Journal of Molecular Catalysis B: Enzymatic 71 (2011) 16–21

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Searching for local biocatalysts: Bioreduction of aldehydes using plant roots of the Province of Córdoba (Argentina)

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ARTICLE INFO

Article history:
Received 19 October 2010
Received in revised form 3 March 2011
Accepted 11 March 2011
Available online 21 March 2011

Keywords:
Biocatalysis
Bioreduction
Conium maculatum
Aromatic aldehydes
Benzylic alcohols

ABSTRACT

A screening for the capacity of wild plants growing in the Province of Córdoba to bioreduce benzaldehyde was carried out. From this study, thirteen species showed quantitative reduction yields to benzyl alcohol, with *Conium maculatum* showing the best reduction efficiency. This plant was also tested against different substituted benzaldehydes, and quantitative yields of substituted benzylic alcohols were obtained, except for vanillin, where only 27% of vanillic alcohol was formed (main product: 2-methoxyphenol at a 73% yield). A scaling study of the reaction using *C. maculatum* and benzaldehyde was carried out, and it was observed that high substrate–catalyst relationships reduced the efficiency of the reaction due to side reactions of oxidation. The bioreduction method presented here permits substituted benzylic alcohols to be obtained using an environmentally friendly methodology, with excellent yields produced on a laboratory scale.

1. Introduction

The reduction of the carbonyl group is among the most important reactions in organic chemistry, with today's organic chemists having a wide range of appropriate reduction systems at their disposal. In general, most of these use heavy metals or their hydrides and organic solvents as the reaction medium, which are able to provide excellent yields of the desired alcohols [1]. However, comparatively few reduction methodologies have been developed taking into account the concept of green chemistry (environmentally friendly reaction systems) in order to avoid the formation of toxic waste that may pollute the environment [2].

In recent years, chemical reactions using plant parts and their cell cultures as biocatalysts have received great attention due to the large biotechnological potential of enzymatic reactions. Some important characteristics of these biocatalysts are their low cost, high versatility and efficiency, in addition to highly desirable chemical aspects such as chemoselectivity, regioselectivity, and enantioselectivity, with the combination of these factors having made biocatalytic reactions very attractive to the industrial sector [3].

Many transformations of different substrates, such as hydroxylation and oxidation reactions (*Gynostemma pentaphyllum*) [4], hydrolysis of esters (*Solanum tuberosum*, *Helianthus tuberosus*) [5], bioreduction of ketones and aldehydes (*Daucus carota*, *Foeniculum vulgare*, *Cucurbita pepo*, *Phaseolus aureus*, *Cocos nucifera*, *Saccharum officinarum*, *Manihot dulcis*, *Manihot esculenta*) [3,6–16], enzymatic lactonization (*Malus sylvestris*, *Helianthus tuberosus*) [17], glycosylation (*Ipomoea batatas*, *Eucalyptus perriniana*) [18], etc., have been performed, and have produced very good results using plants and their cultured cells.

The use of plants as biocatalysts has many advantages. First of all, a large array of taxonomically different plants is available at a very low cost. Another important aspect is that the separation of the product from the reaction mixture can be carried out very easily by filtration/centrifugation and the remaining material is easily disposed of. Moreover, these systems have the advantage of being environmentally friendly, due to the reaction being carried out in water as the solvent and the catalyst being biodegradable [19], as opposed to the classic reactions of organic chemistry where heavy metal disposal may be an issue.

In summary, it can be stated without equivocation that plants represent an alternative source of "new" enzymes for use in organic synthesis.

Recently, as a part of a major program on the study of the flora in the Province of Córdoba, a project was commenced with the

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doi:10.1016/j.molcatb.2011.03.003

aims of finding green alternatives and economically viable procedures to synthesize chemical products of commercial interest using biocatalytic processes.

In the particular case of the benzylic alcohols, several of these are considered to be key starting materials in the synthesis of scented substances for cosmetics, fragrances and the flavour industry [20], which in general are more expensive than the corresponding aldehydes from which they are obtained. However, the reduction of benzaldehydes may be potentially carried out through biocatalytic methodologies, if an efficient and affordable biocatalyst is available which is also capable of generating the desired product in adequate quantities. With this objective in mind, the screening of the native flora was initiated to find plants that could be used as biocatalysts in reduction reactions of aromatic aldehydes.

