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Enzymatic reduction of the α, β-unsaturated carbon bond in citral

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Abstract

Bacteria, yeasts and filamentous fungi were screened for enantio-specific reduction of the α, β-unsaturated carbon bond in citral to produce citronellal. While a traditional aqueous screening system revealed only *Zymomonas mobilis* as positive, citronellal was produced in an aqueous/organic two liquid phase system by 11 of the 46 tested strains, which demonstrates the relevance of applying two-phase systems to screening strategies. *Z. mobilis* and *Citrobacter freundii* formed 1 mM citronellal in 3 h in the presence of a NADPH regenerating system and 20% (v/v) toluene. In comparison to these bacteria, the eukaryotic strains showed at least five-fold lower citral reductase activities. The bacterial strains produced preferentially the (*S*)-enantiomer of citronellal with e.e. values of >99% for *Z. mobilis* and 75% for *Citrobacter freundii*. In contrast the yeasts produced preferentially (*R*)-citronellal, i.e. *Candida rugosa* with an enantiomeric excess value of more than 98%. Many strains formed alcoholic by-products, viz. geraniol, nerol and citronellol. For *Z. mobilis* the production of these alcohols was suppressed in the presence of various organic solvents, e.g. toluene, and further decreased after EDTA addition.

Keywords: Organic/aqueous two-phase biotransformation; Enzymatic reduction of citral to citronellal; α, β-Unsaturated carbonyl; Chiral terpene; *Zymomonas mobilis*

1. Introduction

Citral is an antimicrobial terpene, which imparts the characteristic lemon scent to plants like lemon grass and the Australian lemon myrtle. It is also readily available as an industrial intermediate e.g. in the synthesis of the Vitamins A and E. Several bioconversions of citral have been reported, viz. reduction or oxidation of the aldehyde group [1], acyloin formation [2] and lyase activity [1,3]. The objective of this study was to screen for enzyme activities which reduce the α, β-unsaturated carbon bond in citral to yield the chiral product citronellal (Fig. 1). Citral is a mixture of the *trans*-isomer geranial and the *cis*-isomer neral, but substrate specificity for either isomer might not be crucial as amino acids can catalyse the isomerization [4]. A valuable use for the product (*R*)-(+)-citronellal would be the subsequent ring closure via a Prins reaction into isopulegol followed by hydration to (1*R*,2*S*,5*R*)-(−)-menthol [5]. Non-biological strategies are still challenged by their limited stereo-selectivity (citral has three double bonds) and enantio-selectivity [6].

Enzymes which reduce the carbon double bond of other α, β-unsaturated carbonyls have been reported in the literature. They predominantly belong to the 'Old Yellow Enzyme' family of flavin and NADPH dependant reductases, reviewed by Williams and Bruce [7] with regards to possible biotechnological applications. Another example is carvone reductase from *Rhodococcus erythropolis*, which used an unidentified heat stable cofactor and was not dependant on added NADH or NADPH [8].

In order to detect a bioconversion of citral into citronellal, the corresponding enzyme must be active, electrons for reduction must be available, the very hydrophobic substrate citral must reach the enzyme in sufficient concentrations and the citronellal formation must be faster than any subsequent conversion. Accordingly, a screening strategy was developed to test various microorganisms for citral conversion.

2. Experimental

2.1. Microbial cultures

The 20 yeast strains, 9 strains of filamentous fungi and 17 bacterial strains were obtained from the University of New

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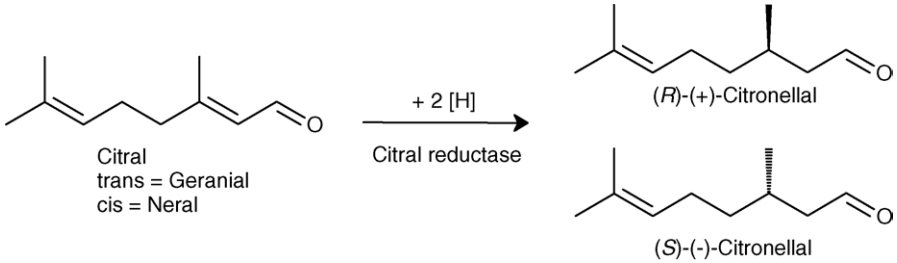

Fig. 1. Biotransformation of citral (geranial and neral) into citronellal.

