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Amide Synthesis *via* Aminolysis of Ester or Acid with an Intracellular Lipase

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ABSTRACT

A unique lipase (SpL) from *Sphingomonas* sp. HXN-200 was discovered as the first intracellular enzyme for the aminolysis of ester or acid to produce amide. Reactions of a series of esters and amines with SpL gave the corresponding amides **3a-g** in high yield with high activity. SpL also showed high enantioselectivity and high activity for enantioselective ester aminolysis, producing amides (*R*)-**3h-j** in high *ee* from the corresponding racemic ester or amine. Moreover, SpL was found to be highly active for the aminolysis of carboxylic acid, which was generally considered infeasible with the known aminolysis enzymes. The aminolysis of several carboxylic acids afforded the corresponding amides **3a**, **3d**, **3k**, **3l** and **3n** in good yield. The intracellular SpL was expressed in *Escherichia coli* cells to give an efficient whole-cell biocatalyst for amide synthesis. Remarkably, high catalytic activity was observed in the presence of water at 2-4% (v/v) for free enzyme and 16% (v/v) for whole cells, respectively. Accordingly, *E. coli* (SpL) wet-cells were used as easily available and practical catalysts for the aminolysis of ester or acid, producing a group of useful and valuable amides in high concentration (up to 103 mM) and high yield. The newly discovered intracellular SpL with unique properties is a promising catalyst for green and efficient synthesis of amides.

KEYWORDS: Amide synthesis, aminolysis of ester, aminolysis of acid, enzyme catalysis, intracellular lipase, whole cells

INTRODUCTION

Amide bonds are present in many drug molecules.[1] Amide synthesis is thus an important task in pharmaceutical industry, evidenced by a recent survey.[2] However, the commonly used methods for amide synthesis *via N*-acylation involve the use of acid chlorides and coupling reagents with poor atom economy.[2-3] In 2005, the ACS Green Chemistry Institute Pharmaceutical Roundtable identified amide bond formation as the most frequently used reaction in pharmaceutical syntheses but requiring green and efficient methods.[3]

Enzyme-catalyzed aminolysis of ester or carboxylic acid offers a green and direct synthesis of amides.[4-6] Compared with chemically used methods, enzymatic amide formation has several advantages such as high selectivity, mild reaction conditions, no use of toxic or shock sensitive reagents, and high atom economy.[4] Three types of serine hydrolases are used for aminolysis of esters - lipase, subtilisin, and penicillin acylase.[5] Since subtilisin requires highly active esters such as 2, 2, 2-trifuoroethyl butyrate as acyl donors and penicillin acylase needs phenyl acetic acid or its α-substituted derivatives as acyl donors, these enzymes are exclusively used for the kinetic resolution of chiral amines *via* the aminolysis of esters.[7-11] In contrast, lipase is useful for amide synthesis in organic solvent with no hydrolysis of amide.[12]

However, there are also challenges associated with lipase-catalyzed amide synthesis. Firstly, the currently used enzymes are exclusively extracellular lipases and thus have to be used in free or immobilized forms, which requires enzyme purification and/or immobilization. Secondly, many enzymes, such as *Candida rugosa* lipase (CRL) and *Pseudomonas alcaligenes* lipase (PAL), have limited substrate scope.[13-14] Thirdly, even the well-known aminolysis enzymes such as *Candida antarctica* lipase B (CALB)[6] show generally low activity towards relatively bulky esters and/or amines.[15-16]

Enzyme-catalyzed aminolysis of acid offers an even more direct synthesis of amides without

the preparation of the ester substrate. However, carboxylic acids were generally considered to be ineffective substrates for enzyme-catalyzed aminolysis reaction due to the unreactive salts formed between carboxylic acids and amines.[4] Only a few examples have been reported for using carboxylic acids as the acyl donor for enzymatic aminolysis reaction. All these examples suffer from incomplete reaction, long reaction time, high enzyme loading or high reaction temperature with poor to moderate amide yield. [17-28]

We have been interested in developing a novel intracellular enzyme that catalyzes the aminolysis of esters with unique substrate specificity for green and efficient amide synthesis. A group of target reactions were selected (Scheme 1-2). While some of the substrates are relatively bulky, the corresponding amides are useful and valuable products. For example, amide **3a** is a structural analogue of capsaicin with much lower pungency for the application in food industry;[12] amide **3c** is a new potent tyrosinase inhibitor;[29] amide **3c**, **3d**, and **3h** are a novel class of 5-HT_3 receptor agonists;[30] the *R* and *S* enantiomer of **3h** showed different anticonvulsant activity towards mice, and (*R*)-**3h** is principally responsible for the anticonvulsant activity.[31] The current syntheses of these compounds are not efficient.[15-16] For instance, Novozyme 435 was reported for the aminolysis reactions to produce amides **3c**[16], **3e**[15], and **3j**,[15] respectively, but with low specific activity.

Here we report the discovery of a novel intracellular enzyme SpL for the aminolysis of ester or acid to prepare amide with broad substrate scope, high activity, and/or high enantioselectivity, the engineering of recombinant *E. coli* expressing the intracellular SpL as active whole-cells catalyst for the aminolysis of ester or acid, and the development of the easily available wet-cells catalyst for practical amide synthesis.

RESULTS AND DISCUSSION

Discovery of SpL as an intracellular lipase for amide synthesis *via* aminolysis of ester

Alkanes are known to be ideal stimulators for lipase expression in many wild type strains.[32-35] Thus, alkane-degrading strains could contain lipases that might catalyze the aminolysis of ester. 40 *n*-octane-degrading strains collected in our lab were thus screened for the aminolysis of methyl hexanoate **1a** with amylamine **2d**.[36-40] *Sphingomonas* sp. HXN-200 was found to be the most active microorganism for the reaction, with a specific activity of 0.055 U/g wet cells.

To identify the enzyme that catalyzes the desired aminolysis of ester, the whole genome of *Sphingomonas* sp. HXN-200 was sequenced, and 11 putative lipases was predicted in the strain based on bioinformatics analysis.[40] Primers were designed to amplify the 11 putative lipases from the genome by polymerase chain reaction (PCR). The amplified fragments were digested with their respective restriction enzymes and ligated to pRSFDuet-1 vector. The resultant plasmid was transformed into chemically competent T7 *E. coli*. 10 out of 11 putative lipases were successfully expressed in *E. coli* and showed activity for hydrolyzing *p*-nitrophenyl butyrate (PNPB). But only 1 lipase (SpL) was found highly active for the aminolysis of ester **1a** with amine **2d** to give *N*-pentylhexanamide.

The gene of SpL consists of 948 bp, encoding a 315 amino acid protein with a predicted molecular weight of 33.81 kDa. A BLASTP with the NCBI protein database showed that SpL shares most similarity (86% amino acid identity) with a putative alpha/beta hydrolase fold-3 protein from *Sphingopyxis alaskensis* RB2256. However, there is no functional study on this protein.

E. coli (SpL) was easily grown in TB (Terrific Broth) medium. As shown in Figure 1a, a cell density of 6.4-7.2 g cdw/L was achieved between 16-20 h. The highest specific activity (350 U/mg cdw) for the hydrolysis of PNPB was achieved at 16-18 h in the early stationary phase. Based on the SDS-PAGE of the CFE (cell-free extract) of the *E. coli* cells taken at 16 h (Figure 1b, Lane 2), SpL is an intracellular enzyme with a molecular weight of 37 kDa. This value is very close to the predicted molecular weight.

E. coli strain overexpressing *N*-terminal his-tagged SpL was then constructed to facilitate the purification of SpL. Cells were cultivated, harvested, and disrupted, and the CFE was subjected to affinity chromatography with a Ni-NTA column. His-tagged SpL was purified to apparent homogeneity as shown in SDS-PAGE (Figure 1b, Lane 4). This purified SpL was used as free enzyme for the aminolysis of ester in the following studies.

SpL-catalyzed aminolysis of ester in the presence of water

Water was generally considered as deleterious to lipase-catalyzed aminolysis of ester due to the undesirable hydrolysis of the acyl donor.[4] Hence, lipase-catalyzed aminolysis of ester was normally carried out in anhydrous organic solvent.[4] *Candida antarctica* lipase B (CALB), by far the most powerful enzyme for aminolysis of ester known thus far, is such an enzyme. Even with only trace amount of water (0.5%; v/v), the performance of CALB dropped significantly. To achieve anhydrous condition, strict pretreatment of the solvent, substrate, and enzyme with activated molecular sieve is often required. Thus, it is of advantages that an enzymatic reaction could be performed without the needs of anhydrous condition. Other type of enzyme such as acyl transferase is able to catalyze amide formation in aqueous condition *via* one-step acylation of amine by using ester as acyl donor in the presence of co-solvent.[41] However, there are only three examples of lipase-catalyzed ester aminolysis in presence of water, but all involving the use of the strong α nucleophiles (hydroxylamine or hydrazine) as acyl acceptor.[14, 42-43] To our surprise, SpL was found to be active in presence of water for the aminolysis of ester **1a** with amine **2a**. As shown in Figure 1c, SpL showed no catalytic activity in totally anhydrous *n*-hexane. With trace amount of water (0.5%; v/v) added, apparent activity was observed. Increase of water content from 0.5% to 2% (v/v) increased both the specific activity and final amide yield. 2%-4% (v/v) water content was the optimal.