2. Experimental

2.1. General methods

Benzaldehyde, benzoic acid, 2-methoxyphenol and substituted benzaldehydes were purchased from the Sigma–Aldrich Chemical Company (Argentina). 4-(*N*,*N*-dimethylamino) benzaldehyde was obtained from Fluka. Benzyl alcohol and substituted benzyl alcohols were purchased from the Sigma–Aldrich Chemical Company. 4-(*N*,*N*-dimethylamino)benzyl alcohol, 2-methylthiobenzyl alcohol and vanillic alcohol were synthesized by a methodology described in the literature [21], and sterile deionized water was used as the solvent in all experiments. The crude reaction products were extracted with ethyl acetate, the organic solutions were evaporated, and the products were filtered on a short column with silica gel (70–230 mesh) using ethyl acetate as the eluent. GC analyses were made on a Shimadzu GC-14B instrument, with FID detector and GC–MS analyses were carried out on a Shimadzu GC-17A/QP-5000 instrument. ^{1}H NMR spectra were recorded on a Bruker AC 200 MHz using $CDCl_3$ as the solvent. All products were characterized by comparison of their GC retention time (GC Rt) with authentic samples, and by comparison of their MS and ^{1}H NMR spectra with literature data [21–26].

2.2. GC-FID and GC–MS analyses

The GC separations were performed on a Hewlett Packard HP-5 fused silica capillary column (Crosslinked 5% PhMe Siloxane, 30 m, 0.32 mm, 0.25 μm film thickness) with GC conditions of: split 1/50, injector 220 °C, detector FID: 220 °C, carrier gas: N_2 to 1 mL/min, temp: $T_1 = 50$ °C (5 min), $\Delta T = 5$ °C/min, $T_2 = 150$ °C (5 min). The yields of the reactions were determined by GC using the normalized peak areas without a correction factor. The GC–MS (70 eV) analyses were performed using the same conditions as those used in the GC analysis and the same capillary column.

2.3. Biocatalysts

Healthy and intact plants were collected in the Punilla Valley (Province of Córdoba, Argentina) and identified by a botanist. To carry out this study, plants were selected whose roots were similar in form and texture to that of carrot. The aerial parts were discarded, and the roots were washed with distilled water to remove traces of soil.

2.4. Bioreductions

The reactions were conducted immediately after acquisition of the plant to assure the integrity of the enzymatic system. A typical reaction was conducted as follows: fresh plant roots were washed with distilled water and maintained in a 5% sodium hypochlorite aqueous solution for 20 min. Then, they were washed with sterile deionized water and the external layer was removed, with the remaining roots being cut into small thin slices (1 cm) with a sterile cutter. Both the treated and cut plant roots (10 g) were added to a sterile Erlenmeyer flask (250 mL) with sterile deionized water (80 mL), and the corresponding aldehyde (50 mg) was added to this suspension and the reaction carried out by stirring on an orbital shaker at room temperature with the Erlenmeyer flask closed. The reaction's progress was monitored every 24 h for 7 days, and the samples (5 mL, saturated with sodium chloride) were extracted by shaking with ethyl acetate (2 mL). The organic layer was collected, sodium sulfate was added to remove the dissolved water, and the organic solution was filtered and analyzed (1 μL) by GC.

2.5. Scaling study

This study was carried out using treated and cut roots (10 g), sterile deionized water (80 mL) and an orbital shaker at room temperature. In this system, the concentration of the substrate and the reaction time were modified to optimize the conditions, with the evolution of the reactions being periodically monitored by GC-FID analysis. The crude reaction mixture described in Table 3 (entry 6) was filtered and the aqueous solution was extracted with ethyl acetate (3× 20 mL). Then, the combined organic layer was dried over calcium sulfate, and the solution was preconcentrated on a rotary evaporator. The crude solution was filtered on a short column with silica gel (70–230 mesh) using ethyl acetate as eluent, and benzyl alcohol was isolated (192 mg, 96% yield). The presence of benzoic acid in the reactions (Table 3; entries 9 and 10) was determined by GC, using a standard sample of benzoic acid and through GC–MS analysis by comparing the obtained spectra with library data.

2.6. Spectroscopic and GC data

2.6.1. Benzyl alcohol

GC Rt: 13.5 min (benzaldehyde GC Rt: 10.7 min), MS *m*/*z*: 109 (M^+ +1, 5%), 108 (M^+, 60%), 107 (41%), 91 (13%), 79 (100%), 78 (13%), 77 (62%), 65 (10%), 63 (10%), 53 (14%), 52 (14%), 51 (50%), 50 (27%). ^{1}H NMR δ (ppm): 2.30 (s, 1H), 4.61 (s, 2H), 7.20–7.40 (m, 5H).

