several days using the solid/gas continuous reactor. The thermodynamic activity of hexanal is of the same order in the gas phase (0.06–0.012) and in the aqueous phase (0.077 calculated from UNIFAC program) and was thus found not to be responsible of the enormous gain of stability at the solid/gas interface. Lyophilized-rehydrated cells were exposed to a relatively low water activity (0.7), and as a result, thermoinactivation was reduced at 60 °C [12,30]. However, as previously suggested in *S. cerevisiae* [42], it could be hypothesized that np-ADH is stabilized within the cellular matrix.

As a conclusion, this work confirmed that *R. erythropolis* is a simple to handle tool for redox reactions. More importantly, we showed using a reactional approach that major limitations of the redox biocatalyst were overcome using 1-butanol-grown cells in the continuous gas phase. This work was a prerequisite to further exploit the np-ADH and *R. erythropolis* potentialities notably in non-conventional gas phase.

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