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ARTICLE TYPE

An efficient condensation of substituted salicylaldehyde and malononitrile catalyzed by lipase under microwave irradiation

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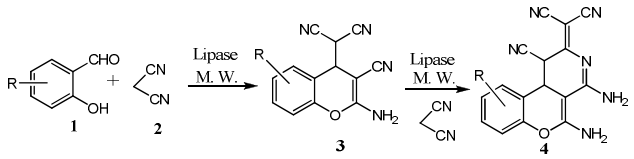
The present work illustrates the condensation of substituted salicylaldehyde and malononitrile catalyzed by lipase under microwave irradiation. The reaction obtains two different products by a delicate control of the substrate molar ratio and reaction time. This protocol has the advantages of high yield, short reaction time and environmental friendliness.

In the past few years, enzymes have attracted much attention for their abilities to catalyze chemical reactions fully different to the physiological ones which is termed catalytic promiscuity [1-5]. The progress in the field of promiscuous reactions has provided a new green synthetic protocol for organic synthesis [6-8]. However, one of the major drawbacks in this field is the low reaction rate mainly due to the unnatural substrates, which may limit its industrial application seriously. Therefore, it is necessary to find a suitable method to accelerate the reaction.

It's known that the current tools of enzyme engineering can increase the catalytic performance of lipase, such as immobilization, medium engineering and chemical modification [9-16]. As an efficient and environmentally benign technique, microwave irradiation can also accelerate many reactions in several minutes which typically required hours or days under conventional heating [17]. More attractively, microwave irradiation has been successfully introduced into the enzymatic reactions in the past decade, and can enhance the enzyme activity and selectivity greatly [18-21]. However, no research has been reported so far on the application of microwave irradiation in enzyme-catalyzed promiscuous reaction.

Our recent interest has been focused on the development of new enzyme promiscuous applications in the synthesis of heterocyclic compounds using lipase as catalyst. 2-Iminochromene appears as a ubiquitous structural motif in many heterocyclic compounds. Synthetic analogues were developed over the years, some of them displaying extensive bioactivities, such as antifungal and antimicrobial activity [22-23]. Herein, we report an efficient condensation of substituted salicylaldehyde and malononitrile to synthesize **3** and **4** catalyzed by lipase under microwave irradiation for the first time (Scheme 1). A comparison between conventional heating and microwave irradiation is conducted and the effect of the reaction conditions on the condensation has also been investigated.



Scheme 1 Lipase-catalyzed condensation of substituted salicylaldehyde and malononitrile under microwave irradiation

In order to find the most efficient biocatalyst, five enzymes from different resources (CALB, PPL, CSL, BSL2 and APE1547) were screened for the condensation of substituted salicylaldehyde and malononitrile. Initially, the condensation of salicylaldehyde **1a** and malononitrile **2** was selected as the model reaction in this study. As shown in Table 1, after 1 h of incubation in 60 °C, the moderate yields of **3a** (38.1-58.3%) could be achieved when enzymes were selected as catalyst. The highest yield (58.3%) was achieved by using CSL as catalyst under conventional heating.

To improve the catalytic performance of enzyme, microwave irradiation was adopted on the enzymatic condensation. Compared with conventional heating, the reaction time was dramatically shortened to 2 min with the high yields (68.5-91.3%) when the reaction was preceded under microwave irradiation at 60 °C. These results demonstrate that microwave irradiation is an efficient method to improve the catalytic performance of lipase. Among of the selected enzymes, BSL2 exhibited the highest catalytic performance under microwave irradiation instead of CSL under the conventional heating. The possible explanation may be that BSL2 could be changed to be a more active conformation under microwave irradiation than CSL.

