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Resolution of N-protected amino acid esters using whole cells of *Candida parapsilosis* ATCC 7330

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ABSTRACT

Whole cells of *Candida parapsilosis* ATCC 7330 were used for the resolution of *N*-acetyl amino acid esters. Excellent enantioselectivities (E = 40 to >500) were achieved for the resolution of N-protected protein and non-protein amino acid esters giving good yields (28–50%) and high enantiomeric excesses (up to >99%) for both enantiomers.

1. Introduction

Enantiomerically pure natural and unnatural α-amino acids and their derivatives are useful intermediates in the pharmaceutical, biotech, and chemical industries.[1] Therefore, it is important to synthesize enantiomerically pure α-amino acids in high yields and enantiomeric excesses.[2] Among the various biocatalytic methods, the enzymatic resolution of DL-amino acids is an efficient method for the preparation of amino acids as it allows the recovery of both enantiomers. Isolated hydrolase enzymes such as proteases and lipases have been used extensively for the resolution of racemic amino acids through the enantioselective hydrolysis of their esters.[3] Detergent (alkaline) proteases used for the resolution of amino acid esters are mostly from bacterial origin, and require cost-intensive technologies to obtain a microorganism-free enzyme preparation.[3m] Using a whole cell biocatalyst is advantageous because the enzymes in the whole cell system are regarded as immobilized by entrapment or attachment to the cell wall[8a] and no isolation or purification of the enzyme is needed. Industrial production of amino acids mostly involves microbial processes because cell disruption and concentration of the enzyme solution are dispensable in whole cell-catalyzed reactions.[4] For example, whole cells of recombinant *Escherichia coli* are reported for the asymmetric hydrolysis of amino acid hydantoins[5] and *N*-acyl amino acids[6] to produce enantiomerically pure amino acids. There are very few reports exploring the hydrolytic properties of whole cells for the resolution of amino acid esters. In whole cell-mediated enzymatic resolution, baker's yeast (*Saccharomyces cerevisiae*) was employed for the resolution of racemic *N*-acetyl amino acid esters. The unreacted substrates were recovered, but the corresponding product *N*-acetyl-L-amino acids were not isolated.[7] Baker's yeast cross-linked with glutaraldehyde and immobilized with calcium alginate was used for the enantioselective hydrolysis of methyl esters of racemic *N*-acetyl-α-amino acids in reverse micellar media.[8] Immobilized whole cells of *Sulfolobus solfataricus*, a thermoacidophilic archaeobacterium, trapped in sodium alginate were reported for the hydrolysis of the methyl esters of natural racemic α-amino acids.[9] Given the advantages of using whole cells and expanding upon the scope of *Candida parapsilosis* ATCC 7330 which is known to deracemize the racemic α,β-hydroxy esters and allylic alcohols, and reduce α- and β-oxo-esters and aryl imines in an enantioselective manner,[10,17] we herein report the resolution of natural and unnatural *N*-acetyl amino acid esters using this biocatalyst. Both the enantiomers were isolated in high yields and enantiomeric excess (ee).

2. Results and discussion

In preliminary studies, the racemic *N*-free phenylalanine ethyl ester **1** was subjected to hydrolysis using the whole cells of *C. parapsilosis* ATCC 7330 (Scheme 1). The natural enantiomer L-phenylalanine ethyl ester was hydrolyzed into its L-acid. At 25 °C, 38% conversion was reached in 10 min and the reaction led to rather low enantiomeric excesses of both the remaining substrate D-ester **2** (35% ee) and the L-acid **3** (57% ee) with an E ratio of 5.1. A similar type of hydrolysis with low E ratio (E = 1.2–4.2) was reported using *Aspergillus oryzae* protease for the methyl esters of racemic *N*-free amino acids[11] and in this case the highest enantioselectivities were obtained by employing esters bearing a longer alkyl chain (isobutyl ester).

When the amino group was replaced by the acetylamino group an increase in the enantioselectivity was observed that is, *N*-acetyl-DL-phenylalanine ethyl ester was hydrolyzed with good enantiose-

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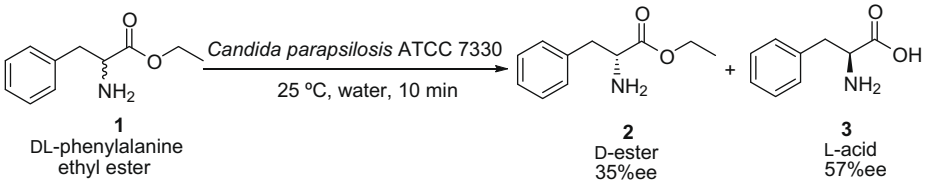


