Single-step enzymatic synthesis of (*R*)-2-*O*-α-D-glucopyranosyl glycerate, a compatible solute from micro-organisms that functions as a protein stabiliser†‡

Thornthan Sawangwan, Christiane Goedl and Bernd Nidetzky*

Received 26th June 2009, Accepted 3rd July 2009
First published as an Advance Article on the web 12th August 2009
DOI: 10.1039/b912621j

Regioselective glucosylation of *R*-glycerate catalysed by sucrose phosphorylase in the presence of sucrose as the donor substrate provided the natural compatible solute (*R*)-2-*O*-α-D-glucopyranosyl glycerate with complete regioselectivity in an optimised synthetic yield of 90% *R*-glycerate converted and a concentration of about 270 mM.

Introduction

Microbial life under extremes of temperature, osmotic pressure and pH is facilitated by the intracellular accumulation of a diverse class of small molecules that are often referred to as compatible solutes.[1] A conserved characteristic for many of these compounds is a glycosidic chemical structure (Fig. 1).

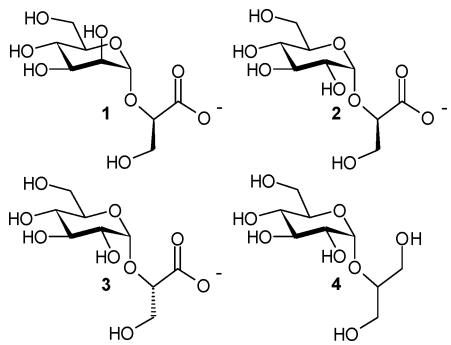


Fig. 1 The microbial compatible solutes mannosyl glycerate (**1**), glucosyl glycerate (**2**) and a non-natural isomer thereof (**3**), as well as glucosyl glycerol (**4**).

Mannosyl glycerate[2] (**1**) is widely spread in (hyper) thermophilic micro-organisms where it contributes to osmotic adaptation and may serve a role as an extrinsic stabiliser of proteins and other cell components at high temperatures. Glucosyl glycerate (**2**), a close structural analogue of **1**, has also been reported from a range of microbial lineages.[3]

Exhaustive studies on the protein-stabilising capacity of a series of compatible solutes have led to the proposal that negatively charged compounds such as **1** or **2** are the most physiologically effective in coping with the denaturing effects of heat stress.[4,5] Exploitation of **1** or **2** in biotechnology as protein-protecting reagents is therefore immediately suggested. However, benchmarking of the use of these glycosyl glycerates against that of established solutes such as glycerol, ectoine and α,α-trehalose raises concern that improved functional efficacy of **1** and **2** may be invalidated by disproportionally high compound production costs. Chemical synthesis of **1** has not been reported and appears too laborious to present an economically viable option for **2**.[6,7] Likewise, despite notable efforts, technologically mature bioprocesses for microbial or enzymatic production[2] of **1** and **2** are currently not available. Therefore, removing the bottleneck of availability of **1** and **2** as fine chemicals could not only promote their development into industrial protein stabilisers but might also open up new fields of application for these compounds, in cosmetic products for example.

We report here on a biocatalytic process that provides **2** in excellent synthetic yield using a single-step enzymatic transformation of simple starting materials, sucrose and *R*-glycerate. Glucosylation of *R*-glycerate is catalysed by sucrose phosphorylase and is shown to occur with absolute regioselectivity for the 2-OH of the acceptor substrate. Using optimised reaction conditions, about 90% of *R*-glycerate (300 mM) was converted into stereochemically pure **2**. When *S*-glycerate was employed as acceptor for enzymatic glucosyl transfer from sucrose, a non-natural form of glucosyl glycerate, (*S*)-2-*O*-α-D-glucopyranosyl glycerate (**3**), was synthesised in likewise useful amounts. Although we have in recent work identified sucrose phosphorylase as a powerful catalyst for glucosylation of glycerol to produce the uncharged solute 2-*O*-α-D-glucopyranosyl *sn*-glycerol (**4**),[8] it was unexpected in light of earlier reports on the specificity of the enzyme[9] that group transfer to glycerate took place in a fully regioselective manner. The preparative yield of **2** in >98% purity was ≥60%.