South Wales culture collection (World Culture Collection number 248) and from BASF. Yeast strains, filamentous fungi, and the bacteria *Zymomonas mobilis* and *Zymobacter palmae* were grown in YPG medium (3 g/l yeast extract, 5 g/l peptone, 10 g/l D-glucose, pH 6.9). Lactic acid bacteria were grown in MRS (de Man, Rogosa, Sharpe) broth from Oxoid (pH 6.2). Nutrient broth from Oxoid (pH 7.4) was used for all other bacteria. For *Planococcus citreus* and *Vibrio harveyi* nutrient broth was supplemented with 3% (w/v) NaCl. The growth temperature was 30 or 37 °C, depending on which was closer to the reported optimal growth temperature. Cultures were agitated in an orbital shaker at 150 rpm except for non-agitated cultures of *Lactobacillus casei, Leuconostoc mesenteroides* and *Propionibacterium freundenreichii*. All cultures were grown to the stationary phase. For generating permeabilized cells, the biomass was washed twice in buffer (50 mM MOPS/KOH pH 7) and resuspended in half of the volume buffer (for yeast and filamentous fungi) or one quarter of the volume (bacterial cultures). The suspensions were freeze thawed three times using liquid nitrogen and a 25 °C water bath and were then stored in aliquots at −20 °C.

2.2. Culture screening in aqueous systems

Citral (5 mM) was added in form of a 0.2 M solution in isopropanol. The 2.5% (v/v) isopropanol fulfilled two functions: increasing the solubility of the hydrophobic substrate citral and potentially acting as a substrate for intracellular alcohol dehydrogenases to regenerate NAD(P)H. Each culture was subjected to three experiments.

(a) Whole cells in culture medium
Five milliliters of fresh medium were added to 5 ml of a stationary phase culture without change of temperature, agitated at 150 rpm for 1 h and then citral in isopropanol was added. After 3 h and after 24 h, 1 ml broth was extracted with 0.2 ml freshly prepared solution of 0.3% (v/v) 1-octanol (internal GC standard) in chloroform. The organic phase was recovered, diluted with isopropanol and analysed by GC.

(b) Permeabilized cells + NADH
Two milliliters of screw-cap glass vials agitated on an orbital shaker at 150 rpm and 30 °C contained 0.5 ml thawed permeabilized cells in a total volume of 1 ml, with final concentrations of 50 mM MOPS buffer at pH 7, 10 mM NADH, 5 mM citral and 2.5% (v/v) isopropanol. After 3 h the complete sample was extracted with chloroform/octanol as above.

(c) Permeabilized cells + NADPH regenerating system
The procedure was the same as in (b) except that NADH was replaced by a NADPH regenerating system with final concentrations of 1.5 mM $NADP^+$, 10 mM glucose-6-phosphate, 3.3 mM $MgCl_2$, and 0.4 U/ml glucose-6-phosphate dehydrogenase.

2.3. Culture screening in a two-phase system

Prior to the biotransformation, 0.5 ml of permeabilized cells were vortexed with 0.15 ml toluene in 2 ml screw capped glass vials and incubated for 10 min at room temperature. The other components were then added to give 1 ml of aqueous phase with the same composition as the aqueous/NADPH system, with only citral and isopropanol omitted. The reaction was started by addition of 0.05 ml of 0.4 M citral in toluene and the vials were turned vertically on a wheel at 30 °C. After 3 h the complete sample was extracted with 0.4 ml freshly prepared solution of 0.15% (v/v) 1-octanol (internal GC standard) in chloroform. After centrifugation 0.4 ml of the lower organic phase (toluene/chloroform mixture) was removed for GC analysis. Strains that formed citronellal were compared in a toluene and a methyl tertiary-butylether (MTBE) two-phase system as detailed below.

2.4. Biotransformations

The biotransformations shown in Figs. 2–4 were carried out as for the two-phase culture screening but with 0.8 ml aqueous phase and 0.2 ml organic solvent in 4 ml screw capped glass vials, magnetically stirred to maintain an emulsion of the organic solvent in the aqueous phase. Details for composition and conditions are given in the respective figure captions. Prior to the start of the reaction, 0.5 ml of washed cells at a defined biomass concentration were stirred with 0.15 ml of the respective organic solvent for 30 min on ice. The reaction was started by addition of 0.05 ml of 0.4 M citral in the same organic solvent.

