Autodisplay of Active Sorbitol Dehydrogenase (SDH) Yields a Whole Cell Biocatalyst for the Synthesis of Rare Sugars

Joachim Jose*[a] and Steffen von Schwichow[a, b]

Whole cell biocatalysts are attractive technological tools for the regio- and enantioselective synthesis of products, especially from substrates with several identical reactive groups. In the present study, a whole cell biocatalyst for the synthesis of rare sugars from polyalcohols was constructed. For this purpose, sorbitol dehydrogenase (SDH) from Rhodobacter sphaeroides, *a member of the short-chain dehydrogenase/reductase (SDR) family, was expressed on the surface of* Escherichia coli *using Autodisplay. Autodisplay is an efficient surface display system for Gram-negative bacteria and is based on the autotransporter secretion pathway. Transport of SDH to the outer membrane was monitored by SDS-PAGE and Western blotting of different cell fractions. The surface exposure of the enzyme could be verified by immunofluorescence microscopy and fluorescence activated cell sorting (FACS). The activity of whole cells displaying SDH at the surface was determined in an optical test. Specific activities were found to be 12 mU per 3.3×10^8 cells for the conversion of* D*-glucitol (sorbitol) to* D*-fructose, 7 mU for the conversion* D*-galactitol to* D*-tagatose, and 17 mU for the conversion of* L*-arabitol to* L*-ribulose. The whole cell biocatalyst obtained by surface display of SDH could also produce* D*-glucitol from* D*-fructose (29 mU per 3.3×10^8 cells).*

Introduction

Unnatural monosaccharides and polyalcohols are valuable compounds in terms of organic synthesis or pharmaceutical applications.[1, 2] Typical examples of such rare naturally occurring compounds are sorbitol (D-glucitol) and D-tagatose. Sorbitol serves as the starting point for the chemical synthesis of ascorbic acid, the so-called Reichstein synthesis,[3] and can be used as an artificial sweetener with reduced metabolism. In addition to further pharmaceutical applications, sorbitol is widely utilized as a plasticizer and exipient in tablet formulations.[4] Of all natural sugar substitutes, D-tagatose, an isomer of galactose, is the most similar in taste and physical properties to sucrose. Since D-tagatose is poorly absorbed, it yields only 1.5 cal g^{-1} instead of 4 compared to sucrose, and shows no laxative effect, there has recently been great interest in this sugar substitute as a potential dietary supplement.[5] Efforts have been made to produce these valuable polyols by using chemical reactions. The chemical route, however, is time-consuming, requires many steps and costly chemicals, and often produces unnecessary by-products.[1] Therefore, it does not seem worthwhile for production on an industrial scale. Simple methods are required that would allow increasing production of rare sugars and polyalcohols, and, in particular, that would use substances which are cheap and abundant in nature.[6] In this respect, biocatalysis using enzymes with high regio- and stereoselectivity could afford a solution. Biocatalysis has already been successfully employed to synthesize sugars and polyalcohols.[7, 8]

A surface display system, "Autodisplay", has been developed by our group for *Escherichia coli*, which allows expression of a wide variety of proteins, including enzymes, on the cell surface.[9–13] In biocatalytic applications, the cellular surface display of enzymes has some interesting advantages. A substrate to be processed has free access to its enzyme and does not need to cross a membrane barrier. The enzyme itself needs no purification, but can be applied in the form of a whole cell biocatalyst to synthesis reactions. It can be removed again by a simple centrifugation step. Moreover, when connected to a matrix, in this case the cell envelope, enzymes are usually more stable and active than as free molecules. Therefore, the aim of this study was to combine the advantages of cellular surface display with the benefits of biocatalysis in the synthesis of rare sugars and polyalcohols.

The phototrophic bacterium *Rhodobacter sphaeroides* is capable of utilizing a variety of sugar alcohols, including D-mannitol and sorbitol.[14] One of the most interesting polyol converting enzymes that have been isolated and characterized from this organism is sorbitol dehydogenase (SDH; EC

[a] *Priv.-Doz. Dr. J. Jose, Dr. S. von Schwichow*
Pharmazeutische und Medizinische Chemie
Naturwissenschaftlich-Technische Fakultät III
Universität des Saarlandes, P.O. Box 151150
66041 Saarbrücken (Germany)
Fax: (+49) 681-3024386
E-mail: j.jose@mx.uni-saarland.de

[b] *Dr. S. von Schwichow*
Current address:
Pfizer Global Manufacturing, Illertissen
P.O. Box 2064
89252 Illertissen (Germany)
Fax: (+49) 681-3024386

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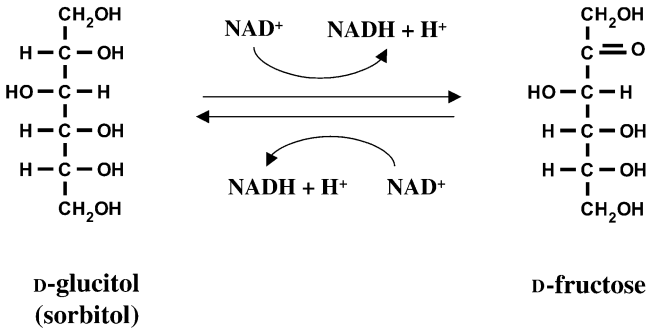
1.1.1.14).[15] SDH from *R. sphaeroides* belongs to the short-chain dehydrogenase/reductase (SDR) family. It is a dimer with a subunit molecular mass of 29 000 kDa and is active on different substrates with sorbitol being the preferred substrate (Scheme 1).[16, 17] It also converts galactitol into D-tagatose and



Scheme 1. *Preferred reactions of sorbitol dehydrogenase (SDH) from* R. sphaeroides.*[16]*

L-arabitol into L-ribulose.[16] In the study reported herein, we report on the Autodisplay of SDH in *E. coli*. The enzyme was expressed in high numbers in an active conformation on the cell surface. This resulted in a new whole cell biocatalyst, which was investigated as regards its activity towards the enzymatic formation of various sugar alcohols including D-glucitol, D-tagatose, and L-ribulose.