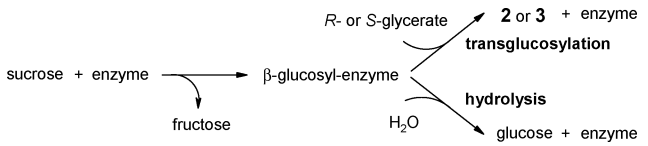
Results and discussion

The catalytic mechanism underlying the enzymatic substrate transformation into **2** or **3** is shown in Scheme 1. Sucrose phosphorylase promoted a transglucosylation reaction in two steps where a β-glucosyl enzyme intermediate formed in the first half-reaction (*enzyme glucosylation*) was intercepted by the 2-OH of *R*-glycerate

Institute of Biotechnology and Biochemical Engineering, Graz University of Technology, Petersgasse 12/1, Graz, Austria. E-mail: bernd.nidetzky@tugraz.at; Fax: +43 316 873 8434; Tel: +43 316 873 8400

† This paper is part of an *Organic & Biomolecular Chemistry* web theme issue on biocatalysis.

‡ Electronic supplementary information (ESI) available: Supplementary data of the influence of Ca^{2+} on the enzymatic synthesis, variation of temperature to optimise the enzymatic synthesis, HPLC analysis of the product mixture obtained by enzymatic conversion of sucrose in the presence of racemic *R*/*S*-glycerate. See DOI: 10.1039/b912621j



Scheme 1 Synthesis of **2** and **3** using transglucosylation catalysed by sucrose phosphorylase (enzyme), and hydrolytic competition in the reaction of the β-glucosyl-enzyme intermediate.

in the second half-reaction to yield the α-configured glucosidic product (*enzyme deglucosylation*). Hydrolysis of the intermediate competed with group transfer to the acceptor substrate. Although synthesis of **2** or **3** proceeded under kinetic control, we show later that there was a marked kinetic hindrance to secondary hydrolysis of **2** catalysed by the enzyme. Formation of **2** appeared therefore as an equilibrium-controlled process which facilitated design of the enzymatic conversion. The physiological reaction of the sucrose phosphorylase is glucosyl transfer from sucrose to phosphate, forming α-D-glucopyranosyl phosphate. To prevent this *phosphorolysis* of sucrose from taking place, it is important that synthesis of **2** (or **3**) occurs in buffer lacking phosphate.

When sucrose phosphorylase was incubated in the presence of sucrose (300–800 mM) and *R*-glycerate (50–600 mM) at 30 °C and pH 7.0, HPLC analysis of samples taken from the reaction mixtures at certain times revealed the appearance of a new compound eluting in a peak baseline—separated and thus clearly distinct from peaks assigned to substrates, fructose and the product of hydrolysis, glucose (Fig. 2A). NMR spectroscopic characterization of a sample in which about 60% of the original *R*-glycerate (300 mM) was converted in the enzymatic glucosylation using 300 mM sucrose gave clear indication of formation of **2**. It also showed the absence, above the detection limit of the method used, of products that would have resulted from group transfer to alternate acceptor sites on *R*-glycerate, (*R*)-3-*O*-α-D-glucopyranosyl glycerate and (*R*)-1-*O*-α-D-glucopyranosyl glycerate. According to literature,[9] both primary hydroxyls and carboxyl groups serve as acceptors of glucosyl transfer catalysed by the enzyme.

