Green Chemistry

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

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Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

COMMUNICATION

An Artificial Enzymatic Reaction Cascade for a Cell-free Bio-system Based on Glycerol†

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

Conversion of glycerol into high-value products is of significant importance for sustainability in the biofuel industry. In this study, pyruvic acid, a central intermediate needed for the production of versatile biomolecules, was produced from glycerol without the addition of any cofactors by the cell-free bio-system composed of alditol oxidase, dihydroxy acid dehydratase, and catalase. (3*R*)-Acetoin was then produced at 85.5% of the theoretical yield from glycerol by α-acetolactate synthase and α-acetolactate decarboxylase. Since other biomolecules can also be produced from pyruvic acid, the cell-free bio-system might serve as a versatile bio-production platform, and support the viability of the biofuel economy.

Although the processes required to produce biofuels constitute extremely inefficient land use,[1] production of biofuels from renewable feedstocks has been increasing throughout our world where utilization of petrol fuels is becoming more expensive and unsustainable.[2-5] Nowadays, the global biofuel market consists of approximately 15% biodiesel and 85% bioethanol.[6] Glycerol is an inevitable byproduct of bioethanol and biodiesel production.[7] For example, during an industrial bioethanol process, the fermentation of sugars by yeasts is accompanied by the generation of significant amounts of glycerol as a fermentation byproduct.[8] During biodiesel production, a large amount of glycerol is generated during the transesterification of fats and oils with an alcohol (1 kg of glycerol per 10 kg of biodiesel produced).[9] It is estimated that the world production of glycerol will reach approximately 2,200,000 tons in 2013 and a projected 4,200,000 tons in 2020 (OECD/FAO 2012).[10] However, the existing market for industrial applications of glycerol was only 1,000,000 tons per year.[6] Thus, new processes that can convert glycerol into high-value products need to be developed for the viability of the biofuel economy.

Glycerol can be converted into higher value products by heterogeneous or homogeneous catalysis or by using biocatalysts.[11-13] Biological conversions are generally preferred to chemical means because of higher chemical selectivity (i.e., higher product yield), more modest reaction conditions, and lower levels of chemical contaminants.[14, 15] Additionally, because of its availability in dilute aqueous solutions, glycerol is usually incompatible with chemical transformations (requires energy-costly purification), but is ideal for bioconversions. However, the multitude of cellular metabolic pathways can often lead to the production of unintended byproducts and hence result in low conversion efficiencies and yields. Despite advances in genetic engineering, it is still prohibitively difficult to obtain optimal product formation at a cellular level because of the high complexity and unpredictability of the cellular metabolic pathways.[16]

One of the possible solutions for the above-mentioned problem is to use a cell-free bio-system, which leaves out the cells and exclusively employs purified enzymes.[17-24] It can eliminate cell-associated process barriers, such as substrate or product toxicity, and the undesired and substrate-induced metabolism pathway that leads to the byproducts. In the cell-free bio-system, glycerol should first be converted into pyruvic acid, a central intermediate from which other biomolecules can be produced with few additional enzymatic steps. During the metabolism of glycerol in organisms, glycerol is dehydrogenated by an NAD-dependent glycerol dehydrogenase (GDH) to dihydroxyacetone (DHA). DHA is then phosphorylated by phosphoenolpyruvate or ATP-dependent DHA kinase (DhaK) to dihydroxyacetone phosphate (DHAP). Alternatively, glycerol is first phosphorylated by ATP and glycerol kinase (GlpK) to glycerol-3-phosphate (G3P) and dehydrogenated by NAD-dependent glycerol-3-phosphate dehydrogenase (GlpD) to DHAP. DHAP will be converted into pyruvic acid through six enzymes (triose phosphofructokinase, glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase, phosphoglyceromutase, enolase, and pyruvate kinase) in the glycolysis pathway. Thus, a cell-free bio-system based on the natural metabolic pathway requires at least a total of eight enzymes and expensive cofactors such as NAD, ADP, and ATP (Fig. 1) to biotransform glycerol into pyruvic acid.[25-30]

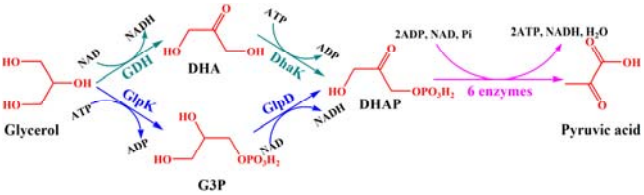

Fig. 1. Bio-system based on the natural metabolism pathway of glycerol in organisms. The enzymes are GDH (glycerol dehydrogenase), DhaK (DHA kinase), GlpK (glycerol kinase), GlpD (glycerol-3-phosphate dehydrogenase), and six additional enzymes including triose phosphofructokinase, glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase, phosphoglyceromutase, enolase, and pyruvate kinase in glycolysis.

