Tetrahedron Letters 55 (2014) 1448–1451

Contents lists available at ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

High yield synthesis of D-phenylglycine and its derivatives by nitrilase mediated dynamic kinetic resolution in aqueous-1-octanol biphasic system

Jian Qiu [a], Erzheng Su [a,b,*], Wei Wang [a], Dongzhi Wei [a,*]

[a] State Key Laboratory of Bioreactor Engineering, New World Institute of Biotechnology, East China University of Science and Technology, Shanghai 200237, PR China
[b] Enzyme and Fermentation Technology Laboratory, College of Light Industry Science and Engineering, Nanjing Forestry University, Nanjing 210037, PR China

ARTICLE INFO

Article history:
Received 29 October 2013
Revised 9 January 2014
Accepted 13 January 2014
Available online 18 January 2014

Keywords:
Nitrilase
Dynamic kinetic resolution
Biphasic system
D-Phenylglycine

ABSTRACT

A strategy of nitrilase mediated dynamic kinetic resolution toward the synthesis of D-phenylglycine was developed, using aqueous-1-octanol biphasic system. Due to the efficient suppression of the decomposition of phenylglycinonitrile, a maximum yield of 81% is obtained. This result indicates that the nitrilase mediated dynamic kinetic resolution is a promising method toward the synthesis of D-phenylglycine and its derivatives.

D-Phenylglycine is an important chiral building block in the synthesis of antibiotics, such as ampicillin and cefalexin.[1] The conventional process for making enantio-pure D-phenylglycine is the crystallization of its diastereomeric salts racemate and (+)-camphor-8-sulfonic acid.[2] The other enantiomer, L-phenylglycine is racemized in a separate step and then recycled. In recent years, many chemo-enzymatic methods have been developed for preparing D-amino acid,[3] such as the use of aminoacylase,[4] amidase,[5] penicillin G acylase,[6] and D-aminotransferase.[7] The well-known hydantoinase/carbamoylase process has been commercially used for the preparation of D-*p*-hydroxyphenylgly-cine.[8] However, D-phenylglycine has not yet been produced through this process on an industrial scale.[9]

Nitrilase (EC 3.5.5.1) is a kind of hydrolase found in many bacteria, fungi, and plants, which can convert nitriles into the corresponding carboxylic acids and ammonia. To date, nitrilases have already been shown to be versatile biocatalysts for the production of chiral pharmaceutical intermediates as well as bulk products, such as (*R*)-mandelic acid, (*R*)-4-cyano-3-hydroxybutyric acid, and nicotinic acid.[10] Chaplin et al.[11] reported two novel approaches of nitrilase-catalyzed dynamic kinetic asymmetric synthesis of aromatic amino acids. One approach was the enantioselective synthesis of D-phenylglycine via dynamitic kinetic resolution of phenylglycinonitrile at pH 10.6. This process is attractive with two features: (i) The method uses α-aminonitriles as raw materials, which are easier to be obtained than α-amino acid derivatives used in other chemo-enzymatic routes; (ii) unlike kinetic resolution routes mentioned above, recovery and racemization of the undesired enantiomer in separated steps are not required. This 'nitrilase process' affords a 100% theoretical yield through continuous in situ racemization under alkaline condition. Although it is a great potential approach, there are few reports on amino acid production involving nitrilases.[12,13] Presumably, this is due to the labilization of α-aminonitriles and the lack of high enantioselective nitrilase. In our previous work (data unpublished), a novel nitrilase SWRW1 mined from *Sphingomonas wittichii* RW1 was used for high enantioselective synthesis of D-phenylglycine by kinetic resolution of phenylglycinonitrile and 46% yield with 95% ee was obtained at pH 6.0. Herein, we reported improved alkaline conditions, under which the nitrilase mediated enantioselective hydrolysis is combined with in situ racemization in aqueous and biphasic aqueous–organic system.

