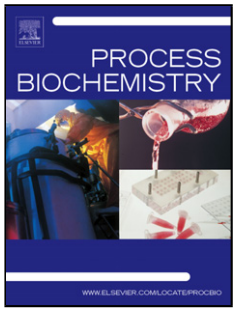

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Selection of microbial biocatalysts for the reduction of cyclic and heterocyclic ketones

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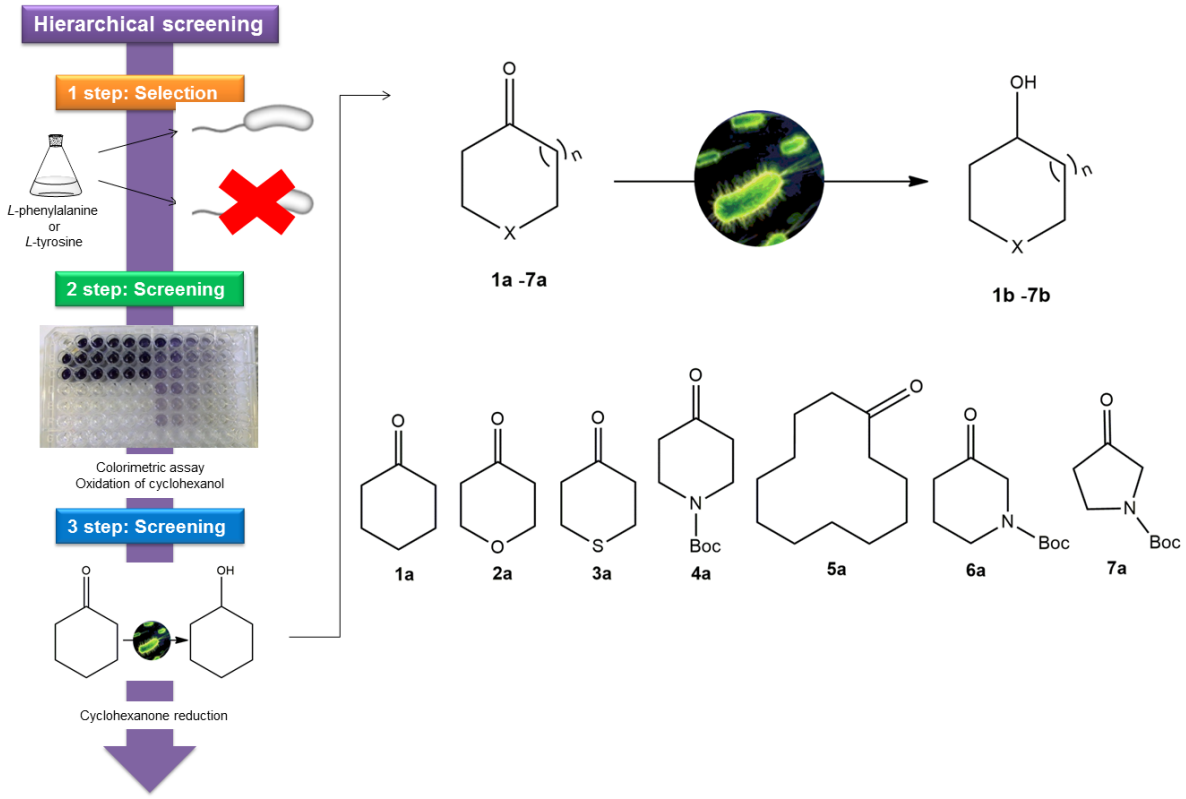
Graphical Abstract



Highlights

- Biocatalysts for prochiral heterocyclic ketone reduction were selected by screening
- *E. chrysanthemi* and *M. racemosus* almost quantitatively reduced cyclododecanone.
- *E. carotovora* produced (*S*)-*N*-Boc-3-pyrrolidinol with 87% yield and 99%ee.
- *X. fragariae* produced (*S*)-1-phenylethanol with 100% yield and 79%ee.
- *G. candidum* produced (*R*)-1-phenylethanol with 55% yield and 93%ee.

Abstract

The reduction of carbonyl compounds plays an important role in the synthesis of complex chiral molecules. In particular, enantiopure substituted cyclic and heterocyclic compounds are useful intermediates for the synthesis of several antiviral, antitumor, and antibiotic agents, and recently, they have also been used as organocatalysts for *C-C* addition. Alcohol dehydrogenases (ADH) are enzymes involved in the transformation of prochiral ketones to chiral hydroxyl compounds. While significant scientific effort has been paid to the use of aliphatic and exocyclic ketones as ADH substrates, reports on (hetero)cyclic carbonyl compounds as substrates of these enzymes are scarce. In

the present study, 109 bacteria and 36 fungi were screened, resulting in 10 organisms belonging to both kingdoms capable of transforming cyclic and heterocyclic ketones into the corresponding alcohols. Among them, *Erwinia chrysanthemi* could quantitatively reduce cyclododecanone and *Geotrichum candidum* could stereoselectively reduce *N*-Boc-3-piperidone and *N*-Boc-3-pyrrolidinone to their corresponding (*S*)-alcohols; however, the anti-Prelog isomer was obtained when acetophenone was the substrate.

Keywords: alcohol dehydrogenases; (hetero)cyclic ketones; microbial reduction; hierarchical screening

1. Introduction

Cyclic and heterocyclic scaffolds are frequently present in many natural and unnatural products with important biological activity, in synthetic intermediates used for the synthesis of many drugs, and in reagents and additives used in different industries [1, 2]. For example, pyrane ring is present in zanamivir, an antiviral drug approved for treatment of influenza [3], and polyhydroxylated piperidines are a class of azasugars called iminocyclitols, which are carbohydrate mimetics used as glycosidase inhibitors [4]. Piperidinols have also been identified as potential antimycobacterial agents that inhibit mycobacterial arylamine *N*-acetyltransferase, an enzyme recognized as the potential target for the treatment of tuberculosis [5]. Heteronucleosides are compounds in which the oxygen in the carbohydrate moiety is replaced by sulfur, nitrogen, and more recently by selenium and have received much attention because of their therapeutic applications. Among them, thiarabine is a potent antitumor agent, and lamivudine was approved for the treatment of HIV infection [6]. Regarding azanucleosides, five-membered pyrrolidine-type azanucleosides have been the most investigated because of their similarity with naturally occurring nucleosides [7].

