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Immobilized *Manihot esculenta* preparation as a novel biocatalyst in the enantioselective acetylation of racemic alcohols

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ABSTRACT

The enzymatic preparation obtained from a discard of *Manihot esculenta* roots has been successfully immobilized on calcium alginate hydrogels. This preparation has been tested as a chiral biocatalyst in the enzymatic acylation of a set of racemic aromatic alcohols. Depending on the reaction conditions, excellent enantioselectivities can be achieved. Some parameters that can alter the biocatalytic properties of the enzyme, such as solvent, temperature, acyl donor and substrate structure have been studied exhaustively in order to establish a deeper knowledge of this novel biocatalyst.

1. Introduction

Nowadays, the use of biotransformations presents a great interest for organic chemists in order to develop the preparation of high added value compounds.[1] It has been recognized that enzymes are able to accept a wide set of substrates catalyzing chemo-, regio- and enantioselective processes and represent an effective and sometimes preferable alternative to chemical synthesis. As a result, biocatalysts can be used in the synthesis of enantiomerically pure compounds with high efficiencies and low by-product formation.

Over the last few years, the search for inexpensive biocatalysts has become one of the main objectives in the field of biotransformations. From several easily available sources, plant cell cultures and whole plant cells have gained an increasing interest as a source from which to obtain novel enzymes.[2] These biocatalysts are easily obtained from markets and manipulated by organic chemists.

Manihot esculenta (cassava) is one of the major sources of carbohydrates in the world. During the treatment of these plant roots in order to obtain flour, several liters of an aqueous phase called *manipueira* are separated from the solid and later discarded. In the last years, it has been discovered that this discard presents some interesting properties as a surfactant and is an important source of enzymes. For example, one (*S*)-hydroxynitrile lyase from *M. esculenta* has been cloned, overexpressed, and studied as a chiral biocatalyst in the synthesis of cyanohydrins, which are interesting synthons in organic chemistry.[3] Recently, crude *manipueira* has been employed in the bioreduction of different aldehydes and ketones, and also in hydrolytic enzymatic processes.[4]

A practical technique to improve enzyme efficiency, operational stability, and performance in organic solvents is biocatalyst immobilization.[5] Among all the immobilization methods that have been described, the entrapment of biocatalysts on calcium alginate hydrogels is a well-known methodology.[6] By forming a solidified water phase in an aqueous-organic two-phase system, hydrogels protect the entrapped biocatalyst against detrimental effects of the environment. It provides an easy, fast, non-toxic, inexpensive, and versatile immobilization technique.

As a part of our ongoing research in the preparation and application of novel enzymatic systems, calcium alginate hydrogels obtained from the roots of *M. esculenta* have been prepared and studied as a biocatalyst in the selective enzymatic acylation of racemic secondary alcohols.

2. Results and discussion

The biocatalyst contained in the *manipueira* discarded from the treatment of the tuber *M. esculenta* was immobilized by the sodium alginate (1% w/v)-calcium chloride (5% w/v) method,[7] and obtained as an active enzyme form encapsulated in alginate spheres. The yield of the immobilization was determined through the concentration of protein present in the initial extract in comparison to the solution of calcium chloride used to precipitate the spheres, using the Hartree method.[8] Only a 3.8% of the active enzyme from the initial extract was not incorporated. The size of the beads was determined by Electronic Microscopy Sweeping, spheres of 1.0 mm diameter, as shown in Figure 1.

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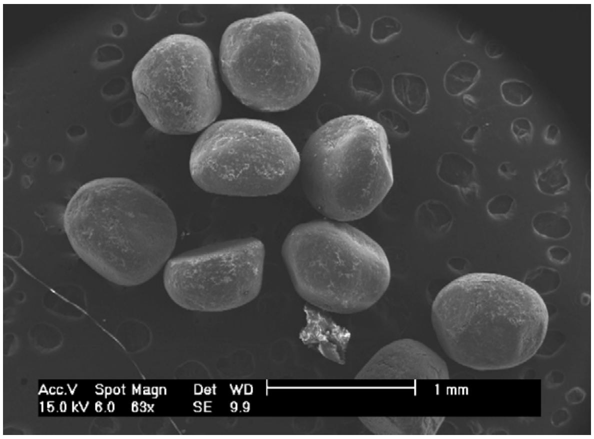


Figure 1. Scanning electron microscopy of *Manihot esculenta* hydrogels prepared by the sodium alginate-calcium chloride method.

Table 1
Acetylation of racemic phenylethan-1-ol catalyzed by immobilized *Manihot esculenta* employing vinyl acetate as acyl donor[a]

Entry	Solvent	Equiv VA	*T* (°C)	Time (h)	ee (*S*)-**1a**[b]	ee (*R*)-**1b**[b]	*c*[c] (%)	*E*[d]
1	Hexane	5	45	118	34	90	27	27
2	Toluene	5	45	116	10	94	14	34
3	$^i\text{Pr}_2\text{O}$	5	45	118	17	96	15	54
4	^{t}BuOMe	5	45	118	18	94	16	39
5	Et_2O	5	45	118	36	94	28	46
6	Vinyl acetate	—	45	118	15	90	14	23
7	THF	5	45	116	12	72	14	7
8	1,4-Dioxane	5	45	116	7	85	6	13
9	MeCN	5	45	116	4	88	5	16
10[e]	Et_2O	5	45	92	36	93	28	40
11[e]	$^i\text{Pr}_2\text{O}$	5	45	92	18	95	16	51
12	Et_2O	10	45	116	40	94	30	50
13	$^i\text{Pr}_2\text{O}$	10	60	116	26	92	25	30
14[f]	Et_2O	10	45	116	21	92	20	31
15[g]	Et_2O	10	45	116	17	74	19	8