It has been previously demonstrated that pH values higher than 10 were required for the racemization of phenylglycinonitrile, and $t_{1/2}$ for the racemization at pH 10.5 was estimated to be 60 min compared to 4 h at pH 9.5.[11] To evaluate the alkaline tolerance of the nitrilase SWRW1, resting cells of a recombinant *Escherichia coli* BL21 expressing nitrilase activity were incubated with phenylglycinonitrile at various pH values. The maximum activity of whole cell biocatalysts was observed at pH 8.0, which was similar to

* Corresponding authors. Tel./fax: +86 25 85428906 (E.S.); tel.: +86 21 64252078; fax: +86 21 64250068 (D.W.).
E-mail addresses: ezhsu@njfu.edu.cn (E. Su), dzhwei@ecust.edu.cn (D. Wei).

0040-4039/$ - see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tetlet.2014.01.044

the result observed with the purified nitrilase. About 50% residual activities were maintained at pH 6.0 and pH 10.0, respectively (Fig. 1), indicating that whole cell biocatalysts displayed more alkaline stability than the purified nitrilase.

The whole cell biocatalysts were then used in the dynamic kinetic resolution reaction optimized, and the results are summarized in Table 1. This dynamic kinetic resolution route is complicated, which mainly involves three reactions (Scheme 1): spontaneous decomposition (reaction 1), enantioselective hydrolysis mediated by nitrilase (reaction 2), and racemization under alkaline conditions (reaction 3). Therefore, the yields and ee values are dependent on the competition of three reaction rates under different conditions.

In our earlier work, kinetic resolution was investigated at pH 6.0. Under this acidic condition, phenylglycinonitrile was stable without any racemization (Table 1, entry 1). In order to accelerate the racemization, different alkaline conditions (pH 9.5–11) were investigated and the ee values were higher than 92% in most cases (Table 1, entries 3–6), except 80% ee at pH 9.5 (Table 1, entry 2). Presumably, this is due to the inefficient racemization, which might lead to the low ee value. A maximum yield of 68% was observed at pH 10.5 (Table 1, entry 4), and the yields decreased along with the pH increase (Table 1, entries 5 and 6). The decreasing yield might be primarily caused by the more rapid decomposition. Other possibilities could be the high concentration of benzaldehyde acting as an enzyme inhibitor,[14] or the deactivation of nitrilase under severe alkaline conditions. The reaction temperature is a key parameter in bioprocess, which can significantly influence the activity, enantioselectivity, and stability of a biocatalyst as well as the equilibrium of a reaction.[15] Although lower temperature (10 °C) was useful in enhancing the enantioselectivity, lower solubility, and reaction rate also decreased the final yield (Table 1, entry 7). The optimal temperature for this reaction was 20 °C (Table 1, entry 8).

Substrate concentration is another very important issue associated with nitriles biotransformation. In general, nitrile compounds

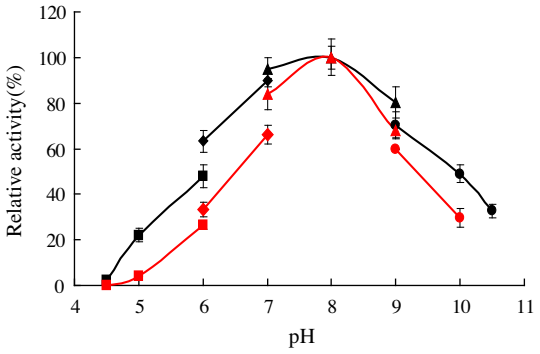
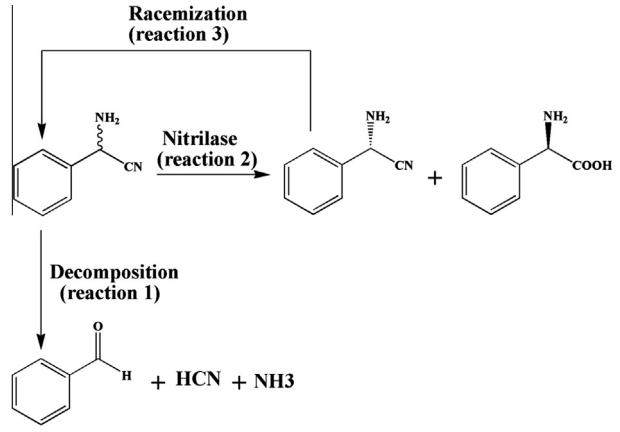


Figure 1. pH stability of the whole cell biocatalysts and the purified nitrilase. The relative activities were expressed as a percentage of maximum activities of whole cell biocatalysts (black line) and purified nitrilase (red line), respectively. Reactions were performed in triplicate, and error bars represented the standard error of the mean. Sodium citrate–citric acid buffer (■), sodium phosphate buffer (♦), Tris–HCl buffer (▲), and carbonate buffer (●).