2.6.2. Benzoic acid

GC Rt: 19.6 min, MS *m*/*z*: 277 (M^+ +1, 9%), 276 (M^+, 93%), 245 (100%), 217 (14%), 90 (24%), 89 (17%), 63 (8.5%).

2.6.3. 4-Chlorobenzyl alcohol

GC Rt: 18.9 min (4-chlorobenzaldehyde GC Rt: 14.9 min), MS *m*/*z*: 143 (M^+ +1, 17%), 142 (M^+, 84%), 125 (24%), 113 (24%), 107 (52%), 89 (11%), 79 (53%), 77 (100%), 51 (25%). ^{1}H NMR δ (ppm): 2.30 (s, 1H), 4.61 (s, 2H), 7.05–7.50 (m, 4H).

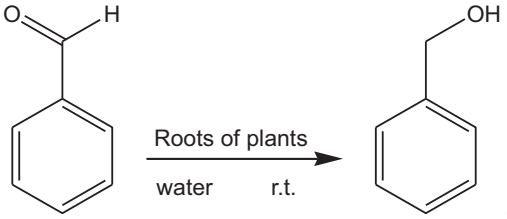
2.6.4. 4-Methoxybenzyl alcohol

GC conditions: $T_1 = 50$ °C (2 min), $\Delta T = 5$ °C/min, $T_2 = 200$ °C (2 min), GC Rt: 17.5 min (4-methoxybenzaldehyde GC Rt: 16.3 min), MS *m*/*z*: 139 (M^+ +1, 10%), 138 (M^+, 100%), 137 (67%), 121 (56%), 109 (71%), 107 (27%), 105 (22%), 94 (17%), 77 (36%), 65 (15%), 63 (16%), 51 (18%), 39 (16%). ^{1}H NMR δ (ppm): 2.30–2.42 (s, 1H), 3.77 (s. 3H), 4.52 (s, 2H), 6.87 (d, 2H), 7.21 (d, 2H).

2.6.5. 4-(N,N-Dimethylamino)benzyl alcohol

GC retention time: 19.6 min (4-(*N*,*N*-dimethylamino)benzaldehyde GC Rt: 21.6 min), MS *m*/*z*: 152 (M^+ +1, 5%), 151 (M^+, 49%), 135 (100%), 134 (67%), 120 (34%), 119 (40%), 118 (42%), 105 (11%), 91 (45%), 89 (19%), 77 (22%), 65 (18%),

Table 1
Screening for biocatalysts: reduction of benzaldehyde to benzyl alcohol carried out by roots of wild plants



Entry	Family	Scientific name	Time (days)	% Benzyl alcohol[a]
1	Alliaceae	*Nothoscordum gracile* (Dryand. ex Aiton) Steam var. *gracile*	4	nr[b]
2	Amaranthaceae	*Alternanthera pungens* Kunth	5	99
3	Apiaceae	*Daucus carota* L.	3	>99
4	Apiaceae	*Pastinaca saliva* L.	4	>99
5	Apiaceae	*Conium maculatum* L.	1	>99
6	Apiaceae	*Eryngium horridum* Malme	4	>99
7	Apocynaceae	*Mandevilla petraea*(A. St.-Hil.) Pichon	4	99
8	Asteraceae	*Trichocline reptans* (Wedd.) Hieron.	4	97
9	Bromeliaceae	*Puya spathacea* (Griseb.) Mez.	5	98
10	Cannaceae	*Canna indica* L.	4	99
11	Euphorbiaceae	*Euphorbia portulacoides* L. var. *portulacoides*	4	nr[b]
12	Fabaceae	*Dalea elegans* Gillies ex Hook. & Arn var. *elegans*.	4	97
13	Iridaceae	*Iris pseudacorus* L.	4	99
14	Nyctaginaceae	*Mirabilis jalapa* L.	5	98
15	Oxalidaceae	*Oxalis articulata* Savigny ssp. *articulata*	4	nr[b]
16	Talinaceae	*Talinum polygaloides* Gillies ex Arn.	6	98

[a] Measured by GC analysis.
[b] nr: No reaction.

63 (15%), 51 (20%), 42 (19%), 39 (15%). ^{1}H NMR δ (ppm): 2.30 (s, 1H), 2.98 (s, 6H), 4.54 (s, H), 6.78 (d, 2H), 7.21 (d, 2H).

