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Continuous Flow Biocatalysis: Synthesis of Coumarin Carboxamide Derivatives by Lipase TL IM from *Thermomyces lanuginosus*

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Abstract: Coumarin carboxamide derivatives are important building blocks for organic synthesis and chemical biology due to their excellent biopharmaceutical properties. In this paper, we demonstrate for the first time a two-step enzymatic synthesis of coumarin carboxamide derivatives. Salicylaldehyde and dimethyl malonate were reacted to obtain coumarin carboxylate methyl derivatives, which were then reacted with various amines under the catalysis of lipase TL IM from *Thermomyces lanuginosus* to obtain coumarin carboxamide derivatives in continuous flow reactors. We studied various reaction parameters on the yields. The important features of this method include mild reaction conditions, a short reaction time (40 min), reduced environmental pollution, higher productivity (STY = 31.2941 g L^{-1} h^{-1}) and enzymes being relatively easy to obtain.

Keywords: enzymatic synthesis; microreactor; coumarin carboxamide derivatives

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1. Introduction

Coumarin is a class of natural and synthetic compound with antioxidant, anti-inflammatory, antithrombotic and antibacterial activities [1–4]. Natural coumarin derivatives show potential pharmacological effects in vivo and in vitro, which provide valuable clues for the further design of more active compounds. Ensaculin and AP 2238 are coumarin scaffold-containing compounds that have shown prospective acetylcholinesterase (AChE) inhibitory activity in clinical studies and are proposed for the treatment of Alzheimer's disease (AD) [5–7]. Umbelliferone compounds can selectively inhibit human carbonic anhydrases (hCAs) for the treatment of cancer [8,9] (Figure 1).

Coumarin carboxamide compounds, as important coumarin derivatives, have attracted much attention in organic synthesis and drug research because of their outstanding biopharmaceutical properties [10–12]. Research has shown that introducing an amide group at the third position of the coumarin can provide it with various biological activities [13]. Robert et al. reported the synthesis of 3-carboxamide-coumarin derivatives and studied their pharmaceutical activities and found that they could selectively inhibit plasmatic activated factor VII (FVIIa), and could be used in anticoagulant drugs [14]. Chidamide and Entinostat are both histone deacetylase inhibitors (HDACIs). Chidamide has been approved by FDA for the treatment of lymphoma or myeloma, and Syndax Pharmaceuticals currently owns the rights to Entinostat, which is still in trials for cancer treatment. Tooba et al. designed and synthesized some new coumarin-based benzamides, substituting

coumarin carboxamide for the benzyl carbamate moiety of Entinostat or the acrylamide moiety of Chidamide to have the same pharmacological effect, and able to be used to treat lymphoma or myeloma [15].

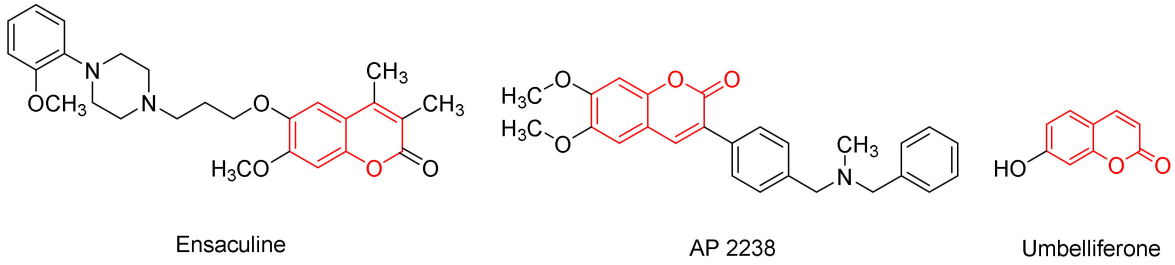


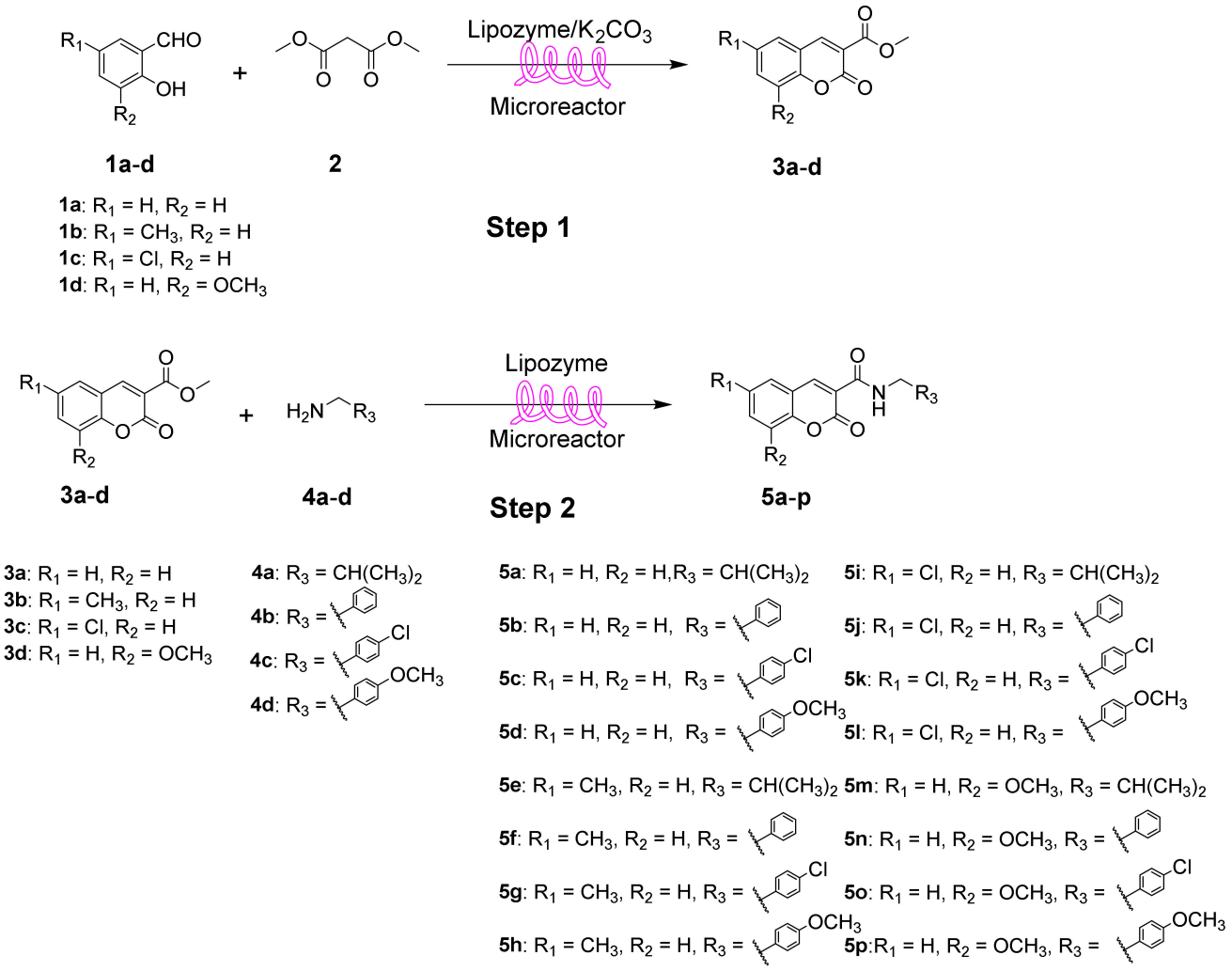
Figure 1. Structures of coumarin derivatives with potential drug activity.

