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Letter

Are endophytic microorganisms involved in the stereoselective reduction of ketones by *Daucus carota* root?

Paula Rodríguez [a,1], María Barton [a,1], Virginia Aldabalde [b,2], Silvia Onetto [b,2], Paola Panizza [a,1], Pilar Menéndez [b,2], David Gonzalez [b,*], Sonia Rodríguez [a,**]

[a] *Laboratorio de Biocatálisis y Biotransformaciones, Departamento de Biociencias, Facultad de Química, Universidad de la República (UdelaR), 11800 Montevideo, Uruguay*
[b] *Laboratorio de Biocatálisis y Biotransformaciones, Departamento de Química Orgánica, Facultad de Química, Universidad de la República (UdelaR), 11800 Montevideo, Uruguay*

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Abstract

Four strains of endophytic microorganisms isolated from carrot root were able to carry out the reduction of the carbonyl group with diverse degree of enantio-, and diasteroselectivity. Furthermore, biotransformation in the presence of bacterial inhibitor affects the stereochemical outcome of the reaction, and the concomitant addition of a yeast inhibitor results in a large decrease in the conversion percentage. These results indicate that endophytic microorganisms might be involved in the enantioselective reduction of ketones and ketoesters with fresh carrot root pieces.
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Despite the vast progress of organic chemistry during the last decades, there is a continuous and increasing demand for new stereoselective transformations. Biocatalytic reactions are efficient, performed under mild conditions, usually in water as a solvent, purification procedures are often simple, and the regio-, and stereoselectivity achieved is very high. These virtues make biocatalysis into a valid green alternative to traditional organic transformations [1–4].

Among the most popular biocatalytic transformations is the stereoselective reduction of ketones and ketoesters to the corresponding secondary alcohol by Baker's yeast (BY) [5]. The reaction was first described in 1918 and has been reviewed several times [6–10].

This microorganism is the preferred and most thoroughly studied source of reductases. BY reductions are amenable to lab work due to operational simplicity, particularly because it does not require aseptic conditions, and because it is widely available at low cost. In addition, reduction of a wide range of aliphatic and aromatic ketones is carried out in good yield and high enantiomeric excess. The stereochemical outcome of the reaction is predictable since it usually follows the empiric Prelog's rule [11].

Many other microorganisms are good reductase sources [12,13], and recently several papers have described the reduction of the carbonyl group by plant cell cultures [14–19], and plant fragments [20–29]. Particularly, common carrot plant (*Daucus carota*) has been mentioned repeatedly since 2000 as an effective agent to perform the stereoselective reduction of many ketones. Enantioselectivity and yield are comparable to those obtained with BY and the simplicity of the procedure and ease of isolation is astounding for the unaware organic chemist. The reported reaction conditions are diverse as well as the actual scope of the reaction. Nevertheless, most researchers perform the reaction at 25 °C, in water, often without the addition of cosolvents, and in a maximum reaction time of 48 h. Extraction and purification of the alcohols is simple with carotene and remaining starting material (if any) being the only detectable impurities.

* Corresponding author. Tel.: +598 2929 0106; fax: +598 2924 1906.
** Corresponding author. Tel.: +598 2924 4209; fax: +598 2924 1906.
E-mail addresses: davidg@fq.edu.uy (D. Gonzalez), soniar@fq.edu.uy (S. Rodríguez).
[1] Tel.: +598 2924 4209; fax: +598 2924 1906.
[2] Tel.: +598 2929 0106; fax: +598 2924 1906.