Fig. 2B displays a typical time course of enzymatic synthesis of **2**, showing the concentrations of all compounds involved in the reaction. As expected from Scheme 1, release of fructose paralleled the consumption of sucrose. Formation of glucose in amounts similar to **2** indicates that glucosylated sucrose phosphorylase reacted with water about as efficiently as with *R*-glycerate under the conditions applied. Due to depletion of sucrose in the reaction, therefore, the maximum conversion of *R*-glycerate in the shown experiment was about 60%. The glucosidic product was fully stable against secondary hydrolysis catalysed by the enzyme for at least 72 h of incubation. Synthesis of **2** was characterised by a relatively fast initial phase of production in which about half the total product concentration was formed and a second phase in which **2** was released at a significantly slower rate (Fig. 2B). The marked decrease in production rate for **2** after about 5 h is explicable on account of the accumulating glucose acting as inhibitor of binding of sucrose to the free sucrose phosphorylase. The inhibition constant for glucose is known from literature as ~0.5 mM.[10] By way of comparison, synthesis of **4** involves hydrolysis to a minimal extent[8] and probably therefore, proceeds at a productivity about 5 times that of production of **2**. Also note that sucrose phosphorylase did not lose a large amount of activity (≤40%) in

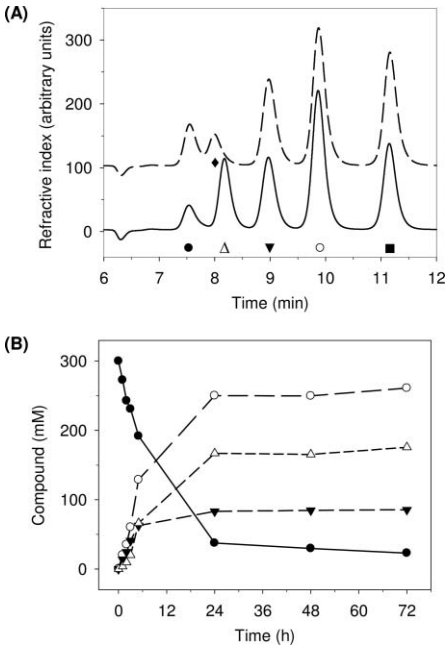


Fig. 2 Enzymatic synthesis of **2**. (A) HPLC method used to monitor the progress of production of **2** (solid line) or **3** (dotted line). (B) Time course analysis for conversion of sucrose and *R*-glycerate, each 0.3 M, at 30 °C and pH 7.0. The symbols indicate sucrose (●), **3** (♦), **2** (△), D-glucose (▼), D-fructose (○), and *R*- or *S*-glycerate (■).

the time-span of the conversion shown in Fig. 2B, indicating that enzyme inactivation is not limiting the overall productivity. The Ca^{2+} introduced with *R*-glycerate acceptor (which is applied as hemicalcium salt in our experiments) did not cause inhibition of sucrose phosphorylase (see Supplementary Table 1 in the ESI‡).

Table 1 summarises results of experiments carried out to optimise the production of **2**. The concentrations of sucrose and *R*-glycerate were systematically varied and the product yield was determined after a 72 h-long incubation. Interestingly, contrary to what one would expect from Scheme 1, an increase in the concentration of *R*-glycerate above the constant concentration of sucrose (300 mM) did not improve utilisation of *R*-glycerate as

Table 1 Optimisation of enzymatic production of **2**

Sucrose [M]	*R*-Glycerate [M]	Product [M] × 10^{-2}	Yield[a] [%]
0.3	0.3	16.7	56
0.4		19.1	64
0.5		23.5	78
	0.05	3.9	78 (13)
	0.10	7.9	79 (26)
	0.15	10.8	72 (36)
	0.20	12.3	62 (41)
0.3	0.30	17.6	59 (59)
	0.40	17.9	45 (60)
	0.50	17.1	34 (57)
	0.60	17.2	29. (57)
0.3	0.3 *S*-form	9.8[b]	33
	0.3 *R*/*S*-form	10.2[c]	34
0.3		17.3	58
0.5	0.3[d]	18.1	60
0.8		27.4	91

[a] Analytical yield based on glycerate (or sucrose) converted. [b] Formation of **3**. [c] **2** is present in excess over **3**. [d] Crude enzyme preparation was used.