Consequently, great effort has been made for designing efficient strategies to prepare substituted heterocyclic compounds [8]. Among them, the reduction of carbonyl compounds plays an important role because the reduction of prochiral ketones to chiral alcohols is an excellent way to generate chirality in the synthesis of a molecule. In addition, the enantiomerically pure alcohol can be easily transformed into other useful functional groups without racemization. Whereas traditional synthetic methods predominantly use toxic metals and expensive complex hydrides [9], biotransformations offer significant advantages for the green and sustainable manufacturing of chemicals. In particular, biocatalysis becomes a preferred approach for asymmetric synthesis because of its excellent chemo-, regio-, and stereoselectivity [10].

Alcohol dehydrogenases (ADHs) [11] and ketone reductases [12], classified under E.C.1.1.X., are enzymes that catalyze the reversible reduction of the carbonyl group. To exhibit catalytic activity, these enzymes require nicotinamide cofactors such as NADH or NADPH. Many dehydrogenases/reductases reduce prochiral ketones following the Prelog rule, in which the hydride ion of NAD(P)H is transferred to the *re*-face of ketones, yielding chiral alcohols with (*S*)-configuration. Anti-Prelog stereoselectivity is uncommon in nature, and the majority of these enzymes are NADPH dependent [13]. Because NADH is much cheaper and more stable than NADPH under operational conditions, NADH-dependent anti-Prelog enzymes are frequently required for practical applications [14].

Microbial transformations using bacteria or fungi as catalysts have been known for many decades. The use of microbial whole cells is economically advantageous as isolated enzymes [15] involve costly enzyme purification and recycling system for expensive coenzymes. Moreover, the use of whole-cell biocatalysts is, in most cases, more desirable for minimizing downstream processing [16]. In particular, yeasts have been applied in biocatalytic redox processes, being *G. candidum* and *Saccharomyces cerevisiae* workhorses in asymmetric ketone transformations [17].

Although some biocatalysts have been reported for the reduction of carbocyclic ketones [18], little attention has been devoted to substrates containing heteroatoms. In this regard, stereoselective HLADH-catalyzed biotransformations of 2-substituted tetrahydropyran-4-ones into *trans* tetrahydropyran-4-ols has been reported [19]; *Daucus carota* was used to reduce *N*-Boc-piperidin-3-one to (*S*)-*N*-Boc-3-hydroxypiperidine with 95% optical purity but low yield [20]; and Ju et al. [21] reported an efficient process for the same substrate but catalyzed by a recombinant ketoreductase with >99%ee.

Five-membered heterocyclic ketones are difficult to reduce in a stereoselective manner, and the majority of the assayed wild type biocatalysts have failed in this purpose [22]. (*R*)-Tetrahydrothiophene-3-ol, a key intermediate in the synthesis of penem-based antibiotics [23], has been prepared with high selectivity, starting from tetrahydrothiophene-3-one using mainly *Penicillium, Aspergillus,* or *Streptomyces* strains [24]. Optically active 3-pyrrolidinol derivatives are also versatile building blocks for the synthesis of pharmaceuticals such as quinolones, *β*-lactam antimicrobial agents, and calcium antagonists*. Devosia riboflavin* (KNK10702) was discovered when it was screened for the stereoselective reduction of *N*-benzyl-3-pyrrolidinone and piperidinone to the (*R*)-alcohol, while (*S*)-*N*-benzyl-3-pyrrolidinol was purified and characterized from *G. capitatum* (JCM3908) [25]. Recently, directed evolution was applied to TbSADH, an ADH from *Thermoethanolicus brockii*, and (*R*)- and (*S*)-selective variants for the reduction of tetrahydrofuran-3-one and tetrahydrothiofuran-3-one were obtained (95-99%ee) [22]. Using these recombinant enzymes, Boc-protected *N*-analogs were reduced only to the (*S)*-isomer.

Screening of wild-type microorganism collections remains one of the most powerful tools for finding novel biocatalytic reduction systems. Therefore, the aim of the present work was to select new sources of useful biocatalysts for the synthesis of cyclic and heterocyclic alcohols.

2. Materials and Methods

2.1. Chemicals and microorganisms

Cyclohexanone, tetrahydro-4*H*-pyran-4-one, tetrahydro-4*H*-thiopyran-4-one, *N*-Boc-4-piperidinone, cyclododecanone, *N*-Boc-3-piperidinone, *N*-Boc-3-pyrrolidinone, and acetophenone were purchased from Sigma-Aldrich (St Louis, MO, USA). All other chemical reagents used were commercially available and of the best analytical grade.

The culture media components were obtained from Britania (CABA, Argentina) or Biopack (Zárate, Argentina). HPLC-grade methanol and acetonitrile were from Carlo Erba (Rodano, Italy) or Sintorgan (Buenos Aires, Argentina). Bacteria were supplied by the *Colección Española de Cultivos Tipo* (CECT), Universidad de Valencia (Spain). Fungi were kindly provided by the Food Mycology Laboratory, Universidad Nacional de Quilmes (Argentina).

2.2. Standard growth conditions

2.2.1. Bacterial strains

Bacterial strains were cultured in 25 mL flasks containing 5 mL liquid media at the optimum temperature (T) and time (t), according to the American Type Culture Collection (ATCC) (see Supplementary material). Growth was measured by determining the optical density at 600 nm ($DO_{600\ nm}$, equivalence: 0.4 DO units: $2x10^8$ cells mL^{-1}).