Furthermore, the denatured BSL2 (denatured by heating) or BSA (Entry 6-7) was used as catalyst in this reaction and the result was similar to the control (Entry 9). To confirm the role of active center of BSL2 in this reaction, a serine protease inhibitor (Phenylmethanesulfonyl fluoride, PMSF) was used to inactivate the lipase. The yield of **3a** for the inactivated BSL2 (inactivated by PMSF) was similar to that resulted from using BSL2 in this reaction (Entry 8). It's well known that the irreversible inhibitor (PMSF) inactivates enzyme by binding specifically to the active site serine residue in a serine protease and the heat-induced denaturation results from a conformational change of the protein. Thus, this reaction might be catalyzed by another site/s of the enzyme structure, instead of the catalytic triad in the active center of lipase, and a proper active conformation of lipase should be

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necessary for this reaction which was suggested by the results obtained above. These results were similar to the report by Izquierdo *et al* [13].

Table 1 Effect of enzyme sources and microwave irradiation for the synthesis of **3a**

Entry	Enzyme	Yield (%) Conventional heating [a]	Yield (%) Microwave irradiation [b]
1	*Candida antarctica* lipase B (CALB)	38.1±2.10	71.3±1.36
2	Porcine pancreas lipase (PPL)	49.6±0.95	80.5±0.89
3	Lipase from *Candida sp*. 99-125 (CSL)	58.3±1.37	83.9±1.85
4	Aeropyrum *pernix* esterase (APE1547)	45.9±1.61	68.5±1.42
5	*Bacillus subtilis* lipase (BSL2)	51.3±1.35	91.3±1.38
6	Bovine serum albumin (BSA)	18.2±1.86	28.4±1.96
7	BSL2, denatured [c]	19.5±2.43	28.0±1.55
8	BSL2, inactivated [d]	45.1±1.89	81.3±1.29
9	No enzyme	17.6±1.28	28.8±1.05

[a] Reaction condition: salicylaldehyde (1 mmol), malononitrile (2 mmol), ethanol (2 mL), enzyme (30 mg), under conventional heating (60 °C, 1 h).

[b] Reaction condition: salicylaldehyde (1 mmol), malononitrile (2 mmol), ethanol (2 mL), BSL2 (30 mg), under microwave irradiation (60 °C, 2min).

[c] BSL2 was denatured by heating it to 100 °C for 24 h in ethanol.

[d] BSL2 was inactivated by a serine protease inhibitor (PMSF).

To evaluate the effect of temperature on this reaction, the reaction was investigated at a temperature range from 35 °C to 75 °C under microwave irradiation. It could be found from Fig.1 that the yield increased as the reaction temperature increased from 35°C to 60°C, and was not further improved by increasing the temperature (60°C ~ 75°C). Compared to other reported lipase catalytic promiscuous reactions under conventional heating [26-28], no obvious denaturation of BSL2 was found at higher temperature. It should be attributed to the short reaction time which may help to avoid the heat-induced destruction of enzyme under microwave irradiation.

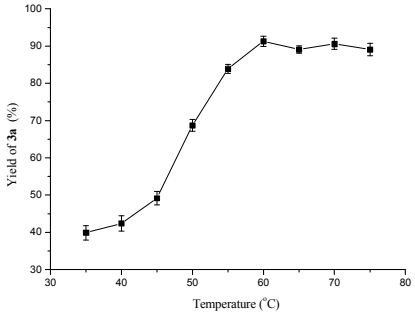

Fig. 1 Effect of temperature for the synthesis of **3a**. Reaction condition: salicylaldehyde (1 mmol), malononitrile (2 mmol), ethanol (2 mL), BSL2 (30 mg), microwave irradiation (2 min).

In order to indicate the importance of the reaction media, several organic solvents were screened and the results were listed in Table 2. Our results demonstrated that the solvent had a significant effect on the yields of compound **3a**. The condensation of salicylaldehyde and malononitrile was faster in protic/polar solvents than in nonprotic/nonpolar solvents presumably. The possible explanation might be that protic/polar solvent can enhance the stabilization of charged intermediates and more-facile proton-transfer reactions [29]. Meanwhile, protic solvent has the excellent microwave-absorbing ability to convert electromagnetic radiation into heat [30]. Therefore, ethanol was selected as the optimal reaction solvent in this work.