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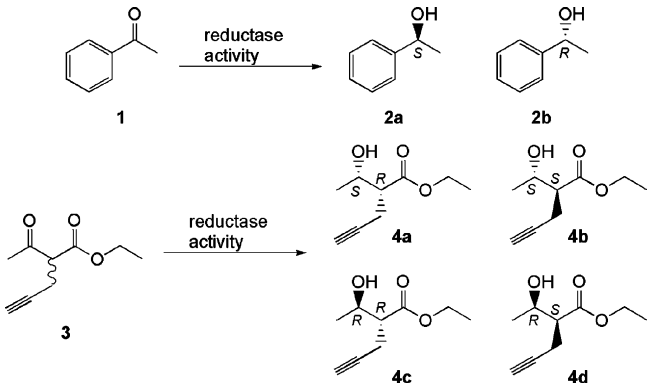

Scheme 1. Reduction of ketones and ketoesters by reductases.

After experimenting several times with this reaction, we considered the question about what the actual biological entity that performs the reaction was. That is to say, what the actual reductase or reductases involved in the biocatalysis are. Recently, Surette et al. [30] have reported the isolation of up to 360 endophytic microorganism strains from two varieties of *D. carota*: Carochoice and Red Core Chantenay. The microorganisms were classified into 28 genus being *Pseudomonas*, *Staphylococcus*, and *Agrobacterium* the predominant ones.

Inspired by this report, we decided to isolate endophytic microorganisms from *D. carota* and test their ability as reducing agents to tests the hypothesis that they might be involved in the biotransformation. In that sense, we also studied the effect of bacteria and yeast inhibitors in the biotransformation results observed with carrot fragments.

With the aim of isolating endophytic microorganisms from carrot root, we tried to reproduce the environment where endophytic microorganisms in *D. carota* may be able to proliferate. A medium consisting of a sterile suspension of triturated carrot root was inoculated with small, clean carrot root fragments,[3] and incubated at 25 °C for 48 h. In this fashion six bacterial strains and a yeast strain were isolated. Then, these same seven microorganisms were screened for reductase activity by testing their ability to reduce acetophenone (**1**), a common oxidoreductase substrate, to the corresponding secondary alcohol **2** (Scheme 1). Three out of six bacterial strains and the yeast strain showed catalytic activity and therefore were selected for further experiments. The former three bacterial strains were identified as *Pseudomonas* sp., *Staphylococcus* sp., and *Micrococcus* sp., whereas the yeast strain was identified as belonging to the genus *Pichia*. Further experiments will be performed in order to complete the identification of these strains.

In an attempt to compare the bioreducing power and stereoselectivity of the endophytic microorganisms versus the carrot root, we performed the reaction with α-propargyl ethyl acetoacetate (2-acetyl-pent-4-ynoic acid ethyl ester) (**3**), which was chemically prepared by a described method [31]. We chose an α-substituted-β-ketoester because the corresponding optically pure alcohols are important chiral synthons [32–34] and because the presence of an unresolved chiral center in the α position provided an extra element to compare the stereochemical outcome of the reaction. Reduction of α-substituted-β-ketoesters can potentially render up to four different stereoisomers (**4a–d**). However, reduction of the chosen substrate with commercial Baker's yeast or carrots delivers a mixture consisting essentially of alcohols **4a** and **4b** with defined stereochemistry on the β position, as directed by the Prelog's rule. The isomers differ in the stereochemistry of the α position with an *R* or *S* configuration resulting from the action of different reductases present in Baker's yeast [35–37]. This is presumably true for the carrot enzyme system too, although only preliminary work has been performed on the reduction of α-substituted-β-ketoesters with carrot root [38]. The reaction was biocatalyzed by each of the four isolated strains independently in sterile carrot broth, at 25 °C and 100 rpm, for 48 h. In addition, two positive control reactions utilizing Baker's yeast and carrot root were carried out under the same conditions as the microorganisms in study. Reduction of the substrate with sodium borohydride provided the racemic mixture for GC analysis and, after careful optimization of the conditions, complete separation of the four stereoisomers was achieved.[4] In a previous work [39], α-propargyl ethyl acetoacetate was reduced with Baker's yeast under standard conditions, and with recombinant *E. coli* strains overexpressing enzymes from baker's yeast [36]. These biotransformations' products were used to assign the *anti* and *syn* diasteroisomers, as well as the (2*R*, 3*S*) and (2*S*, 3*S*) optical isomers. The 3*R* enantiomers were deduced from the previous assignments. The chiral GC analysis of the resulting mixtures provided quantitative information of the relative composition of each mixture of stereoisomers, as well as conversion percentage for the parent ketoesters under these particular reaction conditions. The results for each of the strains and the controls are presented in Table 1.