2.2.2. Fungal strains

Fungal strains were cultured on solid media at the optimal temperature (T) and time (t), according to ATCC (see Supplementary material). After growth in Petri dishes, 25 mL flasks containing 5 mL of the corresponding liquid media were inoculated with $1x10^4$ spores mL^{-1} (counted on a Neubauer chamber), and the cultures were incubated at 26°C for 3-6 days.

2.3. Identification of G. candidum *fungal strain*

2.3.1. Fungal material

G. candidum strain GC1, isolated from dairy products in Santa Fe province, Argentina, was maintained in laboratory on 2% malt extract agar at 23°C. The original species of the strain was identified on the basis of its physio-morphological growth characteristics.

2.3.2. Genomic DNA extraction

Genomic DNA of *G. candidum* strain GC1 was extracted from mycelia grown on 2% malt extract agar at 23ºC for 1 week. Hundred milligrams of mycelia was frozen in liquid nitrogen and pulverized by grounding with mortar and pestle, and total gDNA was purified using DNAeasy plant mini kit (Quigen, Hilden, Germany) according to the manufacturer's instruction. The gDNA yield was quantified on NanoDrop 1000 spectrophotometer (Termo Fisher

Scientific Inc.), and the sample integrity was evaluated by electrophoretic size separation on 1% agarose gel with posterior ethidium bromide staining.

2.3.3. PCR amplification of nuclear ribosomal DNA

Five nanograms of total gDNA was used as template for the PCR amplification of genomic rRNA encoding locus with the universal fungal internal transcribed spacer (ITS) region primers ITS1 and ITS4 (5´-TCCGTAGGTGAACCTGCGG-3´/5´-TCCTCCGCTTATTGATATGC-3´ [26]). The given primer pair amplifies from the 3' of the ribosomal small subunit 18S rDNA till the 5´of the large subunit 28S rDNA. The resultant amplicon includes sequences ITS1, rDNA of 5.8S, and ITS2.

The PCR reaction final volume of 20 µL included 1x Taq buffer with $(NH_4)_2SO_4$, 1 µM of each primer, 1.5 mM $MgCl_2$, 200 µM each of four dNTPs, and 1 U of Taq DNA polymerase. The PCR was done using products of Fermentas (TermoFisher Scientific Inc) and the primers were purchased from Genbiotech, Argentina.

The PCR conditions were as follows: initial denaturation for 5 min at 95°C, 30 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min, followed by a final elongation at 72°C for 7 min. The amplification was performed on Tpersonal thermocycler (Biometra®), and the results of PCR were analyzed by electrophoretic separation on 1% agarose gel and visualized with ethidium bromide staining.

2.3.4. Sequencing and analysis of rDNA-ITS amplicon

The PCR reactions presented a single specific amplification product of approximately 400 bp. The amplicon was purified directly from the PCR reaction mixtures using MinElute PCR Purification Kit (Qiagen, Hilden, Germany) and sequencing was performed by the Sanger dideoxy sequencing method using both ITS1 and ITS4 as sequencing primers. Sequencing service was purchased from Macrogen (Seoul, South Korea). The PCR amplicon was sequenced four times in total, twice from both sequence directions.

The raw sequencing chromatograms were evaluated manually to identify reliable base calling regions and checked for possible ambiguous bases. The trimmed two forward and two reverse sequences were aligned to construct the final 372bp long rDNA-ITS sequence using Clustal Omega online aligning platform (http://www.ebi.ac.uk/Tools/msa/clustalo/). The final sequence was deposited in GenBank under accession no. **KY009607**. The species identity of the fungal strain GC1 was evaluated based on BLAST searches of the rDNA-ITS sequence against public nucleotide collections (nt/nr) using megablast search option (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4. Bacterial screening

2.4.1. Primary Screening

The 109 bacterial strains were cultured in minimal medium (13.6 g/L KH_2PO_4; 2.5 g/L $(NH_4)_2SO_4$; 0.3 g/L $MgSO_4$; 0.5 mg/L $FeSO_4.7H_2O$; 5 mg/L citric acid; 5 mg/L $ZnSO_4.7H_2O$; 1 mg/L $Fe(NH_4)_2(SO_4)_2$; 0.25 mg/L $CuSO_4.5H_2O$; 0.05 mg/L $MnSO_4.H_2O$; 0.05 mg/L H_3BO_3 anhydrous; 0.065 mg/L $(NH_4)_2MoO_4$; 0.4 µM biotin; 30 µM thiamine-HCl; 20 µM calcium pantothenate; 26 µM riboflavin; 23 µM folic acid) containing *L*-tyrosine (0.4 g/L) or *L*-phenylalanine (0.4 g/L) as the sole carbon and energy source at the optimal temperature of each microorganism. Culture growth was determined by measuring $DO_{600\ nm}$ every 24 h for 144 h.

2.4.2. Secondary screening

The strains selected in the primary screening were used to perform a colorimetric assay to identify ADH activity in the corresponding cell extracts [27]. After growing the microorganisms in their optimal medium, treating the cells with lysozyme (1 mg/mL) and DNAse (1 U/mL), and centrifugation, the obtained supernatants were mixed with NAD^+ or $NADP^+$ (225 µM), nitroblue tetrazolium chloride (NBT, 225 µM), phenazine methosulfate (PMS, 22.5 µM), gelatin (0.13% w/w), and cyclohexanol (225 µM) in 50 mM Tris-HCl buffer, pH 8, at 30°C. Different volumes of cell lysates were assayed, which correspond to different concentrations of total proteins in the reaction medium: 0.05, 0.1, 0.15, and 0.2 mg/mL. The reaction was followed spectrophotometrically (λ = 508 nm) in 96-well microtiter plates by monitoring the production of formazan at 1, 24, and 48 h.