E. coli (SpL) cells were then examined in the presence of water for the aminolysis. As shown

in Figure 1d, 16% (v/v) water content was found to be the optimal for achieving the highest amide yield while maintaining high activity.

SpL-catalyzed aminolysis of ester for the synthesis of amides 3a-g

Free SpL was used to catalyze a series of reactions between esters **1a-d** and amines **2a-d**, as shown in Scheme 1. Methyl esters were used in this study to ensure low Sheldon's E factor by producing methanol as byproduct.[17] For comparison, parallel reactions were carried out with free CALB under its optimal conditions. As shown in Scheme 1, SpL accepts esters **1a-d** and amines **2a-d** for the amide synthesis. For the aminolysis of ester **1a-d** with amine **2a**, SpL showed high activities ranging from 26.7 U/g to 115.9 U/g with 91.2% to 99% yield for **3a-d**. These activities and yields are much higher than those of CALB (Table S2). It is worthwhile to note that for the aminolysis of the relatively bulky ester **1a**, **1b** and **1c**, SpL is 45, 76, and 26 times more active than CALB. These values are much higher than the 3.4 fold increase observed in the aminolysis of the relatively less bulky ester **1a**. Apparently, SpL is a better enzyme for accepting relatively bulky acyl donors.

As shown in Scheme 1, SpL showed high activities (45.1 U/g to 108.2 U/g) and yields (84.6% to 86.2%) for the aminolysis of ester **1d** with amine **2b-d** to produce amide **3e-g**. The specific activities are 16.4-155.5 times higher than those of CALB. It is interesting to note that even for the weak nucleophile aniline **2b**, SpL showed a high activity of 45.1 U/g enzyme and a very good final amide yield of 84.6%. This indicates that SpL is a promising enzyme for anilide derivative synthesis.

SpL-catalyzed enantioselective aminolysis of ester for the synthesis of (*R*)-amides 3h-j

Enzyme catalyzed-enantioselective aminolysis of ester is a useful reaction to prepare enantiopure amides that are valuable intermediates in pharmaceutical manufacturing. Thus

far, enzymatic aminolysis is exclusively used for the resolution of chiral esters as well as chiral amines. The use of lipase for enantioselective aminolysis towards the synthesis of enantiopure or enriched amides is rarely reported.

SpL was found to show high enantioselectivity for ester aminolysis to produce chiral amides (Table 1). The enantioselective aminolysis of racemic ester **1e** with amine **2a** gave (*R*)-**3h** in 99.1% *ee* and 19.3% yield, with an *E* of 279. For SpL-catalyzed enantioselective aminolysis of ester **1d** with racemic amine **2e-f**, (*R*)-**3i** and (*R*)-**3j** were obtained in 94.3-94.1% *ee* and 31.7-34.6% yield with *E* of 52-54, respectively. In comparison, CALB gave much lower low *E* values for producing (*R*)-**3h** and (*R*)-**3i**, and much low activity of producing (*R*)-**3j** (Table S3).

Preparation of amide in high concentration *via* ester aminolysis with wet cells of *E. coli* (SpL)

The direct use of whole cells expressing intracellular enzyme provides a readily available and low-cost biocatalyst for potential industrial application.[40-46] Thus far, no whole-cell biocatalyst has been used for amide synthesis *via* ester aminolysis, since all enzymes reported are extracellular.

Since SpL is an intracellular enzyme, it is possible to use cells expressing SpL as catalyst. Considering the fact that lyophilization to produce dry cells is a highly energy-intensive process and *E. coli* (SpL) needs 16% (v/v) of water to show the best performance, directly using wet cells is highly desirable to lower the catalyst cost. Thus, wet cells of *E.coli* (SpL) were used for the synthesis of amides. Based on the measured 86% water content of the wet cell pellet, the water content of the reaction system was controlled to be 16% (v/v) by adjusting catalyst loading to 185 mg wet cells/mL. In a typical reaction, 927 mg of freshly prepared wet cell pellet of *E. coli* (SpL) was mixed with 5 mL *n*-hexane to give 16% (v/v) water content.

Wet cells of *E. coli* (SpL) were demonstrated as efficient catalyst for the synthesis of the useful and valuable amides **3a-g** and (*R*)-**3h** at high yield and high substrate concentration. As shown in Table 2, the aminolysis of ester **1a-b** with amine **2a** gave the corresponding amide **3a-b** in >99% yield with 80-100 mM. The reaction was very fast and finished within 0.5 h. For the aminolysis of ester **1c** with amine **2a** and of **1d** with **2c** at high substrate concentration of 120 mM, amide **3c** and **3f** were obtained in 80.7% and 85.9% yield, respectively within 2 h. 79.9% and 71.5% yields of amide **3d-e** were obtained for the aminolysis between ester **1d** and amine **2a-b**, respectively. In comparison, the yield of 71.5% (71.5 mM) for **3e** was much better than the 25-55% yield by using Novozyme 435 or *Pseudomonas stutzeri* lipase (PSL) after 20 h at 50 °C.[15] The superior ability of *E. coli* (SpL) cells to accept aniline **2b** as a nucleophile for the synthesis of amide **3e** at high substrate concentration offers its promising application in the synthesis of anilide derivatives in industry. Wet cells of *E. coli* (SpL) also catalyzed the enantioselective aminolysis of racemic ester **1e** (50 mM) with amine **2a** (60 mM) to give (*R*)-**3h** in 97.9% *ee* with 23.2% yield. During the biotransformation, no side reaction was observed, indicating no undesired catalytic activity of the cells. The water was confined inside the cells, and no aqueous-organic two-phase was observed in the reaction mixture.

Preparative biotransformations were carried out with 4.6 g wet cells of *E.coli* (SpL) in 25 mL *n*-hexane at 30 °C and 500 rpm for 2 h to produce amides **3a** and **3b**. Extraction with chloroform and purification by flash chromatography gave pure amide products in 90-94% isolated yield.

Mechanistic study on SpL-catalyzed aminolysis of ester

Since water is involved in SpL-catalyzed aminolysis of ester, it is interesting to check whether carboxylic acid is the intermediate (Figure 2a). The aminolysis of ester **1d** with amine **2a** using SpL in partially hydrated *n*-hexane (4%, v/v) was chosen as the model reaction. As shown in Figure 2b, in the first 30 min, carboxylic acid **4a** was accumulated at

0.91 mM, indicating the occurring of ester hydrolysis. The carboxylic acid concentration was then gradually reduced to 0.34 mM at 2 h, suggesting the possible formation of amide **3d** from carboxylic acid **4a**. Thus, pathway II in Figure 2a should be involved in the amide synthesis.

To investigate whether pathway II is the major pathway for amide formation, the hydrolysis of ester **1d** with SpL in the same medium was investigated. As shown in Figure 2c, only small amount of carboxylic acid **4a** was produced, the rate at 10 min is 84.1 μmol ($min^{-1} \cdot g^{-1}$ protein). This value is 10 times lower than the measured total reaction rate for SpL-catalyzed aminolysis of ester **1d** with amine **2a** (849 μmol ($min^{-1} \cdot g^{-1}$ protein)), demonstrating that the pathway II is the minor one (10%).

SpL-catalyzed aminolysis of carboxylic acid for the synthesis of amide

The unique ability of SpL to catalyze aminolysis of carboxylic acid to give amide was confirmed. SpL-catalyzed aminolysis of carboxylic acid **4a** and amine **2a** in partially hydrated *n*-hexane was shown in Figure 2d. The reaction of 10 mM of carboxylic acid **4a** with 20 mM of amine **2a** finished in 1 h to give 9.9 mM amide **3d** in 99% yield. This reaction rate is even 1.76 times faster than that using the methyl ester as the substrate, suggesting that carboxylic acid is a more suitable substrate for SpL-catalyzed aminolysis. This unique character of SpL allows for amide formation directly from carboxylic acid without the need of the preparation of ester as the substrate. So far, only several examples have been reported for aminolysis of carboxylic acid for the enzymatic amide synthesis, but required long reaction time, high enzyme loading and gave poor to moderate yields.[17-28] On the other hand, many well-known enzymes such as CAL-B require anhydrous condition to avoid hydrolysis. This is not suitable for the aminolysis of acid, which produces water as a by-product. In comparison, SpL-catalyzed amide formation via aminolysis of carboxylic acid benefits from the unique water tolerance of the enzyme. Possibly, water was excluded from the catalytic site, which was more accessible to the

amine. In our future study, we plan to investigate the protein structure of SpL and explore the structural basis of its water tolerance.

