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Non-Conventional Yeasts as Sources of Ene-Reductases for the Bioreduction of Chalcones

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Received: 9 October 2019; Accepted: 18 February 2020; Published: 21 February 2020

Abstract: Thirteen Non-Conventional Yeasts (NCYs) have been investigated for their ability to reduce activated C=C bonds of chalcones to obtain the corresponding dihydrochalcones. A possible correlation between bioreducing capacity of the NCYs and the substrate structure was estimated. Generally, whole-cells of the NCYs were able to hydrogenate the C=C double bond occurring in (*E*)-1,3-diphenylprop-2-en-1-one, while worthy bioconversion yields were obtained when the substrate exhibited the presence of a deactivating electron-withdrawing Cl substituent on the B-ring. On the contrary, no conversion was generally found, with a few exceptions, in the presence of an activating electron-donating substituent OH. The bioreduction aptitude of the NCYs was apparently correlated to the logP value: Compounds characterized by a higher logP exhibited a superior aptitude to be reduced by the NCYs than compounds with a lower logP value.

Keywords: Non-Conventional Yeasts; whole-cell biocatalysis; bioreduction; ene-reductase; chalcones; dihydrochalcones

1. Introduction

The chemoselective bioreduction of α,β-unsaturated alkenes represents an important tool in the synthesis of a lot of fine chemicals and pharmaceuticals [1–3]. Ene-reductases (ERs) belong to the flavin-containing "Old Yellow Enzyme" family (OYE, EC 1.6.99.1), which includes a class of I flavin-dependent oxidoreductases, which have been extensively studied for their ability to catalyze the asymmetric reduction of electronically activated C=C bonds, possessing electron-withdrawing substituents in the presence of cofactor-recycling systems for NAD(P)H [4–14]. Intracellular ER homologues from bacteria, yeasts, filamentous fungi and higher plants have been isolated and characterized since the 1990s [15–25].

Chalcones represent an interesting class of bioactive open-chain flavonoids, exhibiting α,β-unsaturated carbonyl groups in their scaffolds. Some studies proved their important antitubercular [26], antioxidant [27], antifungal [28] and anticancer [29] activities.

Recently, whole-cells and enzymes catalyzing the C=C hydrogenation and C=O reduction of representative chalcones have been studied for obtaining compounds possessing noteworthy bioactivities. In particular, some dihydrochalcones (achieved via bioreduction of the C=C double bond) have been found to express antioxidant, UV-protective and pro-health activities, which could be interesting for pharmaceutical and cosmetic industries [25,30]. Moreover, their sweet taste make them attractive for producing sweeteners [31,32]. In addition, the dihydrochalcone obtained from bioreduction of (*E*)-1,3-diphenylprop-2-en-1-one has been isolated from the leaves of *Leptoderris fasciculata* [33], a woody liana used in traditional medicine for the treatment of dropsy, edema, pulmonary disorders and as a laxative [34].

Recent studies revealed that Non-Conventional Yeasts (NCYs) are able to express a number of promising biotechnological properties [35–37], including the ability to express important ERs activities. In this framework, due to the presence of cofactor-recycling systems for NAD(P)H at the level of cell metabolism, biotransformation processes catalyzed by whole-cells of NCYs could be considered as useful and cheaper alternatives in place of using purified enzymes for reducing α,β-unsaturated alkenes including chalcones [38–42].

Aiming to identify new possible substrates for the NCYs expressing ERs activity, the present paper reports a study on the ability of lyophilized cells of NCYs to bioreduce the activated C=C double bonds of chalcones. A Structure–Activity Relationship (SAR) approach was used.

2. Materials and Methods

2.1. Chemicals and Culture Media

A set of chalcones were used as substrate for bioreduction by NCYs. A Structure–Activity Relationship (SAR) approach was used by means of different substituents on the B-ring: **1a** = (*E*)-1,3-diphenylprop-2-en-1-one; **2a** = (*E*)-1-(4-chlorophenyl)-3-phenylprop-2-en-1-one; **3a** = (*E*)-1-(4-hydroxyphenyl)-3-phenylprop-2-en-1-one (4-hydroxychalcone); (Figure 1A). Besides, two α,β-unsatured ketones, i.e., (3*E*)-4-phenylbut-3-en-2-one (**4a**) and (3*E*)-4-(4-chlorophenyl)but-3-en-2-one (**5a**), were used for comparison (Figure 1B). They were from Sigma-Aldrich Co, USA.

