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Continuous Indirect Electrochemical Regeneration of Galactose Oxidase

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Abstract—The development of an indirect anaerobic electrochemical regeneration of galactose oxidase (GOase) allows the prevention of the undesired production of the enzyme inhibitor hydrogen peroxide, which is generated under aerobic regeneration conditions during synthetic applications of GOase. The pH optimum for the electrochemical regeneration of GOase with polyethyleneglycol-modified ferrocene mediators in carbonate buffer is 10.8. Total turnover numbers achieved by either electrochemical or aerobic regeneration of GOase are almost the same. The electrochemical regeneration is half as fast as the aerobic regeneration. It is not necessary to work under anaerobic conditions, because at pH 10.8 the aerobic regeneration of GOase is prevented. The enzyme can be stabilized most effectively by immobilization on an aminopropylated polysiloxane (DELOXAN®) via the glutaric dialdehyde procedure with good activity yields up to 37%. Buffers containing amino groups proved to be fatal for long-term GOase stability.

Introduction

Galactose oxidase (GOase) (E.C. 1.1.3.9) from the fungus *fusarium* NRRL 2903, formerly known as *dactylium dendroides*,[1] oxidizes a wide range of primary alcohol substrates to the corresponding aldehydes.[2] The product of the GOase reaction with D-galactose is *meso*-galactodialdose and not D-galactohexodialdose (Fig. 1), which is a common error in the literature.

The synthetic application of GOase is of interest due to its broad substrate tolerance, its chemoselective oxidation of primary hydroxy functions in polyols, and its stereoselectivity. For example, the aerobic GOase reaction has been utilized for the synthesis of rare carbohydrate.[3–7] Only in one case was the GOase recovered by ultrafiltration.[7] However, in the aerobic process hydrogen peroxide is formed as the coproduct which has to be destroyed by the addition of large amounts of catalase.

It was our goal to develop a continuous regeneration system for GOase, which guarantees the reuse of GOase for preparative purposes and at the same time avoids the generation of the enzyme inhibitor hydrogen peroxide, the natural coproduct of the aerobic GOase regeneration (Schemes 1 and 2).

This regeneration system should be applicable to a reactor similar to the electrochemical enzyme membrane reactor described before.[8] The principle of the electrochemical enzyme membrane reactor has been successfully applied before on three non-immobilized enzymes.[9,10] In this reactor, a continuous process is made possible by an ultrafiltration membrane, which keeps the enzyme and a polymermodified mediator within the reaction cycle and permits the low molecular components, such as product, unreacted substrate and buffer solution, to pass through. An alternative to the normal ultrafiltration membrane could be the use of a charged mediator in combination with a charged membrane. The mediator is regenerated in an electrochemical flow-through cell, which is a part of the reaction cycle.

Results

All experiments were performed with xylitol as model substrate, which is oxidized to L-xylose by GOase. In aqueous solution, xylose consists of the two anomeric forms α- and β-L-xylopyranose, which can be easily distinguished from xylitol and quantified by silylation and subsequent GC. The reaction product L-xylose is commercially available. Therefore, the determination of a GC calibration curve is easily achieved. In addition to this, the turnover of xylitol is by far less influenced

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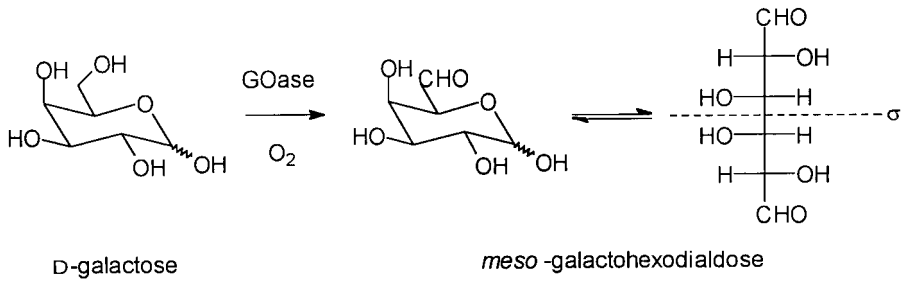
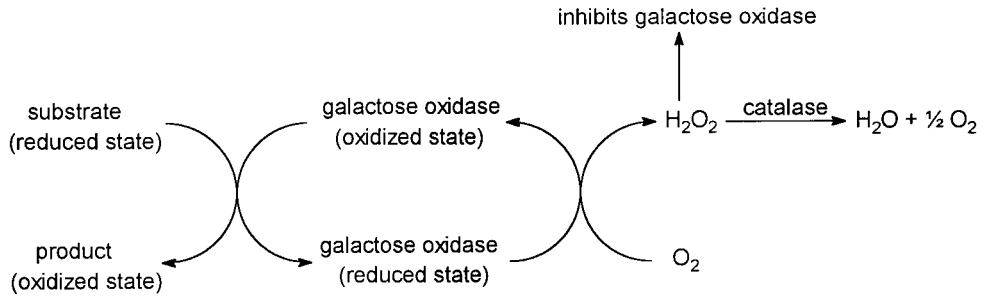
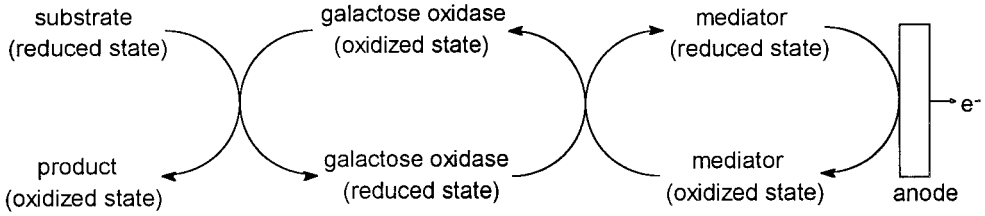