2.6.6. Vanillic alcohol

GC conditions: $T_1 = 50\,^\circ$C (2 min), $\Delta T = 6\,^\circ$C/min, $T_2 = 200\,^\circ$C (2 min), GC Rt: 19.6 min (vanillin GC Rt: 18.3 min), MS *m*/*z*: 155 (M^+ +1, 9%), 154 (M^+, 100%), 137 (32%), 135 (9%), 125 (31%), 123 (21%), 122 (28%), 107 (13%), 93 (31%), 77 (16%), 65 (42%), 63 (15%), 53 (19%), 51 (21%), 50 (17%), 39 (22%). ^{1}H NMR δ (ppm): 3.74 (s, 3H), 4.38 (s, 2H), 5.03 (s, 1H), 6.71 (d, 2H), 6.89 (s, 1H), 8.79 (s, 1H).

2.6.7. 2-Methylthiobenzyl alcohol

GC Rt: 24.4 min (2-methylthiobenzaldehyde GC Rt: 23.5 min), MS *m*/*z*: 155 (M^+ +1, 10%), 154 (M^+, 100%), 139 (50%), 137 (34%), 136 (35%), 135 (34%), 111 (32%), 109 (25%), 105 (22%), 91 (8%), 77 (43%), 52 (11%), 51 (20%), 50 (12%), 45 (18%), 39. ^{1}H NMR δ (ppm): 2.41 (s, 3H), 4.62 (s, 2H), 7.07–7.30 (m, 3H), 7.33–7.41(m, 1H).

2.6.8. 4-Methylthiobenzyl alcohol

GC Rt: 25.6 min (4-methylthiobenzaldehyde GC Rt: 24.3 min), MS *m*/*z*: 155 (M^+ +1, 12%), 154 (M^+, 100%), 137 (22%), 125 (16%), 122 (11%), 109 (35%), 107 (24%), 91 (8%), 79 (25%), 77 (34%), 51 (13%), 45 (18%). ^{1}H NMR δ (ppm): 2.21 (s, 1H), 2.47 (s, 3H), 4.60 (s, 2H), 7.23 (m, 4H).

2.6.9. 3-Nitrobenzyl alcohol

GC Rt: 20.8 min (3-nitrobenzaldehyde GC Rt: 16.9 min), MS *m*/*z*: 154 (M^+ +1, 26%), 153 (M^+, 3%), 137 (29%), 136 (60%), 124 (9%), 108 (40%), 107 (100%), 105 (66%), 88 (30%), 77 (90%), 76 (78%), 74 (75%), 62 (28%), 51 (24%), 50 (46%), 49 (17%), 39 (11%). ^{1}H NMR δ (ppm): 2.55 (s, 1 H), 4.8 (s, 2H), 7.40–7.65 (m, 2H), 8.00–8.20 (m, 2H).

2.6.10. 2-Methoxyphenol

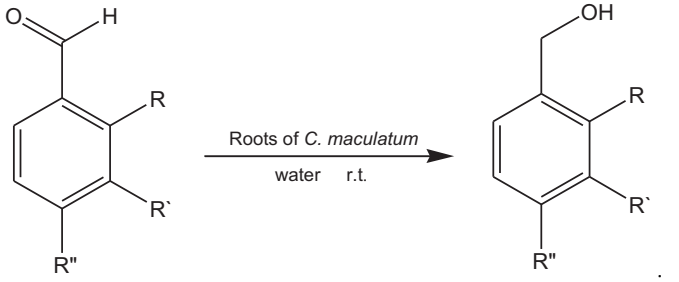
GC Rt: 11 min MS *m*/*z*: 125 (M^+ +1, 10%), 124 (M^+, 94%), 110 (20%), 109 (100%), 81 (80%), 63 (10%), 54 (15%), 53 (35%), 51 (23%). ^{1}H NMR δ (ppm): 3.80 (s, 3H), 5.81 (s, 1H), 6.80–6.95 (m, 4H).

3. Results and discussion

3.1. Screening of plants for the bioreduction of benzaldehyde

With the dual purpose of firstly developing economically viable and environmentally friendly reaction systems, and secondly, of finding a use for the local flora, the study of the biocatalytic reduction of benzaldehyde was performed on roots of plants that grow wild in Córdoba Province. Sixteen species of naturalized or native plants from thirteen families were collected, and studies were carried out using a methodology similar to one previously reported [6], with benzaldehyde as the model substrate. Results are shown in Table 1.