glucosyl acceptor. It seems therefore that kinetic competition between glucosyl transfer and hydrolysis did not occur solely at the level of binding of the acceptor to the β-glucosyl enzyme intermediate but also after the complex of glucosylated sucrose phosphorylase and *R*-glycerate had been formed. In this scenario, saturating concentrations of acceptor would partly, however, not completely suppress hydrolysis as a side reaction. The yield of **2** (based on *R*-glycerate converted) was therefore best improved by using a saturating level of *R*-glycerate (300 mM) in combination with 2.5-fold molar excess of donor over acceptor substrate. Variation of the temperature in the range 25–40 °C did not affect the yield of **2**. The production rate was however enhanced ~2-fold at 35 °C, as compared to 25 °C (see Supplementary Table 2 in the ESI‡)

The performance of sucrose phosphorylase in the synthesis of **2** was not dependent on biocatalyst purity. We replaced isolated enzyme by crude cell extract of the *Escherichia coli* strain producing recombinant phosphorylase without introducing a negative effect on production of **2**. Using optimised substrate concentrations (800 mM sucrose, 300 mM *R*-glycerate), the analytical yield of **2** was 91% based on the limiting amount of glucosyl acceptor converted.

Product **2** (sodium salt) was isolated in >98% purity (HPLC, NMR) and 60% yield using a two-step procedure of elution chromatography in which anion exchange and adsorption to activated charcoal were employed for separation. It was obtained as white powder after freeze drying, and its chemical structure was confirmed by ^{1}H and ^{13}C NMR analysis.

The absolute regioselectivity of sucrose phosphorylase for glucosylation of the 2-OH of glycerate was contrasted by a similar, however, not identical reactivity of the enzyme toward the *R* and *S* antipodes of the acceptor (Table 1). Fig. 2A shows that upon incubation of the enzyme in the presence of sucrose and *S*-glycerate, a glucosyl transfer product was formed which eluted in HPLC analysis differently from **2**. The product thus obtained was purified identically as described for **2**. Relevant NMR data confirmed the expected chemical structure of **3**. Sucrose phosphorylase preferred *R*- over *S*-glycerate as a substrate for glucosyl transfer (Table 1). It is interesting therefore that in the case of using a racemic mixture of *R*/*S*-glycerate as a glucosyl acceptor, the overall yield of transfer product (abundance of **2** is visible in Supplementary Fig. 1 in the ESI‡) was about that obtained when employing enantiomerically pure *S*-glycerate. Because the amount of sucrose utilised in the experiments was comparable irrespective of the use of *R*, *S* or *R*/*S*-glycerate, we conclude that the extent to which the complex of glucosylated enzyme and acceptor was converted *via* "error hydrolysis" was significantly (~2-fold) lower for the *R*-configured substrate. Subtle differences in positioning of the *R*- and *S*-antipodes of glycerate at the acceptor binding site of sucrose phosphorylase could determine the observed changes in enzymatic chemo-selectivity, that is, the reactivity ratio for group transfer and hydrolysis.

Purified **2** (100 mM) was examined as a donor substrate for glucosyl transfer to phosphate (50 mM; potassium salt) and fructose (50 mM) catalysed by sucrose phosphorylase. The turnover frequency (k_{cat}) measured under these conditions was low ($\leq 2 \times 10^{-3}$ s^{-1}) and explains the observed high kinetic stability of **2** in the synthesis experiment. It can be compared to reported k_{cat} values of ~100 s^{-1} for analogous reactions of the enzyme[11] in which sucrose (transfer to phosphate) or α-D-glucopyranosyl phosphate (transfer to fructose) are glucosyl donors. These results show that the rate of enzyme glucosylation was slowed $\geq 5 \times 10^{4}$-fold when the *R*-glycerate of **2** replaced the fructose of the natural sugar substrate sucrose as a leaving group of the biocatalytic reaction.

Conclusions

In summary, this paper describes a stereoselective and efficient single-step biocatalytic synthesis of the microbial compatible solute **2** as well as its non-natural isomer **3**. The enzymatic process opens up the first promising route towards **2** as an industrial fine chemical. Applications for compatible solutes having a charged glycosidic structure, like the use as a stabilising reagent or excipient in protein formulation technology,[4,6] appear therefore realistic for **2**.