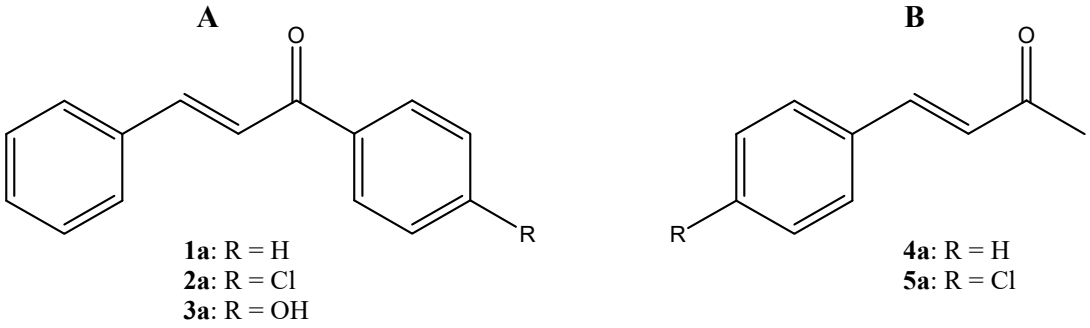


Figure 1. Chemical structures of substrates (from **1a** to **5a: A**, chalcones; **B**, α,β-unsatured ketones) with different substituents (R) used for checking NCY ene-reductase activity.

The following microbiological culture media were used: YEPG: yeast extract 10 g L^{-1}, peptone 10 g L^{-1}, glucose 20 g L^{-1} and agar 15 g L^{-1}; and Carvone Medium (CM) [39]: yeast extract 3 g L^{-1}, malt extract 3 g L^{-1}, peptone 5 g L^{-1}, glucose 10 g L^{-1} and pH 6.5. Ingredients of the culture media were from Difco (Franklin Lakes, NJ, USA).

2.2. Yeast Strain

Thirteen NCY strains belonging to ascomycetous and basidiomycetous species (genera *Candida*, *Cyberlindnera*, *Goffeauzyma*, *Hanseniaspora*, *Kazachstania*, *Naganishia*, *Pichia*, *Scheffersomyces*, *Solicoccozyma* and *Wickerhamomyces*) were used. They were preliminarily selected from a few hundred of

environmental strains isolated worldwide for their ability to catalyze the biotransformation of α,β-unsaturated alkenes [39–41]. All strains are conserved at the Industrial Yeasts Collection DBVPG of the University of Perugia, Italy. Salient information on strains are reported in Table 1 and are available on the DBVPG website (www.dbvpg.unipg.it). NCY strains were maintained in frozen form (−80 °C), while working cultures were routinely grown on YEPG agar slants at 20 or 25 °C, depending on their psychrophilic or mesophilic aptitudes.

Table 1. Salient information on the Non-Conventional Yeast (NCY) strains used in the present study.

Species	Origin	Location
Candida freyschussii DBVPG 6208	Wood pulp	Sweden
Cyberlindnera amylophila DBVPG 6346	Frass of *Pinus taeda* (loblolly pine)	USA
Goffeauzyma gastrica DBVPG 4709	Sub-glacial debris of the Sforzellina glacier	Italy
Goffeauzyma gilvescens DBVPG 4712	Supra-glacial debris of the Sforzellina glacier	Italy
Hanseniaspora guilliermondii DBVPG 6790	Trachea of bee	France
Kazachstania exigua DBVPG 6469	Soil	South Africa
Kazachstania naganishii DBVPG 7133	Decaying leaves	Japan
Kazachstania spencerorum DBVPG 6746	Soil	South Africa
Kluyveromyces lactis DBVPG 6854	Rain forest drosophilids	Brazil
Naganishia diffluens DBVPG 6237	Soil of vineyard	Hungary
Pichia kluyveri DBVPG 5826	Soil close to plum tree	Algeria
Scheffersomyces shehatae DBVPG 6850	Rain forest drosophilids	Brazil
Wickerhamomyces canadensis DBVPG 6211	Ground wood pulp	Sweden

2.3. Preparation of the Lyophilized NCYs Whole-Cells Biocatalyst

Lyophilized NCYs whole-cells were obtained as previously reported [40]. Briefly, aliquots (200 µL) of 24 h cell suspensions, calibrated to A_{580} = 0.5 (approx. 10^6 cells mL^{-1}), were used to inoculate 110 mL of CM [39]. After incubation for 48 h at 20 °C, the NCYs' biomass and supernatants were separately harvested. Cells were washed 3 times by using 50 mM phosphate buffer (pH 6.5), centrifuged each time for 15 min at 4000 rpm, snap frozen (−80 °C) and lyophilized for 48 h in a Lyophilizer Modulyo (Edwards, Irvine, CA, USA).