The reported synthesis methods of coumarin carboxamide derivatives have the following problems: (1) they were usually prepared by condensation of substituted salicylaldehyde and malonate, and then undergo hydrolysis, halogenation and other steps, and finally acylation with amines, which is a cumbersome process; (2) they were commonly catalyzed by chemical catalysts such as NaOH, piperidine or DMAP, and cause pollution to the environment; (3) these reactions always require complex post-treatment, a long reaction time and high temperature. [16–19] Therefore, looking for a greener, environmentally friendly and efficient synthesis method of coumarin carboxamide derivatives, and quickly constructing a compound library of coumarin carboxamide derivatives for later drug activity screening attracted our attention. As efficient natural catalysts, enzymes have been introduced into the chemical reaction process, a series of chemical reaction steps have been replaced by biotransformation, creating a "greener" route for the design and synthesis of drugs and chemicals [20–23]. Several studies have been reported on the enzymatic synthesis of coumarin derivatives [24,25]. Hossein et al. reported the synthesis of dicoumarin compounds catalyzed by lipase PPL from *porcine pancreas* [26]. Dawei et al. first discovered and identified the coumarin C-glucosyltransferase (CGT) MaCGT from *Morus alba*, and then synthesized coumarin C-glycosides by whole-cell biotransformation of *Escherichia coli* [27]. However, the enzymatic synthesis of coumarin derivatives has the following problems: the reaction needs to be carried out in a specific environment, the alternative enzymes are difficult to obtain and the reaction time is longer. Therefore, finding more efficient, green and easier-to-obtain enzyme sources for coumarin carboxamide derivative synthesis has attracted our attention.

The combination of continuous flow microreactor and enzyme yields an influential new process [28–30]. The application of continuous flow microreactors in biocatalysis increases the overall reaction efficiency, facilitates the recycling of biocatalysts (without mechanical stirring) and simplifies the reaction process (reaction steps and subsequent processing) [31–33]. In the fields of medicine, daily chemicals and food, there are many research reports on continuous flow enzymatic reaction technology. Our laboratory also studied the enzyme-catalyzed synthesis of sugar-containing coumarin derivatives in continuous flow, and achieved good results. Whether the continuous flow enzymatic reaction technology can be used for the synthesis of coumarin carboxamide derivatives has aroused our attention. The purpose of this thesis is to utilize the Lipozyme® TL IM, a lipase from *Thermomyces lanuginosus* immobilized on porous polymeric beads and produced by Novozymes, to realize the continuous flow synthesis of a variety of coumarin carboxamide derivatives and investigate the influence of different reaction conditions on reaction yield.

The synthesis of coumarin carboxamide derivatives 5a–5p were outlined in Scheme 1. The first step is to obtain intermediates coumarin 3 carboxylate methyl derivatives through the reaction of salicylaldehyde 1 derivatives with dimethyl 2 malonates in continuous flow microreactors [34]. In the second step of the reaction, coumarin 3 carboxylate methyl

derivatives were directly reacted with various amines 4 catalyzed by lipase TL IM in continuous flow microreactors to obtain coumarin carboxamide derivatives 5a–5p.



Scheme 1. Synthesis of coumarin carboxamide derivatives in the continuous flow microreactors.

2. Results

2.1. Synthesis of Intermediates Coumarin Carboxylate Methyl Derivatives

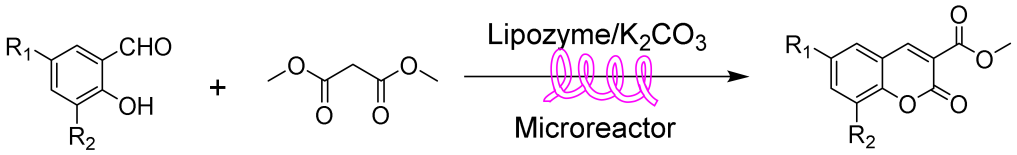
We investigated the reaction of coumarin carboxylate methyl derivatives co-catalyzed by the thermophilic fungus lipase TL IM and potassium carbonate in the continuous flow microreactors. By studying the synthesis of intermediate coumarin carboxylate methyl derivatives, we found that the reaction was catalyzed by a mixed catalyst (25 mg K_2CO_3/120 mg lipozyme TL IM) to give the best yield after 10 min at 40 °C (Table 1); the STY was 210.4 gh^{-1} L^{-1} and the biocatalyst yield was 4.06.

2.2. Effect of Reaction Medium and Catalyst

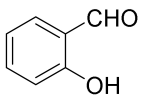
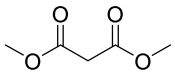
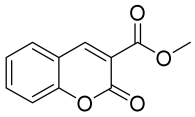
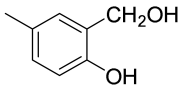
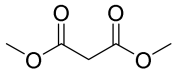
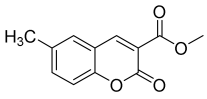
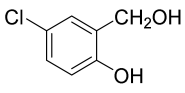
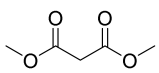
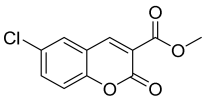
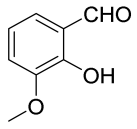
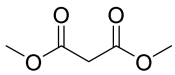
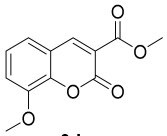
Generally, the reaction medium affects the catalytic performance of the enzyme. In this work, we tested coumarin-3-carboxylate methyl ester (3a) and isobutylamine (4a) for the synthesis of isobutyl-coumarin-3-carboxamide (5a) in the continuous flow reactors. We did a blank control test and got results that would not react in the absence of the enzyme. Different enzymes (Lipozyme TL IM, Novozym® 435) were used as biocatalysts in different organic solvents (*tert*-amyl alcohol, methanol, DMSO, isopropanol, acetonitrile, acetone, toluene). Highly polar solvents may strip essential water from the protein and disrupt the functional structure of the enzyme, which may reduce enzyme activity. In

addition, some organic solvent molecules may change the conformation of the enzyme by entering the active center of the enzyme, thereby changing the performance of the enzyme [35,36]. Table 2 indicates that lipozyme TL IM in *tert*-amyl alcohol was the best catalyst and provided the highest yield (66%).