Results

Construction of a SDH-autotransporter fusion protein

To obtain surface display of a recombinant passenger protein by Autodisplay, it is necessary to construct an artificial precursor by genetic engineering.[9, 11] The resulting precursor protein consists of a signal peptide, which is cleaved off during transport across the inner membrane, the recombinant passenger, the β-barrel for outer membrane translocation, and a linking region in between that is needed to achieve full surface access. For Autodisplay we use the β-barrel and the linking region of the AIDA-I adhesin and the signal peptide of the cholera toxin β subunit (CTB).[13] The recombinant, cytoplasmic expression of SDH from *R. sphaeroides* in *Escherichia coli* has been reported previously.[17] We used the plasmid pMSFG12d7 – 19 (described in the previous report) for PCR amplification of the SDH gene. The PCR reaction with the primers jj007 and jj008 added an *Xba*I site at the 5′ end and a *Bgl*II site at the 3′ end, which were used to connect the SDH gene, in frame, to the autotransporter domains needed for Autodisplay. The autotransporter domains were delivered by the plasmid pJJ369, which is a pBR322-derived high copy number plasmid.[18] This plasmid directs the expression of the AIDA-I autotransporter under control of the constitutive P_{TK} promoter. Insertion of the SDH-PCR fragment yielded plasmid pJJ-SDH08 (Figure 1), which encodes a fusion protein consisting of the CTB signal peptide, SDH, and the AIDA-I β-barrel, including a linker region that previously proved to be sufficient for full surface access. The fusion protein encoded by pJJ-SDH08 has a predicted molecular mass of 77.3 kDa after processing by the signal peptidase and was named FP77. After DNA sequence analysis of the ligation product, pJJ-SDH08 was transformed into *E. coli* UT5600, an OmpT-negative mutant, which proved to be beneficial for Autodisplay,[19] and expression of FP77 was analyzed. As a result of the cloning procedure, FP77 contains four amino acids in addition to SDH; two are derived from CTB and two are due to the introduction of the *Xba*I site (Figure 1).

Expression of the SDH-autotransporter fusion protein FP77 and surface display of the SDH domain

Most of the available *E. coli* host strains possess an outer membrane protease (OmpT) that catalyses the sequence-specific release of surface-exposed proteins.[20] As the linker in our SDH-$Aida_{\beta}$ fusion protein contains an OmpT protease-specific cleavage site,[9, 11] it was necessary to use an *omp*T-negative strain (UT5600) for SDH surface display. The expression of FP77, encoded by pJJ-SDH08, was monitored by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of outer membrane-preparations followed by Western blot analysis. As can be seen in Figure 2 (lane 2), a protein of the correct size could be detected in outer membrane preparations of UT5600 pJJ-SDH08, but not in control cells (lane 1). Neither the cytoplasmic cell fraction nor the inner membrane fraction, obtained during outer membrane preparation with both strains, contained a protein that was recognized by the SDH specific antiserum (not shown).

To find out whether the SDH domain of FP77 is directed to the surface, immunofluorescence microscopy was performed with whole cells of *E. coli* UT5600 pJJ-SDH08.[9] A polyclonal anti-SDH rabbit antiserum was used for specific labeling. Externally added antibodies are too large to pass through the outer membrane of *E. coli*. Therefore, if cells are labeled by the SDH-specific antiserum, the enzyme must be exposed to the cell surface. Figure 3 shows that UT5600 cells harboring pAT-SDH08 were uniformly fluorescent after the addition of FITC-labeled secondary antibody (anti-rabbit), whereas UT5600 cells without plasmid, applied as control, were not (not shown). This clearly indicates that the SDH passenger domain of the autotransporter fusion protein AT-SDH08 was accessible by externally added antibodies and therefore must be directed to the cell surface.

Overexpression of FP77

In order to increase the amount of FP77 within the outer membrane, the corresponding gene was expressed under the control of an inducible promoter. For this purpose, the SDH encoding region was excised from plasmid pJJ-SDH08 by the *Xba*I and *Bgl*II sites, and inserted into plasmid pETSH3.[13] This plasmid encodes all domains necessary for Autodisplay but in the context of a pET vector backbone. The commercially available pET vector series consist of high copy number plasmids that direct the expression of recombinant proteins by a promoter which is recognized by T7 RNA polymerase.[21] Accordingly, to achieve expression of the recombinant protein,