Table 1
Bioreduction of α-propargyl ethyl acetoacetate with carrot's root isolated strains

Bioreducing agent	Conv. (%)	*syn* (%)		*anti* (%)	
		2*R*, 3*S*	2*S*, 3*R*	2*R*, 3*R*	2*S*, 3*S*
Staphylococcus sp.	11	32.9	2.2	1.1	63.9
Micrococcus sp.	8	28.5	–	–	71.5
Pseudomonas sp.	8	25.3	1.2	0.8	72.6
Pichia sp.	>99	93.7	0.4	0.3	5.6
Baker's yeast (control 1)	96	53.2	1.3	0.3	45.1
D. carota (control 2)	85	25.8	–	–	74.5

From the data in Table 1 it is noteworthy that the results obtained for the bacterial strains are very similar. In all cases, there is a preference for the *anti* isomers but only in approx-

[3] The carrot root was thoroughly washed, disinfected (10% NaClO, 5′), and rinsed with plenty of sterile distilled water. The external layer was peeled off, followed by a second disinfection (10% NaClO, 5′). The carrot root was then rinsed with plenty of sterile distilled water, treated with 70 % ethanol (3′), and rinsed again with sterile distilled water. A sterile knife was used for chopping the carrot root.

[4] Analysis was performed in a HP 5890SII GC with a Chrompack CP chirasil-Dex CB (0.25 mm × 25 M) column. Conditions: 70 °C (2 min) to 130 °C (5 min) at 1 °C/min, then 10 °C/min to 180 °C (10 min) using He as the carrier gas.

Table 2
Chloramphenicol and cycloheximide effect on the bioreduction of α-propargyl ethyl acetoacetate by carrot's root

Bioreducing agent	Inhibitor	Conv. (%)	*syn* (%)		*anti* (%)	
			2*R*, 3*S*	2*S*, 3*R*	2*R*, 3*R*	2*S*, 3*S*
D. carota (control)	None	85	25.8	–	–	74.5
D. carota	Chloramphenicol	84	42.6	0.9	1.7	54.9
D. carota	Cycloheximide	74	25.1	–	–	74.9
D. carota	Chloramphenicol and cycloheximide	4	40	–	–	60

imately 70/30 ratio. In the already mentioned conditions, the percentage of conversion provided by the bacterial strains was low and the enantioselectivity was dictated by the Prelog's rule. The overall stereoselectivity is similar to the one observed for control 2 (*D. carota*). The isolated yeast strain resulted in an outstanding catalyst and delivered the *syn* 2*R*, 3*S* compound in 94/6 ratio (88% de), and >98% ee, with a very high (>99%) degree of conversion. This is notable, particularly if compared with commercial Baker's yeast (control 1) that rendered the same compound as the major isomer, but with poor diasteroselectivity (55/45 ratio (10% de)). The high value of diasteroselectivity for this strain was also observed in preliminary results for the reduction of other α-substituted-β-ketoesters, and deserves further study.

The similar diasteroselectivity exhibited by the isolated bacterial strains and the carrot root, favors the hypothesis that endophytic microorganisms may be involved in biotransformations with this biocatalyst. On the other hand, the isolated yeast strain presented the opposite diasteroselectivity. Consequently, we can conclude that it is not present in the vegetable root in a significant amount to influence the stereochemical outcome of the reaction. Although we isolated seven strains, the diversity of endophytic microorganisms in carrot is much larger and it is likely that other strains also contribute to the outcome of the biotransformation.