[a] For reaction conditions, see Section 4.
[b] Determined by HPLC or GC.
[c] Conversion, $c = \text{ee}_s/(\text{ee}_s + \text{ee}_p)$.
[d] Enantiomeric ratio, $E = \ln[1 - c(1 + \text{ee}_p)]/\ln[1 - c(1 - \text{ee}_p)]$.
[e] Reactions were performed with double amount of biocatalyst.
[f] Resolution was performed with 0.7 equiv of NEt_3.
[g] The reaction was carried out in the presence of 0.7 equiv of 4-DMAP.

Once obtained, after the immobilized enzyme preparation, the system was subjected to study as a chiral catalyst in a set of biocatalyzed acylations of racemic aromatic alcohols. Firstly, the enzymatic acetylation of phenylethan-1-ol (±)-**1a** was studied using 5 equiv of acetic anhydride with immobilized *M. esculenta*. Reactions were performed in different organic solvents at 45 or 60 °C, being observed after 120 h conversions from 5% in 1,4-dioxane to 37% when using toluene. Very low enantioselectivities were achieved ($E \approx 1.0$)[9] for all the conditions employed. The acetylations performed in absence of the biocatalyst led to the recovery of the starting material, indicating that the lack of enantioselectivity is due to the enzyme biocatalytic properties.

In view of these results, we decided to employ a less reactive reagent, vinyl acetate (5 equiv) as an acyl donor in the enzymatic resolution of (±)-**1a**. First, we studied the influence of the organic solvent on the biocatalytic properties of the enzyme. In all the tested conditions, the (*R*)-enantiomer of the starting material was acetylated, to give an alcohol with an (*S*)-configuration. Thus it can be established that this enzyme preparation follows Kazlauskas' rule for the acylation of secondary alcohols.[10] Enzymatic reactions were performed at 45 °C, due to the low activity shown by the hydrogels of *M. esculenta* at lower temperatures.

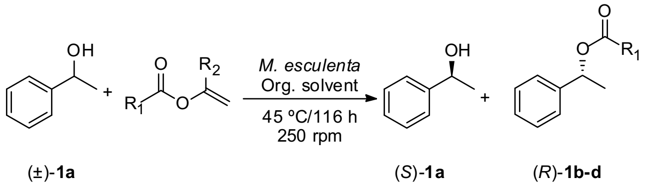
In order to determine if alginate beads could have any contribution in the acetylation reactions, experiments were carried out using only this polymer in the absence of *manipueria*. When the enzymatic acylation of (±)-**1a** was performed with vinyl acetate, only starting material was recovered after long reaction times. Compound (±)-**1b** was obtained with only 4.0% conversion after 120 h when acetic anhydride was used as the acyl donor, hence the presence of the alginate beads can affect slightly the enantioselectivities achieved when using this latest compound.

Table 1 summarizes the results achieved in the enzymatic acetylation of racemic phenylethan-1-ol **1a** employing different organic solvents. As shown in entry 3, the highest enantioselectivity was obtained with $^i\text{Pr}_2\text{O}$ ($E = 54$) in a process with moderate conversion ($c = 15\%$ after 118 h). The use of Et_2O (entry 5) led to the formation of (*R*)-**1b**. Good selectivity ($E = 46$), was observed with an increase in activity ($c = 28\%$) when compared with $^i\text{Pr}_2\text{O}$. Immobilized *M. esculenta* also exhibited good enantioselectivities when working with either toluene or ^{t}BuOMe (entries 2 and 4, respectively), but moderate reaction rates (conversions close to 15% after 5 days). When employing vinyl acetate as an acyl donor and solvent in the resolution of (±)-**1a** (entry 6), moderate conversion and enantioselectivity were measured. For the rest of the solvents tested, lower selectivities were found, in addition to a loss in the enzymatic activity when employing solvents with high hydrophilic character such as THF, 1,4-dioxane, or acetonitrile (entries 7–9).

Once $^i\text{Pr}_2\text{O}$ and Et_2O were established as the best solvents for the enzymatic resolution of (±)-**1a**, other parameters that could influence the biocatalytic properties of *M. esculenta* were studied. As shown in entries 10 and 11, the enzymatic resolution was performed using double the amount of biocatalyst hydrogels. Under these conditions, only a slight increase was observed in the conversion. The enzymatic acylation of (±)-phenylethanol was developed at 45 °C with 10 equiv of vinyl acetate (entry 12) resulting in a slight increase in enzymatic activity ($c = 30\%$ after 116 h). Taking into account that the temperature can have a marked effect on the activity and selectivity of enzymes, the resolution of (±)-**1a** was performed at 60 °C when using $^i\text{Pr}_2\text{O}$ as a solvent (entry 13), it being possible to obtain an increase in the enzymatic activity ($c = 25\%$ after 116 h) but with a significant loss in its selectivity. Finally, in order to improve the reaction rate obtained when performing the acetylations in Et_2O, different amines were used as additives in the resolution of (±)-**1a**.[11] The presence of 0.7 equiv of triethylamine or 4-DMAP (entries 14 and 15) led to a decrease in both conversion and selectivity with repect to the reaction without amine. This loss is especially significant when using 4-DMAP, a very low *E* value in the resolution of phenylethan-1-ol being observed.

