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Highly Efficient Synthesis of (*R*)-3-Quinuclidinol in a Space–Time Yield of 916 g L^{-1} d^{-1} Using a New Bacterial Reductase *Ar*QR

Wen-Xia Zhang, Guo-Chao Xu, Lei Huang, Jiang Pan, Hui-Lei Yu,* and Jian-He Xu*

Laboratory of Biocatalysis and Synthetic Biotechnology, State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, People' s Republic of China

huileiyu@ecust.edu.cn; jianhexu@ecust.edu.cn

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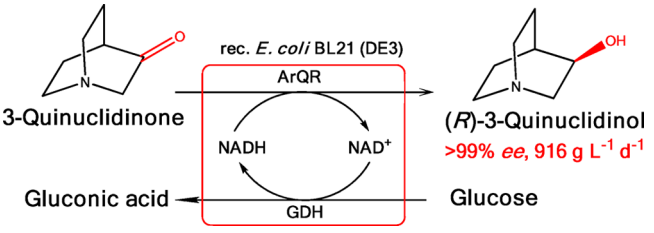
ABSTRACT



A new keto reductase (*Ar*QR), identified from *Agrobacterium radiobacter* ECU2556, can efficiently reduce 3-quinuclidinone in excellent enantioselectivity and high space–time yield for the synthesis of (*R*)-3-quinuclidinol, a chiral building block of many antimuscarinic agents. This is the first time that a high yield of (*R*)-3-quinuclidinol up to 916 g L^{-1} d^{-1} using a bioreduction approach is reported.

Biocatalysis has been growing rapidly as a practical and environmentally friendly alternative to traditional chemical synthesis to access secondary alcohols,[1] which are intermediates of many pharmaceuticals. For example, optically pure (*R*)-3-quinuclidinol is an important chiral building block of many antimuscarinic agents for the treatment of COPD (chronic obstructive pulmonary disease),[2] such as talsaclidine, revatropate, solifenacin, and aclidinium bromide.[3]

There are various methods to synthesize (*R*)-3-quinuclidinol, including chemical synthesis, enzymatic kinetic resolution, and asymmetric bioreduction. Chemical synthesis is one of the most efficient routes to produce (*R*)-3-quinculidinol. For example, asymmetric hydrogenation of 3-quinuclidinone with $RuBr_2$[(S,S)-xylskewphos](pica) in a base containing ethanol has afforded (*R*)-3-quinuclidinol in 88–90% ee.[4] A combined catalyst system of $RuCl_2$[(*S*)-binap][(*R*)-iphan] and *t*-C_4H_9OK in 2-propanol afforded the chiral alcohol in 97–98% ee,[5] but the trace metal contamination in the product and the incredible fluctuation in the price of rare metals over the past decade quite rightly make a cause for concern in the pharmaceutical industry. Enzymatic resolution of the racemic quinuclidinol ester is a reliable procedure to obtain (*R*)-3-quinuclidinol in high optical purity. *Aspergillus melleus* protease was used for hydrolyzing (±)-3-(butyryloxy)quinuclidinium butyrate, giving (*R*)-3-quinuclidinol in 96% ee and 42% overall yield,[6] despite the necessary modification of the substrate prior to enzymatic reaction and the unsatisfactory ≤50% yield, which limits

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the potential of the enzyme. In recent years, more researchers have been paying attention to the bioreduction approach of producing (*R*)-3-quinuclidinol, since biocatalyzed asymmetric reductions can offer highly selective reactions, environmentally benign processes, and energy-effective operations.[7] *Rr*QR from *Rhodotorula rubra*,[8] QNR and BacC from *Microbacterium luteolum* JCM 9174,[9] and *Nocardia* sp. WY1202[10] can convert 3-quinuclidinone stereospecifically to (*R*)-3-quinuclidinol, but the common disadvantages of all these reductases were their poor substrate tolerance and low volumetric productivity, which limits their industrial application.

Herein, a new reductase *Ar*QR (GenBank accession no.: YP_002542435.1) was identified from *Agrobacterium radiobacter* ECU2556 through the screening of various microorganisms stocked in our labratory. After heterologous expression in *E. coli*, it can efficiently convert 3-quinuclidinone to (*R*)-3-quinuclidinol with high activity and excellent enantioselectivity.