Table 1. Reaction results of intermediates coumarin carboxylate methyl derivatives under continuous-flow conditions [a].



1 **2** **3**

Entry	Salicylaldehyde Derivative	Dimethyl Malonate	Product	Yield [b] (%)
1	 **1a**	 **2**	 **3a**	86.5 ± 1.7
2	 **1b**	 **2**	 **3b**	89.3 ± 1.3
3	 **1c**	 **2**	 **3c**	82.6 ± 0.8
4	 **1d**	 **2**	 **3d**	95.5 ± 1.5

[a] General experimental conditions: in the continuous flow reactors, feed A, dissolve 5 mmol of salicylaldehyde derivatives in 10 mL DMSO; feed B, dissolve 10 mmol diethyl malonate in 10 mL DMSO; catalyze with mixed catalyst K_2CO_3/lipase TL IM; react in continuous flow microreactors. [b] Isolated yield. Yield: 100 × (actually obtained amount/calculated amount). Data are presented as mean ± SD of three experiments.

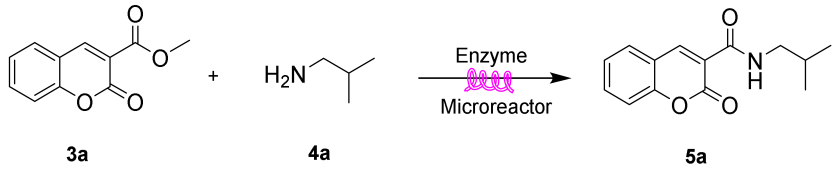
2.3. Effect of Reaction Temperature

Another parameter examined was temperature, which plays significant roles in enzymatic reactions. We investigated the effect of temperature (30 °C to 60 °C) on the reaction (Figure 2); the maximum reaction yield of reaction was observed at 50 °C. As can be seen in Figure 2, the reaction yield declines at 55 °C, which may be due to a decrease in enzyme activity at higher temperatures [37].

2.4. Effect of Substrate Ratio

In order to evaluate the effect of the molar ratio on the reaction, six conditions were evaluated. We found that with the increase in isobutylamine (4a), the synthesis efficiency of the product improved correspondingly (Figure 3). When the substrate ratio of coumarin-3-carboxylate methyl ester (3a) to isobutylamine (4a) was 1:2, the reaction yield was the best at 73%. Therefore, we decided to choose coumarin-3-carboxylate methyl ester (3a):isobutylamine (4a) = 1:2 as the optimal substrate ratio.

Table 2. The effect of reaction medium and catalysts on the synthesis of coumarin carboxamide derivatives under continuous-flow conditions [a].



3a 4a 5a

Entry	Solvent	Catalysts	Log p	Yield [b] (%)
1	*tert*-amyl alcohol	None	0.60	n.d.
2	Methanol	Lipozyme TL IM	−0.76	45.4 ± 2
3	*tert*-amyl alcohol	Lipozyme TL IM	0.60	66.8 ± 1.5
4	DMSO	Lipozyme TL IM	−1.3	n.d.
5	Isopropanol	Lipozyme TL IM	0.39	35.3 ± 0.5
6	Acetonitrile	Lipozyme TL IM	−0.33	42.4 ± 0.8
7	Acetone	Lipozyme TL IM	−0.23	36.7 ± 1.2
8	Toluene	Lipozyme TL IM	2.5	33.2 ± 0.7
9	*tert*-amyl alcohol	Novozym® 435	0.60	21.8 ± 0.3
10	Methanol	Novozym® 435	−0.76	16.1 ± 0.6
11	Isopropanol	Novozym® 435	0.39	n.d.
12	Acetonitrile	Novozym® 435	−0.33	n.d.
13	DMSO	Novozym® 435	−1.3	n.d.

[a] General experimental conditions: in the continuous flow reactors, feed 1, dissolve 5 mmol of coumarin-3-carboxylate methyl ester (3a) in 10 mL solvent; feed 2, dissolve 5 mmol of isobutylamine (4a) in 10 mL solvent: 45 °C, flow rate 20.9 μL min^{-1}, residence time 30 min, enzyme 870 mg. [b] Isolated yield. Yield: 100 × (actually obtained amount/calculated amount). The data are presented as average ± SD of triplicate experiments. n.d.—no data.

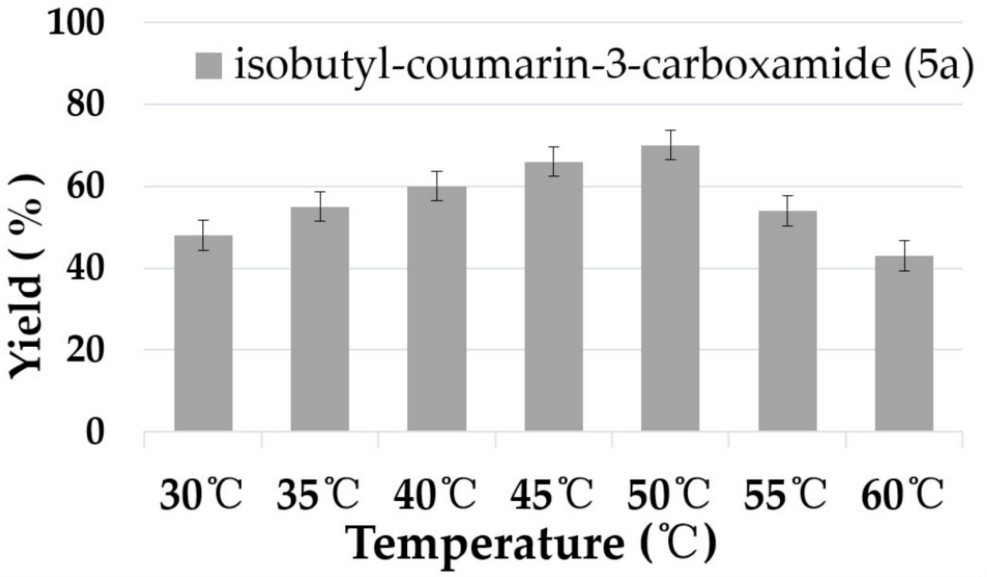


Figure 2. The effect of temperature on the enzymatic synthesis of coumarin carboxamide derivative under continuous-flow conditions.