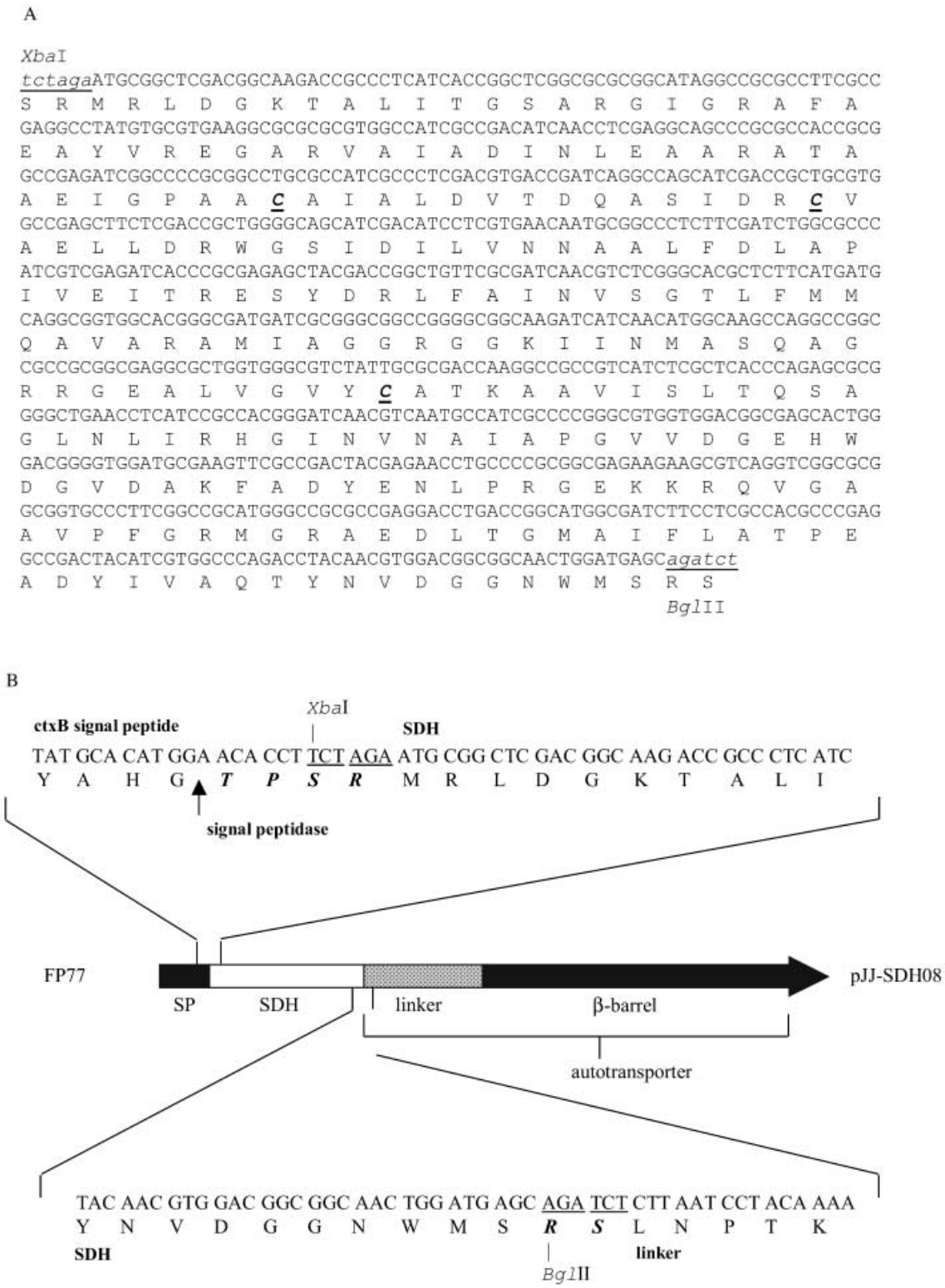


Figure 1. *A) Nucleotide and amino acid sequence of SDH as amplified by PCR using the primers jj007 and jj008. B) Structure of the fusion protein FP77 encoded by pJJ-SDH08. The environment of the fusion sites is given as sequences. The four amino acids at the N terminus and the two amino acids at the C terminus that were added to SDH due to the cloning procedure are shown in italics. The signal peptidase cleavage site is indicated. Restriction sites used for cloning are underlined. SP, signal peptide.*

the corresponding host strain must contain the gene for T7 RNA polymerase. We supplied our host strain *E. coli* UT5600 with the *DE3* gene,[22] which encodes T7 RNA polymerase under the control of an inducible *lac* promoter by phage transduction as described in the experimental section. The resulting strain UT5600(DE3) was transformed with plasmid pETSH3-SDH08 and expression of FP77 was analyzed after differential cell fractionation. As shown in Figure 4 A, induction with isopropyl-β-D-

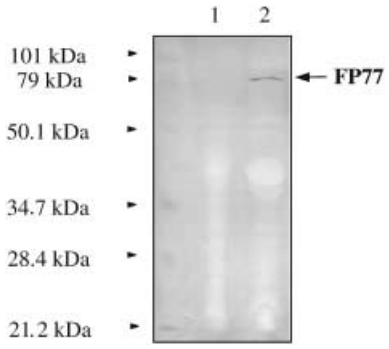


Figure 2. *Western blot analysis of outer membrane preparations from* E. coli *UT5600 (lane 1) and UT5600 pJJ-SDH08 (lane 2). Molecular weights of prestained marker proteins are indicated at the left. A rabbit antiserum specific for SDH from* R. sphaeroides *and a horseradish peroxidase linked goat anti-rabbit IgG secondary antibody were used for detection.*

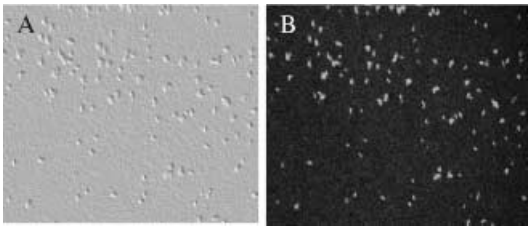


Figure 3. *Immunofluorescence microscopy of E. coli UT5600 pJJ-SDH08. Transmission (A) and fluorescence (B) view of whole cells treated with the SDH-specific rabbit antiserum and an FITC-labeled secondary anti-rabbit antibody as obtained by confocal laser scanning microscopy. The visual field is identical for both parts. Because an antibody is too large a molecule to pass through the outer membrane, labeling of whole cells with the SDH-specific antiserum indicates the surface exposure of SDH.*