Next, the influence of the type of acyl donor used in the biocatalytic acetylation of (±)-**1a** was analyzed (Table 2). Thus, it was observed that isopropenyl acetate was also a suitable donor for the transesterification of racemic phenylethan-1-ol, both in $^i\text{Pr}_2\text{O}$ and Et_2O (entries 1 and 2), lower activities and selectivities were measured when compared with vinyl acetate. By using this acyl donor, a slightly higher conversion and *E* value were obtained in Et_2O ($E = 26$ and conversion of 14% after 116 h). Ethyl methoxyacetate has also been employed as an acetylating reagent in the enzymatic resolution of alcohols and amines,[12] but when phenylethan-1-ol was treated with 10 equiv of this compound in Et_2O at 45 °C in the presence of immobilized *M. esculenta*, no reaction was observed after long times (entry 3). Finally, a less reactive vinyl ester such as vinyl benzoate was tested in the acylation of (±)-**1a** in both $^i\text{Pr}_2\text{O}$ and Et_2O (entries 4 and 5), but no formation of the corresponding ester was achieved.

Table 2
Enzymatic resolution of (±)-phenylethan-1-ol catalyzed by immobilized *Manihot esculenta* with different acyl donors[a]



Entry	R_1	R_2	Solvent	ee_S[b] (%)	ee_P[b] (%)	c[c] (%)	E
1	Me	CH_3	$^iPr_2O$	12	90	11	21
2	Me	CH_3	Et_2O	14	92	14	26
3	$MeOCH_2$	H	Et_2O	—	—	$\leqslant 1.0$	—
4	Ph	H	Pr_2O	—	—	$\leqslant 1.0$	—
5	Ph	H	Et_2O	—	—	$\leqslant 1.0$	—

[a] Reactions were carried out employing 10 equiv of acyl donor, except for entries 1 and 2 (5 equiv). For other details, see Section 4.
[b] Determined by GC or HPLC.
[c] Conversion, $c = ee_s/(ee_s + ee_p)$.

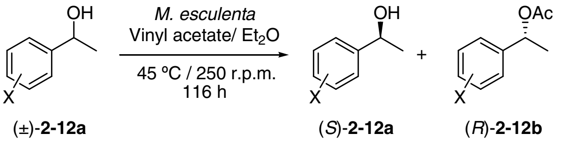
At this point, we decided to study the behavior of *M. esculenta* hydrogels in the biocatalyzed acetylation of racemic phenylethan-1-ols presenting different substituents in the aromatic moiety. The best reaction conditions found for the enzymatic resolution of (±)-**1a** (10 equiv of vinyl acetate, Et_2O, and 45 °C) were applied in the acetylation of different substituted phenylethan-1-ols, as summarized in Table 3. When employing compounds with strong electron-withdrawing groups (4-NO_2 and 3-NO_2) as substrates, it was observed that the presence of the nitro group at the *meta*-position increased to a great extent the enantioselectivity of the process, being achieved an excellent E value ($E > 200$) in the resolution of (±)-**3a** with a conversion of 12% after 116 h. When the nitro group was located at the *para*-position, the enantioselectivity decreased ($E = 53$) in a reaction with a higher conversion ($c = 18\%$). Also, the acetylation of 3-trifluoromethylphenylethan-1-ol (±)-**4a** led to the formation of (*S*)-**4a** and (*R*)-**4b** with excellent enantioselectivity in a process with moderate conversion. Some halide-substituted 1-phenylethan-1-ols were analyzed; the 4-bromo- and 4-chloro-derivatives [(±)-**5a** and (±)-**8a**] gave high E values ($E > 100$) with conversions close to 30% after 116 h, while for the *meta*-substituted alcohols (±)-**6a** and (±)-**9a**, an important loss in both conversions and enantioselectivities was observed (E values close to 50). The effect of the *ortho*-substitution was also analyzed by reacting racemic 2-chlorophenylethan-1-ol **7a** with vinyl acetate and immobilized *M. esculenta* in Et_2O. After 116 h, (*R*)-**7b** was obtained with an 18% conversion and a low enantioselectivity. We can establish that there is a clear influence of the halide atom position on the biocatalytic properties of the enzyme, higher enantioselectivities for *para*-derivative, and low E values for the *ortho*-one being obtained.

Next, the enzymatic acylation of racemic 3-methoxyphenylethan-1-ol **10a** was performed with good enantioselectivity and moderate conversion. When a phenylethan-1-ol derivative with an electron-donating group at the *para*-position was employed as a substrate of *Manihot esculenta* [(±)-**11a**], a moderate E value and conversion were achieved. The enzymatic acetylation of 4-hydroxyphenylethan-1-ol (±)-**12a** led to the formation of the monoester (*R*)-**12b** with good selectivity ($E = 50$) and moderate conversion. Only trace amounts of the diacetylated product (less than 1%) were observed when the crude reaction was analyzed by GC.