2.5. Analytical methods

Concentrations of terpenes were determined by gas chromatography (GC) using a capillary column (Chrompack CP-SIL 5B from Varian, 50 m × 0.25 mm, 0.12 μm phase thickness) with nitrogen as carrier gas (0.98 ml/min) and a flame ionization detector (250 °C) with hydrogen (20 ml/min) and air

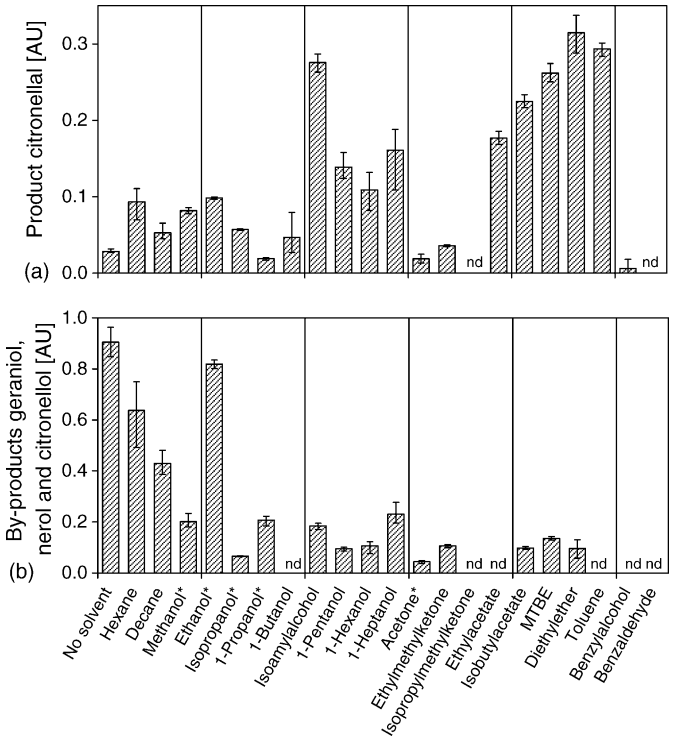

Fig. 2. Biotransformation of citral by *Zymomonas mobilis* cells in the presence of a NADPH regenerating system and various solvents: (a) product citronellal (b) sum of by-products geraniol, nerol and citronellol. Initial conditions: 20% (v/v) organic solvent, 20 mM citral, 5 mM dithiothreitol, 3.3 mM $MgCl_2$, 1.5 mM $NADP^+$, 10 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, approx. 14 g/l DCM *Z. mobilis*, 50 mM MOPS/KOH, pH 7.0, 3 h at 30 °C. *Solvents marked with asterisk dissolved in water, all other solvents formed emulsions. MTBE = methyl tertiary-butylether, nd = not detected. AU = arbitrary units: GC area of compound divided by the GC area of internal standard. Average values of three replicates are given and error bars indicate highest and lowest results. For toluene the ratio of areas corresponded to a concentration of 1.4 mM citronellal.