Aminolysis of carboxylic acid to the synthesis of amide 3a, 3d, 3k-n with wet-cells of *E. coli* (SpL)

As SpL was shown to be able to catalyze amide synthesis *via* aminolysis of carboxylic acid, the wet cells of *E. coli* (SpL) were used to explore the amide synthesis. As shown in the Table 3, the aminolysis of acid **4a** (80 mM) with amine **2a** (90 mM) in n-hexane containing wet cells (185 mg cells/mL) gave amide **3d** in 99% yield in 1 h, with a specific activity of 99.8 U/g cdw. The method was further examined with 4 aliphatic fatty acids **4b-e** (40 mM) with amine **2a** (45 mM). For the aminolysis of the acids with in medium chain **4b-d**, *E. coli* (SpL) showed similar specific activity (55.1-57.0 U/g cdw) and afforded amides **3a**, **3k** and **3l** in 55-75% yield within 8 h (Scheme 3). For the aminolysis of oleic acid **4e** of long fatty acid chain (C18), *E. coli* (SpL) cells showed lower activity (3.4 U/g cdw). Nevertheless, amide 3m was obtained in 19% yield after 24 h. It is interesting to note that the cells could also catalyze the aminolysis of cyclic carboxylic acid **4f** with amine **2a** to afford amide **3n** in 51% yield within 8 h. The results suggested a broad substrate scope of SpL-catalyzed aminolysis of acid, including aromatic, aliphatic and cyclic carboxylic acids.

The practical potential of amide synthesis from acid by using the wet cells of *E. coli* (SpL) was demonstrated by the preparation of amides **3d**, **3a** and **3k-n** in 25 mL *n*-hexane with 4.6 g wet cells of *E.coli* (SpL) at 30 °C and 500 rpm. After 24 h reaction, the amide **3d**, **3a**, **3k** and **3n** were extracted with chloroform and purified by simple washing with base and acid, giving amide products in 53%, 60%, 55% and 39% yield, respectively. For the preparation of amide **3m**, the amide product was isolated through flash chromatography in 11% isolated yield. The structures of the prepared amides were confirmed with NMR (Fig. S6-S10) and high resolution MS (S14-S18).

CONCLUSIONS

The first intracellular enzyme that catalyzes the aminolysis of ester or acid to produce amide has been developed. Intracellular SpL from *Sphingomonas* sp. HXN-200 showed high activity for the aminolysis of a series of esters with different amines, accepting relatively bulky substrates and broadening the substrate scope of enzymatic ester aminolysis. SpL catalyzed also the highly enantioselective reactions with either racemic ester or racemic amine, providing a new method for the synthesis of chiral amides in high *ee*. Moreover, SpL is able to catalyze the aminolysis of carboxylic acid, giving rise to the high-yielding enzymatic synthesis of amide directly from acid without the need of preparing ester as the substrate. The amide synthesis with SpL could be performed in the presence of water, without the need of anhydrous reaction medium. While cells of *E. coli* expressing intracellular SpL was proven to be efficient catalyst for amide synthesis, the use of easily available wet-cells of *E. coli* (SpL) allows for even more practical synthesis of amide in high concentration and yield. The newly discovered intracellular SpL is a potentially useful enzyme for green synthesis of amides in pharmaceutical manufacturing.

EXPERIMENTAL SECTION

Screening of microorganisms for the aminolysis of ester 1a with amine 2d to produce *N*-pentylhexanamide

40 octane degrading strains stored in our lab were first inoculated into 10 mL LB medium and grown at 30 °C, and 250 rpm for 24 h. 10 mL of the seed culture was then inoculated into 100 mL M9 medium and grown on octane vapor as the sole carbon source as described previously.[35] After growth for 3 days, the cells were harvested by centrifugation, washed with deionized water, and then subjected to activity test.

The activity test was carried out with 200 mg wet cell pellet, 1 mL *n*-hexane, 10 mM methyl hexanoate **1a** and 20 mM amylamine **2d** in 2 mL safe-lock tube on a mixing block at 30 °C, and 1000 rpm for 24 h. After centrifugation, the supernatant was taken for GC analysis to determine the formation of *N*-pentylhexanamide.

Construction of recombinant *E. coli* (SpL) and *E.coli* (his-tagged SpL)

The whole genomic DNA of *Sphingomonas* sp. HXN-200 was prepared by using the DNeasy Blood & Tissue Kit from Qiagen and used as the PCR template. The following primers were used for amplifying SpL: Forward Primer: ACT GCA TAT GAC CGA CAG CAC GAC CCA TTA CAC (NdeI restriction site is underlined); Reverse Primer: ACT GAG ATC TTC ATG CCG CTC CGG TAG CCT CGG CG (BglII restriction site is underlined).

To facilitate the purification of SpL, the following primers were used to engineer an N-terminal his-tagged SpL: Forward Primer: ACT GGG ATC CGA TGA CCG ACA GCA CGA CCC ATT ACA (BamHI restriction site is underlined); Reverse Primer: ACT GCT CGA GTC ATG CCG CTC CGG TAG CCT CGG CG (XhoI restriction site is underlined).

PCRs were carried out using a Biorad Mycycler^{TM} thermo cycler with the following program: initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 72 °C for 30 s, extension at 72 °C for 45 s, total 33 cycles, final extension at 72 °C for 10 min. PCR products were purified on 1% agarose gel in TAE buffer and then subjected to double digestion with appropriate restriction enzymes followed by ligation to pRSFDuet-1 vector. The ligation product was then used to transform *E.coli* T7 express competent cells and the positive transformants were selected on LB agar plate containing 50 μg/mL of kanamycin. DNA sequencing confirmed the successful construction of the recombinant *E.coli* (SpL) and *E.coli* (his-tagged SpL).

Cell growth, specific activity, and enzyme expression of *E. coli* (SpL)

The recombinant *E. coli* (SpL) was firstly grown in LB medium containing kanamycin (50 μg/mL) at 37 °C for 4-6 h and then inoculated into TB (Terrific Broth) medium containing kanamycin (50 μg/mL). When OD_{600} reached 0.6-0.8, IPTG (0.25 mM) was added to induce the expression of protein. The cells continued to grow for 14−16 h at 22 °C. The cells were harvested by centrifugation (5000 g, 5 min), washed with deionized water, and used for biotransformation. During the cell growth, the culture was taken at different time points for measuring OD_{600}, specific activity for hydrolyzing PNPB and SDS-PAGE analysis.

Purification and characterization of his-tagged SpL from *E. coli* (his-tagged SpL)

The cells of *E. coli* (his-tagged SpL) expressing SpL with a 6×his-tag at the N-terminal were cultivated and harvested at 16-18 h using the same method for *E.coli* (SpL). The cells were then resuspended in KP buffer (10 mM, pH 8.0) and let pass through a cell homogenizer (Stansted fluid power LTD) twice, followed by centrifugation (23000 g, 4 ^{o}C) for 50 min to remove the cell debris. The cell-free extract (CFE) was filtered with 0.2 μm Supor® membranes to remove small particles present and then subjected to FPLC for protein purification on an AKTA purifier with a HisPrep FF 16/10 column, and UV detection at 280 nm at 4 ^{o}C. The untagged proteins were washed out with a wash buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.4) containing 30 mM imidazole. Afterwards, a wash buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.4) containing 300 mM imidazole was used to wash out the his-tagged SpL. The salts and imidazole in the collected enzyme fraction were removed by washing with deionized water in an Amicon Ultra-15 Centrifugal Filter Units (20 kD). The purity and molecular weight of

his-tagged SpL were checked by SDS-PAGE and MALDI-TOF-MS respectively. Free SpL was prepared by lyophilization of purified his-tagged SpL.

Amide synthesis *via* aminolysis of ester with free SpL in presence of water

A mixture consisting of 10 mM methyl hexanoate **1a**, 20 mM benzylamine **2a**, and 10 mg free SpL in 5 mL *n*-hexane containing 0-12% (v/v) water was shaken at 250 rpm and 30 ^{o}C. For the case of 0% (v/v) water, 200 mg of activated 4A molsieve was added to ensure totally anhydrous condition. Specific activity was measured at 30 min, and amide yield was determined at 20 h.

General procedure for aminolysis of ester to give amide 3a-g and (*R*)-3h-j with free SpL

10 mg free SpL was added to a 10 mL shaking flask containing 5 mL *n*-hexane, 200 μL deionized water, and 20 mM corresponding amine (**2a-f**). The reaction was initiated by adding 10 mM corresponding ester (**1a-e**), and the mixture was shaken at 30 °C, and 250 rpm for certain reaction time. 5 mL *tert*-butyl methyl ether (MTBE) was added to dissolve any possible insoluble amide, and anhydrous sodium sulfate was added to remove water. 1 mL reaction mixtures were taken for centrifugation, followed by mixing 200 μL supernatant with 200 μL MTBE (2 mM benzyl alcohol as internal standard). The samples were analyzed by HPLC for quantification of the concentration of amide **3a-g** and concentration and *ee* of amide **3h-j**. The specific activity and amide yield were calculated based on the product concentration.

