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Product distribution in the microbial biogeneration of raspberry ketone from 4-hydroxybenzalacetone

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Abstract

The enzymic saturation of the double bond of 4-hydroxybenzalacetone **5**, of practical importance as an entry to the natural modification of raspberry ketone **6**, has been studied using a variety of microorganism.

Keywords: Natural raspberry ketone; Microbial reduction

1. Introduction

In the microbial transformation of multifunctional substrates such as the alpha,beta-unsaturated ketones **1** the major problems are the distribution of the products and the enantiomeric composition of the educts bearing a stereogenic center. This complication conceivably arises from the multiplicity of the enzymic capacities present in a living system and, secondly, from the circumstance that sometime a compound is substate for two enzymes acting with opposite stereochemistry. For example, using baker's yeast from **1** products **2**, **3** and **4** are formed in ratios strongly depending upon the nature of the substituents R, R′ and R″ and the amount of unreacted alpha, beta-unsaturated ketone still present in the incubation medium (Scheme 1) [1]. Carbinols **3** and **4** are usually produced from **1** by bioreduction in optically active form. However, absolute configuration and enantiomeric composition depend upon the microorganism used and the substitution pattern [2,3]. From a synthetic point of view, of the products of Scheme 1, unsaturated and saturated carbinols **3** and **4** seem of major interest, since they can be used as starting materials in the preparation of enantiomerically pure forms of biologically active substances [3].

Conversely, the bioconversion of ketone **1**, in which R′ = H, to saturated ketone **2** is not offering advantages over a chemical process, based, i.e., on catalytic hydrogenation. However, there is a research area, namely, the biogeneration of flavor materials, in which also a chemically simple transformation such as the saturation of the double bond of a ketone like **1** to provide **2**

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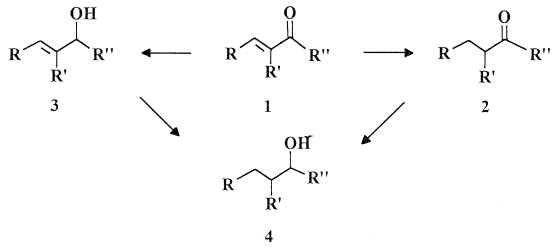


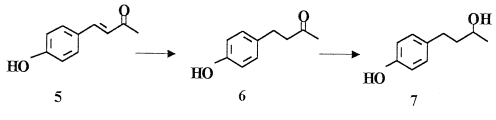
Scheme 1. Biotransformation of alpha,beta-unsaturated ketones.

is of preparative significance if performed enzymatically. Indeed, a recent legislation discriminates chemically identical food aroma components in *natural* and *natural-identical* products [4]. The consumer's preferences for the first set of materials, has stimulated studies designed to obtain from abundant natural extractive precursors through enzymic procedures substantial quantities of potent aroma substances present in nature in trace amounts and not accessible by extraction [5]. The products obtained in this way are indeed considered *natural* by any means and legislation.

In this context, we recently reported on the obtainment of the *natural* modification of raspberry ketone **6**, impact flavor of raspberry fruit [6], upon baker's yeast mediated reduction of the unsaturated precursor **5** prepared from 4-hydroxybenzaldehyde of botanical origin and acetone from sugars fermentation [7] (Scheme 2).

An alternative approach to *natural* raspberry ketone **6** is based on the microbial oxidation of **7**, containing an excess of the (*R*) enantiomer, obtained, in turn, by enzymic hydrolysis of the corresponding beta-D-glucoside. This material, named betuloside, is quite widespread in nature and can be extracted, for example, from the bark of *Betula alba* [8].

However, a drawback which is common to both the reductive and oxidative approaches to



Scheme 2. Mode of reduction of 4-hydroxybenzalacetone **5** by micro-organisms.

natural **6** is the complex composition of the final incubation mixture, leading to low isolated yields of precious **6**. In baker's yeast, under incubation conditions assuring an almost complete consumption of **5**, there is, to a large extent, reduction of the saturated ketone **6** to carbinol **7**. Conversely, the biooxidation of **7** to **6** show a kinetic dependence from the enantiomeric composition of the alcohol and seldom is complete [8]. Also, carbinol **7** obtained in baker's yeast from **6** contains an excess of the (*S*) enantiomer, a circumstance unfavourable for the back oxidation to **6**, due to the preference for the (*R*) enantiomer of **7** of microbial systems effective to this end such as *Candida boidinii* and *Beauveria bassiana*, respectively [8,9].