The effect of the substrate structure on the biocatalytic properties of the enzyme was also analyzed by testing phenyl alcohols possessing larger alkyl chains than the methyl group, as shown in Table 4. When racemic 1-phenylpropan-2-ol **13a** was acetylated in the presence of immobilized *M. esculenta*, (*S*)-**13a** and the corresponding ester (*R*)-**13b** were obtained in a process with low conversion ($c = 12\%$ after 116 h) and moderate enantioselectivity, lower values than those observed with racemic phenylethan-1-ol. The acetylation of longer chain alcohols, as for example (±)-**14a**, led to the formation of (*S*)-**14a** and (*R*)-**14b** with a higher conversion ($c = 20\%$) and lower enantioselectivity. The acetylation of an allyl alcohol such as (±)-1-phenylbut-3-en-1-ol gave a low activity and selectivity, with only 6% of (*R*)-**15b** after 116 h being obtained. The biocatalyzed acylation of racemic 2-hydroxy-2-phenylacetonitrile took place with low conversion and enantioselectivity, with (*S*)-**16b** being obtained only 4% after a long reaction time. When the alcohol moiety is located further away from the aromatic ring, there was also an important loss in the biocatalytic properties of immobilized *M. esculenta*, as shown in Table 4 for 1-phenylpropan-2-ol (±)-**17a**. Finally, the immobilized form of *Manihot* was tested in the enzymatic acetylation of a tertiary alcohol, but no reaction was observed when 2-phenylpropan-2-ol **18a** was treated with vinyl acetate in Et_2O at 45 °C for 116 h.

To prove the efficiency of this biocatalyst, we have also investigated the feasibility of reusing the immobilized *M. esculenta* under

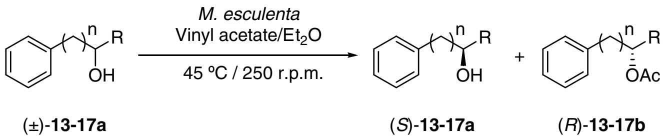
Table 3
Immobilized *M. esculenta*-catalyzed acetylation of substituted phenylethan-1-ols[a]



Compound	X	ee_s[b] (%)	ee_p[b] (%)	c[c] (%)	E
1a	H	40	94	30	50
2a	4-NO_2	21	95	18	53
3a	3-NO_2	13	99	12	>200
4a	3-CF_3	9	99	12	>200
5a	4-Cl	33	97	28	105
6a	3-Cl	11	96	12	51
7a	2-Cl	19	88	18	19
8a	4-Br	35	97	28	111
9a	3-Br	13	96	16	59
10a	3-OMe	31	95	27	55
11a	4-OMe	31	91	28	29
12a	4-OH	18	95	16	50

[a] Reactions were carried out employing 10 equiv of vinyl acetate. For other details, see Section 4.
[b] Determined by GC or HPLC.
[c] Determined by ^1H NMR.

Table 4
Immobilized *Manihot esculenta*-catalyzed acetylation of phenyl alcohols[a]

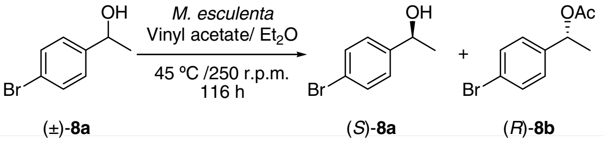


Compound	R	n	ee_s[b] (%)	ee_p[b] (%)	c[c] (%)	E
13a	Et	0	9	93	12	32
14a	Bu	0	13	58	20	4
15a	Allyl	0	5	85	6	12
16a[d]	CN	0	2	62	4	4
17a	Me	1	12	9	6	1

[a] For reaction details, see Section 4.
[b] Determined by GC or HPLC.
[c] Determined by ^1H NMR.
[d] Absolute configuration is reversed due to a change in the substituent priority according to the sequence rules.

Table 5

Reuse of immobilized *Manihot esculenta*-catalyzed in the enzymatic acetylation of 4-bromophenylethan-1-ol[a]



Cycle no.	ee$_s$[b] (%)	ee$_p$[b] (%)	c[c] (%)	E
1	30	98	25	103
2	32	97	25	94
3	35	97	26	90
4	20	97	17	72
5	24	91	17	24
6	8	71	10	7
7	5	71	6	6

[a] Reactions were carried out employing 10 equiv of vinyl acetate. For other details, see Section 4.

[b] Determined by GC or HPLC.

[c] Determined by ^{1}H NMR.

the optimal reaction conditions found for the acetylation of 1-(4-bromophenyl)ethan-1-ol, (±)-**8a**: Et_2O as organic solvent, 10 equiv of vinyl acetate as acylating agent, and 116 h of reaction time at 45 °C at 250 rpm. As shown in Table 5, the reuse of the immobilized biocatalyst afforded almost the same enantioselectivity and activity in the second and third cycles, with only a slight loss in these biocatalytic properties for the fourth one ($E = 72$ and $c = 17\%$). When the biocatalyst was employed in the fifth cycle, the conversion obtained was the same as the previous cycle, but the enantioselectivity in the resolution of (±)-**8a** decreased by a large extent ($E = 24$). For the next two cycles, the enzyme seemed to be almost completely inactivated [(*S*)-**8b** was obtained with only 6% of conversion and $E = 6$ for the seventh cycle].