Table 2
Ability of *C. maculatum* to reduce different substituted benzaldehydes



Entry	R	R′	R″	Time (days)	% Alcohol[a]
1	H–	H–	H–	1	>99
2	H–	H–	Cl–	1	>99
3	H–	H–	CH_3–O–	1	>99
4	H–	HO–	CH_3–O–	2	27
5	CH_3–S–	H–	H–	1	>99
6	H–	H–	CH_3–S–	1	>99
7	H–	NO_2–	H–	1	>99
8	H–	H–	$(CH_3)_2$–N–	7	>99

[a] Measured by GC analysis.

As can be seen in Table 1, thirteen of the sixteen species studied produced excellent results: *Alternanthera pungens*, *Pastinaca sativa*, *Conium maculatum*, *Mandevilla petraea*, *Trichocline reptans*, *Eryngium horridum*, *Puya spathacea*, *Canna indica*, *Iris pseudacorus*, *Dalea elegans*, *Mirabilis jalapa* and *Talinum polygaloides* showed quantitative yields for the formation of benzyl alcohol. The reaction using *D. carota* as a model bioreducer of carbonyl compounds was conducted as well, and as with other members of the family, this reaction was also quantitative (Table 1, entry 3).

It can be observed in Table 1 (entries 3–6) that the Apiaceae family was able to reduce benzaldehyde with a high efficiency, which appears to be a common feature of this family. In contrast, no reduction reaction was found using *Euphorbia portulacoides*, *Nothoscordum gracile* or *Oxalis articulata* as catalysts (Table 1, entries 1, 11 and 15).

3.2. Bioreduction of substituted benzaldehydes by C. maculatum: advantages and scope

Although most of the species studied showed almost quantitative yields for the reduction reaction, *C. maculatum* (Apiaceae) was selected for the experiments because the reaction for the reduction of benzaldehyde occurred in the shortest period of time (Table 1, entry 5). In addition to this, *C. maculatum,* a weed that grows abundantly in the Province of Córdoba and is available throughout most of the year, is not used industrially and can be discarded as a livestock feed due to its toxicity. It is commonly known as *hemlock*, and is an annual herb of 50–300 cm high which presents green stalks with slightly violet specks and parsley-like leaves. Its thick vertical root is like a carrot, with some branching, and is yellowish-white in colour. It is highly toxic due to the presence of alkaloids, a neurotoxin and coumarins [27–29]. Hemlock is in fact better known as an active ingredient in the preparation of poisons [30].

With the aim of establishing the ability and the scope of *C. maculatum* to reduce substituted benzaldehydes to the corresponding substituted benzyl alcohols, studies were conducted and the results are listed in Table 2, where it can be seen that *C. maculatum* proved to be a very efficient biocatalyst for the reduction of substituted benzaldehydes. In this process, all of the aldehydes tested, except vanillin, produced alcohols of excellent yield, better than the results reported for coconut water [10] and comparable to those of *M. esculenta*, *M. dulcis* [13], sugar cane juice [14], *Brassica oleracea*, *Beta vulgaris* and *Spinacia oleracea* [20].

It is also noteworthy that, for these working conditions, there were no limitations as concerning the position or nature of the substituent, with the reaction giving quantitative yields, regardless of the relative position of the substituent with respect to the aldehyde function, with the substituents being either electron withdrawing or donating groups.

In the particular case of 3-nitrobenzaldehyde (Table 2, entry 8), it was observed that *C. maculatum* produced the reduction of the aldehyde group without reducing the nitro group, although there are studies on other plant species reporting the reduction of the nitro group to an amine [10,31,32]. When vanillin, however, was used as the substrate (Table 2, entry 5), the reaction produced only 27% of the corresponding vanillic alcohol. Here, it was observed that the major reduction product was 2-methoxyphenol, at a 73% yield (Fig. 1).

It is worth noting that in previous studies using *Passiflora edulis* [15] and two species of *Manihot* [13] no reduction of vanillin was observed, but vanillic alcohol, as well as the corresponding benzyl methyl ether, were obtained when using sugar cane juice [14] and coconut water [10].

The important increase in reaction time observed for 4-(*N*,*N*-dimethylamino) benzaldehyde (Table 2, entry 8), may have been due to steric factors or to stronger π-donating effects inherent to the *N*,*N*-dimethylamino group [1], or to both, which could have decreased the reactivity of the aldehyde group.