In the light of the above considerations, it seemed of interest to identify biotransformation conditions enabling the selective conversion of **5** into **6**. Some results have been presented [9,10]. Now we add further details of the study, which include also the observation that the enantiomeric composition of carbinol **7** formed from **6** depends upon the microorganism used.

2. Experimental section

2.1. Microbial transformations

The microorganisms grown in medium MPGA (malt 20 g l^{-1}, peptone 5 g l^{-1}, glucose 10 g l^{-1} and agar 15 g l^{-1}) were seeded in 300 ml conical flasks containing 50 ml medium MPGB and incubated on an orbital shaker (200 rpm) at 30°C. After 24 h of incubation addition of 1 g l^{-1} of 4-hydroxybenzalacetone **5** was performed and the biotransformations were carried out under the same conditions for 24–48 h.

2.2. Analytical procedures

At the end of the indicated incubation period, 50 mg of 4-(3-methoxy-4-hydroxyphenyl)-but-

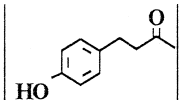
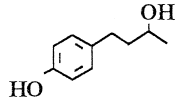
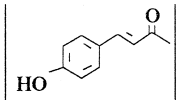
3-en-2-one, as internal standard, are added to the microbial cultures and the whole mixture is extracted twice with an equal volume of methylene chloride. The separated organic extract (filtration under vacuum through a short Celite pad is sometime necessary for the separation of the emulsion) is dried (Na_2SO_4) and submitted to GLC analysis. The identification of the compounds is made by comparison with authentic materials. The samples obtained were analysed using a DANI 8610 with a capillary column DB5 (30 m × 0.25 mm × 0.25 nm), equipped with PTV injector and FID detector. Helium was used as carrier gas at the pressure of 0.9 bar. The oven temperature programme was: 80°C for 1 min, 10°C min^{-1}, 155°C for 1 min, 165°C for 2 min, 15°C min^{-1}, 250°C. The samples were injected in splitless, the injection temperature was set at 250°C, detector temperature 250°C.

Determination of the enantiomeric composition of **7** was performed through HPLC analysis of the diacetate on Chiracel OD: The organic phase is evaporated under vacuum and the residue is treated in 1 ml of methylene chloride with 0.5 ml of acetic anhydride and 0.5 ml of triethylamine 24 h at room temperature. The reaction mixture is evaporated under vacuum and submitted to HPLC analysis. Merck-Hitachi L-6200 equipped with UV detector L-4200 and a D-2500 integrator. HPLC column used was a chiral stationary phase (Chiracel OD, 25 cm × 4 mm, Daicel, Japan). The elution conditions were the following: *n*-hexane/*i*-PrOH 9:1, flow 0.6 ml min^{-1} and the detector was set at 220 nm. The retention times were 9.9 and 11.2 min for the (*R*) and (*S*) enantiomers, respectively.

3. Results and discussion

Inspection of the distribution of products **6** and **7** in the incubation cultures of **5** (1 g l^{-1}, 24 and 48 h) (Table 1) with 14 different microorganisms indicates that only in two instances (entries 5 and 14) there is an almost complete substrate consumption. Conversely, a consistent number of the examined microorganisms (en-

Table 1
Product distribution (%) in the microbial incubation of **5** fed at 1 g l^{-1} and overall (%) recovery

Entry	microorganisms							product recovery	
		24h	48h	24h	48h	24h	48h	24h	48h
1	*Candida boidinii* CBS 2428	6.6	12.8	-	8.3	93.4	78.9	89	84
2	*Candida lipolytica* CBS 2074	22.9	24.0	3.8	17.1	73.3	58.9	92	90
3	*Hansenula anomala* CBS 110	25.5	26.5	40.9	44.5	33.6	29	94	87
4	*Kloeckera saturnis* CBS 5761	3.9	18.5	3.0	23.8	93.1	57.7	95	93
5	*Pichia etchellsii* CBS 2011	68.3	51.3	23.8	47.4	7.9	1.3	87	82
6	*Pichia membranae faciens* CBS 107	21.3		19.9		58.8		94	
7	*Pichia ohmeri* CBS 5367	16.5	30.0	-	26.9	83.5	43.1	88	87
8	*Pichia pastoris* CBS 704	26.8		32.8		40.4			91
9	*Saccharomyces fermentati* CBS 818	8.9	40.1	-	-	91.3	89.9	94	92
10	*Aureobasidium sp.*	3.0	2.3	-	-	97.0	97.7	92	89
11	*Beauveria bassiana* CBS 209.27	30.1	26.7	8.4	8.7	61.5	64.6	91	90
12	*Cladosporium suaveolens* CBS157.58		2.8		-		97.2		95
13	*Geotrichum candidum* CBS 233.76	15.4	32.8	-	2.2	84.6	65.0	92	87
14	*Mucor subtilissimus* CBS 735.70	66.4	50.8	33.6	49.2	-	-	90	89