To yield a stable and technically feasible cell-free bio-system, it is essential to minimize the number of enzymes and eliminate reactions driven by expensive cofactors such as NAD and ATP.[16] Here, we have designed a completely artificial reaction cascade for the conversion of glycerol into pyruvic acid, which requires only three enzymes. Glycerol is first oxidized with oxygen to glyceric acid via glyceraldehyde by the FAD-dependent alditol oxidase (Aldo)-catalyzed two-step oxidation reaction.[31-33] The substrate tolerance of the dihydroxy acid dehydratase (DHAD) in *Sulfolobus solfataricus* was reported recently.[16, 34] Glyceric acid produced from glycerol can be transformed into pyruvic acid and water without oxygen by the DHAD in *S. solfataricus*.[16, 34] The hydrogen peroxide generated in the reaction catalyzed by Aldo can be decomposed into water and oxygen by the catalase.[35-37] As a result, one mole of glycerol plus one mole of oxygen can generate one mole of pyruvic acid and two moles of water without addition of any cofactor (Fig. 2).

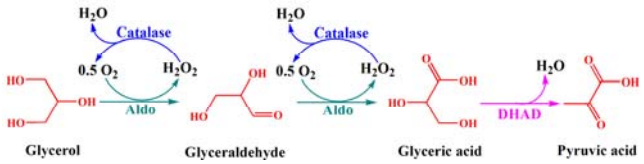

Fig. 2. Bio-system based on artificial reaction cascade for the conversion of glycerol into pyruvic acid. The enzymes are Aldo (EC 1.1.3.41, alditol oxidase with a specific activity of 0.261 U mg^{-1}) in *S. coelicolor* A3; DHAD (EC 4.2.1.39, dihydroxy acid dehydratase with a specific activity of 0.011 U mg^{-1}) in *S. solfataricus*; and catalase (EC 1.11.1.6, with a specific activity of 131,600 U mg^{-1}) in *A. niger*.

To investigate the viability of this approach, Aldo in *Streptomyces coelicolor* A3 (DNA-sequences with optimized *Escherichia coli* codon usage can be found in ESI,†) and DHAD in *S. solfataricus* (GenBank: AE006641.1) were obtained in their recombinant forms after expression in *E. coli* BL21 (DE3) and purified to homogeneity (see ESI,† Table S1–S2, and Fig. S1). The details of recombinant enzyme sequences, expression conditions, and purification methods are presented in the Supporting Information. The catalase in *Aspergillus niger* is commercially available (Sigma-Aldrich). These enzymes were chosen based on their stability and selectivity. The Aldo and catalase used in this study were both thermostable enzymes that still retained 76% and 74% of their initial activities after incubation at 50°C for 12 h (see ESI,† Fig. S2). The substrate tolerance of the thermostable *S. solfataricus* DHAD was reported recently. It had a specific activity of 0.011 U mg^{-1} for pyruvic acid production from glyceric acid.[16] Since enhanced thermostability of the enzymes could allow for a higher rate of substrate diffusion, increase the reaction rates, lower the viscosities of the reaction mixture, and decrease the bacterial contamination, the three thermostable enzymes were put into a bioreactor at 50°C to validate the synthetic pathway design.

As shown in Fig. 3, 9.3 mM pyruvic acid was generated from glycerol (10 mM) as expected, and the average rate of pyruvic acid generation was 0.39 mM of pyruvic acid per liter per hour. The integrated pyruvic acid yield after 24 h was 0.93 mole of pyruvic acid per mole of glycerol (see ESI,† Fig. S3). Next to pyruvic acid, reaction intermediates including glyceric acid, glyceraldehyde, and hydrogen peroxide were also assayed during the course of the reaction. As shown in Fig. 3, glyceric acid accumulated temporarily up to 5.6 mM during the first 3 h of the reaction. No accumulation of glyceraldehyde or hydrogen peroxide could be detected. Notably, undesired side products, such as lactate and acetate that often accumulated at an organism level, were not detected, indicating that the cell-free bio-system did provide the necessary substrate specificity.