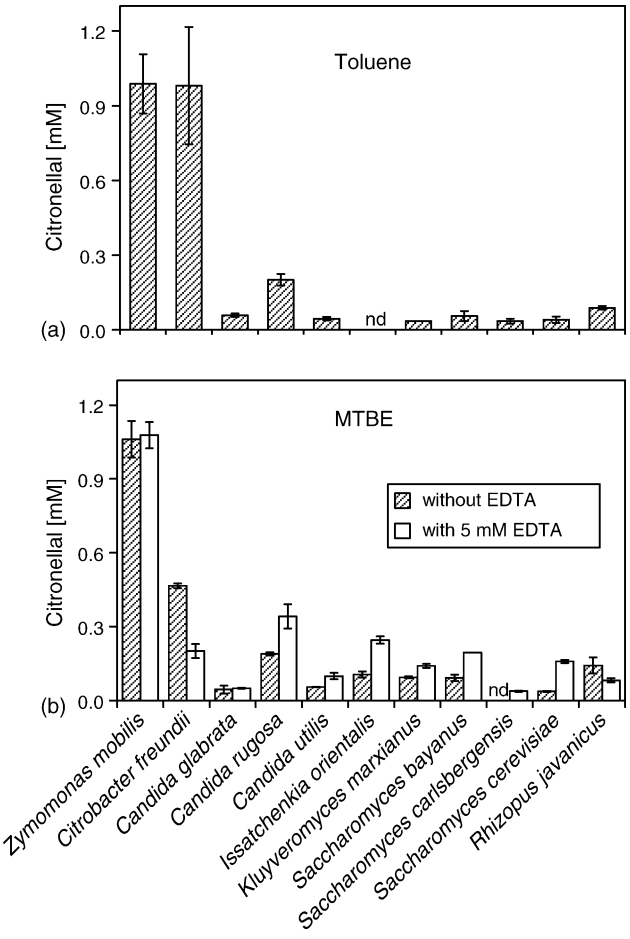

Fig. 3. Comparison of citronellal formation by bacteria, yeasts and a filamentous fungus in the (a) aqueous/toluene two-phase system and (b) aqueous/methyl tertiary-butylether (MTBE) two-phase system (3 h incubation at 30 °C, composition as in Fig. 2 at a biomass concentration of approximately 12 g DCM/l). Average values of two replicates are given and error bars indicate highest and lowest results. nd = not detected.

(300 ml/min). The injection volume was 1 μl with a split ratio of 29:1. The injection temperature was 250 °C and the column temperature was held at 100 °C for 9 min and then increased to 240 °C at 50 °C/min. Using 1-octanol as an internal standard, terpene concentrations were calculated based on standards which have been subjected to the same extraction procedure as the samples. For the separation of citronellal and isopulegol the initial column temperature was 75 °C for 12 min. Nerol and citronellol were not separated under any of the former conditions. Their separation as well as the chiral analysis was performed with a Supelco Beta Dex 225 column (30 m × 0.25 mm, 0.25 μm phase thickness). The split ratio was 1:35, the column flow 2.77 ml/min, the column temperature 95 °C for 35 min, increasing to 160 °C at 5 °C/min. Citronellal was identified by co-elution with a citronellal standard from both GC columns.

3. Results

3.1. Culture screening in aqueous systems

Twenty yeast strains, 9 strains of filamentous fungi and 17 bacterial strains were tested for citral conversion in 3 aqueous systems: using whole cells in culture medium or washed and permeabilized cells with NADH or NADPH. Only permeabilized *Z. mobilis* with NADPH formed citronellal. Both, the NADPH regenerating system or direct addition of NADPH supported the citronellal formation. Geraniol was the major product for many of the tested strains, in whole cell cultures as well as with NADH or NADPH addition to permeabilized cells. Also nerol and/or citronellol were formed by many strains. Thus the enzyme activities for the reduction of the aldehyde group were more prevalent than the required activity for the carbon double-bond reduction.

3.2. Effect of solvents on citral conversion by Z. mobilis

Z. mobilis was used to test the feasibility of an organic/aqueous two-phase system for the conversion of citral into citronellal. Fig. 2a illustrates citronellal formation in the absence and presence of various water soluble (asterisk) and insoluble solvents, sorted by functionality: two *n*-alkanes, nine aliphatic alcohols, three ketones, two esters, two ethers, and three aromatic solvents. Four solvents – isoamylalcohol, MTBE, diethylether and toluene – supported approximately