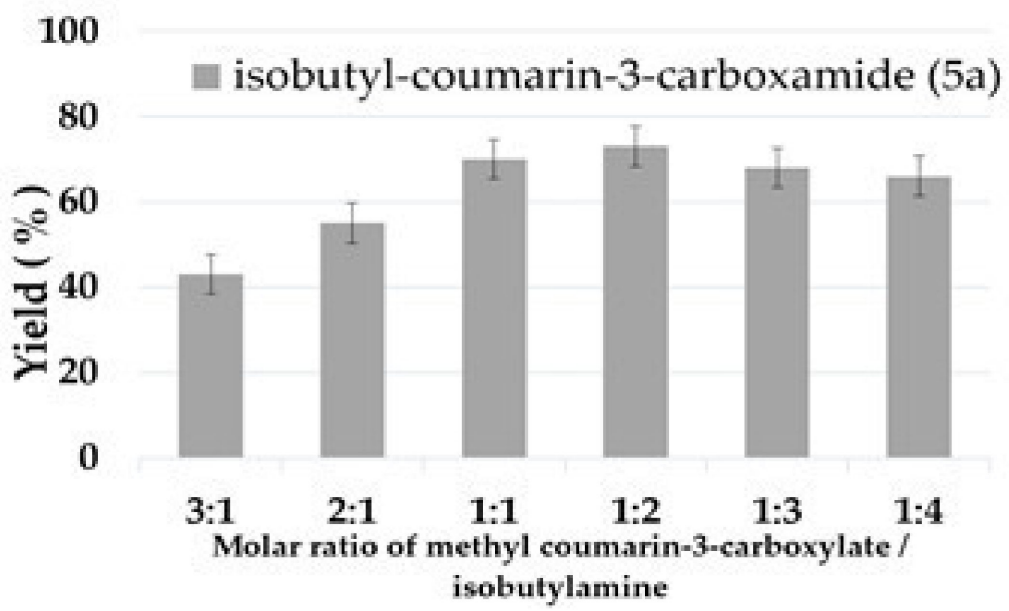


Figure 3. The effect of the molar ratio on the enzymatic synthesis of coumarin carboxamide derivative under continuous-flow conditions.

2.5. Effect of Residence Time

During the continuous-flow reaction, residence time is also very important. We investigated the reaction from 20 min to 50 min (Figure 4). The best yield, 85%, was obtained when the residence time was 40 min (flow rate of 15.6 μL min^{-1}). Therefore, 40 min was selected as the optimal residence time for the following studies.

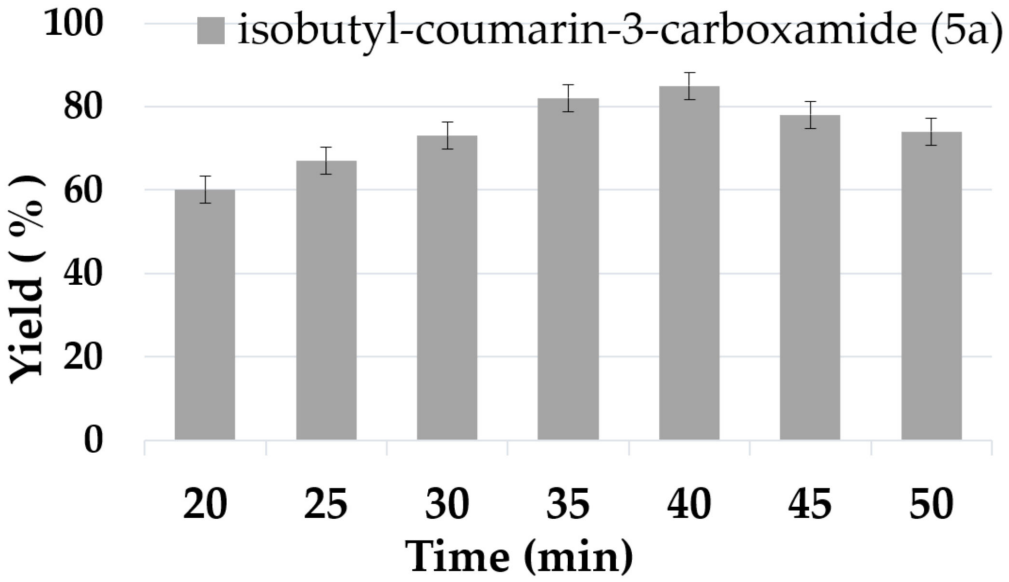


Figure 4. The effect of residence time on the enzymatic synthesis of coumarin carboxamide derivative under continuous-flow conditions.

2.6. The Effect of Enzyme Reusability

The reusability of Lipozyme TL IM was investigated under optimal conditions. After ten catalytic cycles with the same enzyme sample, the reaction retained 62% of its original yield (Figure 5). This result shows that Lipozyme TL IM has reusable properties.

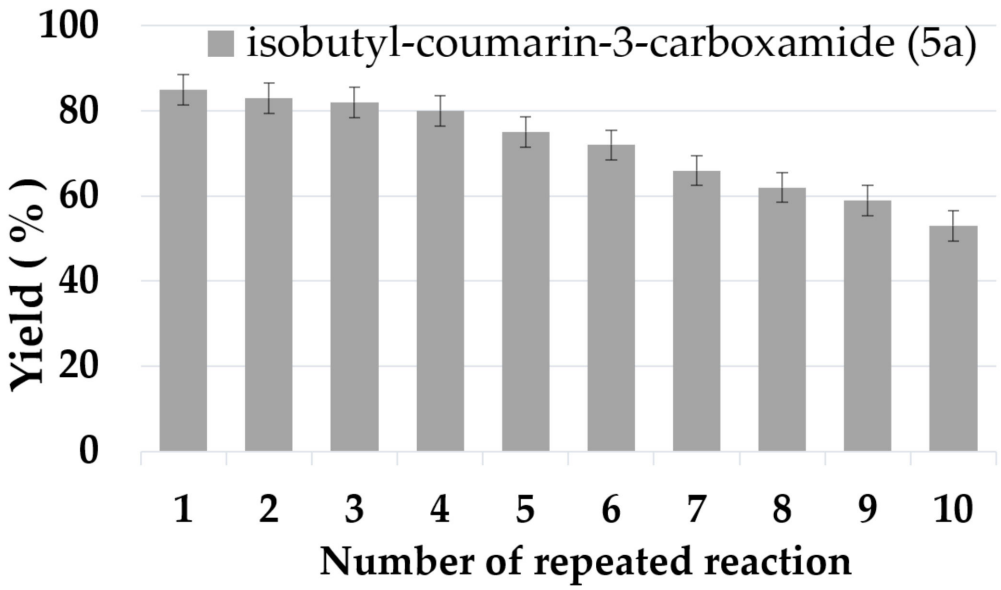
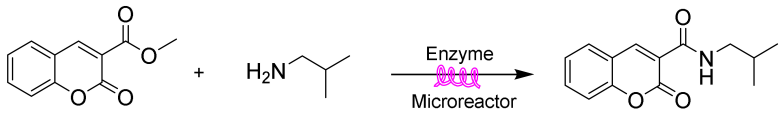