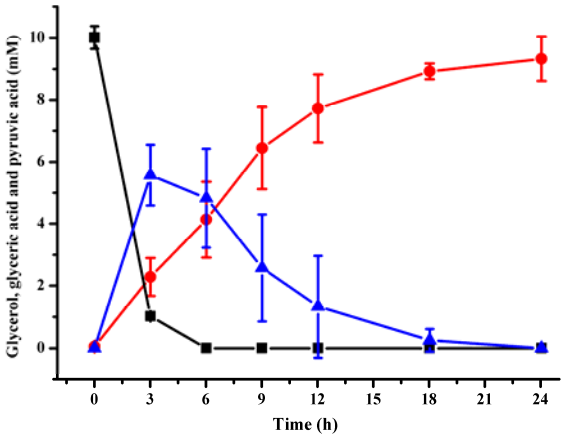

Fig. 3. Conversion of glycerol into pyruvic acid by the artificial reaction cascade. Glycerol(■), glyceric acid (▲), pyruvic acid (●). The reaction was carried out in a 100 mL screw-capped bottle with 30 mL of reaction broth containing 100 mM HEPES buffer (pH 7.4), 10 mM glycerol, 0.3 U mL^{-1} Aldo, 0.1 U mL^{-1} DHAD, and 1000 U mL^{-1} catalase. The reaction was conducted at 50°C and a shaking speed of 300 rpm. Results are means ± SD of three parallel replicates.

Then, we advanced the concept and converted pyruvic acid into (3*R*)-acetoin using only two additional enzymes in a completely cell-free environment. Acetoin was classified as one of the 30 platform chemicals that were given the priority to their development and utilization by the US Department of Energy.[38] It exists in two stereoisomeric forms: (3*R*)-acetoin and (3*S*)-acetoin. Both isomers of acetoin can be widely used in dairy products, cosmetics, pharmaceuticals, and chemical synthesis.[39] Although some microbial fermentation processes were developed, acetoin production through fermentation often resulted in a mixture of both isomers.[40-42] Apart from acetoin, some other end products are synthesized, i.e. 2,3-butanediol, ethanol, acetate, lactate, and succinate, depending on the microorganism and applied conditions (see ESI,† Fig. S4).[40-42] Biocatalysis, which uses 2,3-butanediol or diacetyl as the precursor for chiral acetoin production, suffers from the scarcity of substrates.[43-45]

During the production of acetoin in organisms, thiamine diphosphate-dependent α-acetolactate synthase (ALS) catalyzes the condensation of two molecules of pyruvic acid to yield α-acetolactate. Then, α-acetolactate is converted into (3*R*)-acetoin by α-acetolactate decarboxylase (ALDC) (Fig. 4a).[46] Since some *B. licheniformis* strain 10-1-A can thermophilically produce 2,3-butanediol at 50°C,[47] ALS (GenBank: KM882896) and ALDC (GenBank: KM882897) in *Bacillus licheniformis* 10-1-A were expressed in *E. coli* BL21 (DE3) and purified to homogeneity (see ESI,† Table S1–S3, and Fig. S5) to investigate the feasibility of using glycerol to drive (3*R*)-acetoin production.

The five enzymes, Aldo, DHAD, catalase, ALS, and ALDC, were mixed with glycerol at 50°C. As shown in Fig. 4b, 4.4 mM

(3*R*)-acetoin was generated from 10.4 mM glycerol. The (3*R*)-acetoin yield after 24 h was 85.5% of its theoretical yield. No accumulation of 2,3-butanediol, which is often co-produced with acetoin at the cellular level, could be detected. On the other hand, the stereoisomeric purity of produced (3*R*)-acetoin was 95.4% (see ESI,† Fig. S6), which was only slightly lower than that of the biocatalysis process using (2*R*,3*R*)-2,3-butanediol as the substrate.[44]