2.4.3. Specific Screening

The strains selected in the secondary screening were used as biocatalysts in liquid medium for the reduction of cyclic ketones using cyclohexanone as a model substrate by adapting a methodology previously reported [28]. The microorganisms were cultured as mentioned above (see standard growth conditions), and then cyclohexanone (10 mM) was added. The reaction was carried out at 30ºC in an orbital shaker at 180 rpm for 2 days. After removing the cells by centrifugation, the supernatants were extracted using ethyl acetate or diethyl ether, and the organic layers were analyzed by Gas Chromatography (GC).

2.5. Fungal Screening

Fungal strains were cultured as detailed in Standard Growth Conditions. After 6 days, cyclohexanone (10 mM) was added, and the reaction was carried out at 26ºC in an orbital shaker at 180 rpm for 4 days. After removing the cells

by filtration, the supernatants were extracted using ethyl acetate or diethyl ether. The organic layers were analyzed by GC.

2.6. Standard biotransformation

The selected strains were used as biocatalysts for the reduction of tetrahydro-4*H*-pyran-4-one, tetrahydro-4*H*-thiopyran-4-one, *N*-Boc-4-piperidinone, cyclododecanone, *N*-Boc-3-piperidinone, *N*-Boc-3-pyrrolidinone, and acetophenone using the same experimental conditions as mentioned in Specific Screening.

2.7. Analytical methods

Qualitative analysis was performed by Thin Layer Chromatography using Silicagel 60 F254 plates (Merck, Rahway, NJ, USA) and 9.0/1.0 (v/v) CH_2Cl_2/CH_3OH as mobile phase. Rf: cyclohexanone 0.94, cyclohexanol 0.8, tetrahydro-4*H*-thiopyran-4-one 0.82, tetrahydrothiopyran-4-ol 0.62, tetrahydro-4*H*-pyran-4-one 0.83, tetrahydro-4-pyranol 0.49, *N*-Boc-4-piperidinone 0.86, *N*-Boc-4-piperidinol 0.6, cyclododecanone 0.73, cyclododecanol 0.67, *N*-Boc-3-piperidinone 0.8, *N*-Boc-3-piperidinol 0.6, *N*-Boc-3-pyrrolidinone 0.89, *N*-Boc-3-pyrrolidinol 0.62, acetophenone 0.85, and 1-phenylethanol 0.31.

GC analysis was performed using an Agilent 7890B GC with an Agilent 5977A series GC/MSD and a HP-5MS UI (30 m x 0.25 mm, 0.25 µm) column. The operating conditions were as follows: injector temperature 250ºC; 5 min at 50ºC and 20ºC/min gradient to 280ºC, run time: 16.5 min. MSD: full SCAN: 40-500, injection volume 1 µL, split: 1:50, heaters: 280ºC. Rt (min): cyclohexanone 5.8; cyclohexanol 6.1; tetrahydro-4H-thiopyran-4-one 9.1; tetrahydrothiopyran-4-ol 9.6; tetrahydro-4H-pyran-4-one 6.5; tetrahydro-4-pyranol 7.3; *N*-Boc-4-piperidone 12.1; *N*-Boc-4-piperidinol 12.4; cyclododecanone 12.8, cyclododecanol 13, *N*-Boc-3-piperidone 12.3, *N*-Boc-3-piperidinol 12.2, *N*-Boc-3-pyrrolididone 12, and *N*-Boc-3-pyrrolidinol 12.1.

High-Performance Liquid Chromatography (HPLC) analysis was performed using a Beckman SystemGold HLPC with UV detector and a GraceSmart RP18 4.6 mm x 150 mm, 5 µm particle size column. The operating conditions were as follows: flow 0.9 mL/min, 5 min acetonitrile:water 45:55, 3%/min gradient to acetonitrile:water 80:20, 5 min acetonitrile:water 80:20, run time 22 min, detection λ=245 nm. Rt (min): acetophenone 3.7 and 1-phenylethanol 15.6.

Chiral GC analysis was performed using a Shimadzu GCMS-QP2010 plus with Supelco BetaDEX TM 325 30 m x 0.25 mm x 0.25 µm column. The operating conditions were as follows: (a) injector temperature 220ºC, 5 min at 60ºC, 4ºC/min gradient to 150ºC and 30ºC/min gradient to 220ºC column temperature, run time: 31.00 min. Injection

volume 1 µL. Rt (min): acetophenone 18.5, (*S)*-1-phenylethanol 21.7, and (*R*)-1-phenylethanol 22.0 and (b) injector temperature 220ºC, 5 min at 60ºC, 1ºC/min gradient to 140ºC column temperature, run time: 88.00 min. Injection volume 1 µL. Rt (min): *N*-Boc-3-piperidinone 71.3, (*S*)-*N*-Boc-3-piperidinol 85.0, (*R*)-*N*-Boc-3-piperidinol 85.5, *N*-Boc-3-pyrrolidinone 50.6, (*S)*-*N*-Boc-3-pyrrolidinol 51.8, and (*R*)-*N*-Boc-3-pyrrolidinol 52.

3. Results and discussion

The development of different and accurate screening techniques is one of the most important steps toward detection of new biocatalysts. Although the fastest and easiest route to find a new enzyme is to identify one that exists in commercial libraries, they can have limited diversity and may be economically inaccessible. On the contrary, sources such as microbial collections or gene libraries [29] are readily available and have the advantage of enabling the discovery of unknown enzymes.

3.1. Bacterial screening

In this regard, to find enzymes than can reduce cyclic and heterocyclic ketones, we decided to perform a hierarchical screening procedure using our bacterial collection. This three-step screening consisted of a primary growth selection step on amino acids as the sole energy source, a second step using colorimetric assay for cyclohexanol oxidation, and a third step involving the reduction of cyclohexanone as a model reaction (Fig. 1).