Figure 5. The effect of enzyme reusability on the enzymatic synthesis of coumarin carboxamide derivative under continuous-flow conditions.

2.7. Enzymatic Synthesis of Coumarin Carboxamide Derivative in Continuous Flow Microreactors and Batch Bioreactors

In order to compare the differences between reactions in the continuous flow reactors and the traditional shaker reactors, we carried out experiments in two reactors. As we can see from the Table 3, in shaker reactors, a reaction time of 24 h or above is required to achieve optimal yields (Method B). However, in the continuous flow microreactors, the desired optimum conversion can be achieved in 40 min (method A). Space-time yield (STY) is commonly used to evaluate the productivity of different systems and is normalized to 1 L volume (g h^{-1} L^{-1}). This describes the amount of product formed at a certain flow rate and reaction volume [38–42]. The STY of continuous flow microreactors is much higher than that of shaker reactors. Therefore, the continuous flow reactors can improve the efficiency of enzymatic synthesis of coumarin carboxamide derivatives.

Table 3. Enzymatic synthesis of coumarin carboxamide derivatives in the continuous flow reactors or shaker reactors [a].



3a 4a 5a

Entry	Method	STY (g L^{-1} h^{-1})	Yield [b] (%)
1	A	31.2941	85.7 ± 0.6
2	B	0.6829	66.9 ± 1.2

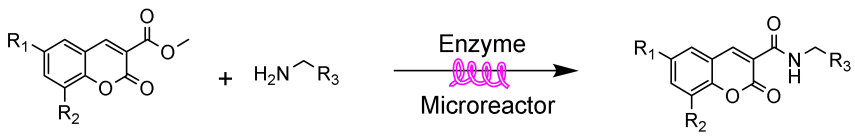
[a] General experimental conditions: method A: in the continuous flow reactors, feed 1, dissolve 5 mmol of coumarin-3-carboxylate methyl ester (3a) in 10 mL *tert*-amyl; feed 2, dissolve 10 mmol of isobutylamine (4a) in 10 mL *tert*-amyl, flow rate 15.6 µL min^{-1}, residence time 40 min, enzyme 870 mg, 50 °C. Method B: shaker reactors, add 5 mmol of coumarin-3-carboxylate methyl ester (3a), 10 mmol of isobutylamine (4a) and 20 mL *tert*-amyl alcohol to a 50 mL Erlenmeyer flask, lipozyme TL IM 870 mg, 180 rpm, 50 °C, 24 h. [b] Isolated yield. Yield: 100 × (actually obtained amount/calculated amount). The data are presented as average ± SD of triplicate experiments.

2.8. The Scope and Limitation for the Coumarin Carboxamide Derivative Synthesis Methodology

We further researched the scope and limitation of the methodology for coumarin carboxamide derivative synthesis catalyzed by lipozyme TL IM. After optimizing the

reaction conditions, the reaction was carried out using coumarin carboxylate methyl derivatives (coumarin-3-carboxylate methyl ester (3a), 6-methyl-coumarin-3-carboxylate methyl ester (3b), 6-chloro-coumarin-3-carboxylate methyl ester (3c) and 5-methoxy-coumarin-3-carboxylate methyl ester(3d)) as acyl donors; its substrate scope was investigated with different amine compounds (isobutylamine (4a), benzylamine (4b), 4-chlorobenzylamine (4c) and 4-methoxybenzylamine (4d)). As shown in Table 4, electron cloud density and steric hindrance affect the reactivity. We found that the reaction yield of aliphatic amine (e.g., entry 1, 85.7%) was higher than that of benzylamine (e.g., entry 2, 71.3%). Furthermore, methoxycoumarins (entry 14, 79.8%) responded better than chlorocoumarins (entry 10, 35.4%). Benzylamine with an electron-donating group (e.g., entry 8, 77.9%) was more reactive than benzylamine with an electron-withdrawing group (e.g., entry 7, 63.4%) under the same parameters. This indicates that the introduction of electron-donating groups is favorable for the reaction, while the presence of electron-withdrawing groups will reduce the reaction yield.

Table 4. The effect of substrate structure to the enzymatic synthesis of coumarin carboxamide derivatives under continuous-flow conditions [a].



3 **4** **5**

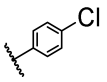
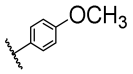
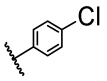
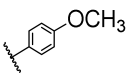
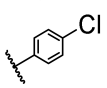
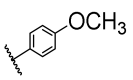
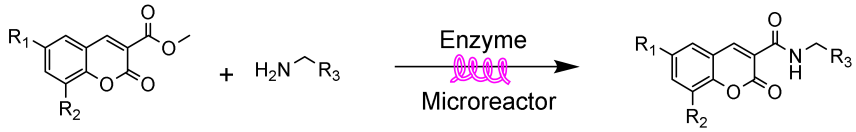
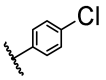
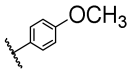
Entry	R_1	R_2	R_3	Product	Yield [b] (%)
1	H	H	$CH(CH_3)_2$	5a	85.7 ± 0.6
2	H	H		5b	71.3 ± 0.8
3	H	H		5c	64.2 ± 1.5
4	H	H		5d	74.6 ± 2
5	CH_3	H	$CH(CH_3)_2$	5e	87.5 ± 0.5
6	CH_3	H		5f	75.8 ± 0.2
7	CH_3	H		5g	63.4 ± 1.1
8	CH_3	H		5h	77.9 ± 0.1
9	Cl	H	$CH(CH_3)_2$	5i	80.6 ± 1.8
10	Cl	H		5j	35.4 ± 1.1
11	Cl	H		5k	<5
12	Cl	H		5l	43.6 ± 1.5
13	H	OCH_3	$CH(CH_3)_2$	5m	90.5 ± 0.5