Table 2 Effect of solvent for the synthesis of **3a**

Entry	Solvent	Yield
1	Acetonitrile	71.5±0.91
2	N,N-dimethyl-Formamide	79.6±1.12
3	Toluene	39.8±0.88
4	Tetrahydrofuran	43.6±1.59
5	Methanol	86.2±1.29
6	Ethanol	91.3±1.38
7	Water	76.3±2.54

Reaction condition: salicylaldehyde (1 mmol), malononitrile (2 mmol), solvent (2 mL), BSL2 (30 mg), under microwave irradiation (60 °C, 2min).

To extend the generality and applicability of this method, the condensation of substituted salicylaldehyde and malononitrile was investigated. ‡ The results are summarized in Table 3. It could be found that this methodology can be applied well when salicylaldehyde with electron-donating groups (methyl, methoxy or hydroxyl group) or electron-withdrawing group (fluoro or chloro group) were employed. However, it must take more reaction time to obtain product **3e** when salicylaldehyde with a strong electron-withdrawing group (nitro group) was employed as the substrate. This could be explained that the strong electron-withdrawing group (nitro group) lessens the nucleophilicity and deactivates the substrate. The structures and purities of all the products were characterized and identified by NMR experiments.

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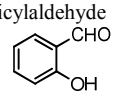
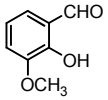
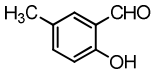
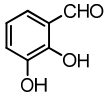
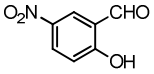
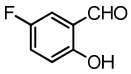
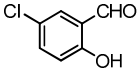
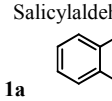
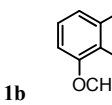
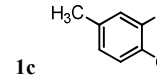
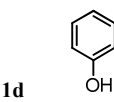
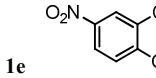
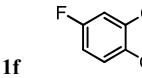
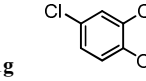
Table 3 Effect of the substituted group of salicylaldehyde for the synthesis of **3**

Entry	Salicylaldehyde	Product	Time (min)	Yield (%)
1	 **1a**	**3a**	2	91.3±1.38
2	 **1b**	**3b**	2	94.4±1.28
3	 **1c**	**3c**	2	90.8±1.32
4	 **1d**	**3d**	2	93.5±0.74
5	 **1e**	**3e**	5	90.4±0.88
6	 **1f**	**3f**	2	93.8±1.27
7	 **1g**	**3g**	5	94.2±1.13

Reaction condition: substituted salicylaldehyde (1 mmol), malononitrile (2 mmol), ethanol (2 mL), BSL2 (30 mg), under microwave irradiation (60 ºC).

In this work, we found a interesting phenomenon that the product **3** would be further reacted to produce compound **4** when the reaction time prolonged to 10 min and the substrate molar ratio (**1** / **2**) was adjusted from 1:2 to 1:3 under microwave irradiation (Table 4). And no other product was observed by further increasing the substrate molar ratio. Meanwhile, considering the low reactivity of the substrate **1e**, we prolonged the reaction time to 30 min under microwave irradiation. However, only **3e** could be obtained instead of **4e** which was confirmed by the spectra of NMR (data not shown). It should be due to the passivating action of nitro group in the compound **3e** which makes a decrease in the reactivity and prevents the further reaction.

Table 4 Effect of the substituted group of salicylaldehyde for the synthesis of 4 [a]

Entry	Salicylaldehyde	Product	Time (min)	Yield (%)
1	 **1a**	**4a**	10	95.2±1.22
2	 **1b**	**4b**	10	96.2±0.82
3	 **1c**	**4c**	10	94.2±0.91
4	 **1d**	**4d**	10	94.9±1.35
5	 **1e**	**4e**	30	Not obtained [b]
6	 **1f**	**4e**	10	92.2±1.08
7	 **1g**	**4f**	10	93.8±1.15

[a] Reaction condition: salicylaldehyde (1 mmol), malononitrile (3 mmol), ethanol (2 mL), enzyme (30 mg), under microwave irradiation (60 ºC).