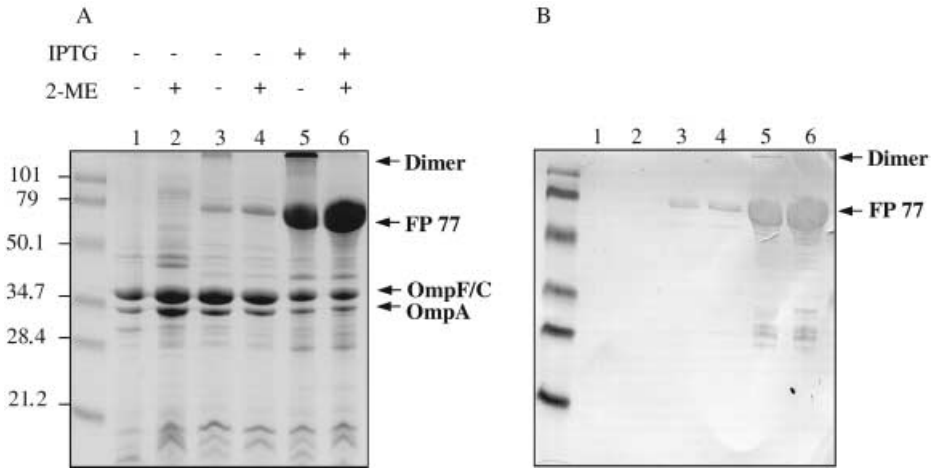


Figure 4. *SDS-PAGE (A) and Western blot analysis (B) of outer membrane preparations from* E. coli *UT5600(DE3) (lanes 1,2) and UT5600(DE3) pETSH3-SDH08 (lanes 3,4,5,6). Molecular weight markers are indicated. IPTG + and − : cells were induced with IPTG (1 mM), or not, prior to outer membrane preparation. 2-ME + : sample buffer contained 2-mercaptoethanol; 2-ME − : sample buffer without 2-mercaptoethanol. Natural outer membrane proteins (Omp) F/C and A are marked by arrows.*

thiogalactopyranoside (IPTG) resulted in huge amounts of FP77 within the outer membrane of UT5600(DE3) pETSH3-SDH08 cells. IPTG induces the production of T7 RNA polymerase, which is then presumed to start transcription of the artificial SDH-autotransporter gene.[21] The amount of FP77 was even higher than that of the natural outer membrane proteins OmpA and OmpF/C. Degradation products of different sizes were detected in Western blot experiments with the SDH-specific antiserum (Figure 4B), presumably due to the extremely high level of expression. Cells of UT5600(DE3) pETSH3-SDH08 not induced with IPTG also exhibited small amounts of FP77, whereas controls cells (UT5600 without plasmid) did not. The small amount expressed under noninducing conditions might be due to the well-known leakiness of the *lac* promoter used in these experiment for controlling the expression of T7 RNA polymerase. Interestingly, when the outer membrane preparations were resolved in nonreducing sample buffer (without 2-mercaptoethanol) prior to SDS-PAGE, an additional protein band double the size of FP77 was detected (Figure 4A). As it was labeled by the SDH-specific antiserum, it appears to consist of dimers of FP77. Natural SDH has been reported previously to be a dimer.[17] As the β-barrel used for outer membrane anchoring of recombinant proteins in Autodisplay is not fixed within the membrane but freely motile, the dimers of FP77 detectable with nonreducing sample buffer might be due to a passenger (SDH) driven dimerization. A similar dimerization has been reported previously in Autodisplay of bovine adrenodoxin.[11] The possibility that proteins expressed from monomeric genes can spontaneously form functional protein dimers at the cell surface, is a special feature of the Autodisplay system and represents a fundamental advantage in comparison to other protein display systems.

Recently we reported a new method for monitoring the surface display of recombinant proteins by fluorescein-maleimide-labeling of their cysteines.[13] We used this new detection tool to verify surface exposure of the SDH domain in over-expressed FP77. The SDH domain contains three cysteines (Figure 1), whereas the autotransporter domains are devoid of any sulfhydryl groups.[19] Whole cells of UT5600 were treated with 2% of 2-mercaptoethanol to resolve any disulfide bonds before they were incubated with fluorescein-maleimide for 15 min, as described in the experimental section. As fluorescein-maleimide cannot pass the outer membrane, it can only react with surface-exposed cysteines by Michael addition of the sulfhydryl group to the double bond of maleimide.[13] The fluorescence of whole cells of UT5600 pETSH3-SDH08 as well as of control cells (UT5600 without plasmid) treated in the same way, were analyzed by flow cytometry. As can be seen in Figure 5, the relative fluorescence of whole cells of UT5600 pETSH3-SDH08 increased to a mean value of 300 in comparison to a mean value of 9

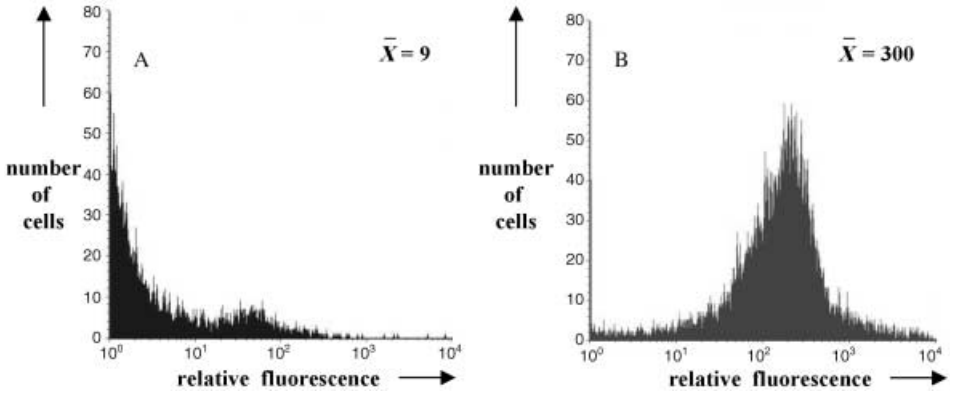


Figure 5. *Flow cytometric analysis of the surface display of SDH, which contains three cysteines.* E. coli *UT5600(DE3) pETSH3-SDH08 cells were labeled with fluoresceine-maleimide and analyzed by flow cytometry[13] (B). The mean fluorescence of these cells, X̄, was significantly increased in comparison to the mean value of fluorescence of UT5600(DE3) cells without plasmid (A), which were treated identically and applied as a control.*

obtained with control cells. This clearly indicates that the SDH domain in over-expressed FP77 is accessible at the cell surface.