To construct the cofactor regeneration system, *Ar*QR and *Bm*GDH (glucose dehydrogenase from *Bacillus megaterium*) were coexpressed in *E. coli* in a tandem mode. Considering that the reduction of 3-quinuclidinone was a rate-limiting step comparing with the oxidation step of glucose, *E. coli* BL21 (DE3) (pET28a-*Ar*QR-*Bm*GDH), rather than *E. coli* BL21 (DE3) (pET28a-*Bm*GDH-*Ar*QR), was chosen for further research. The lyophilized cells were employed as the biocatalyst to perform the reductive reaction of 3-quinuclidinone.

To optimze the reaction conditions, the biocatalyst dosage, substrate loading, and external NAD^+ concentration have to be assessed. Because both the substrate and product are water-miscible, all the reactions were performed in aqueous solution. Initial experiments were performed in 10 mL of potassium phosphate buffer (200 mM. pH 7.0) with 5 mmol of substrate (81 g/L), 1 μmol of NAD^+, 7.5 mmol of glucose, and 0.1 g of lyophilized cells of *E. coli* (pET28a-*Ar*QR-*Bm*GDH), and the pH of the reaction mixture was controlled at 7.0 by titrating 2 M NaOH. Surprisingly, the substrate was completely transformed to the desired product within merely 0.5 h (Table 1, entry 1). Clearly it was possible to transform more substrate, so the substrate loading was raised to 162 g L^{-1} without changing the other conditions, and the reaction was completed easily within 1.5 h (Table 1, entry 2). In a stepwise manner, a further attempt was made to convert 162 g L^{-1} substrate without external supplement of NAD^+. It was found that the conversion also reached 100% in less than 4 h (Table 1, entry 3). When the substrate was further increased up to 242 g L^{-1}, without any external NAD^+, only 44% substrate was converted to product even if the reaction time was extended to 20 h (Table 1, entry 4). The internal cofactor of the *E. coli* cells could probably not meet the need of high substrate loading.

Table 1. Asymmetric Reduction of 3-Quinuclidinone HCl with Coexpressed Cells of *E. coli* (pET28a-*Ar*QR-*Bm*GDH)[a]

entry	substrate (g/L)	cell (g/L)	NAD_+ (mM)	time (h)	conv[c] (%)	ee[c] (%)
1	81	10	0.1	0.5	100	>99
2	162	10	0.1	1.5	100	>99
3	162	10	0	4.0	100	>99
4	242	10	0	20	54	>99
5	242	10	0.1	4.5	100 (90)[b]	>99
6	242	5	0.1	10	>99 (90)[b]	>99

[a] Reaction conditions: 3-quinuclidinone HCl (0.8−2.4 g), dry cells of *E. coli* BL21 harboring pET28a-*Ar*QR-*Bm*GDH (0.05−0.1 g), D-glucose (1.5 equivalent), NAD^+ (0−1 μmol), 10 mL phosphate buffer (200 mM, pH 7.0), 30 °C. pH was kept at 7.0 with 2 M NaOH. [b] Isolated yield of (*R*)-3-quinuclidinol. [c] Determined by GC analysis.

Further reaction was tried using 242 g L^{-1} substrate and 0.1 mM of external NAD^+. As expected, the substrate conversion reached 99% in 4.5 h (Table 1, entry 5), and the space−time yield of (*R*)-3-quinuclidinol reached 916 g L^{-1} d^{-1}. Considering the internal content (ca. 9.7 μmol/g dry cell) of $NAD(H/^+)$ in *E. coli* cells,[11] the total turnover number (TTN) was estimated to be around 7500, which represents the highest among all the 3-quinuclidinone reductases reported to date. The time course of bioreduction showed that increasing the amount of NAD^+ gave faster bioreduction (Figure 1).

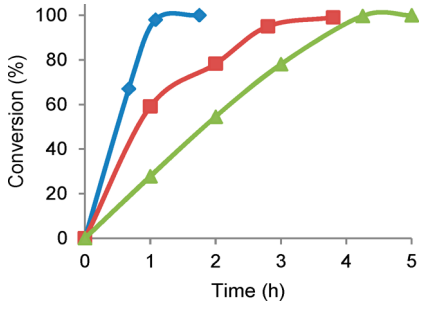

Figure 1. Reduction of 3-quinuclidinone-HCl by *E. coli* transformats harboring pET28a-*Ar*QR-*Bm*GDH: (♦) 162 g/L substrate, 0.1 mM NAD^+ was added; (■) 162 g/L substrate without addition of NAD^+; (▲) 242 g/L substrate, 0.1 mM NAD^+ added.

