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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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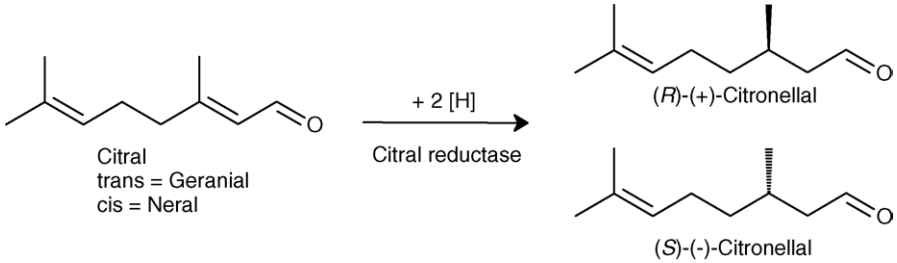

Fig. 1. Biotransformation of citral (geranial and neral) into citronellal.

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