Enzyme activity of surface displayed SDH

The optical test as described by Schneider and Giffhorn was used to measure the activity of whole cells displaying SDH.[15] This test is based on the alteration of absorption at 365 nm by NADH, which is produced in equimolar amounts to the number of polyol molecules oxidized during SDH reaction. We supplied a suspension of whole cells of UT5600 pETSH3-SDH08 with an OD_{578} of 1, with reduction equivalents (NAD^+ for oxidation of polyols, NADH for reduction of sugars), incubated for 5 min at 30 °C for temperature adjustment, and started the reaction by adding the substrate in a final concentration of 0.2 M for sugars and 0.1 M for polyols. Alteration of absorbance at 365 nm was measured for 3 min. Whole cells of *E. coli* UT5600 were used as controls and were applied and treated similarly. Each experiment was repeated independently at least three times. As can be seen in Figure 6, whole cells of *E. coli* displaying SDH were able to oxidize D-glucitol, D-galactitol, and L-arabitol, whereas D-mannitol was not accepted as a substrate. To calculate the enzymatic activity, the slope of reaction obtained by SDH-displaying cells was set against the slope of reaction of control cells for every assay with a different substrate. Using the molar absorption coefficient of NADH at 365 nm, enzymatic activity of 12 mU was determined for D-glucitol, 7 mU for D-galactitol, and 17 mU for L-arabitol. The number of cells within the reaction assay was determined to be 3.3×10^8 cells by plating out adequate dilutions on agar plates. Using this value we calculated a specific activity of 34 pU for D-glucitol, 21 pU for D-galactitol, and 52 pU for L-arabitol as a substrate for every single cell of *E. coli* pETSH3-SDH08. The sugar reducing activity of cells displaying SDH was determined with D-fructose as a substrate. The activity of a solution of UT5600 pETSH3-SDH08 cells with an OD_{578} of 1 was calculated to be 29 mU for D-fructose as a substrate, which means a specific activity of 88 pU per single cell. These data indicate that the SDH enzyme expressed at the cell surface of *E. coli* by Autodisplay is highly active and can be used for the conversion of various substrates.

Comparison of its specificity with that of the purified enzyme as described in the literature,[16] revealed some interesting features of the new whole cell biocatalyst obtained by cellular surface display of SDH (Table 1). The relative activity towards D-galactitol, yielding D-tagatose (Scheme 2), was increased by a factor of almost 4 (16% purified enzyme versus 61% whole cell biocatalyst). The conversion rate for L-arabitol, yielding L-ribulose, was even higher than that for D-glucitol when the whole cell biocatalyst was used, whereas the purified enzyme showed only 8% conversion of L-arabitol in comparison to D-glucitol. This showed that relative activity increased by a factor of 19. Taking these results together, Autodisplay of SDH from *R. sphaeroides* in *E. coli* yields a whole

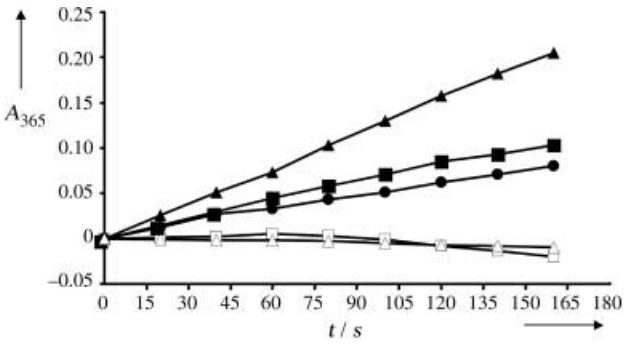
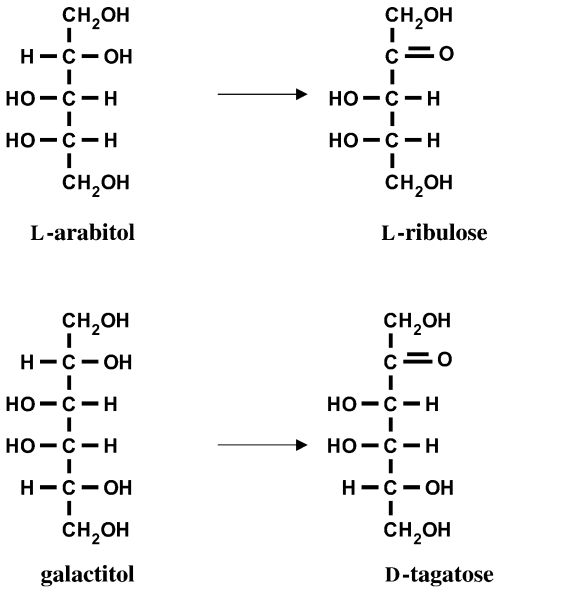


Figure 6. *Enzyme activity of whole* E. coli *cells displaying SDH. An optical test according to Schneider and Giffhorn[15] was used to determine the biocatalytic activity of UT5600(DE3) pETSH3-SDH08 cells. The increase in absorbance at 365 nm was followed photometrically in the presence of NAD^+ (1.8 μmol) and a 100 μL cell suspension in Tris – HCl buffer (100 μmol, pH 9.0, 0.9 mL). Reaction was started by the addition of substrate (100 μmol, 100 μL). Controls were prepared similarly, but UT5600(DE3) cells without plasmid were applied instead. For ease of understanding, the course of absorbance is only given for one control reaction. ▲ = substrate L-arabitol; ■ = D-glucitol, ● = D-galactitol, △ = D-mannitol, □ = control cells with D-glucitol as substrate.*

Table 1. *Substrate specificity of purified SDH from* R. sphaeroides *and substrate specificity of whole cells of* E. coli *displaying SDH at the surface.*

Substrate	Relative enzyme activity [%]	
	Purified enzyme[a]	Whole cell biocatalyst
D-Sorbitol	100[b]	100[b]
Galactitol	16	61
L-Arabitol	8	153
D-Mannitol	–	–
D-Fructose	100[c]	100[c]

[a] According to Schauder et al.[16] [b] Set as 100% for oxidation reactions. [c] Set as 100% for reduction reactions.



Scheme 2. *Substrates and products of the new whole cell biocatalyst obtained by Autodisplay of SDH from* R. sphaeroides *in* E. coli *in addition to* D*-glucitol and* D*-fructose.*

cell biocatalyst with high activity towards the conversion of L-arabitol to L-ribulose, and the conversion of D-galactitol to D-tagatose.

