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MICROBIAL SCREENING FOR THE ENANTIOSPECIFIC PRODUCTION OF ALKYL ESTERS OF 4-CHLORO-3-HYDROXYBUTANOIC ACID

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ABSTRACT

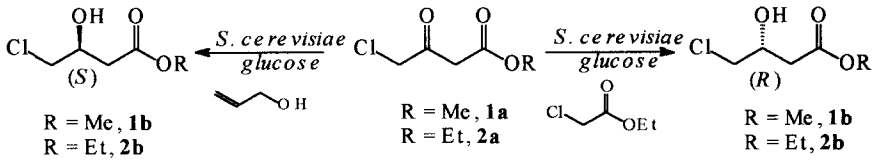
This article describes our preliminary screening results of yeasts and fungi isolated from exotic environments like the Atlantic rain forest, from the roots of Araucaria angustifolia and from a Guava tree . *Rhodotorula glutinis* CCT 2182 and *Cunninghamella echinulata* CCT 4259 revealed high enantioselective reductase activities for the production of alkyl esters of (R)- and (S)-4-chloro-3-hydroxybutanoic acid, respectively.

INTRODUCTION

Enantioselective microbial reduction of β-keto esters has been widely applied to the syntheses of optically active compounds and much effort has been directed towards the screening of different microorganisms, modification of the substrate and the reaction conditions in order to improve the scope of biocatalyzed carbonyl reductions [1].

In the course of our studies on the total syntheses of some insect pheromones [2] we had to face the need to prepare enantiopure 3-hydroxybutanoic esters to be used as our chiral starting material [3]. We have initially examined the performance of commercially available baker's yeast strains (Fleischmann, Brazil and Sigma, USA) under the protocol developed by Nakamura *et al.* [4] which employs enzyme inhibitors to control the stereochemical course of the reduction. Only Sigma baker's yeast was able to efficiently reduce methyl and ethyl 4-chloro-3-oxobutanoates to yield either the corresponding (*S*)-4-chloro-3-hydroxybutanoates (73-79 % e.e.) or (*R*)-4-chloro-3-hydroxybutanoate (60-70 % e.e.) when allyl acohol (1.0 equiv.) or ethyl chloro acetate (1.0 equiv.) were employed as inhibitors, respectively (Scheme 1).

However with Fleischmann baker's yeast we have observed low conversion of the substrates and unsatisfactory enantiomeric excesses [5].



Scheme 1. Biocatalytic reduction of 1a and 2a using *Saccharomyces cerevisiae* and enzyme inhibitors

Due to our recent success in detecting promising epoxide hydrolases in some Brazilian microbial strains [6] and to our ongoing interest on the total synthesis of (*R*)-hexadecanolide from (*S*)-4-chloro-3-hydroxybutanoates we decided to screen the almost unexploited potential of microorganisms isolated from Brazilian exotic environments for the reduction of β-keto esters. Herein we report our initial results concerning the reduction of commercially available **1a** and **2a.**

The choice of the microorganisms relied on genus and species of known redutase activity and we have thus tested different strains of *Rhodotorula glutinis* and *Cunninghamella echinulata* [7]. It should be mentioned that we were particularly attracted to these strains because they were isolated from the Atlantic rain forest, Peruíbe, S.Paulo, Brazil (*Cunninghamella echinulata* CCT 4259), from the roots of *Araucaria angustifolia* growing in Batatais SP Brazil (*C. echinulata* CCT 4424) and from a Guava tree growing at the Fundação Tropical André Tosello SP Brazil (*R. glutinis* CCT 2182). Some representative results of our screening are summarised in Table 1.

Biocatalyst	**Substrate**		**Products**	
	β-keto-ester	**conversion (%)**	**alcohol**	**e.e. (%)**
Fungi				
Cunninghamella echinulata CCT 3140	**1a**	96	**(*S*)-1b**	52
Cunninghamella echinulata CCT 4424	**1a**	52	**(*S*)-1b**	92
Cunninghamella echinulata CCT 4259	**1a**	60	**(*S*)-1b**	94
Cunninghamella echinulata CCT 3140	**2a**	70	**(*S*)-2b**	65
Cunninghamella echinulata CCT 4424	**2a**	34	**(*S*)-2b**	>98
Cunninghamella echinulata CCT 4259	**2a**	61	**(*S*)-2b**	>98
Yeast				
Rhodotorula glutinis CCT 2182	**1a**	98	**(*R*)-1b**	>98
Rhodotorula glutinis CCT 2182	**2a**	85	**(*R*)-2b**	77