Aminolysis and hydrolysis in the aminolysis of ester 1d with amine 2a to give amide 3d with free SpL

5 mg/mL SpL aqueous solution was prepared by dissolving 50 mg free SpL in 10 mL deionized water. 200 uL of the SpL aqueous solution was dispensed into a series of 10 mL

shaking flasks, each containing 5 mL *n*-hexane and 20 mM amine **2a**. The reaction was initiated by adding 10 mM ester **1d** and the mixtures were shaken at 30 °C, 250 rpm for 10 min to 2 h, respectively for monitoring the concentrations of ester **1d**, amide **3d** and carboxylic acid **4a** as the reaction progressed.

Hydrolysis of ester 1d to give carboxylic acid 4a with free SpL

200 uL of 5 mg/mL SpL aqueous solution was dispensed into a series of 10 mL shaking flasks, each containing 5 mL *n*-hexane and 10 mM ester **1d**. The mixtures were shaken at 30 °C and 250 rpm for 10 min to 2 h for monitoring the concentrations of ester **1d** and carboxylic acid **4a**.

Aminolysis of carboxylic acid 4a with amine 2a to give amide 3d with free SpL

200 uL of 5 mg/mL SpL aqueous solution was dispensed into a series of 10 mL shaking flasks, each containing 5 mL *n*-hexane and 20 mM amine **2a**. The reaction was initiated by adding 10 mM carboxylic acid **4a** and the mixtures were shaken at 30 °C and 250 rpm for 10 min to 2 h for monitoring the concentrations of carboxylic acid **4a** and amide **3d**.

Amide synthesis *via* aminolysis of ester with *E. coli* (SpL) cells in presence of water

The harvested cells of *E.coli* (SpL) were lyophilized to give dry cell powder. A mixture consisting of 100 mM methyl hexanoate **1a**, 120 mM benzylamine **2a** and 150 mg *E. coli* (SpL) dry cell powder in 5 mL *n*-hexane containing 0-20% (v/v) water was shaken at 250 rpm and 30 ^{o}C.

General procedure for aminolysis of ester to give amide 3a-g and (*R*)-3h with wet cells of *E. coli* (SpL)

The wet cell pellet of *E. coli* (SpL) was measured to contain 86 wt% of water. 927 mg wet cell pellet was added to 5 mL *n*-hexane to give the optimal 16% (v/v) of water in the system. The mixture was stirred with magnetic stirrer, instead of shaking incubator, to ensure good dispersion of the wet cell pellet. 60-120 mM of amine **2a-d** was added first and the mixture was stirred with a magnetic stirrer at 30 °C and 1400 rpm for 5 min. 50-120 mM of ester **1a-e** was then added to initiate the reaction and the mixture was stirred with a magnetic stirrer at 30 °C and 500 rpm for 30 min for measuring specific activity and 0.5-7 h for measuring amide yield or *ee*.

General procedure for aminolysis of carboxylic acid for the synthesis of amide 3a, 3d and 3k-n with wet cells of *E. coli* (SpL)

927 mg freshly prepared cells (86% water content) were added to 5 mL *n*-hexane (185 mg cells/mL) to give the optimal 16% (v/v) of water in the system. Amine **2a** (45-90 mM) was added, and the reaction mixture was stirred with a magnetic stirrer at 30 °C and 1400 rpm for 5 min. Carboxylic acid **4a-f** (40-80 mM) was then added to initiate the reaction. The reactions were performed at 30 °C and 500 rpm for 24 h and samples were taken at 30 min (**3d**), 2 h (**3a**, **3k-l** and **3n**) or 8 h (**3m**) for measuring specific activity and at 1 h (**3d**), 8 h (**3a**, **3k-l** and **3n**) or 24 h (**3m**) for measuring amide yield.

Preparation of amide 3a and 3b *via* aminolysis of ester using wet cells of *E.coli* (SpL)

4.6 g freshly prepared wet cells of *E.coli* (SpL) was added to 25 mL *n*-hexane at a loading of 185 mg cells/mL in a 100 mL round bottom flask. Amine at specific concentration (90 mM **2a** for **3b** synthesis; 120 mM **2a** for **3a** synthesis) was added, and the reaction mixture was stirring with a mixing propeller at 30 °C and 500 rpm for 5 min. The corresponding ester at a specific concentration (100 mM **1a** for **3a** synthesis; 80 mM **1b** for **3b** synthesis) was added, and the reaction was performed by stirring with the

mixing propeller at 30 °C and 500 rpm for 2 h. After reaction, 25 mL chloroform was added to the reaction mixture, and anhydrous sodium sulfate was added to remove water. The cells were removed by filtration. Crude product was acquired by removing the solvent *via* evaporation. Column chromatography on silica gel (R_f=0.3; *n*-hexane: ethyl acetate=10:1 for **3b;** *n*-hexane: ethyl acetate=5:1 for **3a**) affords product **3a** and **3b**.

3a: obtained as a white solid; yield: 90%; ^{1}H NMR (400 MHz, $CDCl_3$, TMS) [51]: δ 7.35-7.25 (m, 5H), 5.75 (s, br, 1H), 4.44 (d, *J* = 5.6 Hz, 2H), 2.20 (t, *J* = 7.2 Hz, 2H), 1.66 (p, *J* = 7.6 Hz, 2H), 1.33-1.29 (m, 4H), 0.89 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, TMS) [51]: δ 173.0, 138.4, 128.7, 127.8, 127.5, 43.6, 36.8, 31.5, 25.4, 22.4, 13.9. TOFMS-ESI (*m/z*): $[M+H]^+$, $[M+Na]^+$ and $[M+Na+ACN]^+$ calculated for $C_{13}H_{19}NO$, 206.154, 228.136, 269.163; Found, 206.153, 228.135, 269.161.

3b: obtained as a white solid; yield: 91%; ^{1}H NMR (400 MHz, $CDCl_3$, TMS) [52]: δ 7.35-7.25 (m, 5H), 5.72 (s, br, 1H), 4.44 (d, *J* = 5.6 Hz, 2H), 2.11 (tt, *J* = 11.8, 3.5 Hz, 1H), 1.91-1.66 (m, 5H), 1.51-1.42 (m, 2H), 1.31-1.19 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, TMS) [52]: δ 175.9, 138.5, 128.7, 127.7, 127.4, 45.6, 43.4, 29.7, 25.7. TOFMS-ESI (*m/z*): $[M+H]^+$, $[M+Na]^+$ and $[M+Na+ACN]^+$ calculated for $C_{14}H_{19}NO$, 218.154, 240.136 and 281.163; Found, 218.154, 240.133 and 281.160.

Preparation of amides 3a, 3d, 3k-n *via* aminolysis of carboxylic acid 4a-f and amine 2a with *E. coli* (SpL)

4.6 g freshly prepared wet cells of *E.coli* (SpL) was added to 25 mL *n*-hexane at a loading of 185 mg cells/mL in a 100 mL round bottom flask. Amine at specific concentration (90 mM **2a** for **3d** synthesis; 45 mM **2a** for **3a** and **3k-n** synthesis) was added, and the reaction mixture was stirring with a mixing propeller at 30 °C and 500 rpm for 5 min. The corresponding carboxylic acid at a specific concentration (80 mM **4a** for **3d** synthesis; 40 mM **4b-f** for **3a** and **3k-n** synthesis) was added, and the reaction was

performed by stirring with the mixing propeller at 30 °C and 500 rpm for 2 h (24 h for **3m** synthesis). After reaction, 25 mL chloroform was added for dissolving any possibly insoluble amide, and anhydrous sodium sulfate was added to remove water. The cells were removed by filtration. Crude product was acquired by removing the solvent *via* evaporation.

Column chromatography on silica gel was used to purify **3d** (R_f=0.3; *n*-hexane: ethyl acetate=5:1) and **3m** (*n*-hexane-ethyl acetate, 10:1-5:1). For the amide **3a**, **3k**, **3l** and **3n**, crude products were dissolved in 15 mL chloroform, washed 3 times with 1% Na_2CO_3 solution to remove the unreacted carboxylic acid, and then 3 times with 0.1 M HCl to remove remaining amine. The organic phase was separated, dried with anhydrous sodium sulfate and the solvent was removed by rotatory evaporator to give the amide products.

3d: obtained as a white solid; yield: 94% ^{1}H NMR (400 MHz, $CDCl_3$, TMS) [53]: δ 7.32-7.26 (m, 5H), 7.23-7.14 (m, 5H), 5.62 (s, br, 1H), 4.40 (d, *J* = 5.7 Hz, 2H), 3.00 (t, *J* = 7.6 Hz, 2H), 2.52 (t, *J* = 7.6 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$, TMS) [53]: δ 171.8, 140.7, 138.1, 128.6, 128.5, 128.4, 127.7, 127.4, 126.2, 43.6, 38.5, 31.7. TOFMS-ESI (*m/z*): $[M+H]^+$ and $[M+Na]^+$ calculated for $C_{16}H_{17}NO$, 240.138, 262.121; Found 240.136, 262.120.