3. Conclusion

In this paper, an enzymatic preparation obtained from the discard waters of the treatment of *M. esculenta* roots was immobilized with high efficiency by the alginate-calcium chloride method in order to form the corresponding hydrogels. This preparation has been employed as a chiral biocatalyst in the enzymatic resolution of different alcohols. Analysis of the different reaction parameters in the resolution of phenylethan-1-ol led to the highest activities and selectivities when using 10 equiv of vinyl acetate as an acyl donor in Et_2O or $^iPr_2O$ and 45 °C. Depending on the substrate structure, excellent enantioselectivities can be achieved with certain substituted phenylethan-1-ols presenting electron-withdrawing groups, while the enzyme presented high preference for the *para*-position when using bromo- and chlorophenylethan-1-ols. The enzymatic resolution of longer alkyl chain alcohols took place with moderate to low selectivities. This enzymatic preparation has been demonstrated to be reusable for four cycles with only a slight loss in the activity and selectivity. Hence, by properly immobilizing a food manufacture discard, we have been able to obtain a really promising biocatalyst for the enzymatic resolution of racemic alcohols.

4. Experimental

4.1. General

Chemical reactions were monitored by analytical TLC, performed on Merck Silica Gel 60 F_{254} plates, and visualized by UV irradiation. Flash chromatography was carried out with Silica Gel 60 (230–240 mesh, Merck). IR spectra were recorded on a Perkin–Elmer 1720-X infrared Fourier transform spectrophotometer using KBr pellets. Optical rotations were measured using a Perkin–Elmer 241 polarimeter and are quoted in units of 10^{-1} deg cm^2 g^{-1}. ^{1}H NMR, ^{13}C NMR, and DEPT spectra were recorded with TMS (tetramethylsilane) as the internal standard using Bruker AC-300 (^{1}H, 300.13 MHz and ^{13}C, 75.4 MHz) and Bruker AC-300-DPX (^{1}H, 300.13 MHz and ^{13}C: 75.4 MHz) spectrometers. The chemical shift values (δ) are given in ppm and the coupling constants (J) in Hertz (Hz). APCI$^+$ using a Hewlett Packard 1100 chromatograph mass detector or EI$^+$ with a Hewlett Packard 5973 mass spectrometer were used to record mass spectra (MS).

GC analyses were performed on a Hewlett Packard 6890 Series II chromatograph equipped with a CP-Chiralsil DEX CB column (30 m × 0.25 mm × 0.25 μm, 1.0 bar N_2) from Varian for determining the enantiomeric purities and a HP-1 (crosslinked methyl siloxane, 30 m × 0.25 mm 0.25 μm, 1.0 bar N_2) from Hewlett–Packard for measuring the conversion values. For all the analyses, the injector temperature is 225 °C and the FID temperature is 250 °C. HPLC analyses were developed with a Hewlett Packard 1100 LC liquid chromatograph equipped with a Chiralcel OD (0.46 cm × 25 cm), Chiralcel OB-H (0.46 cm × 25 cm), or Chiralpak IA (0.46 cm × 25 cm) chiral column from Daicel.

Alcohols (±)-**1a**, (±)-**8a**, (±)-**9a**, (±)-**11a**, (±)-**13a**, (±)-**15–17a**, **18a**, and 4-hydroxyacetophenone were purchased from Sigma–Aldrich–Fluka. Compounds (±)-**2a**, (±)-**5-7a**, (±)-**14a**, and 3-nitroacetophenone were products from Alfa Aesar. 3-Methoxyacetophenone and (±)-**4a** are available at Acros Organics. Starting racemic alcohols **3a**, **10a**, and **12a** were prepared from commercial ketones by treatment with $NaBH_4$ in methanol. In all cases, high yields were achieved. Racemic esters (±)-**1**–**17b** and **18b** were synthesized by the chemical acetylation of the corresponding alcohols (yields higher than 80%) employing acetic anhydride and triethylamine in CH_2Cl_2. Preparation of racemic **1c** and **1d** was performed by the addition of pyridine and benzoic anhydride or methoxyacetyl chloride, respectively, to a solution of (±)-**1a** in CH_2Cl_2, with good yields being obtained. Solvents were distilled over an appropriate desiccant under nitrogen. All other reagents and solvents were of the highest quality grade available, supplied by Sigma–Aldrich–Fluka.

Absolute configurations of alcohols **1a**, **8a**, and **16a** were determined by comparison of their GC chromatograms with samples obtained from the commercial chiral alcohols (*S*)-**1a**, (*S*)-**8a**, and (*R*)-**16a**. For compounds **2a**,[2a] **3a**,[13a] **4a**,[12b] **5a**,[2a] **6a**,[13b] **7a**,[13b] **9a**,[13c] **10a**,[13d] **11a**,[2a] **12a**,[2a] **13a**,[13e] **14a**,[13e] **15a**,[13f] and **17a**,[13g] the absolute configurations were established by comparison of the specific rotation measured with the ones previously reported.