Table 1
Reaction conditions optimized in aqueous system

Entry	Substrate concentration (mM)	pH	Temperature (°C)	Time (h)	Yield (%)	ee (%)
1[a]	25[c]	6.0	20	5	46[e]	95
2[a]	25[c]	9.5	20	6	51	80
3[a]	25[c]	10.0	20	6	55	92
4[a]	25[c]	10.5	20	6	68	95
5[b]	25[c]	11.0	20	10	60	95
6[b]	25[c]	11.5	20	10	57	95
7[b]	25[c]	10.5	10	8	60	97
8[b]	25[c]	10.5	20	6	68	95
9[b]	25[c]	10.5	30	6	59	95
10[a]	25[c]	10.5	20	6	68	95
11[b]	50[c]	10.5	20	8	65	95
12[b]	50[d]	10.5	20	11.5	37	N.D.
13[b]	75[c]	10.5	20	14	52	N.D.

N.D.: not determined, due to low yield.
[a] 300 mg dry cells were used as the whole cell biocatalysts.
[b] 600 mg dry cells were used as the whole cell biocatalysts.
[c] 10% methanol as the cosolvent.
[d] 20% methanol as the cosolvent.
[e] The reaction was terminated at the conversion near to 50% because of the kinetic route of resolution (Fig. S1).



Scheme 1. Production of D-phenylglycine by dynamic kinetic resolution.

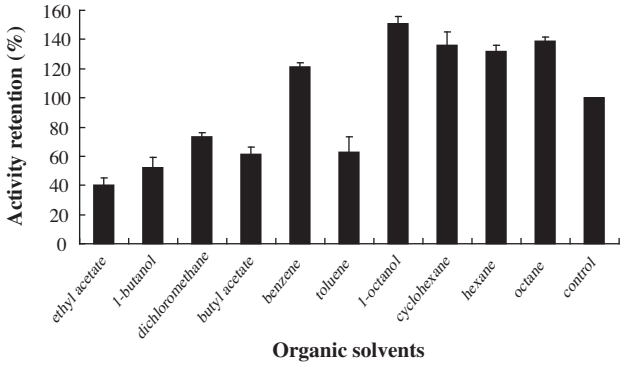


Figure 2. Effect of various organic solvents on the activity retention of the whole cells.

Table 2
Reaction conditions optimized in water–1-octanol biphasic system

Entry	Substrate concentration (mM)	Water content (%)	Temperature (°C)	Time (h)	Yield (%)	ee (%)
1[a]	50	50	20	14	70	95
2[a]	50	20	20	20	73	95
3[a]	50	10	20	24	80	95
4[a]	50	2	20	48	57	97
5[a]	100	10	20	48	78	95
6[a]	150	10	20	48	69	N.D.
7[a]	100	10	20	48	78	95
8[a]	100	10	25	48	81	95
9[a]	100	10	30	48	73	N.D.

N.D.: not determined, due to low yield.
[a] 600 mg dry cells were used as the whole cell biocatalysts.

Table 3
High yield synthesis of D-phenylglycine derivatives in aqueous-1-octanol biphasic system

Entry	Reaction system	Substrate concentration (mM)	pH	Temperature (°C)	Time (h)	Yield (%)	ee (%)
1[a]	Aqueous system	25[b]	6.0	20	20	42[d]	91
2[a]		25[c]	6.0	20	16	49[d]	93
3[a]	Aqueous-1-octanol	25[b]	10.5	25	48	59	90
4[a]	Biphasic system	25[c]	10.5	25	48	70	93