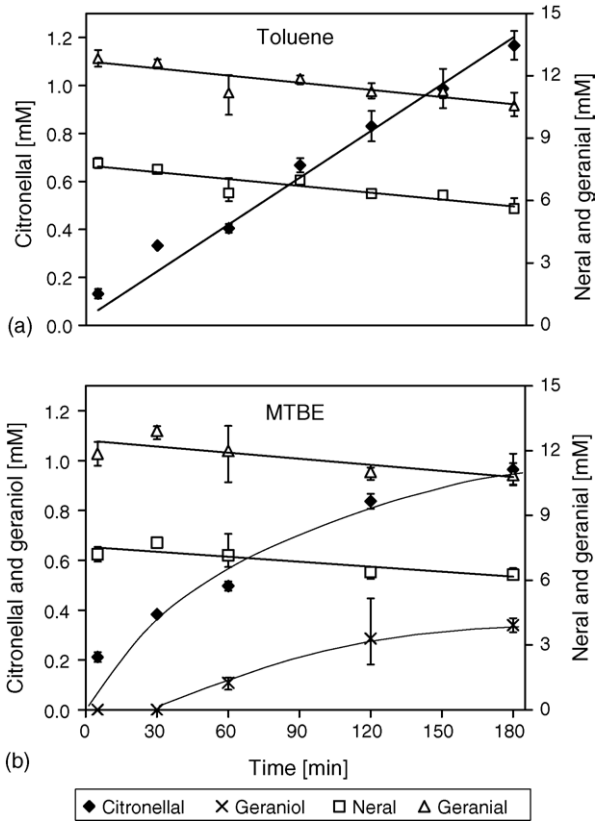

Fig. 4. Citral biotransformation by *Zymomonas mobilis* in the (a) aqueous/toluene and (b) aqueous/methyl tertiary-butylether (MTBE) two-phase systems with NADPH regenerating system (30 °C, composition as in Fig. 2 at a biomass concentration of approximately 14 g DCM/l). Average values of three replicates are given and error bars indicate highest and lowest results.

10-fold higher product levels in comparison to the control without any organic solvent. As an additional advantage, most solvents caused decreased levels of the by-products geraniol, nerol and/or citronellol (Fig. 2b). In a control experiment, the extraction of geraniol, nerol and citronellol standards, confirmed that their extraction relative to the internal standard was not affected by the presence of e.g. isopropanol, toluene or MTBE in the samples. Therefore decreased by-product levels would have been due to decreased enzymatic reduction of the aldehyde group. Toluene and MTBE were chosen for further experiments.

3.3. Culture screening in two-phase systems

In the aqueous/toluene screening system citronellal was formed from citral by several strains, which were not identified earlier in the aqueous screening system. For comparison these cultures were adjusted to a similar biomass concentration and citronellal production for the toluene and the MTBE two-phase system is illustrated in Fig. 3. In the toluene system the two bacterial strains achieved at least five-fold higher concentrations of citronellal than the nine eukaryotic strains. In the MTBE system, *C. freundii* formed less citronellal than in the presence of toluene. A positive result was obtained for *Issatchenkia orientalis*, which did not form citronellal in the toluene system. The addition of 5 mM EDTA resulted in higher citronellal concentrations for most strains but it had an opposite effect for *C. freundii* and the filamentous fungus *Rhizopus javanicus* (Fig. 3b). Citronellal formation by *Z. mobilis* was unaffected by EDTA.

Table 1
Enantiomeric excess of citronellal formed by the various strains in the aqueous/MTBE two-phase system containing 5 mM EDTA (Fig. 3b)

Strain	% e.e.	Enantiomer
Zymomonas mobilis	>99	(*S*)-
Citrobacter freundii	75	(*S*)-
Candida rugosa	>98	(*R*)-
Saccharomyces bayanus	>97	(*R*)-
Saccharomyces cerevisiae	>96	(*R*)-
Kluyveromyces marxianus	>95	(*R*)-
Candida utilis	>94	(*R*)-
Issatchenkia orientalis	64	(*R*)-
Rhizopus javanicus	>94	(*R*)-

With respect to by-product formation in the MTBE/EDTA system, the yeast strains and the bacterium *C. freundii* produced mostly geraniol, some nerol and small amounts of citronellol. In particular, the *Saccharomyces* strains formed up to 13 mM geraniol. Omitting EDTA increased the formation of alcohol by-products for the *Saccharomyces* strains by approximately 10% and for *Z. mobilis* even nine-fold. Isopulegol was not detected in any of the experiments.

The enantiomeric excess (e.e.) values for the product citronellal are listed in Table 1. The bacterial enzymes had a preference for forming the (*S*)-enantiomer with e.e. values of >99% for *Z. mobilis* and 75% for *C. freundii*. In contrast the yeast strains produced predominantly (*R*)-citronellal with *Candida rugosa* for example resulting in an e.e. value of more than 98%.

3.4. Citral biotransformation profile

Fig. 4 illustrates the profiles of citral biotransformation by *Z. mobilis* in the aqueous/toluene and in the aqueous/MTBE two-phase system. For the former the increase of citronellal concentration continued rather linearly over the 3-h period. When the concentration of the NADPH regenerating system was doubled, the same final citronellal concentration was achieved. This indicates that the reaction was not limited by the NADPH regenerating system. Concentrations of both citral isomers, geranial and neral, decreased over time. The alcohols nerol, geraniol and citronellol were not detected. For the aqueous/MTBE two-phase system, citronellal formation was observed to slow down over time, though both of the citral isomers, geranial and neral, were consumed linearly over time. Of the alcohol by-products, 0.34 mM of geraniol and less than 0.1 mM of citronellol were formed. The molar balance after 3 h indicated a loss of 16% of the initial substrate in the toluene system and 10% in the MTBE system. In comparison, controls without biomass showed no loss of citral or citronellal over 3 h. However, controls with boiled biomass lost citral and citronellal to a similar extend as observed in the biotransformations.