Table 4. *Cont.*



14	H	OCH_3		5n	79.8 ± 0.4
15	H	OCH_3		5o	67.9 ± 1.2
16	H	OCH_3		5p	80.2 ± 0.1

[a] General experimental conditions: in the continuous flow reactors, feed 1, dissolve 5 mmol coumarin carboxylate methyl derivatives in 10 mL *tert*-amyl; feed 2, dissolve 10 mmol amine compounds in 10 mL *tert*-amyl, flow rate 15.6 μL min^{-1}, residence time 40 min, enzyme 870 mg, 50 °C. [b] Isolated yield. Yield: 100 × (actually obtained amount/calculated amount). The data are presented as average ± SD of triplicate experiments.

3. Materials and Methods

3.1. Materials

All compounds were purchased from commercial sources unless otherwise stated. Lipozyme® TL IM and Novozym® 435 was purchased from Novo Nordisk (Copenhagen, Denmark). Salicylaldehyde, 5-methylsalicylaldehyde, 5-chlorosalicylaldehyde, isobutylamine and 4-methoxybenzylamine were purchased from Aladdin (Shanghai, China). Dimethyl malonate, benzylamine, 4-chlorobenzylamine were purchased from Energy Chemical (Shanghai, China). A quantity of 2-hydroxy-3-methoxybenzaldehyde was purchased from J&K Scientific (Beijing, China). PHD 2000 syringe pumps were purchased from Harvard (Holliston, MA, USA).

3.2. Experimental Setup and Experiment Conditions

3.2.1. General Procedure of the Synthesis of Coumarin Carboxamide Derivatives

Figure 6 depicts the enzymatic synthesis of coumarin carboxamide derivatives from coumarin carboxylate methyl derivatives and amine compounds in a continuous-flow microreactor. The equipment consists of an injection pump, coil reactor and Y-type mixer (φ = 1.8 mm). A syringe pump was used to deliver reagents from the reactant syringe to the Y-mixer and a microchannel reactor (consists of 100 cm × 2 mm PFA tube) used for reaction. The silica gel tubing was filled with lipozyme TL IM (870 mg) and immersed in a constant temperature water bath to maintain the reaction temperature. Lipozyme TL IM was supplied as silica particles; the reactivity was 250 IUN/g and the particle diameter is 0.3–1.0 mm. A quantity of 5 mmol coumarin carboxylate methyl derivatives were dissolved in 10 mL *tert*-amyl alcohol (feed 1) and 10 mmol amines were dissolved in 10 mL *tert*-amyl alcohol (feed 2). Feed 1 and 2 were put into a 10 mL injector, and after being delivered to the Y-mixer at a flow rate of 15.6 μL min^{-1}, the reaction was carried out through a microchannel reactor at 50 °C; the residence time was 40 min. The product was chromatographed on silica gel (200–300 mesh) and the target product was confirmed by ^{1}H NMR, ^{13}C NMR and ESI-MS.

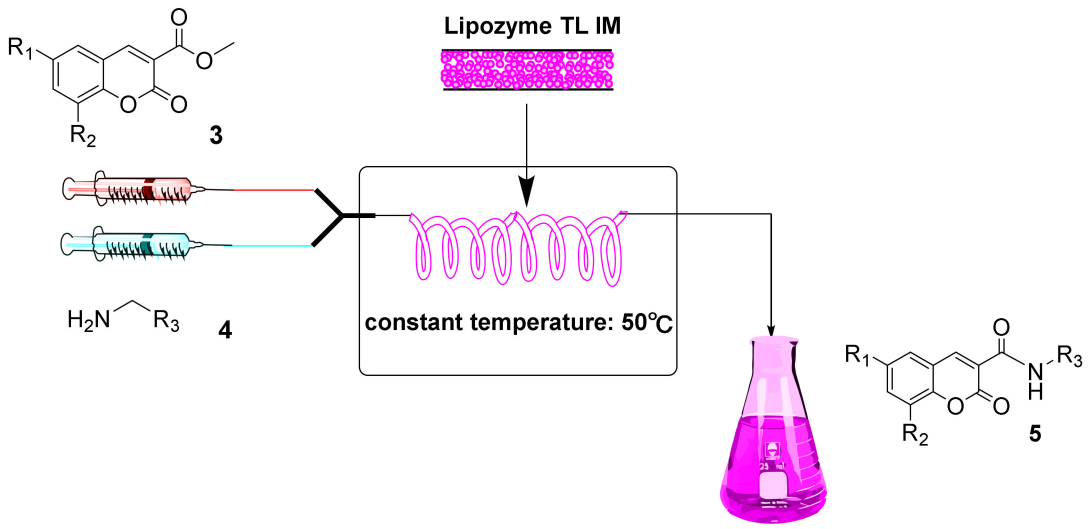


Figure 6. Experimental setup for coumarin carboxamide derivative synthesis in microreactors catalyzed by lipase TL IM.

3.2.2. Experimental Conditions for Enzyme Reusability

The reusability of Lipozyme TL IM was investigated under optimal conditions. A quantity of 5 mmol of coumarin-3-carboxylate methyl ester (3a) was dissolved in 10 mL *tert*-amyl alcohol (feed 1) and 10 mmol of isobutylamine (4a) was dissolved in 10 mL *tert*-amyl alcohol (feed 2). Feed 1 and 2 were put into a 10 mL injector, and after being delivered to the Y-mixer at a flow rate of 15.6 μL min^{-1}, the reaction was carried out through a microchannel reactor at 50 °C; the residence time was 40 min. After each reaction batch, a wash was performed with cold *tert*-amyl alcohol to remove any unconverted reactants and/or product molecules. The same conditions were employed for the next reaction batch for a total of ten catalytic cycles.

3.3. Analytical Methods

3.3.1. Thin-Layer Chromatography (TLC)

For TLC analysis, ethyl acetate/petroleum ether = 1:3 (by vol) was used as the eluent. Results were determined under 254 nm UV irradiation.

3.3.2. Nuclear Magnetic Resonance (NMR) and Electrospray Ionization Mass Spectrometry (ESI/MS)

The product was purified by column chromatography, and the structures of the coumarin carboxamide derivative were confirmed by ^{1}H NMR, ^{13}C NMR and ESI-MS.