2.4. Bio-Reduction Reactions

A total of 30 mg of lyophilized NCYs cells were resuspended in 25 mL sterile vials containing 4.5 mL of 50 mM phosphate buffer (pH 6.5). A total of 0.5 mL of 10% *w/v* glucose, acting as a cofactor-recycling system, was also added. As a final point, chalcone was added at a final concentration of 5 mM and the vials were incubated on an orbital shaker (120 rpm) at 20 or 25 °C (depending on their psychrophilic or mesophilic status) for 120 h. In order to determine whether chalcone was spontaneously reduced in the absence of the NCY cells, blank (cell-free) vials containing 50 mM phosphate buffer + 50 mM glucose and each chalcone were analyzed at 120 h. After incubation, vials were sealed and frozen (−30 °C) until GC–MS analysis.

2.5. GC–MS Analyses

Products obtained after bioconversion were detected via GC–MS after extraction with a solution of octanol (as internal standard) in ethyl acetate 0.1% *v/v* (5 mL). GC–MS analyses were performed on a Hewlett Packard (USA) G1800C Series II gas chromatograph–mass spectrometer equipped with a HP-5 column (25 m × 0.2 mm, 0.5 µm film thickness) coated with (5%)-diphenyl-(95%)-dimethylpolysiloxane copolymer. Compounds derived from biotransformation of substrates were identified by comparing their respective mass fragmentation patterns (EI, 70 eV) with the database library NIST05 (MS Library Software Varian, USA). Temperature program: **1a**, **4a** and **5a**: 120 °C, hold for 2 min, 10 °C/min

to 250 °C, hold for 5 min, Detector 280 °C, Injector 270 °C; 2a: 100 °C, hold for 3 min, 15 °C/min to 180 °C, hold for 3 min, 20 °C/min to 240 °C, hold for 5 min Detector 280 °C, Injector 270 °C; **3a**: 120 °C, hold for 2 min, 15 °C/min to 270 °C, hold for 15 min, Detector 280 °C, Injector 270 °C. Retention times (min): (*E*)-1,3-diphenylprop-2-en-1-one **1a** (12.06), 1,3-diphenylpropan-1-one **1b** (10.70); (*E*)-1-(4-chlorophenyl)-3-phenylprop-2-en-1-one **2a** (15.74), 1-(4-chlorophenyl)-3-phenylpropan-1-one **2b** (14.35); (*E*)-1-(4-hydroxyphenyl)-3-phenylprop-2-en-1-one **3a** (21.07), 1-(4-hydroxyphenyl)-3-phenylpropan-1-one **3b** (17.90); (3*E*)-4-phenylbut-3-en-2-one **4a** (4.56), 4-phenylbutan-2-one **4b** (3.29); (3*E*)-4-(4-chlorophenyl)but-3-en-2-one **5a** (6.87), 4-(4-chlorophenyl)butan-2-one **5b** (5.60).

All the results were expressed as biotransformation yield, i.e., a % of the substrate converted to a given derivative. The concentration of the substrate and product were measured by an internal standard method. All the results represented the average of three independent experiments, and the statistical significance of these average data was assessed via ANOVA.

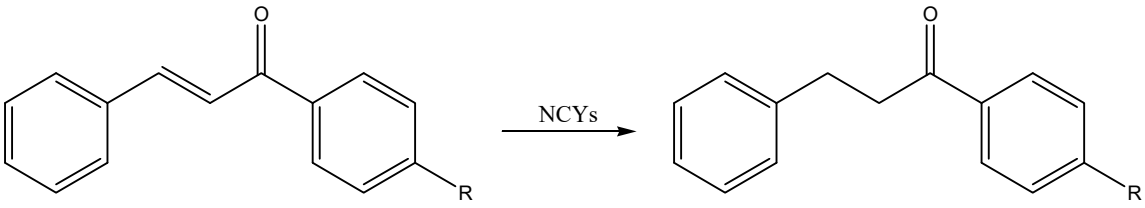
2.6. LogP Calculation

The logP values of chalcones were calculated by the ACD/LogP v.14.06 program in the software package for ACD/Labs 2016 2.2 (Advanced Chemistry Development).