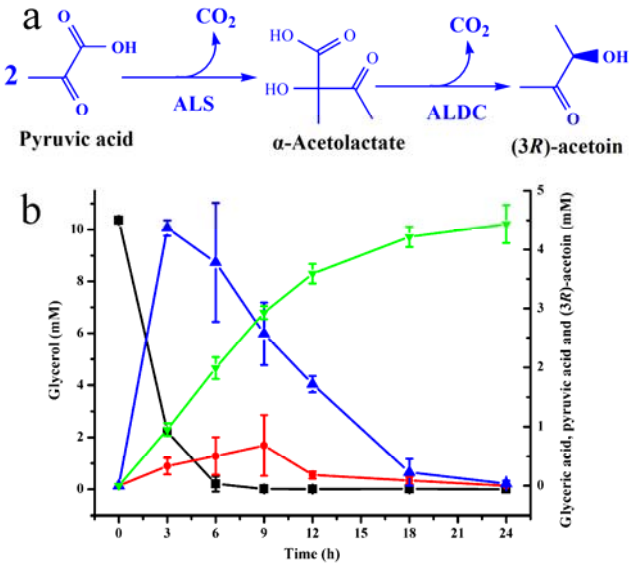

Fig. 4. a) Production of (3*R*)-acetoin by ALS (EC 2.2.1.6, α-acetolactate synthase with a specific activity of 114.55 U mg^{-1}) and ALDC (EC 4.1.1.4, α-acetolactate decarboxylase with a specific activity of 35.47 U mg^{-1}) catalyzed reactions. b) Conversion of glycerol by the artificial reaction cascade. Glycerol (■), glyceric acid (▲), pyruvic acid (●), (3*R*)-acetoin (▼). The reaction was carried out in a 100 mL screw-capped bottle with 30 mL of reaction broth at 50°C and a shaking speed of 300 rpm. The reaction mixture contained 100 mM HEPES buffer (pH 7.4), 10 mM glycerol, 0.2 mM thiamine diphosphate, 0.3 U mL^{-1} Aldo, 0.1 U mL^{-1} DHAD, 1000 U mL^{-1} catalase, 200 U mL^{-1} ALS, and 50 U mL^{-1} ALDC. Results are means ± SD of three parallel replicates.

Hexose and pentose can be used as the substrates for some high-value products *in vitro* by a synthetic enzyme pathway. Recently, Zhang and his collaborators discovered the first polyphosphate-dependent xylulokinase, which did not require the use of the costly ATP. Then polyphosphate, a less costly phosphate donor than ATP, was used to drive H_2 production from glucose or xylose.[22] This breakthrough was very important in *in vitro* synthetic biology since the chemical bond energy stored in the polyphosphate was utilized to drive the phosphorylation required for the decomposition of sugars in lignocellulosic biomass. We now show that glycerol, an unwanted waste of biofuels production, can be converted into pyruvic acid through a synthetic pathway that requires only three enzymes. Additionally, the completely artificial reaction cascade for the conversion of glycerol into pyruvic acid does not require the addition of any cofactors such as NAD(H), ADP, or ATP (Fig. 2). The key to this pathway is the utilization of FAD-dependent Aldo and DHAD, which do not require the use of the costly NAD and ATP, to replace the natural metabolic pathway of glycerol in organisms. This synthetic pathway, which eliminated NAD and ATP-driven reactions, could produce nearly theoretical yields of pyruvic acid from glycerol (93.2%). Accumulation of glyceric acid (up to 5.57 mM) was detected during the reaction, implying the low activity of DHAD to transform glyceric acid into pyruvic acid. However, because of the low specific activity of DHAD (0.011 U mg^{-1}), it is rather difficult to reduce the accumulation of glyceric acid through addition of higher amounts of DHAD in the reaction. With the molecular optimization of DHAD on its activity toward glyceric acid, cell-free biosystems based on glycerol might become an applicable biomanufacturing platform with characteristic high enantioselectivity, high efficiency, and low substrate cost.

Optically pure molecules are essential for the manufacture of many pharmaceuticals, agrochemicals, materials, and food ingredients.[48, 49] In this study, we showed the first report of chiral chemical production through *in vitro* synthetic biology. (3*R*)-Acetoin with an optical purity of 95.4% was produced by the cell-free bio-system. Other important chiral chemicals might also be produced based on the great engineering flexibility of the cell-free bio-systems.