4.2. Plant material and extraction

M. esculenta roots were collected at the Ematerce farm (Caucaia, CE, Brazil). Identification was performed by Professor Edson P. Nunes. Voucher specimen number 34831 has been deposited at the Herbarium Prisco Bezerra (Departamento de Biologia, Universidade Federal do Ceará, Brazil). The roots were rinsed with a 5% aqueous solution of sodium hypochlorite and distilled water and after this, 5.0 kg of *M. esculenta* was grated and then pressed in order to separate the aqueous phase *manipueira* (2.5 L) from the solid residue (3.0 kg).

4.3. Immobilization process

Biocatalyst from *M. esculenta* was immobilized using the calcium alginate technique. *Manipueira* (300 mL) was shaken for 3 h in the presence of 1.0% w/v sodium alginate. After homogenization, this mixture was dropped into 400 mL of a 5.0% w/v solution of calcium chloride, using a syringe with a 1.0 mm internal diameter needle. Upon contact with the solution, the drops were gelled to form constant and defined-size spheres, which remained in the solution for 12 h, under mild agitation, to complete gel formation. After hardening, microspheres were washed several times with water and acetone, and dried at room temperature until constant weight (5.4 g). Determination of the protein content using the Hartree method has been done in triplicate at the initial and final stages of the immobilization process. Experiments allowed the calculation of protein content not incorporated into the beads (3.8%).

4.4. Size of beads

By varying the orifice used for dropping the enzyme-sodium alginate mixture, the dimension of the beads was varied. In this case, the spherical diameter of the particles was determined as 1.0 mm by measuring microscopically the dimensions of beads using Electronic Microscopy of Sweeping (MEV), with the aid of a Philips XL-30 microscope.

4.5. Typical procedure for the enzymatic acetylation of racemic alcohols catalyzed by immobilized *M. esculenta*

Unless otherwise stated, the immobilized preparations of *M. esculenta* (30 mg) and vinyl acetate (5 or 10 equiv, except when used as the solvent) were added to a solution of the corresponding racemic alcohol (±)-**1a**–**17a**, and **18a** (30 mg, 1.0 equiv) in the selected organic solvent (4.0 mL) at room temperature under a nitrogen atmosphere. The resulting mixture was shaken at the established temperature and at 250 rpm on an orbital shaker. The progress of the reaction was monitored by TLC analysis. Once the reaction was finished, the enzyme was filtered off, washed with ethyl acetate, and the solvent evaporated under reduced pressure. The crude residue was then purified by flash chromatography using *n*-hexane/ethyl acetate 7:3 (for compounds **2**–**4a**, **12a**, and **14a**) or 8:2 (**1a**, **5**–**11a**, **13a**, and **15**–**18a**) in order to obtain the optically active alcohols (*S*)-**1**–**15a**, (*R*)-**16a**, (*S*)-**17a**, and **18a** and the corresponding esters (*R*)-**1**–**15a**, (*S*)-**16b**, (*R*)-**17b**, and **18b**.

4.6. Study of enzyme recycling in the acetylation of (±)-8a catalyzed by immobilized *M. esculenta*

To a solution of (±)-**8a** (30 mg, 0.15 mmol) in Et_2O (4 mL) were added 50 mg of immobilized *M. esculenta* and vinyl acetate (138 μL, 1.5 mmol) under a nitrogen atmosphere. The reaction was stirred at 45 °C and 250 rpm for 116 h. After this, the enzyme was filtered off, washed with dry Et_2O, and stored under nitrogen atmosphere in order to start a new cycle. The biocatalyst was used repeatedly in the subsequent enzymatic resolutions, following in all cases the same procedure. In order to determine the conversions and the enantiomeric excesses of the alcohol (*S*)-**8a** and the ester (*R*)-**8b**, flash chromatography was performed using *n*-hexane/ethyl acetate 8:2 as eluent in each cycle.

4.6.1. (*S*)-Phenylethan-1-ol, (*S*)-1a

Determination of the ee by HPLC analysis: Chiralcel OB-H, 20 °C, *n*-hexane/2-propanol (95:5), 0.8 mL/min; t_R (*S*) 10.2 min; t_R (*R*) 15.5 min.

4.6.2. (*R*)-1-Phenylethyl acetate, (*R*)-1b

Determination of the ee by GC analysis. 70 °C (5 min), 3 °C/min, 200 °C; t_R (*S*) 17.5 min; t_R (*R*) 19.1 min.

4.6.3. (*S*)-4-Nitrophenylethan-1-ol, (*S*)-2a

Determination of the ee by HPLC analysis: Chiralpak IA, 20 °C, *n*-hexane/2-propanol (96:4), 0.6 mL/min; t_R (*S*) 35.2 min; t_R (*R*) 33.7 min.

4.6.4. (*R*)-1-(4-Nitrophenyl)ethyl acetate, (*R*)-2b

Determination of the ee by GC analysis. 70 °C (10 min), 2 °C/min, 200 °C; t_R (*S*) 54.9 min; t_R (*R*) 55.9 min.

4.6.5. (*S*)-3-Nitrophenylethan-1-ol, (*S*)-3a

Determination of the ee by HPLC analysis: Chiralcel OB-H, 20 °C, *n*-hexane/2-propanol (95:5), 0.8 mL/min; t_R (*S*) 23.8 min; t_R (*R*) 27.3 min.