Scheme 1. Resolution of DL-phenylalanine ethyl ester **1** using the whole cells of *Candida parapsilosis* ATCC 7330.

lectivity $E = 105$ compared to the *N*-free phenylalanine ethyl ester with an increased time (2 h) of hydrolysis. This led to a further investigation of the hydrolysis of a series of natural and unnatural *N*-acetyl amino acid ethyl esters **4** using the whole cells of *C. parapsilosis* ATCC 7330 (Scheme 2). The natural enantiomer *N*-acetyl-L ester was hydrolyzed into *N*-acetyl-L-acid **6**, while neither the D-enantiomer **5** nor the amide group was affected in both enantiomers. The natural *N*-acetyl amino acid esters phenylalanine **4a**, tyrosine **4g**, and tryptophan **4f** were hydrolyzed to give good ee (70–99%) of the products and excellent ee (95 to >99) of the substrates (Table 1, entries a, f, and g). The enantioselectivities of the *N*-acetyl esters of phenylalanine ($E = 105$) and tryptophan ($E = 40$) were rather low compared to those of the other amino acid esters. The hydrolysis of *N*-acetyl ethyl ester of tyrosine was slow and 49% conversion was reached in 48 h with a high enantiomeric ratio E >500.

The unnatural *N*-acetyl-*p*-F-phenylalanine ethyl ester **4b** was hydrolyzed with an E of ratio >200. Notably, the other halogenated phenylalanines (*p*-Cl and *p*-Br-phenylalanine **4c** and **4d**) were hydrolyzed with good ee for both substrates and products with high enantioselectivity (E >500) (Table 1, entries b–d). This is higher than that reported for the hydrolysis of N-protected *p*-halogenated phenylalanine esters by porcine pancreatic lipase,[12] *Bacillus subtilis*[13], and sulfhydryl proteases.[14] Moreover, the type of halogen atom substituted on the aromatic ring has no effect on the hydrolytic activity of the enzyme(s) involved and all three halogenated amino acids were hydrolyzed within 3–4 h with high ee and conversions close to 50%.

Another important unnatural amino acid *N*-acetyl-L-homophenylalanine **6e** which is a chiral intermediate in the synthesis of angiotensin converting enzyme inhibitors,[15] was obtained in 98% ee by the hydrolysis (E >200) of *N*-acetyl-DL-homophenylalanine ethyl ester **4e** (Table 1, entry e). Different solvent systems have been reported for the enzymatic ester hydrolysis of N-protected homophenylalanine; papain in 20% solution of DMF in phosphate buffer,[14] porcine pancreatic lipase in 15% solution of ionic liquid $[EtPy]^+[CF_3COO]^-$ in water;[3e] 30% acetonitrile in phosphate buffer,[12c] *Bacillus lichenifomis* alcalase in two different ionic liquid solutions of $[EMIM][BF_4]$ and $[EtPy][BF_4]$ in water;[3f] and immobilized α-chymotrypsin and baker's yeast in reverse micellar media.[8b] Herein, the hydrolysis was carried out in water with 7.5% ethanol. The hydrolysis of *N*-acetylphenylglycine ethyl ester **4h** where the side chain is not separated by a $-CH_2-$ group was very slow (entry h). After 96h reaction time, the substrate D-ester was isolated in 62% yield and 36% enantiomeric excess and the product was not isolated.

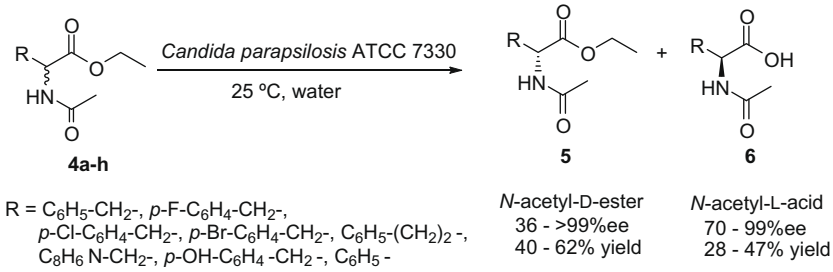
3. Conclusion

This study shows that the whole cells of *C. parapsilosis* ATCC 7330 are efficient biocatalysts in the preparation of enantiomerically pure natural and unnatural amino acids. The enantioselectivity of ester hydrolysis can be increased by substituting the N-free ester with *N*-acetyl amino acid esters. Significantly, both natural and unnatural amino acids, where the R-group is separated by a $-CH_2-$ group, were hydrolyzed with good enantioselectivity (E >500).