In conclusion, a simple artificial reaction cascade was constructed for a cell-free bio-system based on glycerol. Pyruvic acid produced from glycerol was transformed into (3*R*)-acetoin, an important C_4 bulk chemical. Further investigations and applications of processes from glycerol to C_4 chemicals via (3*R*)-acetoin intermediates are ongoing in our laboratory. Since pyruvic acid can be converted into other industrial commodity compounds, such as the (3*R*)-acetoin introduced in this study, the synthetic pathway based on glycerol has the potential to serve as a versatile bio-production system.

Acknowledgements

The work was supported by Chinese National Program for High Technology Research and Development (2014AA021206, 2012AA022104 and 2011AA02A207) and National Natural Science Foundation of China (31170052 and J1103515).

Notes and references

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

1 H. Michel, *Angew. Chem. Int. Ed. Engl.* 2012, **51**, 2516.
2 A. J. Ragauskas, C. K. Williams, B. H. Davison, G. Britovsek, J. Cairney, C. A. Eckert, W. J. Jr Frederick, J. P. Hallett, D. J. Leak, C. L. Liotta, J. R. Mielenz, R. Murphy, R. Templer and T. Tschaplinski, *Science*, 2006, **311**, 484.
3 H. Latif, A. A. Zeidan, A. T. Nielsen and K. Zengler, *Curr. Opin. Biotechnol.* 2014, **27C**, 79.
4 A. S. Heeres, C. S. Picone, L. A. van der Wielen, R. L. Cunha and M. C. Cuellar, *Trends Biotechnol*. 2014, **32**, 221.
5 S. Zinoviev, F. Müller-Langer, P. Das, N. Bertero, P. Fornasiero, M. Kaltschmitt, G. Centi and S, Miertus, *ChemSusChem* 2010, **3**, 1106.
6 M. Simões, S. Baranton and C. Coutanceau, *ChemSusChem* 2012, **5**, 2106.
7 J. M. Clomburg and R. Gonzalez, *Trends Biotechnol*. 2013, **31**, 20.
8 K. D.Rausch and R. L. Belyea, *Appl. Biochem. Biotechnol*. 2006, **128**, 47.