3. Results and Discussion

Figure 1 reports the chemical structures of the substrates used for checking the NCYs' ERs activity. The first substrate, namely (*E*)-1,3-diphenylprop-2-en-1-one (**1a**, Figure 1A), was used as model compound to screen the ability of the lyophilized cells of the NCYs to reduce the α,β C=C double bond. The presence of the conjugate C=O double bond was also considered for assessing the chemoselectivity of the reduction.

The reaction scheme of the bioreduction of chalcones (*E*)-1,3-diphenylprop-2-en-1-one (**1a**), (*E*)-1-(4-chlorophenyl)-3-phenylprop-2-en-1-one (**2a**), and (*E*)-1-(4-hydroxyphenyl)-3-phenylprop-2-en-1-one (**3a**) into dihydrochalcones 1,3-diphenylpropan-1-one (**1b**), 1-(4-chlorophenyl)-3-phenylpropan-1-one (**2b**) and 1-(4-hydroxyphenyl)-3-phenylpropan-1-one (**3b**), respectively, catalyzed by the lyophilized cells of the NCYs is reported in Figure 2. The results of the screening, in terms of conversion yields expressed as molar percentage, are reported in Table 2.



1a: R = H
2a: R = Cl
3a: R = OH

1b: R = H
2b: R = Cl
3b: R = OH

Figure 2. Reaction scheme of the bioreduction of chalcones **1a**, **2a** and **3a** into dihydrochalcones **1b**, **2b** and **3b**, respectively, by NCYs.

With few exceptions, whole-cells of NCYs were able to hydrogenate the C=C double bond occurring in the (*E*)-1,3-diphenylprop-2-en-1-one **1a** scaffold: in particular, nine strains, namely *Cyberlindnera amylophila* DBVPG 6346, *Goffeauzyma gastrica* DBVPG 4709, *Hanseniaspora guillermondii* DBVPG 6790, *Kazachstania exigua* DBVPG 6469, *Kazachstania spencerorum* DBVPG 6746, *Kluyveromyces lactis* DBVPG 6854, *Naganishia diffluens* DBVPG 6237, *Scheffersomyces shehatae* DBVPG 6850 and *Wickerhamomyces canadensis* DBVPG 6211 exhibited bioconversion yields ≥ 94%, with an excellent repeatability and a low standard deviation (Table 2). On the contrary, three strains (i.e., *Candida freyschussii* DBVPG 6208, *Goffeauzyma gilvescens* DBVPG 4712 and *Pichia kluyveri* DBVPG 5826) showed lower bioconversion yields (from 0.7 to 11.5%), but with low repeatability of the bioreduction process (Table 2).

Table 2. Bioreduction yield of substrates **1a**–**5a** to products **1b**–**5b** by NCYs.(*)

DBVPG Accession Numbers	Species	1a Conversion mol% (± SD)	2a Conversion mol% (± SD)	3a Conversion mol% (± SD)	4a Conversion mol% (± SD)	5a Conversion mol% (± SD)
6208	*Candida freyschussii*	2.9 ± 1.5	23.2 ± 13.5	0.0 ± 0.0	0.0 ± 0.0	3.1 ± 0.5
6346	*Cyberlindnera amylophila*	94.0 ± 2.8	88.3 ± 10.2	100.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
4709	*Goffeauzyma gastrica*	100.0 ± 0.0	65.2 ± 56.5	0.0 ± 0.0	3.2 ± 0.6	2.7 ± 1.0
4712	*Goffeauzyma gilvescens*	11. 5 ± 5.6	78.0 ± 6.1	47.8 ± 11.0	7.4 ± 4.0	16.9 ± 1.4
6790	*Hanseniaspora guilliermondii*	96.2 ± 1.6	76.4 ± 22.2	0.0 ± 0.0	0.0 ± 0.0	1.8 ± 1.1
6469	*Kazachstania exigua*	96.3 ± 0.8	17.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
7133	*Kazachstania naganishii*	0.0 ± 0.0	83.5 ± 3.6	0.0 ± 0.0	3.2 ± 0.4	2.7 ± 1.2
6746	*Kazachstania spencerorum*	95.2 ± 2.0	48.4 ± 4.3	100.0 ± 0.0	46.9 ± 7.4	4.7 ± 0.4
6854	*Kluyveromyces lactis*	100.0 ± 0.0	73.3 ± 24.3	100.0 ± 0.0	99.1 ± 0.8	0.0 ± 0.0
6237	*Naganishia diffluens*	99.1 ± 1.6	20.9 ± 20.9	0.0 ± 0.0	15.6 ± 8.3	21.2 ± 4.7
5826	*Pichia kluyveri*	0.7 ± 0.7	98.1 ± 3.4	0.0 ± 0.0	0.0 ± 0.0	1.5 ± 0.5
6850	*Scheffersomyces shehatae*	97.5 ± 4.3	51.6 ± 11.5	0.0 ± 0.0	13.5 ± 3.9	0.0 ± 0.0
6211	*Wickerhamomyces canadensis*	100.0 ± 0.0	8.0 ± 11.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

(*) Chromatograms for biotransformations are available in the supplementary materials.