3k: obtained as a white solid; yield: 60%. ^{1}H-NMR (400 MHz, $CDCl_3$, 25 °C, TMS) [54]: δ = 0.86-0.89 (m), 1.26-1.31 (m), 1.62-1.66 (m), 2.19-2.22 (m), 4.43-4.44 (m), 5.77 (m), 7.26-7.35 (m). ^{13}C-NMR (400 MHz, $CDCl_3$, 25 °C, TMS) [54]: δ = 14.07, 22.60, 25.79, 29,01, 29.28, 31.69, 36.81, 43.58, 127.47, 127.81, 128.69, 138.45, 172.99. TOFMS-ESI (*m/z*): $[M+H]^+$ calculated for $C_{15}H_{24}NO$ 234.186; found 234.186.

3l: obtained as a white solid; yield: 55%. ^{1}H-NMR (400 MHz, $CDCl_3$, 25 °C, TMS) [55]: δ= 0.86-0.89 (m), 1.26-1.31 (m), 1.62-1.66 (m), 2.19-2.22 (m), 4.43-4.44 (m), 5.77 (m), 7.26-7.35 (m). ^{13}C-NMR (400 MHz, $CDCl_3$, 25 °C, TMS) [55]: δ = 14.010, 22.66, 25.79,

29.26, 29.32, 29.35, 29.45, 31.88, 36.82, 43.60, 127.49, 127.82, 128.70, 138.43, 172.99. TOFMS-ESI (*m/z*): $[M+H]^+$ calculated for $C_{17}H_{28}NO$ 262.217; found 262.217.

3m: obtained as a white solid; yield: 11%. ^{1}H-NMR (400 MHz, $CDCl_3$, 25 °C, TMS) [56]: δ= 0.88 (m), 1.28 (m), 1.65 (m), 2.01 (m), 2.24-2.18 (t), 4.44 (d), 5.34 (m), 5.70 (s), 7.37 – 7.24 (m). ^{13}C NMR (100 MHz, $CDCl_3$) [56]: δ = 14.11, 22.68, 25.76, 27.18, 27.23, 29.14, 29.25, 29.29, 29.32, 29.33, 29.52, 29.70, 29.77, 31.91, 36.82, 43.63, 127.52, 127.85, 128.73, 129.75, 130.01, 138.41, 172.98. TOFMS-ESI (*m/z*): $[M+H]^+$ calculated for $C_{25}H_{42}NO$ 372.326; found 372.326.

3n: obtained as a white solid; yield: 39%. ^{1}H-NMR (400 MHz, $CDCl_3$, 25 °C, TMS) [57]: δ=1.54-1.59 (m), 1.72-1.88 (m), 2.52-2.56 (m), 4.42-4.43 (d, J_{Hz}=4 MHz), 5.88 (m), 7.25-7.34 (m). ^{13}C-NMR (400 MHz, $CDCl_3$, 25 °C, TMS) [57]: δ=25.93, 30.47, 43.58, 45.88, 127.42, 127.73, 128.68, 138.57, 176.17. TOFMS-ESI (*m/z*): $[M+H]^+$ calculated for $C_{13}H_{18}NO$ 204.139; found 204.138.

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge on the ACS Publications website. Detailed experimental procedures, characterization of products, NMR spectra and HPLC chromatograms (PDF).

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REFERENCES

(1) Valeur, E.; Bradley, M. Amide Bond Formation: Beyond the Myth of Coupling Reagents. *Chem. Soc. Rev.* **2009**, *38*, 606-631.

(2) Carey, J. S.; Laffan, D.; Thomson, C.; Williams, M. T. Analysis of the Reactions Used For the Preparation of Drug Candidate Molecules. *Org. Biomol. Chem.* **2006**, *4*, 2337-2347.

(3) Constable, D. J. C.; Dunn, P. J.; Hayler, J. D.; Humphrey, G. R.; Leazer, J. J. L.; Linderman, R. J.; Lorenz, K.; Manley, J.; Pearlman, B. A.; Wells, A.; Zaks, A.; Zhang, T. Y. Key Green Chemistry Research Areas-a Perspective From Pharmaceutical Manufacturers. *Green Chem.* **2007**, *9*, 411-420.

(4) van Rantwijk, F.; Hacking, M. A. P. J.; Sheldon, R. A. Lipase-Catalyzed Synthesis of Carboxylic Amides: Nitrogen Nucleophiles as Acyl Acceptor. *Monatsh. Chem.* **2000**, *131*, 549-569.

(5) van Rantwijk, F.; Sheldon, R. A. Enantioselective Acylation of Chiral Amines Catalysed by Serine Hydrolases. *Tetrahedron* 2004, *60*, 501-519.

(6) Gotor-Fernández, V.; Busto, E.; Gotor, V. *Candida antarctica* Lipase B: An Ideal Biocatalyst for the Preparation of Nitrogenated Organic Compounds. *Adv. Synth. Catal.* **2006**, *348*, 797-812.

(7) Wang, Y. F.; Yakovlevsky, K.; Zhang, B.; Margolin, A. L. Cross-Linked Crystals of Subtilisin: Versatile Catalyst for Organic Synthesis. *J. Org. Chem.* 1997, *62*, 3488-3495.

(8) Gutman, A. L.; Meyer, E.; Kalerin, E.; Polyak, F.; Sterling, J. Enzymatic Resolution of Racemic Amines In a Continuous Reactor in Organic Solvents. *Biotech. Bioengin.* **1992**, *40*, 760-767.

(9) van Langen, L. M.; Oosthoek, N. H. P.; Guranda, D. T.; van Rantwijk, F.; Švedas, V.

K.; Sheldon, R. A. Penicillin Acylase-Catalyzed Resolution of Amines in Aqueous Organic Solvents. *Tetrahedron: Asymmetry* **2000**, *11*, 4593-4600.

(10) Guranda, D. T.; van Langen, L. M.; van Rantwijk, F.; Sheldon, R. A.; Švedas, V. K. Highly Efficient And Enantioselective Enzymatic Acylation Of Amines In Aqueous Medium. *Tetrahedron: Asymmetry* **2001**, *12*, 1645-1650.

(11) Basso, A.; Braiuca, P.; De Martin, L.; Ebert, C.; Gardossi, L.; Linda, P. D-Phenylglycine and D-4-Hydroxyphenylglycine Methyl Esters via Penicillin G Acylase Catalysed Resolution in Organic Solvents. *Tetrahedron: Asymmetry* **2000**, *11*, 1789-1796.

(12) Torres-Gavilán, A.; Castillo, E.; López-Munguía, A. The Amidase Activity of Candida Antarctica Lipase B Is Dependent On Specific Structural Features of the Substrates. *J. Mol. Catal. B- Enzym.* **2006**, *41*, 136-140.

(13) Alcántara, A. R.; Domínguez de María, P.; Fernández, M.; Hernaíz, M. J.; Sánchez-Montero, J. M.; Sinisterra, J. V. Resolution Of Racemic Acids, Esters and Amines by *Candida rugosa* Lipase in Slightly Hydrated Organic Media. *Food Technol. Biotech.* **2004**, *42*, 343-354.

(14) Hacking, M. A. P. J.; van Rantwijk, F.; Sheldon, R. A. Lipase Catalysed Synthesis of Diacyl Hydrazines: An Indirect Method for Kinetic Resolution of Chiral Acids. *J. Mol. Catal. B- Enzym.* **2000**, *9*, 183-191.

(15) van Pelt, S.; Teeuwen, R. L. M.; Janssen, M. H. A.; Sheldon, R. A.; Dunn, P. J.; Howard, R. M.; Kumar, R.; Martinez, I.; Wong, J. W. *Pseudomonas stutzeri* Lipase: A Useful Biocatalyst for Aminolysis Reactions. *Green Chem.* **2011**, *13*, 1791-1798.

(16) Whitten, K. M.; Makriyannis, A.; Vadivel, S. K. Enzymatic Synthesis of N-Acylethanolamines: Direct Method for the Aminolysis of Esters. *Tetrahedron Lett.* **2012**, *53*, 5753-5755.

(17) Sheldon, R. A. The E Factor: Fifteen Years On. *Green Chem.* **2007**, *9*, 1273-1283.

(18) Tuccio, B.; Ferré, E.; Comeau, L. Lipase-Catalyzed Syntheses of N-Octyl-Alkylamides in Organic Media. *Tetrahedron Lett.* **1991**, *32*, 2763-2764.

(19) Maugard, T.; Remaud-Simeon, M.; Petre, D.; Monsan, P. Lipase-Catalysed Production of N-Oleoyl-Taurine Sodium Salt in Non-Aqueous Medium. *Biotechnol. Lett.* **1997**, *19*, 751-753.