9 S. S.Yazdani and R. Gonzalez, *Curr. Opin. Biotechnol.* 2007, **18**, 213.
10 Q. M. Viana, M. B. Viana, E. A. Vasconcelos, S. T. Santaella and R. C. Leitão, *Biotechnol. Lett.* 2014, **36**, 1381.
11 C. H. Zhou, J. N. Beltramini, Y. X. Fan and G. Q. Lu, *Chem. Soc. Rev.* 2008, **37**, 527.
12 N. H. Tran and G. S. Kannangara, *Chem. Soc. Rev.* 2013, **42**, 9454.
13 B. Katryniok, S. Paul, M. Capron and F. Dumeignil, *ChemSusChem* 2009, **2**, 719.
14 J. R. Almeida, L. C. Fávaro and B. F. Quirino, *Biotechnol. Biofuels* 2012, **5**, 48.
15 S. Khanna, A. Goyal and V. S. Moholkar, *Crit. Rev. Biotechnol.* 2012, **32**, 235–262.
16 J. K. Guterl, D. Garbe, J. Carsten, F. Steffler, B. Sommer, S. Reiße, A. Philipp, M. Haack, B. Rühmann, A. Koltermann, U. Kettling, T. Brück and V. Sieber, *ChemSusChem* 2012, **5**, 2165.
17 C. You and Y. H. Zhang, *Adv. Biochem. Eng. Biotechnol.* 2013, **131**, 89.
18 Y. H. Zhang, J .Sun and J. J. Zhong, *Curr. Opin. Biotechnol.* 2010, **21**, 663.
19 S. Billerbeck, J. Härle and S. Panke, *Curr. Opin. Biotechnol.* 2013, **24**, 1037.
20 Zhu Z, T. K. Tam and Y. H. Zhang, *Adv. Biochem. Eng. Biotechnol.* 2013, **137**, 125.
21 C. You, H. Chen, S. Myung, N. Sathitsuksanoh, H. Ma, X. Z. Zhang, J. Li and Y. H. Zhang, *Proc. Natl. Acad. Sci. USA.* 2013, 110, 7182.
22 J. S. Martín del Campo, J. Rollin, S. Myung, Y. Chun, S. Chandrayan, R. Patiño, M. W. Adams and Y. H. Zhang, *Angew. Chem. Int. Ed. Engl.* 2013, **52**, 4587.
23 S. Myung, J. Rollin, C. You, F. Sun, S. Chandrayan, M. W. Adams and Y. H. Zhang, *Metab. Eng.* 2014, **24C**, 70.
24 B. Krutsakorn, K. Honda, X. Ye, T. Imagawa, X. Bei, K. Okano and H. Ohtake, *Metab. Eng.* 2013, **20**, 84.
25 Y. Wang, F. Tao and P. Xu, *J. Biol. Chem*. 2014, **289**, 6080.
26 Y. Chao, R. Patnaik, W. D. Roof, R. F. Young and J. C. Liao, *J. Bacteriol.*1993, **175**, 6939.
27 A. Murarka, Y. Dharmadi, S. S. Yazdani and R. Gonzalez, *Appl. Environ. Microbiol.* 2008, **74**, 1124.
28 K. T. Tran, T. Maeda and T. K. Wood, *Appl. Microbiol. Biotechnol.* 2014, **98**, 4757.
29 R. Gonzalez, A. Murarka, Y. Dharmadi and S. S. Yazdani, *Metab. Eng.* 2008, **10**, 234.
30 S. S. Yazdani and R. Gonzalez, *Metab. Eng.* 2008, **10**, 340.
31 D. P. Heuts, E. W. van Hellemond, D. B. Janssen and M. W. Fraaije, *J. Biol. Chem.* 2007, **282**, 20283.
32 S. Gerstenbruch, H. Wulf, N. Mussmann, T. O'Connell, K. H. Maurer and U. T. Bornscheuer, *Appl. Microbiol. Biotechnol.* 2012, **96**, 1243.
33 F. Forneris, D. P. Heuts, M. Delvecchio, S. Rovida, M. W. Fraaije and A. Mattevi, *Biochemistry* 2008 , **47**, 978.
34 S. Kim and S. B. Lee, *J. Biochem.* 2006, **139**, 591.
35 J. Rogalski, J. Fiedurek and A. Gromada, *Acta Microbiol. Pol.* 1998, **47**, 31.
36 J. Fiedurek and A. Gromada, *J. Appl. Microbiol.* 2000, **89**, 85.
37 J. Z. Liu, H. Y. Yang, L. P. Weng and L. N. Ji, Lett. *Appl. Microbiol.* 1999, **29**, 337.
38 See "Top value added chemicals from biomass: vol. I. Results of screening for potential candidates from sugars and synthesis gas" by T. Werpy and G. Petersen at 2004. Available from: <http://www.eere.energy.gov/biomass/pdfs/35523.pdf>.
39 Z. Liu, J. Qin, C. Gao, D. Hua, C. Ma, L. Li, Y. Wang and P. Xu, *Bioresour. Technol.* 2011, **102**, 10741.
40 Z. Xiao and J. R. Lu, *Biotechnol. Adv.* 2014, **32**, 492.
41 Z. Xiao, X. Wang, Y. Huang, F. Huo, X. Zhu, L. Xi and J. R. Lu, *Biotechnol. Biofuels.* 2012, **5**, 88.
42 X. Zhang, R. Zhang, T. Bao, Z. Rao, T. Yang, M. Xu, Z. Xu, H. Li and S. Yang, *Metab. Eng.* 2014, **23**, 34.
43 S. Ui, A. Mimura, M. Ohkuma and T. Kudo, *Lett. Appl. Microbiol.* 1999, **28**, 457.
44 Z. Xiao, C. Lv, C. Gao, J. Qin, C. Ma, Z. Liu, P. Liu, L. Li and P. Xu, *PLoS One* 2010, **5**, e8860.
45 C. Gao, L. Zhang, Y. Xie, C. Hu, Y. Zhang, L. Li, Y. Wang, C. Ma and P. Xu, *Bioresour. Technol.* 2013, **137**, 111.
46 X. J. Ji, H. Huang and P. K. Ouyang, *Biotechnol. Adv.* 2011, **29**, 351.
47 L. Li, L. Zhang, K. Li, Y. Wang, C. Gao, B. Han, C. Ma, P. Xu, *Biotechnol. Biofuels.* **2013**, *6*, 123.
48 G. W. Zheng and J. H. Xu, *Curr. Opin. Biotechnol.* 2011, **22**, 784.
49 R. N. Patel, *Curr. Opin. Drug Discovery Dev.* 2006, **9**, 741.