Isobutyl-coumarin-3-carboxamide (5a). White solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.91 (d, *J* = 14.4 Hz, 2H), 7.77–7.61 (m, 2H), 7.50–7.30 (m, 2H), 3.31 (dd, *J* = 6.8, 5.9 Hz, 2H), 2.00–1.84 (m, *J* = 6.7 Hz, 1H), 1.00 (d, *J* = 6.7 Hz, 6H).; ^{13}C NMR (126 MHz, Chloroform-*d*) δ 161.54, 161.51, 154.38, 148.23, 133.92, 129.76, 125.25, 118.68, 118.59, 116.59, 47.24, 28.44, 20.20. HRMS (ESI): calculated for $C_{14}H_{15}NO_3$ $[M + Na]^+$: 268.1052, found 268.1125.

Benzyl-coumarin-3-carboxamide (5b). White solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.19 (s, 1H), 8.97 (s, 1H), 7.79–7.60 (m, 2H), 7.45–7.33 (m, 7H), 7.30 (s, 1H), 4.69 (d, *J* = 5.8 Hz, 2 H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 161.53, 161.41, 154.47, 148.57, 137.90, 134.09, 129.82, 128.71, 127.71, 127.47, 125.30, 118.65, 118.45, 116.65, 43.87. HRMS (ESI): calculated for $C_{17}H_{13}NO_3$ $[M + Na]^+$: 302.0793, found 302.0785.

4-chlorobenzyl-coumarin-3-carboxamide (5c). White solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.21 (s, 1 H), 8.96 (s, 1H), 7.78–7.62 (m, 1H), 7.50–7.38 (m, 2 H), 7.32 (d, *J* = 2.5 Hz, 3H),

4.65 (d, *J* = 5.9 Hz, 2H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 161.46, 154.49, 148.73, 136.51, 134.21, 133.29, 129.86, 129.09, 128.84, 125.36, 118.62, 116.68, 43.16. HRMS (ESI): calculated for $C_{17}H_{12}ClNO_3$ $[M + Na]^+$: 314.0584, found 314.0915.

4-methoxybenzyl-coumarin-3-carboxamide (5d). White solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.12 (s, 1H), 8.96 (s, 1H), 7.79–7.58 (m, 2H), 7.47–7.35 (m, 2H), 7.30 (s, 2H), 6.89 (d, *J* = 8.6 Hz, 2H), 4.61 (d, *J* = 5.7 Hz, 2H), 3.82 (s, 3H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 161.38, 161.36, 159.01, 154.44, 148.46, 134.03, 130.04, 129.78, 129.10, 125.27, 118.65, 118.51, 116.63, 114.11, 55.30, 43.36. HRMS (ESI): calculated for $C_{18}H_{15}NO_4$ $[M + Na]^+$: 332.0899, found 332.0581.

Isobutyl-6-methyl-coumarin-3-carboxamide (5e). White solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.92 (d, *J* = 7.4 Hz, 1H), 8.87 (s, 1H), 7.51–7.45 (m, 2H), 7.31 (d, *J* = 9.0 Hz, 1H), 3.35–3.28 (m, 2H), 2.45 (s, 3H), 1.94 (dt, *J* = 13.4, 6.7 Hz, 1H), 1.01 (d, *J* = 6.7 Hz, 6H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 161.75, 161.66, 152.63, 148.21, 135.12, 135.10, 129.35, 118.50, 118.46, 116.32, 47.27, 28.48, 20.77, 20.24. HRMS (ESI): calculated for $C_{15}H_{17}NO_3$ $[M + Na]^+$: 282.1101, found 282.1098.

Benzyl-6-methyl-coumarin-3-carboxamide (5f)**.** White solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.23 (s, 1H), 8.92 (d, *J* = 0.7 Hz, 1H), 7.48 (dqd, *J* = 4.2, 2.2, 0.7 Hz, 2H), 7.38–7.26 (m, 6H), 4.68 (d, *J* = 5.9 Hz, 2H), 2.46 (s, 3H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 161.70, 152.68, 148.56, 137.95, 135.27, 135.19, 129.39, 128.70, 127.71, 127.44, 118.43, 118.27, 116.35, 43.84, 20.74. HRMS (ESI): calculated for $C_{18}H_{15}NO_3$ $[M + Na]^+$: 316.0950, found 316.0971.

4-chlorobenzyl-6-methyl-coumarin-3-carboxamide (5g). White solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.25 (s, 1H), 8.91 (s, 1H), 7.49 (d, *J* = 7.7 Hz, 2H), 7.34–7.30 (m, 5H), 4.64 (d, *J* = 5.9 Hz, 2H), 2.46 (s, 3H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 161.81, 161.66, 148.70, 136.57, 135.40, 133.26, 129.42, 129.08, 128.82, 118.39, 118.09, 116.38, 43.14, 20.75. HRMS (ESI): calculated for $C_{18}H_{14}ClNO_3$ $[M + Na]^+$: 350.0662, found 350.0772.

4-methoxybenzyl-6-methyl-coumarin-3-carboxamide (5h). White solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.15 (s, 1H), 8.91 (s, 1H), 7.48 (dd, *J* = 4.7, 2.5 Hz, 2H), 6.89 (d, *J* = 8.7 Hz, 2H), 4.61 (d, *J* = 5.8 Hz, 2H), 3.81 (s, 3H), 2.46 (s, 3H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 161.58, 161.55, 158.99, 152.65, 148.47, 135.22, 135.16, 130.09, 129.37, 129.10, 118.42, 118.31, 116.33, 114.10, 55.30, 43.34, 20.75. HRMS (ESI): calculated for $C_{19}H_{17}NO_4$ $[M + Na]^+$: 346.1050, found 346.1050.

Isobutyl-6-chloro-coumarin-3-carboxamide (5i). White solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.85 (s, 2H), 7.68 (d, *J* = 2.4 Hz, 1H), 7.61 (ddd, *J* = 8.9, 2.4, 1.2 Hz, 1H), 7.37 (dd, *J* = 8.9, 1.1 Hz, 1H), 3.31 (ddd, *J* = 6.8, 5.9, 1.1 Hz, 2H), 1.93 (td, *J* = 6.8, 1.1 Hz, 1H), 1.00 (dd, *J* = 6.7, 1.1 Hz, 6H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 160.97, 160.94, 152.70, 146.96, 133.81, 130.64, 128.73, 119.71, 119.68, 118.06, 47.33, 28.42, 20.19. HRMS (ESI): calculated for $C_{14}H_{14}ClNO_3$ $[M + Na]^+$: 302.0662, found 302.0732.