[a] 600 mg dry cells were used as the whole cell biocatalysts.
[b] 2-Chloro-phenylglycinonitrile was used as substrate.
[c] 4-Chloro-phenylglycinonitrile was used as substrate.
[d] The reaction was terminated at the conversion near to 50%.

have poor solubility in aqueous media, and the polar low-molecular weight solvents, such as methanol and DMSO, are used as cosolvents.[16] However, high concentration of nitriles and cosolvents are both detrimental to nitrilases. To access better efficacy in this approach, substrate concentrations were thus optimized. Conducting the reaction in 50 mM of phenylglycinonitrile (65%, Table 1, entry 11) was as effective as conducting the reaction in 25 mM of substrate (Table 1, entry 10). However, further increasing the substrate concentration made solution turbid immediately, indicating that 10% methanol could not completely dissolve the substrate. 20% methanol was then used in the further test. Unfortunately, this level of methanol inhibited or inactivated the enzyme dramatically and low yield (37%) was obtained within 11.5 h (Table 1, entry 12). When the substrate concentration was increased up to 75 mM, the yield (52%) was not improved further (Table 1, entry 13). This result might be attributed to enzyme inactivated by the substrate or benzaldehyde.

In order to suppress the decomposition of the substrate and improve the yield, aqueous–organic reaction system was investigated. Ten water-immiscible organic solvents, such as ethyl acetate (0.68), 1-butanol (0.8), dichloromethane (0.93), butyl acetate (1.7), benzene (2.0), toluene (2.5), 1-octanol (2.9), cyclohexane (3.2), hexane (3.5), and octane (4.5) were examined for the effect on the activity retention of the whole cells and the results are presented in Figure 2. The activity retentions of more than 100% were obtained in the solvents with the log*P* value ranging from 2.0 to 4.5, except in toluene. It may be due to the more rigid and stable conformational structure of the enzyme in this solvents.[17,18]

The maximum activity retention was observed in 1-octanol, which was 1.5 times greater than that in neat aqueous buffer. Although with similar log*P* value, the activity retention in toluene was much lower, which was opposite to the result observed by Zhang et al.[19] This result indicated that the structure of enzyme is an important factor for its performance in different organic solvents and toluene was harmful to SWRW1 in this work. Accounting for the solubility of the substrate and the product, 1-octanol was chosen as the best organic solvent for further experiments.

To evaluate the performance of the whole cells in aqueous-1-octanol biphasic system, many important factors such as water content, substrate concentration, and reaction temperature were tested as summarized in Table 2. Water content is a vital factor for suppressing the decomposition of the substrate. Conducting the reaction in 50% water content failed to suppress the decomposition reaction, and only 70% yield was obtained, which was similar to that in the aqueous system (Table 1, entries 1 and 2). Decreasing the water content from 50% to 10% significantly enhanced the yield to 80% (Table 2, entry 3). However, further decreasing the water content to 2% lowered the yield to 57%, even when the reaction time was extended to 48 h (Table 2, entry 4). This might be due to the diffusion limitation. Thus, further investigations were carried out in water–1-octanol (1:9, v/v) biphasic system. Substrate concentration also plays an important role in the reaction outcome. Similar yield was observed when the concentration was double to 100 mM, (Table 2, entry 5). Further increasing the concentration decreased the yield (Table 2, entry 6), which might be due to the fact that high concentration of the substrate assembling at the biphasic interface areas deactivated the enzyme. The effect of reaction temperature on yield was also investigated (Table 2, entries 7–9) and showed that 25 °C was the optimal temperature (Table 2, entry 8). It was found that the selectivity of the nitrilase was independent of all the factors mentioned above, and the enantiomeric excess value was greater than 95% in all cases.

Having established the optimal conditions for the hydrolysis of phenylglycinonitrile, other derivatives, such as 2-chloro- and 4-chloro-phenylglycinonitrile were also investigated in the aqueous system and aqueous-1-octanol biphasic system. As shown in Table 3, in both cases, the activity and enantioselectivity decreased (Table 3, entries 1 and 2), which might be due to the electronic and steric effects. Compared to the aqueous system, this biphasic system also improved the yield effectively (42% vs 59%; 49% vs 70%).