Table 3
Scaling study: bioreduction of benzaldehyde by *C. maculatum*.

Entry	Benzaldehyde (mg)	Time (days)	% Benzyl alcohol[a]	% Benzoic acid[a]
1	50	1	>99	–
2	100	4	>99	–
3	150	4	>99	–
4	175	4	>99	–
5	187.5	4	>99	–
6	200	4	>99	–
7	250	7	21	–
8	300	7	9	–
9	400	7	5	19
10	800	7	3	15

[a] Measured by GC analysis.

3.3. Scaling of the bioreduction of benzaldehyde using C. maculatum

A scaling study of the reduction process was carried out using hemlock as the biocatalyst and the results are summarized in Table 3, where it can be seen (entry 6) that the reaction proceeded quantitatively when a substrate ratio of 200 mg per 10 g of catalyst was used in 80 mL of water. This reduction methodology permitted the theoretical acquisition of 2.5 g of benzyl alcohol per liter of reaction, but it should be noted that when a higher substrate/catalyst ratio was used the performance of the reaction lapsed abruptly (Table 3 entries 7–10), with substrate oxidation to the benzoic acid beginning to occur unexpectedly as the main reaction (Table 3, entries 9 and 10). In addition, when the concentration of the substrate was increased, the reaction time was longer (Table 3, entries 1–6).

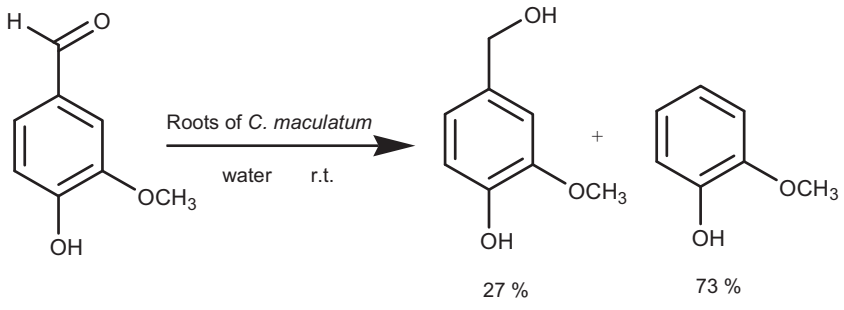


Fig. 1. Bioreduction of vanillin to vanillic alcohol and 2-methoxyphenol catalyzed by *C. maculatum*.

4. Conclusions

The results demonstrate that most of the locally available species studied have enzyme systems with the ability to reduce aldehydes to the corresponding alcohols at high yields, with thirteen species (out of sixteen) showing an excellent ability to reduce benzaldehyde to benzyl alcohol. Moreover, it is noteworthy that *C. maculatum* showed the fastest reaction rate in carrying out this transformation.

C. maculatum was also effective in reducing substituted benzaldehydes and the yield was always quantitative, except for the reaction using vanillin, where 2-methoxyphenol was the main product. Nevertheless, based on the results observed here, the reaction may not be efficient with disubstituted aldehydes, with more extensive studies being required to investigate this hypothesis. Currently, we are conducting studies to determine the ability of *C. maculatum* to produce the decarbonylation of aldehydes similar to vanillin, and to attempt to identify if this type of reaction is common with disubstituted benzaldehydes.

In the study of the scaling reaction using benzaldehyde as a model substrate and *C. maculatum* as a biocatalyst, it was observed that a higher substrate–catalyst ratio reduced the efficiency of the reaction, resulting in side reactions of oxidation to benzoic acid.

The results obtained here using *C. maculatum* for biocatalysis may offer new strategies for the reduction of selected substituted benzaldehydes as a critical step in a synthetic organic pathway, thereby avoiding the use of costly and non-renewable metal reducing agents and organic solvents commonly utilized in organic synthesis. As a result of this study with wild plants, it is clear that an unexpected opportunity has arisen to establish new applications for the native flora, especially for those species which do not have any other reported practical utility and are branded weeds. The bioreduction method presented here allows substituted benzylic alcohols to be obtained using a methodology which is more environmentally friendly than classical reductions of aldehydes, with excellent yields produced on a laboratory scale.

Acknowledgments

These studies were supported by the Ministry of Science and Technology of the Province of Córdoba, Argentina. This work is dedicated to the teaching career of Dr. Rita H. de Rossi. Special thanks are due to Dr Elba Buján for technical assistance in this study, and to Dr. Paul Hobson, native speaker, for revision of the manuscript.

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