4. Discussion

The present study investigates a biological strategy for the stereo- and enantio-specific reduction of the α, β-unsaturated carbon bond of the terpene citral (isomers geranial and neral) to citronellal. The traditional aqueous screening of 46 prokaryotic and eukaryotic microorganisms revealed only the Gram-negative bacterium *Z. mobilis* as capable of forming citronellal. A drawback of the aqueous system was the low solubility of the substrate citral in the aqueous medium.

In the two-phase biotransformation systems with cells of *Z. mobilis*, up to 10 times higher concentrations of citronellal were formed than in a system without added organic solvent. Best results were achieved with the organic solvents toluene, methyl tertiary-butylether (MTBE), isoamylalcohol and diethylether. Organic media in biocatalytic reactions with whole cells offer several advantages [9]. One benefit is an enhanced solubility of hydrophobic substrates and/or products. A separate organic phase allows high overall concentrations of toxic or inhibitory substrates and products in the reactor while maintaining low levels in the aqueous phase. Permeabilization of the cells is another potential benefit of organic solvents. Besides 'freezing and thawing'-cycles, organic solvents like toluene have been used for the permeabilization of many organisms, e.g. yeast *Kluyveromyces* [10] and *Z. mobilis* [11], causing an increased passive flux over the cell membrane. The presence of EDTA can result in a significant increase in permeability of the outer membrane, as was demonstrated for *Escherichia coli* [12].

Using 20% (v/v) toluene or MTBE as organic solvent, 10 formerly negative tested strains out of the 46 proved positive. In comparison with the aqueous system this approach represented a far more successful strategy in screening potential microorganisms for the bioconversion of citral to citronellal. Highest product concentrations were obtained for the prokaryotic strains *Z. mobilis* and *C. freundii* with enantiomeric excess values (e.e.) of >99% and 75% for the (*S*)-enantiomer, respectively. In contrast the eukaryotic strains showed opposite enantio-specificity with e.e. values of more than 98%, 97% and 96% for the (*R*)-enantiomer with *C. rugosa*, *S. bayanus* and *S. cerevisae*, respectively.

One problem encountered in bioconversions using whole cells is the formation of by-products due to numerous catalysts within the cells. Interestingly, most organic solvents tested with *Z. mobilis* caused an overall decrease of the alcohol by-products geraniol, nerol and citronellol. Similarly EDTA decreased alcohol formation for both pro- and eukaryotic strains and in most cases increased citronellal concentrations. These opposite effects on product and by-product formation might indicate that the reduction of the aldehyde group and the reduction of the carbon double bond in citral are catalysed by different enzymes.

Enzymatic reductions of the double bond in α, β-unsaturated carbonyls have been described. In particular oxidoreductases of the "old yellow enzyme (OYE)"-family, flavin dependant enzymes common in yeasts, plants and bacteria [13], accept a large number of α, β-unsaturated aldehydes and ketones [7,14,15]. However conversion of citral by OYE has not been reported. A BLAST P (http://www.ncbi.nlm.nih.gov) search of the yeast OYE 1, 2 and 3 protein sequences was performed against the translated open reading frames of the recently published *Z. mobilis* ZM4 genome [16]. Closest similarity was observed for a putative NADH: flavin oxidoreductase (ZMO1885) with unknown function. However, the amino acid similarity to yeast OYEs was less than 50%. Whether or not citronellal formation is related to enzymes of the OYE family is not known yet and the over-expression of the corresponding genes will be required for the development of a high productivity bioprocess.

5. Conclusions

In this study an aqueous/organic two-phase system was more successful than an aqueous system for identifying microorganisms which are able to reduce citral to citronellal with high enantio-selectivity. Though the use of two liquid phase systems is well established in biotransformation processes, they have not been widely applied for screening purposes. The discovery of biocatalytical activities with contrary stereo-specificity for the reduction of citral to either (*S*)- or (*R*)-citronellal allows for selective use in future bioconversion processes.

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