4.6.6. (*R*)-1-(3-Nitrophenyl)ethyl acetate, (*R*)-3b

Determination of the ee by GC analysis. 70 °C (10 min), 2 °C/min, 200 °C; t_R (*S*) 50.4 min; t_R (*R*) 51.5 min.

4.6.7. (*S*)-3-Trifluoromethylphenylethan-1-ol, (*S*)-4a

Determination of the ee by GC analysis. 70 °C (10 min), 2 °C/min, 200 °C; t_R (*S*) 35.6 min; t_R (*R*) 34.2 min.

4.6.8. (*R*)-1-(3-Trifluoromethylphenyl)ethyl acetate, (*R*)-4b

Determination of the ee by GC analysis. 70 °C (10 min), 2 °C/min, 200 °C; t_R (*S*) 22.4 min; t_R (*R*) 26.1 min.

4.6.9. (*S*)-4-Chlorophenylethan-1-ol, (*S*)-5a

Determination of the ee by GC analysis. 70 °C (10 min), 2 °C/min, 200 °C; t_R (*S*) 45.6 min; t_R (*R*) 44.4 min.

4.6.10. (*R*)-1-(4-Chlorophenyl)ethyl acetate, (*R*)-5b

Determination of the ee by GC analysis. 70 °C (10 min), 2 °C/min, 200 °C; t_R (*S*) 37.8 min; t_R (*R*) 39.9 min.

4.6.11. (*S*)-3-Chlorophenylethan-1-ol, (*S*)-6a

Determination of the ee by HPLC analysis: Chiralcel OB-H, 20 °C, *n*-hexane/2-propanol (95:5), 0.8 mL/min; t_R (*S*) 9.8 min; t_R (*R*) 12.5 min.

4.6.12. (*R*)-1-(3-Chlorophenyl)ethyl acetate, (*R*)-6b

Determination of the ee by GC analysis. 70 °C (10 min), 2 °C/min, 200 °C; t_R (*S*) 34.6 min; t_R (*R*) 36.9 min. $[\alpha]_D^{25} = +15.8$ (*c* 0.80, CH_2Cl_2), ee 96%.

4.6.13. (*S*)-2-Chlorophenylethan-1-ol, (*S*)-7a

Determination of the ee by HPLC analysis: Chiralcel OB-H, 20 °C, *n*-hexane/2-propanol (95:5), 0.8 mL/min; t_R (*S*) 7.4 min; t_R (*R*) 5.7 min.

4.6.14. (*R*)-1-(2-Chlorophenyl)ethyl acetate, (*R*)-7b

Determination of the ee by GC analysis. 70 °C (10 min), 2 °C/min, 200 °C; t_R (*S*) 43.4 min; t_R (*R*) 45.7 min. $[\alpha]_D^{25} = +9.6$ (*c* 0.64, CH_2Cl_2), ee 88%.

4.6.15. (*S*)-4-Bromophenylethan-1-ol, (*S*)-8a

Determination of the ee by GC analysis. 70 °C (10 min), 2 °C/min, 200 °C; t_R (*S*) 50.6 min; t_R (*R*) 49.7 min.

4.6.16. (*R*)-1-(4-Bromophenyl)ethyl acetate, (*R*)-8b

Determination of the ee by GC analysis. 70 °C (10 min), 2 °C/min, 200 °C; t_R (*S*) 43.2 min; t_R (*R*) 45.0 min.

4.6.17. (*S*)-3-Bromophenylethan-1-ol, (*S*)-9a

Determination of the ee by HPLC analysis: Chiralcel OB-H, 20 °C, *n*-hexane/2-propanol (95:5), 0.8 mL/min; t_R (*S*) 11.0 min; t_R (*R*) 14.2 min.

4.6.18. (*R*)-1-(3-Bromophenyl)ethyl acetate, (*R*)-9b

Determination of the ee by GC analysis. 70 °C (10 min), 2 °C/min, 200 °C; t_R (*S*) 39.5 min; t_R (*R*) 41.3 min. $[\alpha]_D^{25} = +13.7$ (*c* 0.75, CH_2Cl_2), ee 96%.

4.6.19. (*S*)-3-Methoxyphenylethan-1-ol, (*S*)-10a

Determination of the ee by HPLC analysis: Chiralcel OB-H, 20 °C, *n*-hexane/2-propanol (95:5), 0.8 mL/min; $t_{\rm R}$ (*S*) 21.5 min; $t_{\rm R}$ (*R*) 31.1 min.

4.6.20. (*R*)-1-(3-Methoxyphenyl)ethyl acetate, (*R*)-10b

Determination of the ee by GC analysis. 70 °C (10 min), 2 °C/min, 200 °C; $t_{\rm R}$ (*S*) 38.8 min; $t_{\rm R}$ (*R*) 40.0 min. $[\alpha]_{\rm D}^{25} = +27.8$ (*c* 0.94, CH_2Cl_2), ee 95%.