Discussion

In the investigation reported herein we were able to show that Autodisplay is an adequate tool to express SDH from *R. sphaeroides* in a functional state at the surface of *E. coli*. The SDH-autotransporter fusion protein was expressed from an artificial, monomeric gene. As SDH had previously shown to be a functional dimer,[16] dimerization at the cell surface was a prerequisite for activity. In outer membrane preparations of cells displaying SDH, a protein band with an apparent molecular weight double that of the monomeric SDH-autotransporter fusion protein was detectable, which indicates that, within the outer membrane, a passenger (SDH) driven dimerization of SDH-autotransporter fusion proteins occurred (Figure 7). This seems to be a special feature of Autodisplay and has been reported previously for Autodisplay-mediated surface display of bovine adrenodoxine,[23] which is also a functional dimer. This might be due to the fact that the β-barrel used for outer membrane translocation and which eventually serves as a membrane anchor in Autodisplay,[18] is freely motile and cannot resist an affinity of passenger proteins. This is a clear advantage of Autodisplay in comparison to other display systems and could also allow the expression of heterodimeric or -multimeric proteins, for example antibodies or receptors, without the need to place a connecting peptide in between the monomers, as currently applied, for example for so-called single chain antibodies.

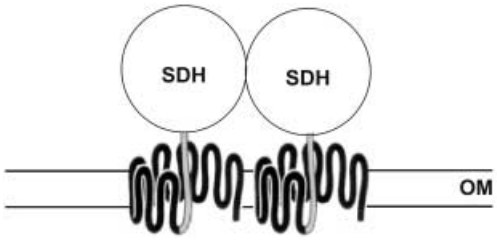


Figure 7. *Schematic illustration of the passenger-driven dimerization of SDH-autotransporter proteins at the surface of* E. coli.

The enzymatic activity of whole cells of *E. coli* displaying SDH was easy to detect. A quantity of 3.3×10^8 cells within a reaction volume of 1 mL exhibited a maximum activity of 29 mU if D-fructose was used as a substrate. Since it would be possible to significantly increase both the cell density and the reaction volume, production on an industrial scale could be feasible. It is not possible to determine the specific activity of the whole cell biocatalyst by dividing its enzyme activity by its protein content, because the vast majority of proteins are inside the cell and do not interfere with enzyme activity. Therefore, we determined the activity per single cell to be 34 pU with D-glucitol as a substrate. The specific activity of purified, recombinant SDH towards D-glucitol has been reported to be 106.5 U mg^{-1} protein.[17] By using this value, the number of substrate molecules converted by a single enzyme molecule is calculated to be 3089, given a molecular weight of the enzyme of 29 000 kDa. For activity of 34 pU, about 6600 of these enzyme molecules would be necessary or, in other words, the activity of one single cell of *E. coli* UT5600 pETSH3-SDH08 corresponds to 6600 free enzyme molecules.

We also tried to estimate the number of SDH molecules on the surface of one single *E. coli* cell by using fluorescein-maleimide coupling to SDH.[13] For this purpose beads coated with different but known numbers of FITC molecules were used to set up a calibration curve in the flow cytometer. The calibration curve gives the number of FITC molecules to obtain a certain mean value of relative fluorescence under constant measuring conditions. Using this calibration curve, a mean value of relative fluorescence of 300, as obtained with SDH-displaying cells (Figure 5), corresponds to 469 000 FITC molecules per single cell. SDH possesses three cysteines. Given that each cysteine has coupled to a fluorescein-maleimide molecule, it can be calculated that there are about 150 000 SDH molecules per single cell. This obviously large discrepancy, 6600 molecules determined by enzyme activity versus 150 000 molecules determined by cysteine labeling, can be explained in two different ways. Either the vast majority of SDH enzymes expressed at the cell surface are inactive or the activity of all SDH enzyme molecules has been significantly (about 23-fold) decreased. At this point, it is important to note that in Autodisplay the passenger proteins are N-terminally fixed to the β-barrel by the linker region. This fixation could restrain flexibility thus causing reduced substrate conversion. On the other hand, the N-terminal constraint could alter the conformation of the enzyme and result in altered access to the active site. The increased relative activity towards D-galactitol and L-arabitol of the surface displayed SDH could be due to altered conformation of the enzyme or altered accessi-

bility of the active site. The influence of the N-terminal fixation on the structure and the activity of the passenger protein in Autodisplay appear to be important aspects and could be examined by changing the linker region between passenger and β-barrel. This is currently under investigation.

Another reason for the reduced enzymatic activity of surface displayed SDH could be the presence of lipopolysaccharide (LPS) at the cell surface close to the enzyme's active site. The sugar molecules of LPS could serve as competitive inhibitors of SDH resulting in the reduced overall enzyme activity. This could be overcome by using mutant strains of *E. coli* that are unable to produce the O-specific side chain of LPS.[24]

We did not determine the configuration of the products generated by the whole cell biocatalyst. This has been determined for the native SDH in the case of D-tagatose, D-fructose, and D-glucitol.[16] It seems very unlikely that the N-terminal fixation of SDH in Autodisplay, although able to alter access to the active site, could change its stereoselectivity. Therefore we propose that the whole cell biocatalyst generates products identical to those produced by the native enzyme from defined substrates.

D-Tagatose and L-ribulose seem to be the most interesting of the compounds produced by the whole cell biocatalyst obtained by SDH surface display. The use of D-tagatose as a sweetener in foods and beverages, especially in diabetic foods, and as a dietary supplement is steadily increasing.[5] Feasible enzymatic processes for D-tagatose production are rather limited.[1] Currently, isomerisation of D-galactose into D-tagatose by L-arabinose isomerase is mainly used.[6] L-Ribulose can be used for the synthesis of modified nucleosides, which have been shown to be potent antiviral agents and are also usable in antigen therapy.[1, 2] Enzymatic processes are available to produce L-ribulose from L-arabinose and ribitol.[25] However, L-arabinose is expensive and also provides a low yield of L-ribulose. Here, the whole cell biocatalyst obtained by surface display of SDH could provide an alternative.