Table 1. Biocatalytic reduction of 1a and 2a using *C. echinulata and R. glutinis*

The absolute configurations of **1b** and **2b** and the enantiomeric excesses were established after capillary GC analyses on a chiral stationary phase and coinjection with enriched mixtures of methyl (*S*)- 4-

chloro-3-hydroxybutanoate ($[\alpha]_D$ - 14.0 (c. 1.5, $CHCl_3$); lit. [8] $[\alpha]_D$ - 8.0 (c. 1.5, $CHCl_3$), 36% e.e.) and ethyl (*S*)-4-chloro-3-hydroxybutanoate ($[\alpha]_D$ - 14.9 (c. 5.7, $CHCl_3$); lit. [9] $[\alpha]_D$ - 11.7 (c. 5.7, $CHCl_3$), 55% e.e.).

In conclusion our results show the application of *C. echinulata* CCT 4259 to the production of methyl and ethyl (*S*)-4-chloro-3-hydroxybutanoates in high enantiomeric excesses while *Rhodotorula glutinis* CCT 2182 is certainly recommended for the production of (*R*)-**1b**.

EXPERIMENTAL SECTION

GENERAL

The reactions were monitored by silica gel TLC (foils Aluminum, 60-F254-Merck) and the visualization was obtained by spraying with *p*-anisaldehyde / sulphuric acid followed by heating at about 120 C with a hot gun. Flash column chromatographies were performed with silica gel Merck 60 (230-400 mesh) using N_2 gas for pressure.Optical rotation values were measured with a Polamat A polarimeter. The reported data refer to the Na-line value using a 1 dm cuvette. Enzymatic reactions were monitored by GC(FID) HP-5890 chromatograph, using hydrogen as carrier gas and or GC/MS -analyses using a HP-5890/5970 system using helium as carrier gas and for the latter the MS were taken at 70 eV. Scanning speed was 0.84 scan/s from m/z 40 to 550. The capillary columns used were either 1) J&W Scientific DB-5 fused silica capillary column (25m x 0.2mm x 0.33 μm) or 2) heptakis-(2,6-methyl-3-penthyl)-β-cyclodextrine (25m x 0.25 mm x 0.25 μm).

GROWTH CONDITIONS FOR MICROORGANISM CULTURES

The microorganisms *Rhodotorula glutinis* CCT 2182 and *Cunninghamella echinulata* CCT 3140, *C. echinulata* CCT 4424 and *C. echinulata* CCT 4259 were obtained from the Culture Collection, Fundação de Pesquisa e Tecnologia André Tosello (Rua Latino Coelho 1301, 13087-010 Campinas, SP, Brazil). Each microorganism was grown at 30° C in culture shaker-flasks and in an appropriate medium as follows: *R. glutinis* (48 h) - yeast extract (3g/L), malt extract (3g/L), glucose (10g/L), peptone (5g/L); *C. echinulata* (60 h) - malt extract (8g/L); The yeast cells were harvested by centrifugation (3000 rpm) and washed twice with potassium phosphate buffer pH 7.0 (0.07 M). The fungi cells were harvested by filtration and washed with potassium phosphate buffer pH 7.0 (0.07 M).

GENERAL PROCEDURE FOR THE ASYMMETRIC REDUCTION OF THE KETO ESTERS 1a AND 2a.

Reduction of keto esters was performed in 100 mL bottles on a rotary shaker (140 rpm). To the bottles containing phosphate buffer pH 7.0 (0.07 M, 20 mL) and washed cells (1.0 to 1.5 g for *C. echinulata* and 3.0 to 4.5 g for *R. glutinis*, wet weight), the keto ester was added (20 to 25 μL). The mixture was shaken at 30°C and the reaction was monitored by chiral GC. Upon reaching the appropriate

conversion degree, the cells were centrifuged, except for the fungi cells which were filtrated, the formed alcohol and remaining keto ester were extracted from the supernatants (previously saturated with NaCl) with ethyl acetate and dried.

e.e. - determination:- Enantiomeric excesses were determined by GC (HP GC-5890 equipped with FID) and the results are depicted in Table 1. Column 2) **1b**: temperature program : 90° C iso, pressure at the head of the column 0.6 bar, Rt (*R*)-**1b** = 7.9 min., (*S*)-**1b** = 8.3 min; **2b**: temperature program :80° C iso, pressure at the head of the column 0,6 bar, Rt (*R*)-**2b** = 23.8 min, (*S*)-**2b** = 24.3 min. The conversions were determined using guaiazulene as an internal standard and correction factors were used to calculate the percent composition taking into consideration the specific detector response [10].

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