[b] **4e** was not obtained, and the product **3e** (91.1%) was the only product.

Kinetic constant in a promiscuous reaction is a key to study the mechanism of the enzymatic reaction [31-33]. Apparent kinetic parameters of the multi-component domino reaction can be used to represent the behaviours of substrates and lipase in general. In this study, we kept the concentration of malononitrile (**2**) at a low constant (0.05M), and salicylaldehyde (**1a**) was selected three concentrations (0.1M, 0.5M, 1.0M) to determine the initial rate and k_{cat}^{app}/K_M^{app} (Table 5). It could be found that the value of k_{cat}^{app}/K_M^{app} was decreased with increasing the concentration of salicylaldehyde. These results indicated that salicylaldehyde might be reacted with malononitrile which binds to the oxyanion hole in the lipase firstly. The catalytic proficiency ($(k_{cat}^{app}/K_M^{app})/k_{non}$ was much lower than 10^8 which demonstrated that the substrates in this reaction was not the native substrates of lipase [34].

Table 5 Kinetic constant for the condensation of salicylaldehyde (**1a**) and malononitrile (**2**) catalyzed by lipase

Concentration (**2**)	0.1M	0.5M	1.0M
k_{cat}^{app}/K_M^{app} ($s^{-1}M^{-1}$)	0.44	0.30	0.21
k_{non} ($s^{-1}M^{-1}$)	4.7×10^{-3}	4.7×10^{-3}	4.7×10^{-3}
$(k_{cat}^{app}/K_M^{app})/k_{non}$	93.6	63.8	44.7

According to the previous reports [32-36], lipase can catalyze the Michael addition and Knoevenagel condensation in the domino reactions. Thus, a plausible mechanism of this reaction was proposed in Scheme 2. Product **3** could be obtained by a Knoevenagel condensation (Step **1**), Michael addition (Step **2**), intramolecular cyclization (step **3**) and isomerization (step **4**). To confirm the effect of lipase in the step **5**, we conducted an experiment using compound **3a** and malononitrile as the

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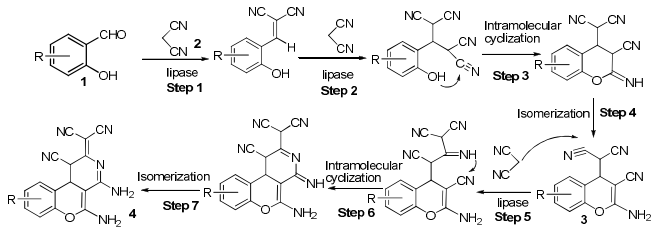
substrate. Compared with the reaction catalyzed by lipase, the reaction rate was dramatically decreased without lipase or catalyzed by the denatured BSL2 (denatured by heating), while the inactivated BSL2 (inactivated by PMSF) demonstrated similar activity to BSL2 in this step (data not shown). These results indicated that lipase with active conformation actually works in the step **5**. Subsequently, the intramolecular cyclization (step **6**) and isomerization (step **7**) were occurred automatically to produce compound **4**.



Scheme 2 Plausible mechanism of Lipase-catalyzed synthesis of **3** and **4**

Conclusions

In conclusion, we have reported a facile and efficient method for the condensation of substituted salicylaldehyde and malononitrile catalyzed by lipase under microwave irradiation. Compared with the reported literatures [37-39], this method is a more convenient and efficient method to obtain the two products **3** and **4** only by changing the reaction time and substrate molar ratio, and the reaction time was dramatically shortened from hours or days to minutes. Thus, this reaction presents several advantages including short reaction time, atom economy, environmental friendliness and simple experimental procedure. This work also extends the application of microwave irradiation to improve the activity of lipase in the study of catalytic promiscuity. It's known that immobilization is a powerful tool to avoid the enzyme aggregation in organic solvent and recover and reuse of the enzyme with high remnant activity [11-13]. Further study of the immobilization enzyme on the lipase-catalyzed condensation of substituted salicylaldehyde and malononitrile is now in progress in our laboratory.