4. Experimental

4.1. General

C. parapsilosis ATCC 7330 was purchased from ATCC Manassas, VA 201018, USA and maintained at 4 °C in yeast malt agar medium that contained 5 g/l peptic digest of animal tissue, 3 g/l malt extract, 3 g/l yeast extract, 10 g/l dextrose, and 20 g/l agar. The unnatural racemic amino acids were purchased from Sigma chemicals, and the natural racemic amino acids were from SRL chemicals, India and used as received. All solvents were of analytical grade and used as received. Thionyl chloride and acetic anhydride were distilled prior to use. HPLC analysis of the *N*-acetyl amino acid esters was carried out on a Jasco PU-1580 liquid chromatograph with a PDA detector using Daicel OJ-H, OB-H, and OD-H chiral columns, Hexane: isopropanol mixture was used as the mobile phase with flow rate 1 ml/min. The proportion of solvents varied for different *N*-acetyl amino acid esters. HPLC analysis of the *N*-free phenylalanine ester and acid was carried out on a Crownpak CR+ column, with $HClO_4$ (pH 2) as the mobile phase with flow rate 0.8 ml/min. Optical rotations were recorded on a Rudolph, Autopol IV digital polarimeter. The characterization of racemic and enantiomerically pure *N*-acetyl amino acid ethyl esters was carried out by ^1H, ^{13}C NMR, and mass spectrometry. ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ or CD_3OD solution on a Bruker AV-400 spectrometer operating at 400 and 100 MHz, respectively.



Scheme 2. Resolution of *N*-acetyl-DL-amino acid ethyl esters **4a–h** using the whole cells of *Candida parapsilosis* ATCC 7330.

Table 1
Resolution of racemic *N*-acetyl amino acid esters **4a**–**h** using the whole cells of *Candida parapsilosis* ATCC 7330

Entry	DL-*N*-Acetyl amino acid ethyl esters **4**	Time (h)	*N*-Acetyl-D-ester **5**			*N*-Acetyl-L-acid **6**			C[d] (%)	E[e]
			Yield[a] (%)	ee_s[b] (%)	Specific rotation	Yield[a] (%)	ee_p[c] (%)	Specific rotation		
a	Phenylalanine	2	45	>99	$[\alpha]_D^{34} = -12.4$ (c 1, EtOH) Lit.[19a]	47	87	$[\alpha]_D^{35} = +28.9$ (c 1, MeOH) Lit.[19b]	53.5	105
b	*p*-F-phenylalanine	4	48	99	$[\alpha]_D^{30} = -72.5$ (c 2, $CHCl_3$) Lit.[7b]	47	98	$[\alpha]_D^{30} = +30.8$ (c 1, MeOH)	50.3	>200
c	*p*-Cl-phenylalanine	4	50	>99	$[\alpha]_D^{34} = -16.8$ (c 2, EtOH)	39	99	$[\alpha]_D^{35} = +47.5$ (c 1, MeOH)	50.3	>500
d	*p*-Br-phenylalanine	3	42	>99	$[\alpha]_D^{34} = -17.5$ (c 1, EtOH)	38	98	$[\alpha]_D^{30} = +35.5$ (c 1, MeOH)	50.5	>500
e	Homophenylalanine	24	50	94	$[\alpha]_D^{30} = -1.8$ (c 2, EtOH)	32	98	$[\alpha]_D^{35} = +14.6$ (c 2, MeOH) Lit.[20]	49.0	>200
f	Tryptophan	24	40	>99	$[\alpha]_D^{30} = -38.7$ (c 1, $CHCl_3$) Lit.[21]	28	70	$[\alpha]_D^{35} = +18.8$ (c 0.5, aq NaOH) Lit.[22]	58.8	40
g	Tyrosine	48	43	95	$[\alpha]_D^{34} = -16.2$ (c 2, EtOH) Lit.[23a]	28	99	$[\alpha]_D^{30} = +44.9$ (c 0.5, MeOH) Lit.[23b]	49.0	>500
h	Phenylglycine	96	62	36	$[\alpha]_D^{34} = -48.6$ (c 2, EtOH) Lit.[24]	nd	nd	—	—	—

nd—not determined.
[a] Isolated yield.
[b] Determined by chiral HPLC.
[c] Determined by chiral HPLC after derivatization into an ethyl ester.
[d] Conversion was calculated according to $ee_s/(ee_s + ee_p)$.
[e] Enantioselectivity ratios were calculated according to $E = \ln[(1 - c)(1 - ee_s)]/\ln[(1 - c)(1 + ee_s)]$,[25] E values in the range 200–500 are mentioned as >200, more than 500 as >500.