In order to use cells displaying SDH at the surface in industrial syntheses, however, solutions must be found to inherent technical obstacles. Both SDH catalyzed reactions, oxidation and reduction, require a cofactor (NAD or NADH) and as surface display precludes support from cellular metabolism, an efficient cofactor recycling scheme needs to be established. One possibility could be the coexpression of a second enzyme that facilitates cofactor regeneration, for example lactate dehydrogenase.[2] In this case a second substrate, lactate or pyruvate, needs to be added. As we have to deal with living bacterial cells, cellular uptake of these substrates as well as added polyols could occur during extended reaction times. This indicates the need to reduce cellular metabolism and uptake during product syntheses, a problem which is under current investigation. Nevertheless, it also suggests an advantage of the whole cell biocatalyst obtained by autodisplay of SDH, which selectively converts substrates that are not able to pass the membrane barrier and therefore are excluded from other whole cell bioconversions. It should be noted that alternative schemes, for example the production of D-tagatose by ketol isomerization,[6] have optimum atom economy and can profit from existing large-scale industrial experience in high fructose corn sirup (HFCS) production from glucose by glucose isomerase. Nevertheless, surface display of SDH and the whole cell biocatalyst thereby obtained provides new enzymatic access to the production of D-tagatose and L-ribulose and suggests the construction of new whole cell biocatalysts for carbohydrate interconversions by autodisplay of other enzymes in active conformations.

In summary, we were able to express SDH from *R. sphaeroides* in an active form on the surface of *E. coli* by Autodisplay. The cells obtained by this strategy can be used as whole cell biocatalysts for the synthesis of rare sugars such as D-tagatose and L-ribulose. The cells can also serve as the starting point for the development of new enzymatic activities by directed evolution,[26] using the short chain dehydrogenase SDH as a scaffold. This is currently being investigated in our laboratory.

Experimental Section

Bacterial strains, plasmids, and culture conditions: *Escherichia coli* strains UT5600 (F$^-$ *ara*14 *leu*B6 *azi*-6 *lac*Y1 *pro*C14 *tsx*-67 *ent*A403 *trp*E38 *rfb*D1 *rps*L109 *xyl*-5 *mtl*-1 *thi*1 Δ*omp*T-fepC266)[19] and UT5600(DE3) [(F$^-$ *ara*14 *leu*B6 *azi*-6 *lac*Y1 *pro*C14 *tsx*-67 *ent*A403 *trp*E38 *rfb*D1 *rps*L109 *xyl*-5 *mtl*-1 *thi*1 Δ*omp*T-fepC266) (DE3)] were used for the expression of autotransporter fusion proteins. UT5600 was chosen as a recipient strain for site-specific integration of λDE3 prophage[21] so as to combine the properties of the established host strain with the possibility of expressing target genes from pET vectors, which are under the control of the T7 promoter and can be induced with isopropyl-β-D-thiogalactopyranoside (IPTG).[22] Therefore, the strain was grown in LB supplemented with maltose (0.2%) and $MgSO_4$ (10 mM) to reach an OD_{578} of 0.5. λDE3 (10^8 pfu), helper phage (B10, 10^8 pfu), and selection phage (B482, 10^8 pfu) were then mixed with host cells (1 μL). The mixture was incubated at 37 °C for 20 min, plated onto agar plates, and incubated at 37 °C overnight. *E. coli* TOP10F′ (F′ {*lac*I^q Tn*10* (Tet^R)} *mcr*A Δ(*mrr*-hsdRMS-*mcr*BC) Φ*lac*ZΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G) and the vector pCR2.1-TOPO, which were used for cloning of PCR products, were obtained from Invitrogen (Groningen, the Netherlands). Plasmid pMSFG12d7 – 19, which encodes SDH from *R. sphaeroides*,[17] was kindly provided by F. Giffhorn (University of Saarland, Saarbruecken, Germany). Plasmid pJJ369, which encodes the AIDA-I autotransporter domains, has been described previously.[18] Plasmid pETSH3 is a derivative of pJJ369 encoding the identical autotransporter domains, but is under the control of a T7 RNA polymerase recognizable promoter.[13]

Bacteria were routinely grown at 37 °C on Luria-Bertani (LB) agar plates containing ampicillin (100 mg L^{-1}). For differential cell fractionation and outer membrane preparations, cells were cultured in the presence of ethylenediaminetetraacetate (EDTA, 10 μM) and 2-mercaptoethanol (10 mM) in liquid LB medium.

Recombinant DNA techniques: For the construction of plasmid pJJ-SDH08 which contains the gene encoding the SDH-autotransporter fusion protein, the SDH gene was amplified by PCR from plasmid pMSFG12d7 – 19[17] by using oligonucleotide primers jj007 (5′-GCT CTA GAA TGC GGC TCG ACG GCA AGA CC-3′) and jj008 (5′-GAA-GAT CTG CTC ATC CAG TTG CCG CCG TC-3′), introducing a *Xba*I and *Bgl*II restriction site, respectively. Preparation of plasmid DNA, ligation, restriction digestion, transformation procedures, and DNA

electrophoresis were performed according to standard procedures.[27]

Differential cell fractionation and outer membrane preparation: Bacteria were grown overnight and a sample (1 mL) of the culture was used to inoculate LB medium (20 mL). Cells were cultured at 37 °C with vigorous shaking (200 rpm) for about 5 h until an optical density at 578 nm (OD_{578}) of 0.7 was reached. After harvesting and washing with Tris – HCl (0.2 M, pH 8), differential cell fractionation was performed according to a modification of the method of Hantke[28] and has been described in detail elsewhere.[11, 12] The outer membrane proteins were washed, dissolved in water, and prepared for SDS-PAGE.