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Notes and references

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‡ A typical enzymatic synthesis of product **3** or **4**: Lipase (30 mg, protein content) was added to a 25 mL round-bottom flask containing substituted salicylaldehyde (1 mmol), malononitrile (2 mmol or 3 mmol) and ethanol (2 mL). The suspension was maintained at 60 ºC for a suitable time under conventional heating or microwave irradiation. The reaction system was then cooled to room temperature and the solid was filtered and washed with ethanol and water to give products **3** or **4** with high purity. The yield was calculated by dividing the final amount of product 3 or 4 by the initial amount of substituted salicylaldehyde. The experiments were performed triplicate, and all data were obtained based on the average values. The structures and purities of all products were characterized by NMR experiments.

[1] I. Nobeli, A. D. Favia, J. M. Thornton, *Nat. Biotechnol.*, 2009, **27**, 157.
[2] U. T. Bornscheuer and R. J. Kazlauskas, *Angew. Chem. Int. Ed.*, 2004, **43**, 6032.
[3] M. Kapoor, M.N. Gupta, *Process Biochem.*, 2012, **47**, 555.
[4] W. Hu, Z. Guan, X. Deng, Y. H. He, *Biochimie.*, 2012, **94**, 656.
[5] X. W. Feng, C. Li, N. Wang, K. Li, W. W. Zhang, Z. Wang, X. Q. Yu, *Green Chem.*, 2009, **11**, 1933.
[7] C. Branneby, P. Carlqvist, A. Magnusson, K. Hult, T. Brinck and P. Berglund, *J. Am. Chem. Soc.*, 2003, **125**, 874.
[8] Z. Wang, C. Y. Wang, H. R. Wang, H. Zhang, Y. L. Su, T. F. Ji, L. Wang, *Chin. Chem. Lett.*, 2014, **25**, 802.
[9] R. C. Rodrigues, C. Ortiz, A. Berenguer-Murcia, R. Torres, R. Fernández-Lafuente, *Chem. Soc. Rev.*, 2013, **42**, 6290.
[10] E. N. Xun, X. L. Lv, W. Kang, J. X. Wang, H. Zhang, L. Wang, Z. Wang, *Appl. Biochem. Biotechnol.*, 2012, **168**, 697.
[11] K. Hernandez, R. Fernández-Lafuente, *Enzyme Microb. Technol.*, 2011, **48**, 107.
[12] C. Garcia-Galan, A. Berenguer-Murcia, R. Fernández-Lafuente, R. C. Rodrigues, *Adv. Synth. Catal.*, 2011, **353**, 2885.
[13] D. F. Lzquierdo, O. Barbosa, M. Isabel Burguete, P. Lozano, S. V. Luis, R. Fernandez-Lafuente, E. García-Verdugo, *RSC Adv.*, 2014, **4**, 6219.
[14] C. A. Godoy, G. Fernández-Lorente, B. de las Rivas, M. Filice, J. M. Guisan, J. M. Palomo, *J. Mol. Catal. B: Enzym.*, 2011, **70**, 144.
[15] R. Jia, Y. Hu, L. Liu, L. Jiang, H. Huang, *Org. Biomol. Chem.*, 2013, **11**, 7192.
[16] Y. Ito, H. Fujii, Y. Imanishi, *Biotechnol. Prog.*, 1994, **10**, 398.
[17] R. S. Varma, *Green Chem.*, 1999, **1**, 43.
[18] S. D. Shinde, G. D. Yadav, *Biochem. Eng. J.*, 2014, **90**, 96.
[19] I. C. R. Costa, F. K. Sutili, G. V. V. da Silva, S. G. F. Leite, L. S. M. Miranda, R. O. M. A. De Souza, *J. Mol. Catal. B: Enzym.*, 2014, **102**, 127.
[20] J. B. Sontakke, G. D. Yadav, *J. Chem. Technol. Biot.*, 2011, **86**, 739.
[21] G. D. Yadav, S. V. Pawar, *Bioresource Technol.*, 2012, **109**, 1.
[22] F. Borges, F. Roleira, N. Milhazes, L. Santana, E. Uriarte, *Curr. Med. Chem.*, 2005, **12**, 887.
[23] G. A. Kraus, I. J. Kim, J. Org. Chem., 2003, **68**, 4517.
[24] L. Wang, J. D. Tai, R. Wang, E. N. Xun, X. F. Wei, Z. Wang, *Biotechnol. Appl. Biochem.*, 2010, **56**, 1.
[25] R. Tian, C. H. Yang, X. F. Wei, E. N. Xun, R. Wang, S. G. Cao, Z. Wang, L. Wang, *Biotechnol. Bioproc. E.*, 2011, **16**, 337.
[26] H. R. Wang, Z. Wang, C. Y. Wang, F. J. Yang, H. Zhang, H. Yue, L. Wang, *RSC adv.*, 2014, **4**, 35686.
[27] J. L. Wang, J. M. Xu, Q. Wu, D. S. Lv, X. F. Lin, *Tetrahedron*, 2009, **65**, 2531.
[28] C. H. Wang, Z. Guan, Y. H. He, *Green Chem.*, 2011, **13**, 2048.
[29] D. B. Ramachary, N. S. Chowdari, C. F. Barbas III, *Angew. Chem. Int. Ed.*, 2003, **42**, 4233.
[30] J. M. Kremsner, C. O. Kappe, *J. Org. Chem.*, 2006, **71**, 4651.
[31] A. Radzicka, R. Wolfenden, *Science.* **267**, 90.
[32] W. N. Li, S. N. Fedosov, T. W. Tan, X. B. Xu, Z. Guo, *ACS Catal.* 2014, **4**, 3294.
[33] W. N. Li, R. Li, X. C. Yu, X. B. Xu, Z. Guo, T. W. Tan, S. N. Fedosov, *Biochem. Eng. J.* 2015, **101**, 99.