3.1.1. Primary screening

The first selection of microorganisms possibly containing redox enzymes was conducted by culturing the bacterial strains in the presence of *L*-phenylalanine or *L*-tyrosine as the sole carbon and energy source. The degradation pathways of these amino acids are known to involve several oxidoreductases, including some dehydrogenases [30, 31]. Therefore, we expected to find microorganisms that can grow either in the presence of one or both tested amino acids, depending on the endogenic enzyme repertoire of each microorganism. For practical purposes, growth was considered positive when $\Delta DO_{600\ nm}$ was higher than 0.3. From the 109 bacterial species tested, 16 grew only on tyrosine, 15 only on phenylalanine, and 38 on both amino acids (Table S1 in Supplementary material). The natural activity of the majority of the microorganisms selected in this primary screening is the degradation of organic matter and soil detoxification, which agrees with the target enzymatic activity.

3.1.2. Secondary screening

Different methodologies for the detection of ADHs using their natural oxidation activity have been reported, including spectrophotometric [32] and colorimetric strategies using tetrazolium salts [26] or *p*-rosaniline [33]. In the present

work, to obtain a rapid and simple detection of bacterial ADHs, the NAD(P)H produced by the oxidation of cyclohexanol to the corresponding ketone was detected by a colorimetric assay on the basis of its reaction with NBT in the presence of PMS [27] (Fig. S2 in Supplementary material). The formation of Formazan, the blue-purple product of the reaction, was colorimetrically determined at 580 nm. This methodology has already been used to detect several dehydrogenases such as lactic [34] and 3-β-hydroxy-Δ^5-steroid [35] dehydrogenases from different biological sources including mammals, bacteria, fungi, and plants. However, to our knowledge, this assay has not been applied before on wild-type bacterial ADHs using cyclic alcohols as enzyme substrates.

Our secondary screening revealed that 38 of the tested bacteria could oxidize cyclohexanol to cyclohexanone. Eight of these depended only on NAD^+, four on $NADP^+$, and the remaining 26 showed no cofactor specificity. This NAD^+ or $NADP^+$ dependence is a useful variable that can be considered for future reaction scaling up. In addition, we evaluated the effect of the reaction time and the cell concentration on the oxidization of cyclohexanol to cyclohexanone. Regarding reaction time, *E. carotovora* (CECT 225) oxidized cyclohexanol after 1 h with the lowest cell extract amount, while the rest of the bacteria performed the biotransformation after 24 h and/or at higher quantities of biocatalyst. Detailed results of the secondary screening are available in Table S1, Supplementary material.

3.1.3. Specific screening

Finally, cyclohexanone was used as substrate for the bacteria selected based on the previous two screening steps. The capacity of the bacteria to reduce the given substrate was evaluated using whole cells under growing conditions to avoid cofactor recycling and enzyme purification. Cyclohexanone was added to the culture media until the bacterial growth had saturated. After 48 h, the formed reaction products were extracted from the culture supernatants, and the product profile was analyzed by GC. Fig. 2a shows the bacterial strains that afforded positive results. The following microorganisms presented conversions higher than 20% and were selected to perform further biotransformations: *E. chrysanthemi* (CECT 509), *E. carotovora* (CECT 314), *E. carotovora* (CECT 225), *Streptomyces* sp. (CECT 3145), and *Xanthomonas fragariae* (CECT 549). There are some previous reports on the use of *Erwinia* [36], *Streptomyces* [37], or *Xanthomonas* [38] for the reduction of different aliphatic ketones or α- and β-ketoesters, but to the best of our knowledge, there are no reports on the reduction of cyclic ketones using these biocatalysts. Moreover, the conversion rates obtained in the present work are relatively high for non-modified wild-type microorganisms.

3.2. Fungal screening

With the aim of selecting new fungal biocatalysts with ADH activity, we applied our screening strategy for fungi isolated from different food sources. Because many fungal species can grow on amino acids as sole carbon and energy source and the complete lysis of their cells is generally not trivial, the methodologies employed in the primary and secondary bacterial screening were not feasible. Therefore, only the specific screening step was conducted for these microorganisms. To optimize the previously reported methodology [28], different variables such as culture age, aeration, initial amount of inoculum, reaction time, and cells state (growing or resting) were evaluated. *Mucor piriformis* (SCR11) was used as a model microorganism in these assays. All the conditions assayed and the results are shown in Table 1.

Because the production of high amounts of *Mucor* spores was difficult and an increase in the initial inoculum (Table 1, entries 1 and 2) did not afford any significant conversion improvement, the experiments were continued using $1x10^4$ spores/mL inoculum. Regarding culture age and reaction time (entries 1, 3, 4, and 5), the highest conversion was achieved using 6-day-old pre-grown fungal cultures reacted for 4 days (entry 5). The conversion value decreased when aeration was enhanced by performing the biotransformation in baffled Erlenmeyer flasks (entry 6). This possibly occurred because of the excess of oxygen in the reaction medium, which shifted the reaction equilibrium toward oxidation. A similar conversion drop was observed when resting cells instead of growing cells were used as biocatalyst (entry 7).

Finally, the experimental conditions detailed in entry 5 (Table 1) were used for fungal screening, and the results are shown in Fig. 2b. The conversions obtained for most of the fungi assayed were higher than those achieved in our bacterial screening (Fig. 2). Most significantly, five of the tested fungi afforded conversions higher than 30%: *M. racemosus* (SCR21, STCC22 and SCG11), *M. piriformis* (SCR11), and *G. candidum* (GC1). To the best of our knowledge, this is the first time that *M. piriformis* has been used for cyclohexanone reduction. Additionally, the conversion obtained in our work using *G. candidum* strain GC1 is higher than what has been previously reported for other strains of this fungus [39].