(20) Maugard, T.; Remaud-Simeon, M.; Petre, D.; Monsan, P. Enzymatic Synthesis of Glycamide Surfactants by Amidification Reaction. *Tetrahedron* **1997**, *53*, 5185-5194.

(21) Maugard, T.; Remaud-Simeon, M.; Petre, D.; Monsan, P. Lipase-Catalyzed Chemoselective N-Acylation Of Amino-Sugar Derivatives in Hydrophobic Solvent: Acid-Amine Ion-Pair Effects. *Tetrahedron* **1997**, *53*, 7587-7594.

(22) Maugard, T.; Remaud-Simeon, M.; Petre, D.; Monsan, P. Enzymatic Amidification for the Synthesis of Biodegradable Surfactants: Synthesis of N-Acylated Hydroxylated Amines. *J. Mol. Catal. B- Enzym.* **1998**, *5*, 13-17.

(23) Maugard, T.; Remaud-Simeon, M.; Monsan, P. Kinetic Study of Chemoselective Acylation of Amino-Alditol by Immobilized Lipase in Organic Solvent: Effect of Substrate Ionization. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.* **1998**, *1387*, 177-183.

(24) Litjens, M. J. J.; J. J. Straathof, A.; A. Jongejan, J.; J. Heijnen, J. Synthesis of Primary Amides by Lipase-Catalyzed Amidation of Carboxylic Acids with Ammonium Salts in an Organic Solvent. *Chem. Commun.* **1999**, *0*, 1255-1256.

(25) Gotor-Fernandez V.; Gotor V. Enzymatic Aminolysis and Ammonolysis Processes in the Preparation of Chiral Nitrogenated Compounds. *Curr. Org. Chem.* **2006**, *10*,1125-1143.

(26) Nechab, M.; Azzi, N.; Vanthuyne, N.; Bertrand, M.; Gastaldi, S.; Gil, G. Highly Selective Enzymatic Kinetic Resolution Of Primary Amines at 80 C: A Comparative Study Of Carboxylic Acids and Their Ethyl Esters as Acyl Donors. *J. Org. Chem.* **2007**, *72*, 6918-6923.

(27) Tufvesson, P.; Annerling, A.; Hatti-Kaul, R.; Adlercreutz, D. Solvent-free Enzymatic Synthesis of Fatty Alkanolamides. *Biotechnol. Bioengin.* **2007**, *97*, 447-453.

(28) Pitzer, J.; Steiner, K. Amides in Nature and Biocatalysis. *J. Biotechnol.* **2016**, *235*, 32-46.

(29) Cho, S. J.; Roh, J. S.; Sun, W. S.; Kim, S. H.; Park, K. D. N-Benzylbenzamides: A New Class of Potent Tyrosinase Inhibitors. *Bioorganic Med. Chem. Lett.* **2006**, *16*, 2682-2684.

(30) Jørgensen, C. G.; Frølund, B.; Kehler, J.; Jensen, A. A. Discovery of Benzamide Analogues as A Novel Class of 5-HT3 Receptor Agonists. *ChemMedChem* **2011**, *6*, 725-736.

(31) Choi, D.; Stables, J. P.; Kohn, H. The Anticonvulsant Activities of Functionalized N-Benzyl 2-Acetamidoacetamides. The Importance of the 2-Acetamido Substituent. *Bioorg. Med. Chem.* **1996**, *4*, 2105-2114.

(32) Boekema, B. K. H. L.; Beselin, A.; Breuer, M.; Hauer, B.; Koster, M.; Rosenau, F.; Jaeger, K.-E.; Tommassen, J. Hexadecane and Tween 80 Stimulate Lipase Production in Burkholderia Glumae by Different Mechanisms. *App. Environ. Microbiol.* **2007**, *73*, 3838-3844.

(33) Kanwar, L.; Gogoi, B. K.; Goswami, P. Production of a Pseudomonas Lipase in N-Alkane Substrate and Its Isolation Using an Improved Ammonium Sulfate Precipitation Technique. *Bioresour. Technol.* **2002**, *84*, 207-211.

(34) Kok, R. G.; Nudel, C. B.; Gonzalez, R. H.; Nugteren-Roodzant, I. M.; Hellingwerf, K. J. *Acinetobacter calcoaceticus* BD413: Fatty Acid Repression of Lipa Expression and Degradation of Lipa. *J. Bacteriol.* **1996**, *178*, 6025-6035.

(35) Kok, R. G.; van Thor, J. J.; Nugteren-Roodzant, I. M.; Brouwer, M. B. W.; Egmond, M. R.; Nudel, C. B.; Vosman, B.; Hellingwerf, K. J. Characterization of the Extracellular Lipase, Lipa, of *Acinetobacter calcoaceticus* BD413 and Sequence Analysis of the Cloned Structural Gene. *Mol. Microbiol.* **1995**, *15*, 803-818.

(36) Liu, Z.; Michel, J.; Wang, Z.; Witholt, B.; Li, Z. Enantioselective Hydrolysis of Styrene Oxide with the Epoxide Hydrolase of *Sphingomonas* sp. HXN-200. *Tetrahedron: Asymmetry* **2006**, *17*, 47-52.

(37) Wang, Z.; Lie, F.; Lim, E.; Li, K.; Li, Z. Regio- and Stereoselective Allylic Hydroxylation of D-Limonene to (+)-Trans-Carveol with *Cellulosimicrobium cellulans* EB-8-4. *Adv. Synth. Catal.* **2010**, *351*, 1849-1856.

(38) Chen, Y.; Lie, F.; Li, Z. Enantioselective Benzylic Hydroxylation with Pseudomonas monteilii TA-5: A Simple Method for the Syntheses of (R)-Benzylic Alcohols Containing Reactive Functional Groups. *Adv. Synth. Catal.* **2009**, *351*, 2107–2112.

(39) Zhang, W.; Tang, W. L.; Wang, Z.; Li, Z., Regio- and Stereoselective Biohydroxylations with a Recombinant *Escherichia coli* Expressing P450pyr Monooxygenase of *Sphingomonas* sp. HXN-200. *Adv. Synth. Catal.* **2011**, *352*, 3380-3390.

(40) Wu, S.; Li, A.; Chin, Y. S.; Li, Z. Enantioselective Hydrolysis of Racemic and Meso-Epoxides with Recombinant *Escherichia coli* Expressing Epoxide Hydrolase from *Sphingomonas* sp. HXN-200: Preparation of Epoxides and Vicinal Diols in High *ee* and High Concentration. *ACS Catal.* **2013**, *3*, 752-759.

(41) Land, H.; Hendil-Forssell, P.; Martinelle, M.; Berglund, P. One-Pot Biocatalytic Amine Transaminase/Acyl Transferase Cascade for Aqueous Formation Of Amides From Aldehydes Or Ketones. *Catal. Sci. Technol.* **2016**, *6*, 2897-2900.

(42) Hacking, M. A. P. J.; Akkus, H.; van Rantwijk, F.; Sheldon, R. A. Lipase And Esterase-Catalyzed Acylation of Hetero-Substituted Nitrogen Nucleophiles in Water and Organic Solvents. *Biotech. Bioengin.* **2000**, *68*, 84-91.

(43) Vaysse, L.; Dubreucq, E.; Pirat, J.-L.; Galzy, P. Fatty Hydroxamic Acid Biosynthesis in Aqueous Medium in the Presence Of the Lipase-Acyltransferase from *Candida parapsilosis*. *J. Biotechnol.* **1997**, *53*, 41-46.

(44) Schmid, A.; Dordick, J. S.; Hauer, B.; Kiener, A.; Wubbolts, M.; Witholt, B. Industrial Biocatalysis Today and Tomorrow. *Nature* **2001**, *409*, 258-268.

(45) Schoemaker, H. E.; Mink, D.; Wubbolts, M. G. Dispelling the Myths-Biocatalysis in Industrial Synthesis. *Science* **2003**, *299*, 1694.

(46) Koeller, K. M.; Wong, C.-H. Enzymes for Chemical Synthesis. *Nature* **2001**, *409*, 232-240.

(47) Tian, K.; Li, Z. High-Yielding, One-Pot, and Green Production of Biodiesel from Waste Grease Using Wet Cells of a Recombinant *Escherichia coli* Strain as Catalyst. *Biochem. Engin. J.* **2016**, *115*, 30-37.

(48) Tian, K.; Tai, K.; Chua, B. J. W.; Zhi, L. Directed Evolution of *Thermomyces lanuginosus* Lipase to Enhance Methanol Tolerance for Efficient Production of Biodiesel from Waste Grease. *Bioresour. Technol.* **2017**, *245*, 1491-1497.

(47) Yan, J. Y.; Li, A. T.; Xu, Y.; Ngo, T. P. N.; Szechao, P.; Li, Z. Efficient Production of Biodiesel from Waste Grease: One-Pot Esterification and Transesterification with Tandem Lipases. *Bioresour. Technol.* **2012**, *123*, 332-337.