Experimental

Materials and methods

Sucrose, *R*-glycerate (*R*-2,3-dihydroxypropanoic acid hemicalcium salt), *S*-glycerate (*S*-2,3-dihydroxypropanoic acid hemicalcium salt), *R*/*S*-glycerate (*R*/*S*-2,3-dihydroxypropanoic acid hemicalcium salt), MES buffer (potassium salt), Dowex® (1 × 2 Cl^- form, strongly basic, 50–100 mesh), activated charcoal (type Norit SX ultra) and Celite® (545 coarse filter aid, treated with sodium carbonate, calcined) were from Sigma-Aldrich, Vienna, Austria. Sodium chloride and sulfuric acid (0.5 M) were obtained from Carl Roth GmbH & Co, Karlsruhe, Germany. Ethanol (absolute for analysis) was from AustrAlco GmbH, Spillern, Austria. Empty chromatography columns (XK 50/30) were obtained from GE Healthcare, Vienna, Austria. The Cation H Micro-Guard pre-column (30 × 4.6 mm) and the HPX-87H column (300 × 7.8 mm; Aminex®) were from Bio-Rad, Vienna, Austria. All other chemicals used were of highest purity available.

Biocatalyst production

Recombinant wild-type sucrose phosphorylase from *Leuconostoc mesenteroides* was produced in *E. coli* DH10B cells and purified to apparent homogeneity according to reported procedures.[12] The preparation had a specific phosphorylase activity of 64 U/mg. Alternatively, crude bacterial cell extract was employed. This was obtained by passing the harvested *E. coli* DH10B cells through an Aminco French press at approximately 150 bar. Cell debris was removed by ultracentrifugation at 80,000 *g* and 4 °C for 15 min. The cell extract contained about 74 mg/ml protein and had volumetric and specific activities of 1,800 U/ml and 24 U/mg, respectively.

Analytical methods

Sucrose phosphorylase activity was measured using a continuous coupled enzymatic assay employing phosphoglucomutase (PGM) and glucose-6-phosphate dehydrogenase (G6P-DH). The formation of NADH with time was followed at 340 nm for 5 minutes. One unit of enzymatic activity is defined as the amount of enzyme liberating 1 μmol of NADH per minute at 25 °C. The standard assay was performed essentially as described elsewhere.[13] Protein concentration was determined using the Bio-Rad

dye-binding method with bovine serum albumin as the standard. Phosphate[14] was determined colorimetrically at 850 nm, and α-D-glucopyranosyl phosphate[15] was assayed in a coupled enzymatic system with PGM and G6P-DH. HPLC analysis was performed using a Cation H Micro-Guard pre-column followed by an Aminex HPX-87H column at 30 °C and a constant flow rate of 0.6 ml/min with 0.005 M sulfuric acid as the mobile phase. All compounds were analysed with a refractive index detector. The obtained chromatograms revealed retention times of 7.6 min for sucrose, 8.0 min for **3**, 8.2 min for **2**, 9.0 min for D-glucose, 9.8 min for D-fructose and 11.1 min for *R*- and *S*-glycerate.

Enzymatic production of 2

Reactions were typically performed in plastic tubes with a total volume of 0.5 ml or in a shake flask with a working volume of 30 ml. Incubations were performed at a controlled temperature (usually 30 °C; range 25–40 °C) and agitation rate (550 rpm) using an Eppendorf Thermomixer comfort. The reaction mixture contained 0.3–0.8 M sucrose and 0.05–0.6 M *R*-glycerate. A 50 mM MES buffer, pH 7.0, was used. Optionally, *R*-glycerate was replaced by 0.3 M *S*-glycerate or 0.3 M *R*/*S*-glycerate. Unless otherwise mentioned, all reactions were carried out using isolated sucrose phosphorylase. Crude *E. coli* cell extract was also used in selected experiments. The volumetric enzyme activity was always 20 U/ml. Reaction times of up to 72 h were used. Samples, taken at appropriate times, were inactivated by heating and centrifuged before further analysis.