3.3. Microbial reduction of non-prochiral cyclic ketones

ADH-catalyzed reduction of exocyclic aromatic ketones, β-ketoesters, or aldehydes [40, 41] have been extensively studied, but very few reports deal with cyclic or heterocyclic ketones as substrates of these enzymes. Therefore, the 10 strains selected in the specific screening step, including 5 different bacteria and 5 fungi, were used for the

reduction of cyclohexanone (**1a**), tetrahydro-4*H*-pyran-4-one (**2a**), tetrahydro-4*H*-pyran-4-one (**3a**), and *N*-Boc-4-piperidinone (**4a**) to analyze heteroatom influence. In addition, cyclododecanone (**5a**) was used for testing steric hindrance (Fig. 3).

As shown in Fig. 4, *Streptomyces* sp. (CECT 3145) and *E. carotovora* (CECT 225) were the only bacteria that could reduce the carbonyl group in the sulfur-containing cyclic ketone (**3a**), while almost all fungi afforded good conversions for this substrate, reaching approximately 80% in the case of the two *M. racemosus* strains evaluated. In contrast, neither bacteria nor fungi were useful biocatalysts for reducing the oxygenated analogue (**2a**). When a cyclic ketone containing *N*-Boc (**4a**) was tested, all the selected biocatalysts could reduce it, with conversions between 20-40%. Remarkably *M. racemosus* (SCG11) achieved conversion values as high as 43% with this substrate.

As mentioned above, the selected biocatalysts reduced (**1a**) yielding cyclohexanol (**1b**) between 25% and 70% conversion. Substrates possessing sterically demanding groups on each side of the carbinol moiety are, for most dehydrogenases, very poor substrates [42]. When using a bulky ketone such as (**5a)** as substrate, the majority of the tested microorganisms could not reduce it. However, *E. chrysanthemii* (CECT 509) and *M. racemosus* (STCC22) yielded 100% and 93% conversions of (**5a**) to (**5b**), respectively. The only previous report regarding ADH-biocatalyzed reduction of compound (**5a**) was published by Carballeira et al. [28]. They used the fungal species *Absidia glauca*, *Gongronella butleri*, and *Pyrenochaeta oryzae,* which afforded 87%, 25%, and 41% conversion, respectively. To the best of our knowledge, there are no previous reports on bacterial reduction of cyclododecanone, which makes the work presented here first of its kind.

3.4. Microbial reduction of prochiral ketones

During the past two decades, significant efforts have been devoted to developing efficient scaffolds for the construction of chiral organocatalysts [43]. The vast majority of organocatalysts are amine compounds [44], which are usually chiral structures that comprise various organic moieties, such as chiral amino alcohols, diamines, prolinamides, and their derivatives.

Thus, we decided to synthesize chiral nitrogen-containing heterocyclic alcohols. Fig. 5 shows the conversion and enantiomeric excess obtained from the biocatalytic reduction of *N*-Boc-3-piperidinone (**6a**) and *N*-Boc-3-pyrrolidinone (**7a**), affording the last compound, better conversion rate and enantiomeric excess. *G. candidum* (GC1) and *X. fragariae* (CECT 549) were the best fungal and bacteria biocatalysts for compounds (**6a**) and (**7a**),

respectively. All the alcohols obtained from these biotransformations were (*S)*-isomers, which is in agreement with previously reported reactions using different biocatalysts, including modified bacteria [45].

Finally, considering that optically pure 1-phenylethanol derivatives are important building blocks in the preparation of fragrances and drugs, it was of interest to us to analyze the microbiological reduction of acetophenone (**8a**). The (*S)*-enantiomer is produced by most carbonyl reducing enzymes; therefore, to obtain inverse stereoselectivity, different screening and protein engineering strategies have been applied. In this sense, ADHs from *Leifsonia* sp., *Pseudomonas* sp., and *Empedobacter brevis* were recently isolated from soil samples [46, 47]. The screening of our biocatalyst collection showed that all the microorganisms tested produced the (*S*)-isomer, as expected, except *G. candidum* (GC1), which yielded the (*R*)-isomer, showing good enantiomeric excess and conversion rate. This fungal species has also been reported to produce (*S*)-isomer in previous studies [48, 49], making the *G. candidum* strain used in the present work of special interest. For these reasons, we decided to perform a molecular-level species identification on *G. candidum* (GC1). The strain was originally identified at species level according to its physio-morphological growth characteristics. DNA-level analysis was performed for re-confirming the taxonomic status of the given strain.

3.5. ITS sequencing results and confirmation of the strain GC1 species identity

PCR amplification using the primers ITS1/ITS4 resulted in a 372-bp amplicon. The manual analysis of the sequencing chromatograms revealed evident intra-genomic nuclear rDNA polymorphism in the strain GC1. This was detected as dual peeks specifically within the ITS1 region. Such intra-nuclear rDNA polymorphism has been reported before for *Galactomyces* sp. [50] and was further studied in detail in *G. candidus* (teleomorph of *G. candidum*) by Alper et al. [51]. Despite the detected dual peeks, the automatized base calling, which reflects the dominant bases at multivariable sequence positions, was accepted. However, one nucleotide (polymorphic position 64) had to be left ambiguous (M=A or C) because of variation in the dominant base-calling results between repeated sequencing reactions.

Blast searches using the final assembled dominant ITS sequence demonstrated that strain GC1 inarguably belongs to the fungal complex of *G. geotrichum*/*G. candidus* (99-98% sequence identity). Current species nomenclature accepted by NCBI taxonomy browser considers these two taxa as one, under the anamorphic species name *G. candidum*. Accordingly, the ITS sequence, deposited under GenBank accession no. **KY009607**, is now linked to *G. candidum* as source organism.

The taxonomic revision of *Geotrichum* and its teleomorphs by de Hoog and Smith [50] has proposed a division of the *G. geotrichum/G. candidus* complex into four independent species: *G. geotrichum*, *G. candidus*, *G. pseudocandidus* sp. nov., and *G. europaeum* sp. nov.