4.2. Synthesis of racemic *N*-free amino acid ethyl esters[16a]

All racemic *N*-free amino acid ethyl esters were prepared in quantitative yields by esterification with thionyl chloride and ethanol at reflux. The salt-free amino acid ethyl ester was obtained by neutralizing the ester hydrochloride with saturated $NaHCO_3$ solution, extracted with ethyl acetate, and concentrated under reduced pressure. The residue was purified by column chromatography and analyzed by ^1H, ^{13}C NMR and mass spectrometry.

4.3. Synthesis of racemic *N*-acetyl amino acid ethyl esters 4a–h[16b]

The *N*-free amino acid ethyl esters (1 mmol) were dissolved in glacial acetic acid and acetic anhydride (1.2 mmol) was added and stirred at rt for 1–2 h. The solvent was removed by rotary evaporation. Further purification by column chromatography yielded the pure N-acetylated amino acid esters.

4.4. Growth conditions for *C. parapsilosis* ATCC 7330[17]

C. parapsilosis ATCC 7330 was grown in yeast malt broth medium (50 ml) in 250 ml Erlenmeyer flasks incubated at 25 °C, 200 rpm. The cells were harvested by centrifuging the 14th h culture broth at 10,000 rpm for 10 min at 4 °C and subsequent washing with distilled water. The process was repeated twice, and the wet cells were used for biotransformation. In total, 1 g wet cells were obtained per 50 ml medium in 250 ml Erlenmeyer flask.

4.5. Resolution of *N*-acetyl-DL-phenylalanine ethyl ester 4a

To 6 g of wet cells of *C. parapsilosis* suspended in 18.5 ml water, 60 mg *N*-acetyl-DL-phenylalanine ethyl ester **4a** dissolved in 1.5 ml ethanol as a co-solvent was added and incubated at 25 °C, 200 rpm for 2 h. The hydrolysis was monitored by determining the ee of the unreacted substrate *N*-acetyl-D-phenylalanine ethyl ester **5a** (ee_s) by chiral HPLC. The ee of the product *N*-acetyl-L-phenylalanine **6a** was determined at the maximum ee_s. After hydrolysis, the cells were centrifuged and the supernatant was adjusted to pH 9 with saturated $NaHCO_3$ solution, extracted with ethyl acetate, and concentrated under reduced pressure to give **5a** which was purified by column chromatography with 1:1 hexane/ethyl acetate mixture to give an isolated yield of 45% (27 mg) and ee >99% {specific rotation of **5a** $[\alpha]_D^{34} = -12.4$ (c 1, EtOH) (Lit.[19a] $[\alpha]_D^{25} = -12.9$ to -14.5 (c 1, EtOH)}.

The basic aqueous layer was then acidified with 6 M HCl to pH 2–3, extracted with ethyl acetate, and concentrated under reduced pressure to give **6a**, which was purified by column chromatography with 1:4 methanol/dichloromethane mixture to give an isolated yield of 47% (25 mg) and ee 87% {specific rotation of **6a** $[\alpha]_D^{35} = +28.9$ (c 1, MeOH) Lit.[19b] $[\alpha]_D^{22} = +40.0$ (c 1, MeOH)}. The ee of the D-ester **5a** and L-acid **6a** (ethyl ester) was determined by chiral HPLC with Chiralcel OBH column, 95:5 hexane/isopropyl alcohol mixture with a flow rate of 1 ml/min.

4.6. General method for the resolution of racemic *N*-acetyl amino acid ethyl esters 4a–h

To 6 g of wet cells of *C. parapsilosis* suspended in 18.5 ml water, 60 mg racemic *N*-acetyl amino acid ethyl ester **4** dissolved in 1.5 ml ethanol as a co-solvent was added and incubated at 25 °C, 200 rpm. The hydrolysis was monitored by determining the ee of the unreacted substrate D-ester **5** (ee_s) by chiral HPLC. The ee of the product *N*-acetyl-L-acid **6** was determined at the maximum ee_s. After hydrolysis, the cells were centrifuged and the supernatant was adjusted to pH 9 with saturated $NaHCO_3$ solution, extracted with ethyl acetate to give *N*-acetyl-D-amino acid ethyl ester. The basic aqueous layer was then acidified with 6 M HCl to pH 2–3, and extracted with ethyl acetate to give the *N*-acetyl-L-amino acid. The ee of the D-ester **5** was determined by chiral HPLC with Chiralcel OB-H, OJ-H, and OD-H columns.[18] The L-acid **6** product was esterified with $SOCl_2$/EtOH and analyzed by chiral HPLC.

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