For inducible expression, cells were grown overnight in LB medium containing ampicillin (50 mg L^{-1}) and a sample (1 mL) of this overnight culture was used to inoculate LB medium (20 mL). Cells were cultured at 37 °C under vigorous shaking (200 rpm) until an OD_{578} of 0.6 was reached and IPTG (1 mM; Roth, Karlsruhe, Germany) was added for induction. After 60 min cells were harvested and outer membranes were prepared according to the rapid isolation method of Hantke as described above.

Specific anti-SDH rabbit antiserum: Purified SDH from *R. sphaeroides*, which was prepared according to the method of Schauder et al.,[16] and which had a final specific activity of 49 U mg^{-1}, was used to immunize two rabbits. After a second boost with the enzyme preparation after 4 weeks, the antiserum of both rabbits was collected and their specificity tested with Western blot experiments without further purification. Both antisera were highly specific and could be used best in a dilution of 1:5000.

SDS-PAGE and Western blot analysis: For SDS-PAGE, outer membrane isolates were diluted 1:2 with sample buffer which consisted of Tris – HCl (100 mM, pH 6.8) containing SDS (4%), bromophenol blue (0.2%), 2-mercaptoethanol (2%), and glycerol (20%), boiled for 5 min, and analyzed on 12.5% acrylamide gels. For Western blot analysis, gels were electroblotted onto polyvinylidene-difluoride (PVDF) membranes and the blotted membranes were blocked in PBS with dried-milk powder (3%) overnight. For immunodetection, membranes were incubated with the SDH-specific antiserum, diluted 1:5000 in PBS with bovine serum albumin (3%) for 3 h. The immunoblots were washed three times with PBS, the secondary antibody was added, and the blots were incubated for 2 h at room temperature. Antigen – antibody conjugates were visualized by treatment with horseradish peroxidase linked goat anti-rabbit IgG secondary antibody (Sigma, Deisenhofen, Germany), diluted 1:2000 in PBS. A color reaction was achieved by adding a solution consisting of 4-chloro-1-naphthol (3 mg mL^{-1}) in ethanol (2 mL), PBS (25 mL), and H_2O_2 (30%, 10 μL).

Spectrophotometrical enzyme assays: SDH activity was measured spectrophotometrically at 365 nm by determining the change in NADH concentration according to Schneider and Giffhorn[15]. For this purpose, cells from an overnight liquid culture were used to inoculate a fresh culture. After induction with IPTG and harvesting by centrifugation, cells were suspended in the appropriate buffer to reach an OD_{578} of 10. This suspension (100 μL) was used routinely in the standard assay for substrate oxidation[15] containing Tris – HCl (100 μmol, pH 9.0) and NAD^+ (1.8 μmol) in a final volume of 0.9 mL at 30 °C. The reaction was started by addition of D-glucitol (100 μmol, 100 μL; Sigma – Aldrich, Steinheim, Germany) or other polyols (Sigma – Aldrich, Steinheim, Germany). For substrate reduction, 0.9 mL of the assay at 30 °C contained potassium phosphate (100 μmol, pH 6.5) and NADH (0.28 μmol), with a cell suspension adjusted to an OD_{578} of 10 (100 μL). The reaction was started by the addition of D-fructose (200 μmol, 100 μL; Sigma – Aldrich, Steinheim, Germany).

Whole cell immunofluorescence: For whole cell immunofluorescence, cultures of *E. coli* strains, containing the respective expression plasmid, were grown at 37 °C overnight and cultured in a dilution of 1:20 at 37 °C until they reached an OD_{578} of 2.0. Cells were harvested, washed three times with PBS, and suspended in PBS with fetal calf serum (FCS, 3%) to reach an OD_{578} of 1.0. A sample (50 μL) of cell suspension was then transferred onto round cover slips and incubated at 37 °C until the slips were completely dry. The cover slips were placed into a 24-well plate with the cells facing up, and fixed for 20 min with formaldehyde (2.5%, in PBS). After washing three times with PBS and blocking with FCS (1%, in PBS) for 5 min, SDH-specific rabbit antiserum (15 μL) diluted 1:5000 in PBS was added and the cover slips were incubated on ice for 1 h. Washing three times with PBS for 15 min was followed by addition of FITC-labeled secondary anti-rabbit antibody (50 μL; Sigma, Deisenhofen, Germany) and incubation on ice for 1 h. Finally, cells were washed three times for 15 min with PBS (500 μL), and the cover slips were transferred onto microscope slides and fixed with mounting medium. Analysis was carried out under a confocal laser scanning microscope (Bio-Rad, Munich, Germany).

Flow cytometric analysis: For flow cytometric analysis, cultures of *E. coli* cells with or without plasmid were grown at 37 °C overnight and cultured in a dilution of 1:20 at 37 °C until they reached an OD_{578} of 0.6. Expression of the target gene was then started by addition of IPTG. Cells were harvested, washed three times with PBS, suspended in PBS, and incubated with the SDH-specific antiserum, diluted 1:5000 in PBS, for 1 h. After the cells were washed again three times with PBS, the secondary FITC-linked goat anti-rabbit IgG secondary antibody (Sigma, Deisenhofen, Germany) was added and the mixture was incubated for 1 h at room temperature. Cells were then washed three times with PBS and suspended in PBS to reach a final OD_{578} of 0.05 for subsequent FACS analysis. For each experiment at least 10000 cells were counted with a FACSCalibur Cytometer (Becton Dickinson, Heidelberg, Germany) using 488 nm as the excitation wavelength and FACS-FLOW (Becton Dickinson, Heidelberg, Germany) as sheath fluid. The threshold trigger was set on side scatter to eliminate background noise and analyze intact cells.

Labeling cysteines on the surface of intact cells:[13] For this purpose cells were induced with IPTG for 60 min and samples (1 mL) of a cell suspension with an OD_{578} of 1.0 were washed with PBS three times. Cells were then incubated with fluorescin-5-maleimide (500 μM) for 15 min at room temperature. After stopping the reaction and removing excess labeling reagent by repeated washing with buffer, cells were diluted to reach a final OD_{578} of 0.05 and subjected to flow cytometry using a FACSCalibur flow cytometer.

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