4.6.21. (*S*)-4-Methoxyphenylethan-1-ol, (*S*)-11a

Determination of the ee by HPLC analysis: Chiralcel OB-H, 20 °C, *n*-hexane/2-propanol (95:5), 0.8 mL/min; $t_{\rm R}$ (*S*) 25.2 min; $t_{\rm R}$ (*R*) 37.7 min.

4.6.22. (*R*)-1-(4-Methoxyphenyl)ethyl acetate, (*R*)-11b

Determination of the ee by GC analysis. 70 °C (10 min), 2 °C/min, 200 °C; $t_{\rm R}$ (*S*) 41.0 min; $t_{\rm R}$ (*R*) 42.5 min.

4.6.23. (*R*)-4-Hydroxyphenylethan-1-ol, (*S*)-12a

Determination of the ee by HPLC analysis: Chiralcel OB-H, 20 °C, *n*-hexane/2-propanol (95:5), 0.8 mL/min; $t_{\rm R}$ (*S*) 34.8 min; $t_{\rm R}$ (*R*) 41.1 min.

4.6.24. (*R*)-1-(4-Hydroxyphenyl)ethyl acetate, (*R*)-12b

Determination of the ee by HPLC analysis: Chiralcel OB-H, 20 °C, *n*-hexane/2-propanol (95:5), 0.8 mL/min; $t_{\rm R}$ (*S*) 32.3 min; $t_{\rm R}$ (*R*) 22.3 min. $[\alpha]_{\rm D}^{25} = +11.2$ (*c* 1.00, CH_2Cl_2), ee 95%.

4.6.25. (*S*)-1-Phenylpropan-1-ol, (*S*)-13a

Determination of the ee by HPLC analysis: Chiralcel OB-H, 20 °C, *n*-hexane/2-propanol (95:5), 0.8 mL/min; $t_{\rm R}$ (*S*) 7.7 min; $t_{\rm R}$ (*R*) 9.2 min.

4.6.26. (*R*)-1-Phenylprop-1-yl acetate, (*R*)-13b

Determination of the ee by GC analysis. 70 °C (5 min), 3 °C/min, 200 °C; $t_{\rm R}$ (*S*) 20.0 min; $t_{\rm R}$ (*R*) 20.9 min.

4.6.27. (*S*)-1-Phenylpentan-1-ol, (*S*)-14a

Determination of the ee by HPLC analysis: Chiralcel OB-H, 20 °C, *n*-hexane/2-propanol (97:3), 0.5 mL/min; $t_{\rm R}$ (*S*) 15.0 min; $t_{\rm R}$ (*R*) 17.0 min.

4.6.28. (*R*)-1-Phenylpentan-1-yl acetate, (*R*)-14b

Determination of the ee by HPLC analysis: Chiralcel OB-H, 20 °C, *n*-hexane/2-propanol (97:3), 0.5 mL/min; $t_{\rm R}$ (*S*) 12.2 min; $t_{\rm R}$ (*R*) 9.0 min.

4.6.29. (*S*)-1-Phenylbut-3-en-1-ol, (*S*)-15a

Determination of the ee by HPLC analysis: Chiralcel OB-H, 20 °C, *n*-hexane/2-propanol (98:2), 0.6 mL/min; $t_{\rm R}$ (*S*) 17.3 min; $t_{\rm R}$ (*R*) 22.8 min.

4.6.30. (*R*)-1-Phenylbut-3-en-1-yl acetate, (*R*)-15b

Determination of the ee by HPLC analysis: Chiralcel OB-H, 20 °C, *n*-hexane/2-propanol (98:2), 0.6 mL/min; $t_{\rm R}$ (*S*) 10.8 min; $t_{\rm R}$ (*R*) 9.8 min.

4.6.31. (*R*)-2-Hydroxy-2-phenylacetonitrile, (*S*)-16a

Determination of the ee by GC analysis after derivatization by acetylation with acetic anhydride. 70 °C (5 min), 3 °C/min, 200 °C; $t_{\rm R}$ (*S*) 26.9 min; $t_{\rm R}$ (*R*) 28.7 min.

4.6.32. (*S*)-Cyano(phenyl)methyl acetate, (*R*)-16b

Determination of the ee by GC analysis. 70 °C (5 min), 3 °C/min, 200 °C; $t_{\rm R}$ (*S*) 26.9 min; $t_{\rm R}$ (*R*) 28.7 min.

4.6.33. (*S*)-1-Phenylpropan-2-ol, (*S*)-17a

Determination of the ee by HPLC analysis: Chiralpak IA, 25 °C, *n*-hexane/2-propanol (98:2), 0.3 mL/min; $t_{\rm R}$ (*S*) 38.9 min; $t_{\rm R}$ (*R*) 41.3 min.

4.6.34. (*R*)-1-Phenylprop-2-yl acetate, (*R*)-17b

Determination of the ee by GC analysis. 70 °C (5 min), 3 °C/min, 200 °C; $t_{\rm R}$ (*S*) 21.8 min; $t_{\rm R}$ (*R*) 22.8 min.

4.6.35. 2-Phenylpropan-2-ol, 18a

Determination of the degree of conversion by GC analysis, HP-1, 60 °C (5 min) 2 °C/min 200 °C; $t_{\rm R}$ 6.7 min.

4.6.36. 2-Phenylprop-2-yl acetate, 18b

$t_{\rm R}$ 14.2 min.

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