Furthermore, if endophytic microorganisms are involved in the biotransformation with carrot root, the addition of bacterial or yeast inhibitors should influence the outcome of the biotransformation. As a consequence, we decided to perform the biotransformation using chloramphenicol as a bacterial inhibitor [40], and cycloheximide as a yeast inhibitor [41]. The results obtained in these experiments are presented in Table 2.

As observed, the addition of chloramphenicol did not affect the overall yield of the reaction, although it had an important effect on the diastereoselectivity. An evaluation of the number of microorganisms at the end of the biotransformation (Table 3), indicates that the bacterial population decreased by four orders, while the yeast population increased considerably. Furthermore, only two bacterial strains were detected indicating that the diversity of the population was also affected since only resistant strains survived. The switch in diastereoselectivity is in agreement with a larger contribution of the isolated endophytic yeast strain to the outcome of the biotransformation. Interestingly, a different endophytic yeast strain was also present in large number in this biotransformation and was isolated for future studies.

As previously stated, the difference in diastereoselectivity observed for the isolated yeast strain and the carrot root (Table 1) indicates that this strain is not present in the vegetable root in a significant amount to influence the stereochemical outcome of the reaction. This conclusion is furtherly supported by the fact that the addition of cycloheximide alone, scarcely affects the biotransformation result (Table 2). However, when bacterial population is affected by the addition of chloramphenicol, the yeast population increases affecting the result of the biotransformation.

The most interesting result emerged from the concomitant addition of chloramphenicol and cycloheximide. The biotransformation yield was drastically reduced, resulting in only 4% conversion. This result was very puzzling since none of the inhibitors alone produced a similar effect. However, a closer look at the population of microorganisms at the end of the biotransformation indicates that when only one inhibitor is present, the total population of microorganisms remains close to 10^7 although its composition is altered. The addition of chloramphenicol lowers the diversity of bacterial strains since only resistant strains survive. Moreover, yeast strains find a favorable environment outnumbering bacterial strains. This change in the diversity of microorganisms impinges on the diastereoselectivity of the reaction while the conversion percentage is scarcely affected. When chloramphenicol and cycloheximide are added together, only chloramphenicol resistant strains survive and they probably do not make a large contribution to the outcome of the biotransformation as only a very modest conversion is achieved.

Table 3
No. of microorganisms, bacteria and yeast, at the end of the biotransformations (48 h reaction)

Bioreducing agent	Inhibitor	No. of microorganisms		
		Bacteria	Yeast	Total
D. carota (control)	None	1×10^7	–	1×10^7
D. carota	Chloramphenicol	1×10^3	3×10^3	3×10^6
D. carota	Cycloheximide	1×10^7	–	1×10^7
D. carota	Chloramphenicol and cycloheximide	1×10^4	–	1×10^4

The results presented in Tables 2 and 3, strongly support the hypothesis that endophytic microorganisms are involved in the biotransformation with carrot root. The isolation of bacterial and yeast strains capable of reducing the tested substrates reinforce this hypothesis. As already mentioned, although we isolated seven strains, the diversity of endophytic microorganisms in carrot is much larger and it is likely that other strains also contribute to the outcome of the biotransformation.

In summary, four out of seven endophytic microorganisms isolated from carrot's root showed reductase activity yielding the Prelog alcohols **4a** and **4b** as the major products. Interestingly, the bacterial strains and the carrot root, exhibited similar diasteroselectivity. Furthermore, biotransformations in the presence of bacterial inhibitors affect the stereochemical outcome of the reaction, and the concomitant addition of a yeast inhibitor, results in a large decrease in the conversion percentage. Altogether, these results strongly support the hypothesis that endophytic microorganisms may play a role in biotransformations with carrot's root.

Moreover, the isolation from carrot of active endophytic strains and particularly a yeast strain with not only very strong reducing capabilities but also extremely sharp stereoselectivity, opens a door to a potential source of biocatalysts. Further experiments such as full characterization of biocatalysts isolated from "bioactive plants" and the reduction of ketones with endophytic-free carrot tissue are ongoing and will be reported in due course.

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