(48) Li, A. T.; Ngo, T. P. N.; Yan, J. Y.; Tian, K. Y.; Li, Z. A Novel and Robust Recombinant Pichia Pastoris Yeast Whole Cell Biocatalyst with Intracellular Overexpression of a *Thermomyces lanuginosus* Lipase: Preparation, Characterization and Application in Biodiesel Production. *Bioresour. Technol.* **2012**, *114*, 725-729.

(49) Nechab, M.; Azzi, N.; Vanthuyne, N.; Bertrand, M.; Gastaldi, S.; Gil, G. Highly Selective Enzymatic Kinetic Resolution Of Primary Amines at 80 C: A Comparative Study Of Carboxylic Acids and Their Ethyl Esters as Acyl Donors. *J. Org. Chem.* **2007**, *72*, 6918-6923.

(50) Tufvesson, P.; Annerling, A.; Hatti-Kaul, R.; Adlercreutz, D. Solvent-free Enzymatic Synthesis of Fatty Alkanolamides. *Biotechnol. Bioengin.* **2007**, *97*, 447-453.

(51) Nordstrøm, L.U.; Vogt, H.; Madsen, R. Amide Synthesis from Alcohols and Amines by the Extrusion of Dihydrogen. *J. Am. Chem. Soc.* **2008**, *130*, 17672-17673.

(52) Vora H.U.; Rovis T. Nucleophilic Carbene and HOAt Relay Catalysis in an Amide Bond Coupling: an Orthogonal Peptide Bond Forming Reaction. *J. Am. Chem. Soc.* **2007**, 129, 13796-13797.

(53) Allen, C.L.; Davulcu, S.; Williams, J.M. Catalytic Acylation of Amines with Aldehydes or Aldoximes. *Org. Lett.* **2010**, *12*, 5096-5099.

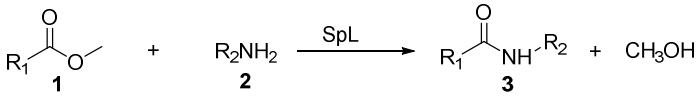
(54) Rolfe, A.; Probst, D.A.; Volp, K.A.; Omar, I.; Flynn, D.L.; Hanson, P.R. High-load, oligomeric dichlorotriazine: A versatile ROMP-derived reagent and scavenger. *J. Org. Chem.* **2008**, *73*, 8785-8790.

(55) Toledo, H.; Pisarevsky, E.; Abramavich, A.; Szpilman, A. Organocatalytic Oxidation of Aldehydes to Mixed Anhydrides. *Chem. Commun.* **2013**, *49*, 4367-4369.

(56) D'Oca, C.D.R.M.; Coelho, T.; Marinho, T.G.; Hack, C.R.L.; da Costa Duarte, R.; da Silva, P.A.; D'Oca, M.G.M. Synthesis and Antituberculosis Activity of New Fatty Acid

Amides. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 5255-5257.

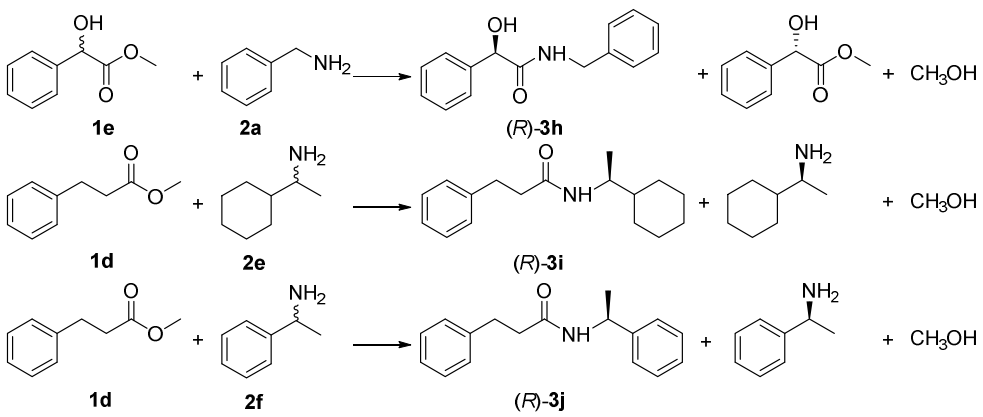
(57) Lafrance, D.; Bowles, P.; Leeman, K.; Rafka, R. Mild Decarboxylative Activation of Malonic Acid Derivatives by 1, 1'-Carbonyldiimidazole. *Org. Lett.* **2011**, *13*, 2322-2325.



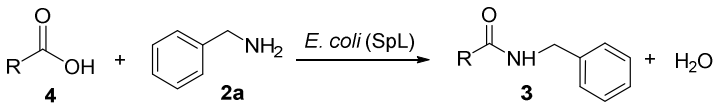
			SpL[a]	
			Activity (U/g) [b]	Yield (%) [c]
1a: R_1 = n-C_5H_{11}	**2a**: R_2 = $PhCH_2$	**3a**: R_1 = n-C_5H_{11}; R_2 = $PhCH_2$	72.1	91.3
1b: R_1 = Cy	**2a**	**3b**: R_1 = Cy; R_2 = $PhCH_2$	39.9	91.2
1c: R_1 = Ph	**2a**	**3c**: R_1 = Ph; R_2 = $PhCH_2$	26.7	92.6
1d: R_1 = $PhCH_2CH_2$	**2a**	**3d**: R_1 = $PhCH_2CH_2$; R_2 = $PhCH_2$	116	>99
1d	**2b**: R_2 = Ph	**3e**: R_1 = $PhCH_2CH_2$; R_2 = Ph	45.1	84.6
1d	**2c**: R_2 = $PhCH_2CH_2$	**3f**: R_1 = $PhCH_2CH_2$; R_2 = $PhCH_2CH_2$	108	86.2
1d	**2d**: R_2 = n-C_5H_{11}	**3g**: R_1 = $PhCH_2CH_2$; R_2 = n-C_5H_{11}	74.1	85.3

[a] The reaction was performed with 10 mM of ester **1a-d** and 20 mM of amine **2a-d** in 5 mL partially hydrated *n*-hexane (4 %; v/v) containing 10 mg of free enzyme. [b] Specific activity (U/g enzyme) was determined after 30 min. [c] Yield (%) of the amide was determined after 20 h.

Scheme 1. Synthesis of amide **3a-g** *via* aminolysis of ester **1a-d** with amine **2a-d** by using free SpL.



Scheme 2. Synthesis of amide (*R*)-**3h-j** *via* enantioselective aminolysis of ester **1d-e** with amine **2a**, **2e** and **2f** by using free SpL.



4a: R = $PhCH_2CH_2$
4b: R = *n*-C_5H_{11}
4c: R = *n*-C_7H_{15}
4d: R = *n*-C_9H_{19}
4e: R = $CH_3(CH_2)_7CH{=}CH(CH_2)_7$
4f: R = Cyclopentyl

3d: R = $PhCH_2CH_2$
3a: R = *n*-C_5H_{11}
3k: R = *n*-C_7H_{15}
3l: R = *n*-C_9H_{19}
3m: R = $CH_3(CH_2)_7CH{=}CH(CH_2)_7$
3n: R = Cyclopentyl

E. coli (SpL) [a]	
Activity (U/g) [b]	Yield (%) [c]
99.8	99
55.1	75
57.0	60
55.5	55
3.4	19
59.1	51

[a] The reaction was performed with 40-80 mM acids **4a-f** with 45-90 mM amine **2a** and 927 mg wet cells in 5 mL *n*-hexane. [b] Specific activity was determined at 30 min (**3d**), 2 h (**3a**, **3k-l** and **3n**) or 8 h (**3m**). [c] Yield was determined at 1 h (**3d**), 8 h (**3a, 3k-l** and **3n**) or 24 h (**3m**).

Scheme 3. Synthesis of amide **3d**, **3a** and **3k-n** *via* aminolysis.

Table 1. Enantioselective Aminolysis of Ester with Amine to Synthesize Chiral Amides by Using Free SpL.

Entry [a]	Enzyme	Ester	Amine	Product	Time (h)	Activity (U/g enzyme) [b]	ee_p (%) [c]	Yield (%) [c]	E [d]
1	SpL	**1e**	**2a**	(*R*)-**3h**	2	16.2	99.1	19.3	279
2	SpL	**1d**	**2e**	(*R*)-**3i**	1.5	43.0	94.3	31.7	52.0
3	SpL	**1d**	**2f**	(*R*)-**3j**	1	51.5	94.1	34.6	54.0

[a] The reaction was performed with 10 mM of ester and 20 mM of amine in 5 mL partially hydrated *n*-hexane (4 %; v/v) with 10 mg of pure enzyme. [b] Specific activity (U/g enzyme) was determined after 30 min. [c] ee_p (%) and yield (%) of the amide were determined after the time stated in the table. [d] E was calculated according to the equation $E=\ln [1-c (1+ee_p)]/\ln [1-c (1-ee_p)]$, c is the conversion.