RSC Advances Accepted Manuscript

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[34] M. Svedendahl, K. Hult, P. Berglund, *J. Am. Chem. Soc.*, 2005, **127**, 17988.
[35] F. J. Yang, H. R. Wang, L. Y. Jiang, H. Yue, H. Zhang, Z. Wang, L. Wang, *RSC adv.*, 2015, **5**, 5213.
[36] F. J. Yang, Z. Wang, H. R. Wang, H. Zhang, H. Yue, L. Wang, *RSC Adv.*, 2014, **4**, 25633.
[37] M. Costa, F. Areias, L. Abrunhosa, A. Venâncio, F. Proença, *J. Org. Chem.*, 2008, **73**, 1954.
[38] C. N. O'Callaghan, T. B. H. McMurry, J. E. O'Brien, *J. Chem. Soc. Perkin Trans. I.* 1995, **4**, 417.
[39] C. N. O'Callaghan, T. B. H. McMurry, J. E. O'Brien, S. M. Draper, *J. Chem. Research (S)*, 1997, **9**, 312.

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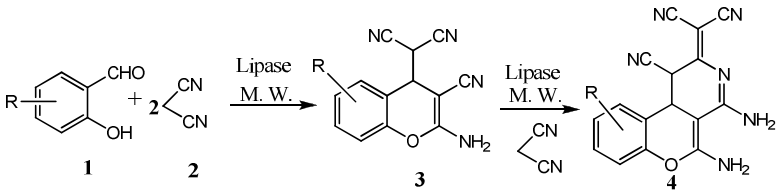

Lipase-catalyzed condensation of substituted salicylaldehyde and malononitrile under microwave irradiation

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