tries 1, 7, 9, 10, 11 and 13) transform **5** to a modest extent, to give **6** as prevalent product. However, when the transformation of unsaturated ketone **5** took place consistently (entries 5 and 14), the amount of the unwanted carbinol **7**, derived from raspberry ketone **6**, increases with the incubation time. *Hansenula anomala* (entry 3) and *Pichia pastoris* (entry 8), at variance with baker's yeast, [7] produce consistent amounts of carbinol **7** while unsaturated ketone **5** is still present in the incubation mixture to a large extent. This observation suggests that in these instances the alpha,beta-unsaturated ketone is not acting as inhibitor of the enzyme(s) presiding over the carbonyl reduction [11].

Incubation of **5** in *Mucor subtilissimus* for 14 h provides starting material **5**, the saturated ketone **6** and the saturated carbinol **7** in 2.6:87.9:9.5 ratio. After 18 h only **6** and **7** are present in the mixture in 86.2:13.8 ratio, respectively. Moreover, growing cultures of *M. subtilissimus*, previously adapted to **5** by addition of 0.1% of product to the solid medium used in the early stages of the growth, rapidly reduces to **7** the saturated ketone **6** produced, in turn, from **5**. Indeed, already at 16 h incubation ketone **6** and carbinol **7**, in 25:75 ratio, are the only product present in the mixture when **5** was fed at 1 g l^{-1}. Conversely, the three products, i.e., **5, 6** and **7**, in 44.2:149.2:6.6 ratio, have been identified when the identical culture was fed with 2 g l^{-1} of **5**. The above product distribution is not significantly changed extending the incubation period up to 36 h, thus indicating that alpha, beta-unsaturated **5** still inhibits the reduction of **6** to **7** at higher concentrations.

Finally, the enantiomeric composition of carbinol **7** produced from **5** in different microorganism at 24 h incubation (1 g l^{-1}) was determined. Surprisingly enough, the stereochemical course of the reduction changes significantly with the microorganism used. The alcohol obtained in *M. subtilissimus* is nearly racemic, whereas the samples formed in *P. etchellsii* and *H. anomala* possess the (*R*) and (*S*) configuration, respectively, and 0.76 and 0.97 *ee*. Interestingly enough, carbinol **7** is present in different plants in the free form or as glucoside in both the (*R*) and (*S*) enantiomeric forms [12].

The different mode of the microbial reduction of **6** to **7** thus observed might have some practical consequence, for example, in the choice of the microorganism. Indeed, in the manufacture of *natural* raspberry ketone **6** by bioreduction of **5** it would be of interest oxidize back to **6**, in a separate microbial process, the alcohol **7** formed at the expenses of **6**, due to the difficulties encountered in the separation. It is reasonable to possibly select a microorganism producing from **6** carbinol **7** in the enantiomeric form, which is the preferred one by the oxidizing system to be used in the oxidative step [8,9].

Seen together, these results thus further support the utility of microorganisms in the biogeneration of *natural* flavor materials. [13] However, only a moderate number of microorganisms seems useful for the biogeneration from 4-hydroxybenzalacetone **5** of raspberry ketone **6**, since when the saturation of the double bond takes place to a significant extent, the amount of carbinol **7** produced is also high, thus decreasing the yield and raising problems of separation. The most effective from a practical point of view seems to be *M. subtilissimus*. This microorganism, at short incubation times with a concentration of the substrate of 1 g l^{-1}, is rather effective in producing **6**, while keeping acceptably low the amount of accompanying **7**. The enantiomeric composition of **7** is strongly dependent upon the microorganism used, thus lending further support to the unpredictability of the steric outcome of microbial carbonyl reductions.

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