Table 2. Synthesis of Amides **3a-g** and (*R*)-**3h** *via* Aminolysis of Esters with Wet Cells of *E. coli* (SpL)

Entry [a]	Ester	Ester conc. (mM)	Amine	Amine conc. (mM)	Amide	Time (h)	Activity (U/g cdw) [b]	Yield (%) [c]	ee_p (%) [c]
1	**1a**	100	**2a**	120	**3a**	0.5	132	>99	-
2	**1b**	80	**2a**	90	**3b**	0.5	105	>99	-
3	**1c**	120	**2a**	120	**3c**	2	114	80.7	-
4	**1d**	80	**2a**	90	**3d**	2	81.9	79.9	-
5	**1d**	100	**2b**	120	**3e**	7	34.5	71.5	-
6	**1d**	120	**2c**	120	**3f**	2	130	85.9	-
7	**1d**	50	**2d**	60	**3g**	3	16.4	59.1	-
8	**1e**	50	**2a**	60	(*R*)-**3h**	1	7.3	23.2	97.9

[a] The reaction was performed in 5 mL *n*-hexane with 926.7 mg wet cells of *E. coli* (SpL), which corresponds to 127 mg of dry cells of *E. coli* (SpL). [b] Specific activity (U/g cdw) was determined after 30 min. [c] Amide yield (%) and ee_p (%) were determined after the time stated in the table.

Table 3. Preparation of Amide **3d**, **3a** and **3k-n** *via* Aminolysis of Carboxylic Acid **4a-f** and Amine **2a** with Wet Cells of *E. coli* (SpL)

Entry[a]	Acid	Acid conc. (mM)	Amine	Amine conc. (mM)	Amide	Time (h)	Isolated yield (%)
1	**4a**	80	**2a**	90	**3d**	1	94
2	**4b**	40	**2a**	45	**3a**	8	53
3	**4c**	40	**2a**	45	**3k**	8	60
4	**4d**	40	**2a**	45	**3l**	8	55
5	**4e**	40	**2a**	45	**3m**	24	11
6	**4f**	40	**2a**	45	**3n**	8	39

[a] The reaction was performed in 25 mL *n*-hexane with 4.6 g wet cells of *E. coli* (SpL), which corresponds to 630 mg of dry cells of *E. coli* (SpL).

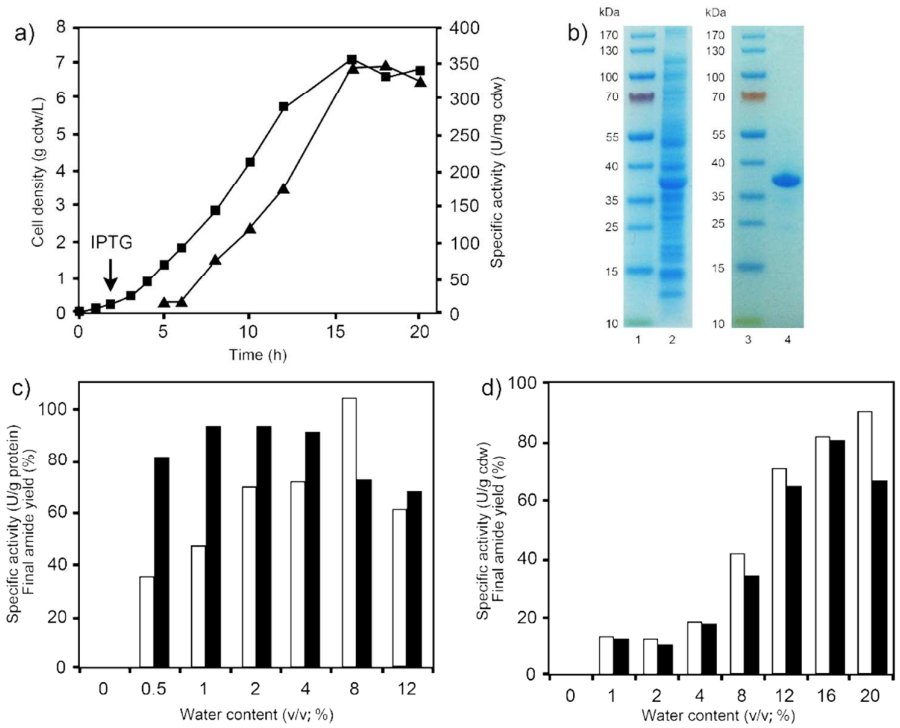


Figure 1. a) Cell growth and specific activity for the hydrolysis of PNPB of *E. coli* (SpL). ■: cell density; ▲: specific activity. Cells were initially grown at 37 °C, induced by the addition of IPTG (0.25 mM) at 1 h 50 min, and then grown at 22 °C. **b)** SDS-PAGE. Lane 1: Marker. Lane 2: Cell-free extract of *E. coli* (SpL). Lane 3: Marker. Lane 4: Purified his-tagged SpL. **c)** Effect of water content on aminolysis of methyl hexanoate **1a** with benzylamine **2a** catalyzed by free SpL in *n*-hexane. **d)** Effect of water content on the aminolysis of 100 mM methyl hexanoate **1a** with 120 mM benzylamine **2a** by *E. coli* (SpL) in *n*-hexane. Specific activity was calculated after 30 min. Final amide yield was determined after 20 h. □: Specific activity (U/g protein or cdw); ■: Final amide yield (%).

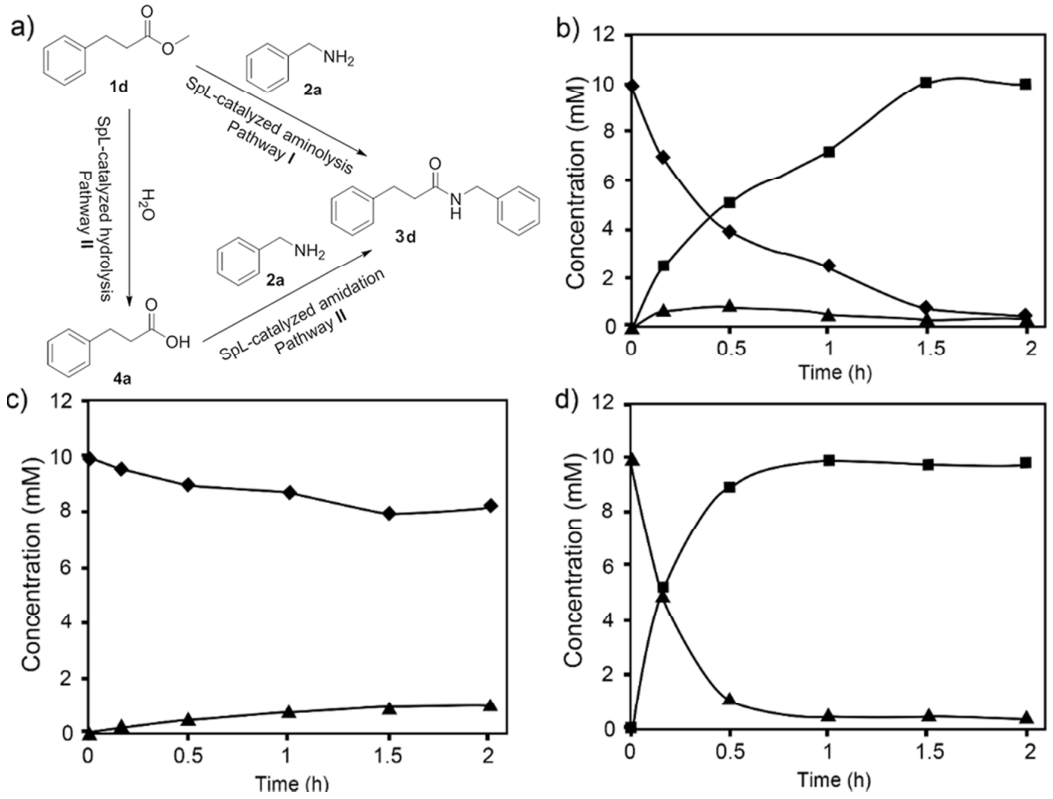


Figure 2. **a)** Possible pathways of SpL-catalyzed aminolysis of methyl 3-phenylpropionate **1d** with benzylamine **2a**. **b)** Time course of free SpL-catalyzed aminolysis of methyl 3-phenylpropionate **1d** with benzylamine **2a**. **c)** Time course of free SpL-catalyzed hydrolysis of methyl 3-phenylpropionate **1d**. **d)** Time course of free SpL-catalyzed aminolysis of 3-phenylpropionic acid **4a** and benzylamine **2a**. ■: **3d**; ▲: **4a**; ◆: **1d**. All reactions were performed in 5 mL partially hydrated *n*-hexane (4 %; v/v) with 1 mg free SpL.

Abstract graphic for TOC