Isolation of 2

The product mixture (49.6 g/l **2**; 110 ml) was loaded onto an anion exchange chromatography column (XK 50/30, V_{bed} = 150 ml) packed with a 1 × 2 Cl^- Dowex® material at a flow rate of 20 ml/min and 0.8 mPa. Elution was performed using a 3-step gradient with 0.6 l of H_2O, followed by 0.6 l of 0.1 M and 0.6 l of 0.5 M of sodium chloride. The eluted fractions were analyzed by HPLC and concentrated by evaporation. The glucosyl glycerate product and the glycerate substrate co-eluted at 0.5 M of sodium chloride. For the second chromatographic step, an activated charcoal column (XK 50/30, V_{bed} = 150 ml), packed with a 1:1 mixture of activated charcoal Norit (type Norit SX ultra) and calcined Celite® 501 was used at a flow rate of 20 ml/min and 0.5 mPa. Elution was carried out employing a 3-step gradient with 0.3 l of 0%, 0.6 l of 10% and 0.2 l of 15% of ethanol. Fractions that eluted at 10% of ethanol and contained the product were pooled, concentrated by evaporation (65.5 g/l **2**; 50 ml), lyophilized, and stored at −21 °C. The chemical product structure of **2** was confirmed by NMR analysis using procedures reported elsewhere in more detail.[11]

NMR data

Assignment of chemical shifts in (*R*)-2-*O*-α-D-glucopyranosyl glycerate (2).[7] δ_H (400 MHz, 300 K, D_2O) glucose: 4.87 (d, 1H, J = 3.6 Hz, H-1), 3.41 (dd, 1H, J = 9.8, 3.8 Hz, H-2), 3.69 (dd, 1H, J = 9.8, 9.8 Hz, H-3), 3.29 (dd, 1H, J = 9.8, 9.1 Hz, H-4), 3.66 (ddd, 1H, J = 9.1, 5.1, 2.8 Hz, H-5), 3.72 (dd, 1H, J = 12.5, 2.8 Hz, H-6a), 3.62 (dd, 1H, J = 12.5, 5.1 Hz, H-6b); glycerate: 4.07 (dd, 1H, J = 6.2, 3.0 Hz, H-2′), 3.69 (dd, 1H, J = 12.0, 6.2 Hz, H-3a′), 3.74 (dd, 1H, J = 12.0, 3.0 Hz, H-3b′). δ_C (100 MHz, 300 K, D_2O) glucose: 97.4 (C-1), 71.6 (C-2), 73.2 (C-3), 69.4 (C-4), 72.2 (C-5), 60.4 (C-6); glycerate: 177 (C-1′), 79.0 (C-2′), 62.0 (C-3′).

Assignment of chemical shifts in (*S*)-2-*O*-α-D-glucopyranosyl glycerate (3). δ_H (400 MHz, 300 K, D_2O) glucose: 4.95 (d, 1H, J = 4.0 Hz, H-1), 3.46 (dd, 1H, J = 9.9, 3.7 Hz, H-2), 3.75 (m, 1H, H-3), 3.36 (dd, 1H, J = 10.0, 9.8 Hz, H-4), 3.76 (m, 1H, J = n.d., H-5), 3.67 (m, 1H, J = n.d., H-6a), 3.64 (m, 1H, J = n.d., H-6b); glycerate: 3.94 (dd, 1H, J = 6.8, 3.6 Hz, H-2′), 3.74 (m, 1H, J = n.d., H-3a′), 3.68 (m, 1H, J = n.d., H-3b′). δ_C (100 MHz, 300 K, D_2O) glucose: 99.2 (C-1), 71.7 (C-2), 73.0 (C-3), 69.3 (C-4), 72.7 (C-5), 60.0 (C-6); glycerate: 177 (C-1′), 81.4 (C-2′), 62.5 (C-3′).

Chemical shifts are referenced to external acetone (δ_H 2.23 ppm and δ_C 31.5 ppm).

Acknowledgements

Financial support from the Austrian Science Fund (project L586-B03 and the DK Molecular Enzymology W901-B05) and the OEAD (to T. S.) is gratefully acknowledged. We thank Prof. Dr Lothar Brecker, University of Vienna, Austria for the NMR spectroscopic analysis.