In conclusion, a new nitrilase mediated dynamic kinetic resolution toward the synthesis of D-phenylglycine and its derivatives in aqueous-1-octanol biphasic system was developed. Several important parameters were evaluated in detail, and showed that 10% water content was essential for the suppression of the decomposition of substrate. The 81% maximum yield was obtained under the optimized conditions, which is 1.2 times greater than that in the aqueous system. These results indicated that the nitrilase mediated dynamic kinetic resolution could be used as a promising approach toward D-phenylglycine and its derivatives.

Acknowledgments

This work was supported by the National Basic Research Program of China (No. 2012CB721003), the Fundamental Research Funds for the Central Universities and the National Major Science and Technology Projects of China (No.2012ZX09304009).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2014.01.044.

References and notes

1. Ma, J. S. *Chim. Oggi* **2003**, *21*, 65–68.
2. Wegman, M. A.; Janssen, M. H. A.; Rantwijk, F.; Sheldon, R. A. *Adv. Synth. Catal.* **2001**, *343*, 559–576.
3. Leuchtenberger, W.; Huthmacher, K.; Drauz, K. *Appl. Microbiol. Biotechnol.* **2005**, *69*, 1–8.
4. Wakayama, M.; Yoshimune, K.; Hirose, Y.; Moriguchi, M. *J. Mol. Catal. B Enzym.* **2003**, *23*, 71–85.
5. Yamaguchi, S.; Komeda, H.; Asano, Y. *Appl. Environ. Microbiol.* **2007**, *37*, 5370–5373.
6. Gong, X. Y.; Su, E. Z.; Wang, P. X.; Wei, D. Z. *Tetrahedron Lett.* **2011**, *52*, 5398–5402.
7. Kobayashi, J.; Shimizu, Y.; Mutaguchi, Y.; Doi, K.; Ohshima, T. *J. Mol. Catal. B: Enzym.* **2013**, *94*, 15–22.
8. May, O.; Verseck, S.; Bommarius, A.; Drauz, K. *Org. Process Res. Dev.* **2002**, *6*, 452–457.

9. *Synthesis of β-Lactam Antibiotics: Chemistry, Biocatalysis & Process Integration*; Bruggink, A., Ed.; Kluwer Academic Publishers: Dordrecht, 2001.
10. Gong, J. S.; Lu, Z. M.; Li, H.; Shi, J. S.; Zhou, Z. M.; Xu, Z. H. *Microb. Cell Fact.* **2012**, *11*, 142–159.
11. Chaplin, J. A.; Levin, M. D.; Morgan, B.; Farid, N.; Li, J.; Zhu, Z. L.; McQuaid, J.; Nicholson, L. W.; Rand, C. A.; Burk, M. J. *Tetrahedron: Asymmetry* **2004**, *15*, 2793–2796.
12. Choi, S. Y.; Goo, Y. M. *Arch. Pharm. Res.* **1986**, *9*, 45–47.
13. Bhalla, T. C.; Miura, A.; Wakamoto, A.; Ohba, Y.; Furuhashi, K. *Appl. Microbiol. Biotechnol.* **1992**, *37*, 184–190.
14. Zhang Z. J. *Study on nitrilase catalyzed preparation of enantiopure mandelic acid and rational design of hydroxynitrile lyase*. Ph.D. Thesis, East China University of Science and Technology, 2013, p 42.
15. Zhang, Z. J.; Xu, J. H.; He, Y. C.; Ouyang, L. M.; Liu, Y. Y.; Imanaka, T. *Process Biochem.* **2010**, *45*, 887–891.
16. Laane, C.; Boeren, S.; Vos, K.; Veeger, C. *Biotechnol. Bioeng.* **1987**, *30*, 81–87.
17. Zaks, A.; Klibanov, A. M. *Proc. Natl. Acad. Sci.* **1985**, *82*, 192–196.
18. Ohya, Y.; Miyaoka, J.; Ouchi, T. *Macromol. Rapid Commun.* **1996**, *17*, 871–874.
19. Zhang, Z. J.; Pan, J.; Liu, J. F.; Xu, J. H.; He, Y. C.; Liu, Y. Y. *J. Biotechnol.* **2011**, *152*, 24–29.