One of the main drawbacks of using whole-cells as catalysts is sometimes their poor chemoselectivity when C=C versus C=O bond reduction is catalyzed. It is worth noting that in any case, the screened NCYs did not exhibit any reduction of the C=O bond (Figure 2). In a previous study, we observed that many of the yeasts herein screened possess carbonyl reductase activity (alcohol dehydrogenase, ADH), being able to reduce the carbonyl group of molecules such as ketoisophorone [40]. The high chemoselectivity in the bioreduction of chalcones observed in the present study is probably not due to the lack of ADH activity in the yeast strains, rather to the steric hindrance given by the bulky phenyl substituents or to enzyme localization (not accessible to the substrates). Anyway, the bioreduction of C=O bond in ketones bearing bulky substituents was found in bacteria whole cells [43], as well as in other microorganism [44–47] such as algae, filamentous fungi, yeasts and in plant tissues [48].

Considering these encouraging results, the ability of NCYs to bioreduce chalcones substituted with both deactivating and activating groups on the B-ring ((*E*)-1-(4-chlorophenyl)-3-phenylprop-2-en-1-one **2a** and (*E*)-1-(4-hydroxyphenyl)-3-phenylprop-2-en-1-one **3a**, respectively) was also checked. The results are reported in Table 2. Worthy bioconversion yields were obtained when the substrate exhibited the presence of a deactivating electron-withdrawing Cl substituent on the B-ring (**2a**): all the NCYs exhibited the ability to reduce C=C double bond of **2a** with bioconversion yields ranging from 8% to 98% (Table 2). On the contrary, in the presence of the activating electron-donating substituent OH (**3a**), no conversion was generally found, with the sole exception of *Goffeauzyma gilvescens* DBVPG 4712 (yield = 47.8%), and *Cyberlindera amylophila* DBVPG 6346, *Kaz. spencerorum* DBVPG 6746 and *K. lactis* DBVPG 6854, which totally reduced the chalcone **3a** (yield = 100%) (Table **1**). Interestingly, the last three NCYs also exhibited worthy bioconversion yields of **1a** and **2a** (falling into the range from 94.0% to 100% and from 48.4% to 88.3%, respectively). On the contrary, *Pichia kluyveri* DBVPG 5826 showed a bioconversion yield of the chlorocalcone **2a** ≥ 95%, but no or very low activity versus **1a** and **3a**. Taking into account the above few exceptions, the bioreduction aptitude of NCYs was apparently correlated to the logP value, which is an indirect measure of the lipophilic degree of a given compound: The substrates **1a** and **2a**, which were characterized by a higher logP (4.01 and 4.78, respectively), exhibited a superior aptitude to be reduced by NCYs than the chalcone **3a** (logP = 3.65). This trend could be justified by considering how the different molecules can go across the yeast cell membrane. Due to the lyophilized nature of the whole cells of the NCYs herein used, the passage of molecules, including **1a**, **2a** and **3a,** across the cell membrane to reach the intracellular ERs should be much simpler. In fact, some studies reported that dehydration–rehydration cycles can determine a significant decrease of cell sizes together with a strong folding of membranes, thus leading to an increased permeability in lyophilized cells [49,50]. In this framework, the hypothesis postulated by some authors [51,52] that higher lipophilic molecules (characterized by a higher logP, i.e., (*E*)-1,3-diphenylprop-2-en-1-one **1a** and (*E*)-1-(4-chlorophenyl)-3-phenylprop-2-en-1-one **2a**) could more easily go across yeast plasma membranes by using the free diffusion mechanism (thus easily reaching cytoplasm ERs) differently from the lesser lipophilic ones (characterized by a lower logP, i.e., (*E*)-1-(4-hydroxyphenyl)-3-phenylprop-2-en-1-one **3a**) could justify the superior aptitude of **1a** and **2a** to be bioreduced by ERs occurring at the cytoplasm level of the lyophilized cells of the NCYs. In addition, the presence of an OH substituent on the B-ring of **3a** (Figure 1) could affect its capability to form hydrogen bonds with hydrophilic components occurring in the cell surface, thus reducing the rate of passage across the membrane.