G. candidum would be the anamorphic state linked to *G. candidus*, while the anamorph of *G. geotrichum* remains taxonomically unidentified. More importantly, these two *Galactomyces* sibling species cannot be identified using molecular taxonomy tools, such as ITS sequencing. Nonetheless, according to the *Galactomyces* sp. identification key by de Hoog & Smith [52], these closely related species can be distinguished by further growth phenotypic testing. This involves differential temperature tolerance, *G. candidus* being able to grow at 35°C, while *G. geotrichum* does not present growth at such an elevated temperature.

The growth capacity of the strain GC1 was tested at this restrictive temperature on solid 2% malt extract media. No growth was detected after 1 week of cultivation, which strongly suggests that according to *Galactomyces* sp. definition key established by de Hoog & Smith [52], the tested GC1 is a strain of *G. geotrichum*.

Presently, *G. geotrichum* does not have independent species status. Under official species nomenclature (http://www.catalogueoflife.org/), *G. geotrichum* and *G. candidus* are considered synonymous names, which actually refer to *Dipodascus geotrichum* (E.E. Butler & L.J. Petersen) Arx, 1977, the officially accepted and oldest scientific species name of the given fungal taxon.

4. Conclusions

There are no existing reports on the use of hierarchical screening approach to detect the reduction capacity of heterocyclic ketones among wild-type bacteria and fungi. According to our results, the tested fungal species were more efficient biocatalysts than the bacteria. However, while fungi are the most widespread source of oxidoreductases, the main disadvantage of using wild-type fungal cells instead of bacterial ones at a preparative scale is the downstream processing steps that are required [53].

Although the heteroatom present in the cyclic ketones did not significantly affect reaction conversions, general trends were observed: oxygen replacement afforded lower conversions and bacteria showed lower acceptance of sulfur-containing heterocycles than fungi.

Each microorganism selected on the basis of the results of our specific screening step showed relevant characteristics as a biocatalyst. *E. chrysanthemi* (CECT 509) and *M. racemosus* (STCC22) could reduce a bulky ketone with very high efficiency, resulting in substrate conversions of 100% and 93%, respectively. *G. candidum*

(GC1), *E. carotovora* (CECT 225), and *X. fragariae* (CECT 549) resulted in good conversions and enantiomeric excess for prochiral ketone substrates; *E. carotovora* (CECT 225) afforded (*S*)-*N*-Boc-3-pyrrolidinol with 87% conversion and more than 99%ee, and *X. fragariae* (CECT 549) quantitatively generated (*S*)-1-phenylethanol with 79%ee. Finally, *G. candidum* (GC1) reduced all prochiral ketones tested in our study. Most importantly, this strain of the fungus reduced acetophenone and resulted in the anti-Prelog isomer, making it of special interest for further studies and applications.

Moreover, the use of recombinant microorganisms that overexpress highly active reductases may lead to greater productivity. Therefore, enzyme overexpression of the most interesting biocatalysts identified in this study is currently in progress.

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Figure captions

Fig 1 General scheme of enzymatic reduction of (hetero)cyclic ketones. X=CH_2, *S*, *N*-Boc, O; n=0, 1, 7

Fig 2 Specific screening results. Biocatalyzed reduction of cyclohexanone to cyclohexanol by (**a)** bacterial and (**b)** fungal strains under growing conditions. Conversions determined by GC

Fig 3 (Hetero)cyclic ketones used as substrates. (**1a**) cyclohexanone, (**2a**) tetrahydro-4*H*-pyran-4-one, (**3a**) tetrahydro-4*H*-pyran-4-one, (**4a**) *N*-Boc-4-piperidone, (**5a**) cyclododecanone, (**6a**) *N*-Boc-3-piperidone, (**7a**) *N*-Boc-3-pyrrolidinone

Fig 4 Biocatalyzed reduction of non-prochiral (hetero)cyclic ketones. Biotransformations were carried out using microorganisms selected in the specific screening. Conversions were determined by GC. (**1b**) cyclohexanol, (**2b**) tetrahydro-4*H*-pyran-4-ol, (**3b**) tetrahydro-4*H*-pyran-4-ol, (**4b**) *N*-Boc-4-piperidinol, (**5b**) cyclododecanol

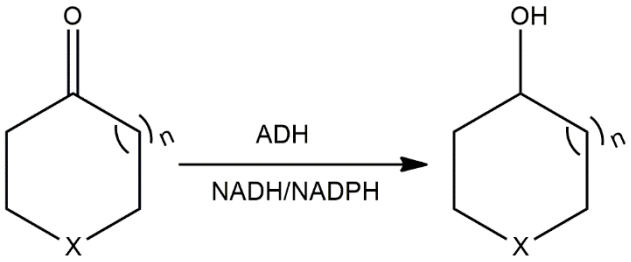
Fig 5 Biocatalyzed reduction of prochiral ketones. Biotransformations were carried out using microorganisms selected in the specific screening. Conversions were determined by GC. %ee (expressed inside square brackets) was obtained from chiral GC and confirmed against standard samples. (**6b**) *N*-Boc-3-piperidinol, (**7b**) *N*-Boc-3-pyrrolidinol, (**8b**) 1-phenylethanol.