Otherwise, to be a good catalyst to render a chemical munufacturing process feasible, it needs to meet the requirement of ≥100 g L^{-1} substrate loading, ≤5 g L^{-1} biocatalyst loading, ≤24 h reaction time, ≥98% conversion, and ≥99% ee.[13]

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Table 2. Comparison of *Ar*QR with Other Reductase Biocatalysts

biocatalyst	coenzyme	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	substrate (g/L)	% conversion (time/h)	% ee (config)	space–time yield (g L^{-1} d^{-1})	ref
*Rr*QR[a]	NADPH	145	n.d.[g]	100	100 (21)	>99.9 (*R*)	90[h]	Uzura et al.[8]
*Dn*TR1[b]	NADPH	8.713	0.0032	n.d.[g]	n.d.[g]	n.d.[g]	n.d.[g]	Chen et al[12]
WY1202 cell[c]	n.d.[g]	n.d.[g]	n.d.[g]	16	95.3 (48)	>99 (*R*)	6[h]	Wang et al[10]
WY1406 cell[d]	n.d.[g]	n.d.[g]	n.d.[g]	10	95 (48)	>99 (*S*)	4[h]	Wang et al[10]
QNR[e]	NADH	6.5	5.6[h]	51	100 (12)	>99.9 (*R*)	80[h]	Isotani et al[9]
BacC[e]	NADH	13.8	0.083[h]	51	94 (12)	>99.9 (*R*)	75[h]	Isotani et al[9]
***Ar*QR**[f]	**NADH**	**0.4**	**290**	**242**	**100 (4.5)**	**>99.9 (*R*)**	**916**	**This work**

[a] Quinuclidinone reductase from *Rhodotorula rubra*. [b] Tropinone reductase from *Dendrobium nobile* Lindl. [c] *Nocardia* sp. WY1202 cell. [d] *R. erythropolis* WY1406 cell. [e] Quinuclidinone reductase from *Microbacterium luteolum* JCM 9174. [f] Lyophilized cells of *E. coli* (pET28a-*Ar*QR-*Bm*GDH). [g] n.d. = not determined. [h] Calculated according to the references.

Satisfactorily, the substrate was completely reduced to give >99% conversion and >99% ee within 10 h even if the cell dosage was cut down to 5 g L^{-1} (Table 1, entry 6). In the practical case, the space time yield of (*R*)-3-quinuclidinol still reached 408 g L^{-1} d^{-1}, indicating the great potential of *Ar*QR for industrial usage.

To investigate its intrinsic properties, the recombinant reductase *Ar*QR with an *N*-terminal His-tag was purified to electrophoretic homogeneity by nickel affinity chromatography. The specific activity of the crude cell-free extract was 78 U mg^{-1} powder, and it reached 198 U mg^{-1} protein with an increase of 2.5 folds after purification. Kinetic parameters were measured using varied concentrations of 3-quinuclidinone and NADH. The K_m measured of *Ar*QR for 3-quinuclidinone was about 0.4 mM, which is one to 2 orders of magnitudes lower than those of all the quinuclidinone reductases ever reported. It indicates that the enzyme has excellent affinity to substrate 3-quinuclidinone, implying that the reaction velocity will be high even with a low substrate concentration, while for the enzymes with high K_m, such as *Rr*QR, only 46% of the maximum velocity was exhibited when the concentration of substrate was 120 mM. The catalytic efficiency (k_{cat}/K_m) of *Ar*QR is 290 s^{-1} mM^{-1}, as listed in Table 2, which is also outstanding as compared to other quinuclidinone reductases, explaining the higher reaction velocity and shorter reaction time of *Ar*QR than the others.

To make the biocatalysis-based processes scalable, one has to fulfill a certain requirement for throughput (volume efficiency) which is industrially acceptable.[14] Among all the enzymes or cells that can reduce 3-quinuclidinone for the synthesis of (*R*)-3-quinuclidinol (Table 2), *Ar*QR is the only one that can reduce >200 g L^{-1} substrate in a space time yield as high as 916 g L^{-1} d^{-1}, demonstrating its great potential in industrial manufacturing.

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Supporting Information Available. Experimental procedures and compound characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.