The degree of lipophilicity of a given molecule is just one of the parameters determining its passage across the membrane and its interaction with enzymes. Thus, the α,β-unsaturated ketones (3*E*)-4-phenylbut-3-en-2-one (**4a**) and (3*E*)-4-(4-chlorophenyl)but-3-en-2-one (**5a**) (Figure 1B), exhibiting lower logP (2.17 and 2.70, respectively), but also lesser steric hindrance and substituents unable to form hydrogen bonds, were studied for their ability to be bioreduced by NCYs (Figure 3). The results are reported in Table 2. Overall, with the sole remarkable exception of *K. lactis* DBVPG 6854 on substrate **4a** (bioreduction yield = 99.1%), the aptitude of both substrates **4a** and **5a** to be bioreduced is

significantly lower than those of the substrates **1a**–**3a**. These results seem to confirm the importance of the lipophilicity and that the steric hindrance is a less important factor in the determining the aptitude to be bioreduced.

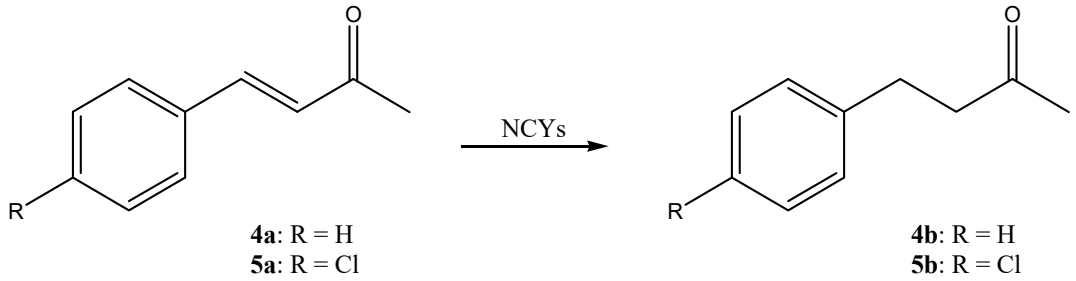


Figure 3. Reaction scheme of the bioreduction of substrates **4a** and **5a** to products **4b** and **5b**, respectively, by NCYs.

In close analogy with previous literature [53], the results herein reported underline that the use of lyophilized yeast cells could be considered preferable for enhancing the permeability of a membrane to the substrate. Indeed, as supposed by some authors [39,54], the ERs that reduced the α,β-unsatured carbonyl compounds could be exclusively associated with yeast biomass, with no release of extracellular enzymes.

4. Conclusions

Our study has shown that lyophilized NCYs whole cells are useful biocatalysts to obtain dihydrochalcones from corresponding chalcones. In particular, we have identified nine strains (namely, *Cyberlindnera amylophila* DBVPG 6346, *Goffeauzyma gastrica* DBVPG 4709, *Hanseniaspora guillermondii* DBVPG 6790, *Kazachstania exigua* DBVPG 6469, *Kazachstania spencerorum* DBVPG 6746, *Kluyveromyces lactis* DBVPG 6854, *Naganishia diffluens* DBVPG 6237, *Scheffersomyces shehatae* DBVPG 6850 and *Wickerhamomyces canadensis* DBVPG 6211) that exhibited high bioconversion yields, with an excellent repeatability and a low standard deviation

The bioreduction aptitude of the NCYs was affected by both the structure of chalcones and the logP value of tested substrates: NCYs were able to better reduce compounds characterized by a higher logP than ones with a lower logP value. Due to their sweet taste, dihydrochalcones are interesting derivatives for the food industry for the production of new sweeteners. Furthermore, some dihydrochalcones have been found to express interesting biological activities, which could be important for the pharmaceutical and cosmetic industries.

Supplementary Materials: The following are available online at http://www.mdpi.com/2311-5637/6/1/29/s1, Figure S1: chromatograms for biotransformations.

Author Contributions: Conceptualization, L.F. and P.B.; methodology, L.F. and P.B.; formal analysis, S.F., G.T., F.-Z.K.L., B.T. and M.R.C.; data curation, S.F., G.T., F.-Z.K.L., B.T. and M.R.C.; writing—original draft preparation, all authors; writing—review and editing, L.F. and P.B.; supervision, L.F. and P.B.; project administration, L.F. and P.B. All authors have read and agree to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

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