Table 1. Reduction of cyclohexanone to cyclohexanol biocatalyzed by *Mucor piriformis* (SCR11). Optimization of the experimental conditions used in fungal screening. Conversions were determined by GC

Entry	Culture age (days)	Reaction time (days)	Initial inoculum (x10^4 spores/mL)	Aeration	Cell state[#]	Cyclohexanol conversion (%)
1	3	3	1	No	G	25
2	3	3	7.5	No	G	28
3	3	4	1	No	G	27
4	6	3	1	No	G	29
5	6	4	1	No	G	39
6	6	4	1	Yes	G	16
7	6	4	1	No	R	12

[#] G: growing; R: resting

Fig 1



1a -7a **1b -7b**

Fig 2

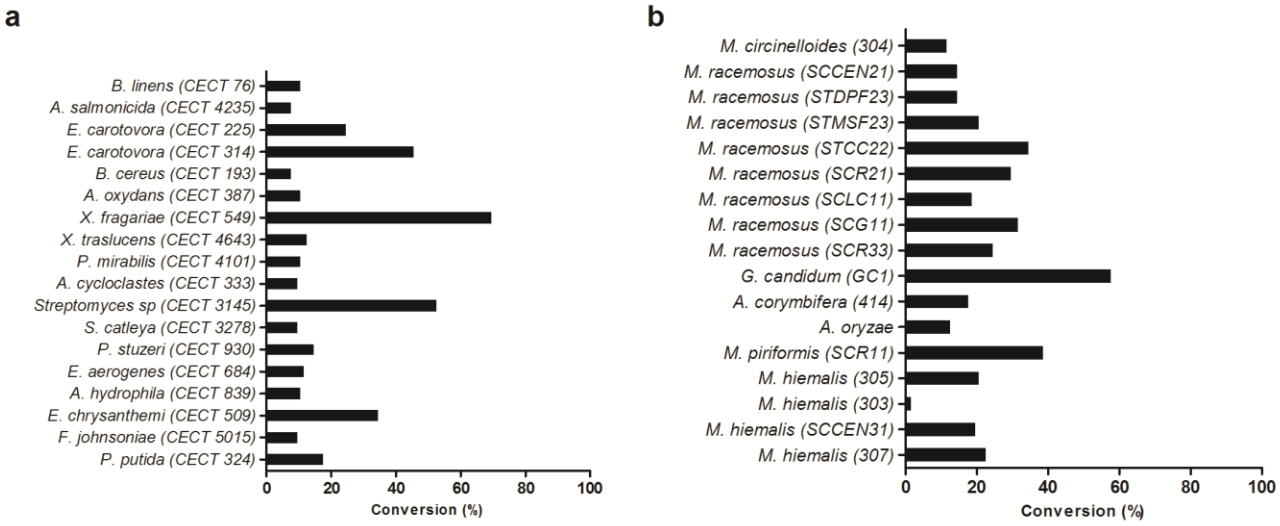


Fig 3

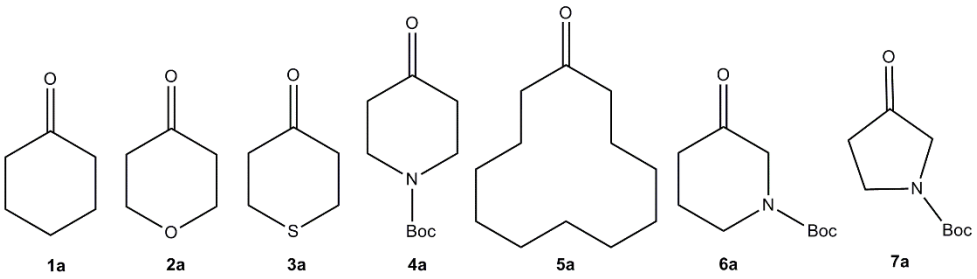


Fig 4

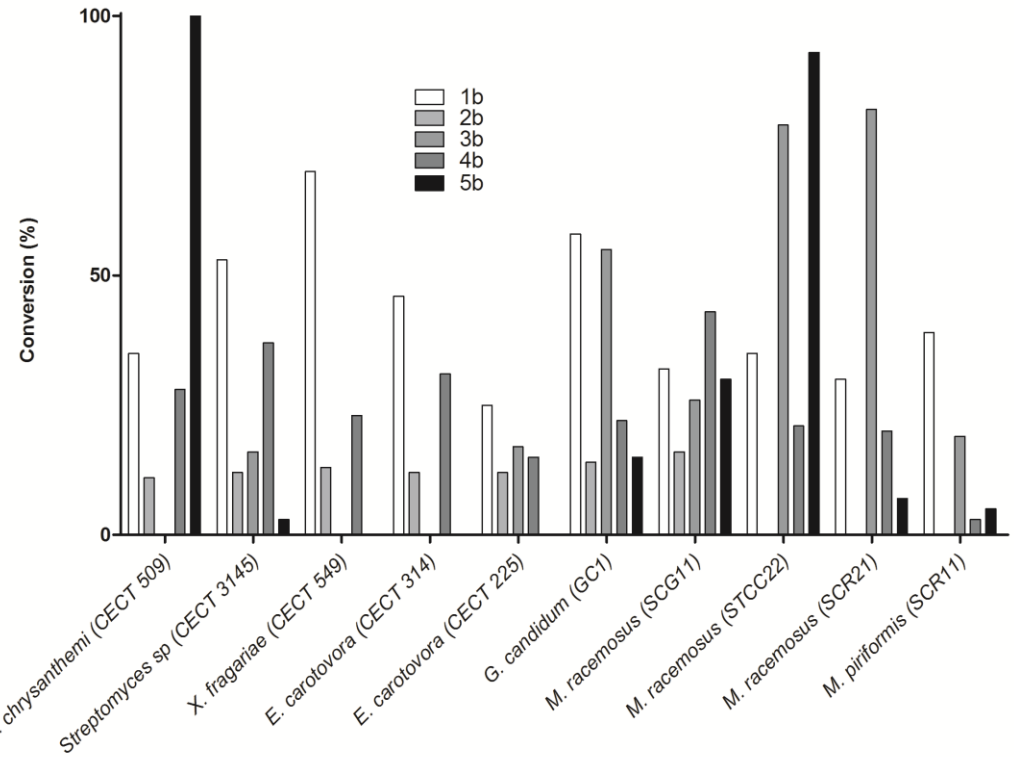


Fig 5