References

1 V. Muller, R. Spanheimer and H. Santos, *Curr. Opin. Microbiol.*, 2005, **8**, 729–736; H. Santos and M. S. da Costa, *Environ. Microbiol.*, 2002, **4**, 501–509; G. Lentzen and T. Schwarz, *Appl. Microbiol. Biotechnol.*, 2006, **72**, 623–634; M. B. Burg and J. D. Ferraris, *J. Biol. Chem.*, 2008, **283**, 7309–7313.
2 N. Empadinhas and M. S. da Costa, *Syst. Appl. Microbiol.*, 2008, **31**, 159–168; K. Egorova, T. Grudieva, C. Morinez, J. Kube, H. Santos, M. S. da Costa and G. Antranikian, *Appl. Microbiol. Biotechnol.*, 2007, **75**, 1039–1045.
3 R. Goude, S. Renaud, S. Bonnassie, T. Bernard and C. Blanco, *Appl. Environ. Microbiol.*, 2004, **70**, 6535–6541; J. Costa, N. Empadinhas, L. Goncalves, P. Lamosa, H. Santos and M. S. da Costa, *J. Bacteriol.*, 2006, **188**, 1022–1030; J. Costa, N. Empadinhas and M. S. da Costa, *J. Bacteriol.*, 2007, **189**, 1648–1654; C. Fernandes, N. Empadinhas and M. S. da Costa, *J. Bacteriol.*, 2007, **189**, 4014–4019; S. Pospíšil, P. Halada, M. Petricek and P. Sedmera, *Folia Microbiol.*, 2007, **52**, 451–456.
4 N. Borges, A. Ramos, N. D. Raven, R. J. Sharp and H. Santos, *Extremophiles*, 2002, **6**, 209–216.
5 T. Q. Faria, S. Knapp, R. Ladenstein, A. L. Macanita and H. Santos, *ChemBioChem*, 2003, **4**, 734–741.
6 T. Q. Faria, A. Mingote, F. Siopa, R. Ventura, C. Maycock and H. Santos, *Carbohydr. Res.*, 2008, **343**, 3025–3033.
7 B. K. Hunter, S. L. Mowbray and D. J. Walton, *Biochemistry*, 1979, **18**, 4458–4465.
8 C. Goedl, T. Sawangwan, M. Mueller, A. Schwarz and B. Nidetzky, *Angew. Chem., Int. Ed.*, 2008, **47**, 10086–10089.
9 K. Sugimoto, K. Nomura, H. Nishiura, K. Ohdan, T. Nishimura, H. Hayashi and T. Kuriki, *J. Biosci. Bioeng.*, 2007, **104**, 22–29; K. Nomura, K. Sugimoto, H. Nishiura, K. Ohdan, T. Nishimura, H. Hayashi and T. Kuriki, *Biosci. Biotechnol. Biochem.*, 2008, **72**, 82–87; S. Kitao and H. Sekine, *Biosci. Biotechnol. Biochem.*, 1994, **58**, 419–420.
10 J. J. Mieyal, and R. H. Abeles, in *The Enzymes*, Academic Press, New York, 1972, pp. 515–532.
11 A. Schwarz, L. Brecker and B. Nidetzky, *Biochem. J.*, 2007, **403**, 441–449.
12 C. Goedl, A. Schwarz, A. Minani and B. Nidetzky, *J. Biotechnol.*, 2007, **129**, 77–86; A. Schwarz and B. Nidetzky, *FEBS Lett.*, 2006, **580**, 3905–3910.
13 A. Weinhäusel, R. Griessler, A. Krebs, P. Zipper, D. Haltrich, K. D. Kulbe and B. Nidetzky, *Biochem. J.*, 1997, **326**, 773–783.
14 S. Saheki, A. Takeda and T. Shimazu, *Anal. Biochem.*, 1985, **148**, 277–281.
15 C. Eis and B. Nidetzky, *Biochem. J.*, 1999, **341**, 385–393.