Benzyl-6-chloro-coumarin-3-carboxamide (5j). White solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.90 (t, *J* = 5.7 Hz, 1H), 8.69 (s, 1H), 7.95 (d, *J* = 2.2 Hz, 1H), 7.60 (dd, *J* = 8.5, 2.3 Hz, 1H), 7.35–7.26 (m, 6H), 7.29–7.22 (m, 1H), 4.48 (dt, *J* = 5.8, 0.9 Hz, 2H). ^{13}C NMR (125 MHz, Chloroform-d) δ 166.55, 160.09, 152.03, 138.21, 135.03, 132.90, 130.40, 128.75, 128.50, 127.70, 127.60, 120.62, 119.09, 115.11, 44.07. HRMS (ESI): calculated for $C_{17}H_{12}ClNO_3$ $[M + Na]^+$: 336.0506, found 336.0556.

4-methoxybenzyl-6-chloro-coumarin-3-carboxamide (5k). White solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.89 (t, *J* = 5.7 Hz, 1H), 8.69 (s, 1H), 7.95 (d, *J* = 2.2 Hz, 1H), 7.60 (dd, *J* = 8.5, 2.3 Hz, 1H), 7.32 (d, *J* = 8.5 Hz, 1H), 7.19 (dt, *J* = 8.4, 1.0 Hz, 2H), 6.88–6.82 (m, 2H), 4.51 (dt, *J* = 5.7, 0.9 Hz, 2H), 3.78 (s, 3H). ^{13}C NMR (125 MHz, Chloroform-*d*) δ 166.54, 160.09, 158.96,

152.03, 135.03, 132.90, 131.78, 130.40, 129.18, 128.75, 120.62, 119.09, 115.11, 113.92, 55.32, 43.62. HRMS (ESI): calculated for $C_{18}H_{14}ClNO_4$ $[M + Na]^+$: 366.0611, found 366.0821.

Isobutyl-8-methoxy-coumarin-3-carboxamide (5m). White solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.89 (s, 2H), 7.34–7.24 (m, 2H), 7.20 (dd, *J* = 7.9, 1.6 Hz, 1H), 4.00 (s, 3H), 3.31 (dd, *J* = 6.9, 5.9 Hz, 2H), 1.93 (dt, *J* = 13.5, 6.7 Hz, 1H), 1.00 (d, *J* = 6.6 Hz, 6H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 161.53, 161.06, 148.41, 147.07, 144.09, 125.10, 120.91, 119.37, 118.80, 115.46, 56.38, 47.28, 28.47, 20.22. HRMS (ESI): calculated for $C_{15}H_{17}NO_4$ $[M + Na]^+$: 298.1050, found 298.1041.

Benzyl-8-methoxy-coumarin-3-carboxamide (5n). White solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.21 (s, 1H), 8.93 (s, 1H), 7.37–7.31 (m, 5H), 7.28 (s, 4H), 7.21 (dd, *J* = 7.9, 1.5 Hz, 1H), 4.68 (d, *J* = 5.8 Hz, 2H), 4.00 (s, 3H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 161.58, 160.92, 148.75, 147.09, 144.15, 137.94, 128.68, 127.72, 127.42, 125.14, 120.95, 119.32, 118.63, 115.62, 56.38, 43.85. HRMS (ESI): calculated for $C_{18}H_{15}NO_4$ $[M + Na]^+$: 332.0899, found 332.0926.

4-chlorobenzyl-8-methoxy-coumarin-3-carboxamide (5o). White solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.24 (s, 1H), 8.93 (s, 1H), 7.35–7.28 (m, 6H), 7.26–7.20 (m, 2H), 4.63 (d, *J* = 6.0 Hz, 2H), 4.01 (s, 3H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 161.70, 160.98, 148.94, 147.08, 144.13, 136.53, 133.24, 129.10, 128.93, 128.81, 125.23, 120.96, 119.26, 118.42, 115.68, 56.37, 43.15. HRMS (ESI): calculated for $C_{18}H_{14}ClNO_4$ $[M + Na]^+$: 366.0611, found 366.0536.

4-methoxybenzyl-8-methoxy-coumarin-3-carboxamide (5p). White solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.14 (s, 1H), 8.93 (s, 1H), 7.32–7.27 (m, 4H), 7.20 (dd, *J* = 7.8, 1.6 Hz, 1H), 6.91–6.87 (m, 2H), 4.61 (d, *J* = 5.8 Hz, 2H), 4.00 (s, 3H), 3.81 (s, 3H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 161.45, 160.90, 159.01, 148.69, 147.07, 144.13, 130.10, 129.13, 125.14, 120.94, 119.32, 118.69, 115.55, 114.11, 56.37, 55.31, 43.36. HRMS (ESI): calculated for $C_{19}H_{17}NO_5$ $[M + Na]^+$: 362.1107, found 362.0997.

4. Conclusions

In conclusion, we demonstrated the enzymatic synthesis of coumarin carboxamide derivatives in two steps for the first time, from salicylaldehyde derivatives with dimethyl malonate, to get the intermediates coumarin carboxylate methyl derivatives which were then reacted directly with various amines (isobutylamine, benzylamine, 4-chlorobenzylamine, 4-methoxybenzylamine) catalyzed by lipozyme TL IM from *Thermomyces lanuginosus* under continuous-flow microreactors. We investigated the effect of various reaction parameters (reaction medium, catalyst, temperature, substrate ratio, residence time and reactant structure) on the coumarin carboxamide derivative synthesis performance under the continuous-flow microreactors. Compared with the traditional method, this method has a short reaction time (40 min), mild reaction conditions (*tert*-amyl alcohol) and higher productivity (STY = 31.2941 g L^{-1} h^{-1}). What is more, using enzymes as catalysts results in reduced environmental pollution and avoids tedious reaction processes and complicated follow-up processing. This work presents information that facilitates the design and synthesis of new coumarin derivatives for subsequent drug screening.

Author Contributions: M.-J.Y. and L.-H.D. conceptualized the studies, carried out data collection, data analysis, drafted and revised the manuscript; M.-J.Y. and P.-F.C. conducted background research and data collection; Y.P., L.-Y.Z., S.-Y.Z. and Z.-K.S. carried out data collection; M.-J.Y., L.-H.D. and X.-P.L. analyzed the data and revised the manuscript. All authors gave final approval for publication. All authors have read and agreed to the published version of the manuscript.

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