Figure 1. Oxidation of D-galactose to meso-galactohexodialdose by GOase.



Scheme 1. Aerobic GOase regeneration.



Scheme 2. Indirect electrochemical GOase regeneration.

by product inhibition than that of most other GOase substrates.[3]

With the polyethyleneglycol-modified ferrocene mediator α,ω - bis - methylferrocene - polyethyleneglycol(20 000), developed earlier by our group,[11] we were able to continuously regenerate GOase by mediated anodic oxidation. By application of cyclovoltammetric measurements under anaerobic conditions the pH optimum for the electrochemical regeneration in Tris/HCl buffer was found to be 9.7, while for the aerobic regeneration the literature reports a pH optimum of 6.5[12] or 7–7.3[13] (see Discussion). However, the intensive study of the buffer system showed that the enzyme GOase is quite instable in Tris/HCl buffer and other buffers containing amino groups like glycine/NaOH especially at higher pH values (Fig. 2). This seems to be due to complexation of the copper ion in the active site of the enzyme by amino groups in the buffer. In contrast to this, almost no activity loss could be detected after 10 days storage in 50 mM carbonate buffer of pH 10–11 or in borate buffer at pH 9–11. The pH optimum for the electrochemical reactivation of GOase shifted from pH 9.7 in Tris/HCL buffer to 10.8. in carbonate buffer. Therefore, the carbonate buffer of pH 10.8 was applied in all further electrochemical studies.

The reasons for the immense loss of enzyme activity (100% loss within 1 day) during initial batch electrolyses with native enzyme could be found in the denaturation of the enzyme due to direct contact with the counter electrode, and the lability of the enzyme against shearing forces. We could overcome these problems by immobilizing the enzyme on the aminopropylated polysiloxane DELOXAN®[14] by the glutaric dialdehyde procedure. The best reproducible activity yield we achieved was 37%, with a specific activity of 2.4 U per g DELOXAN TAP III®.[14] The specific activity could be increased to 3 U per g DELOXAN DAP III®,[14] but the overall activity yield was decreased to 20%. By immobilization we could improve the enzyme stability under electrolysis conditions to 50% activity loss within 40 days, compared to 100% within 1 day.

Cyclovoltammetric measurements of the catalytic current were performed under aerobic conditions with different ferrocene mediators: α,ω-bis-methylferrocene-polyethyleneglycol(20 000)[11] [$E \approx 285$ mV versus Ag/AgCl], poly(polyethyleneglycol(3,400)-co-1,1′-bis-hydroxymethyl-ferrocene)[11] [$E \approx 330$ mV versus Ag/AgCl], ferrocene monocarboxylic acid [$E \approx 340$ mV versus Ag/AgCl], ferrocene dicarboxylic acid [$E \approx 440$ mV versus Ag/AgCl], ferrocene sulfonic acid [$E \approx 425$ mV

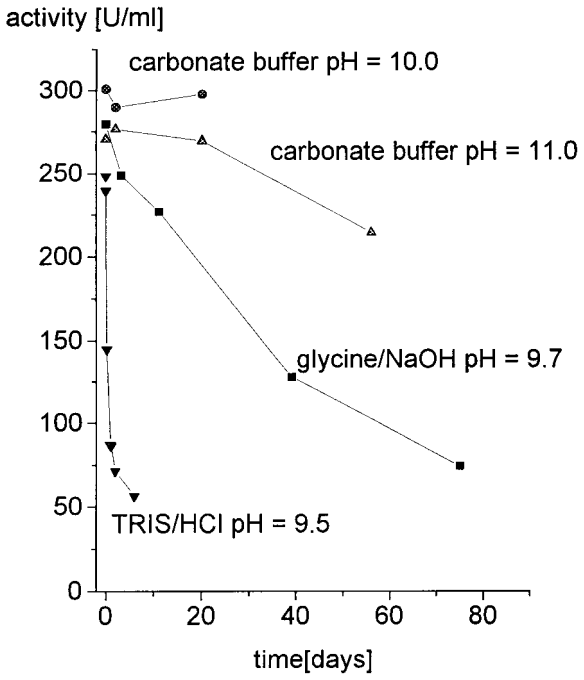


Figure 2. Stability of GOase in different buffer systems under strongly basic conditions.

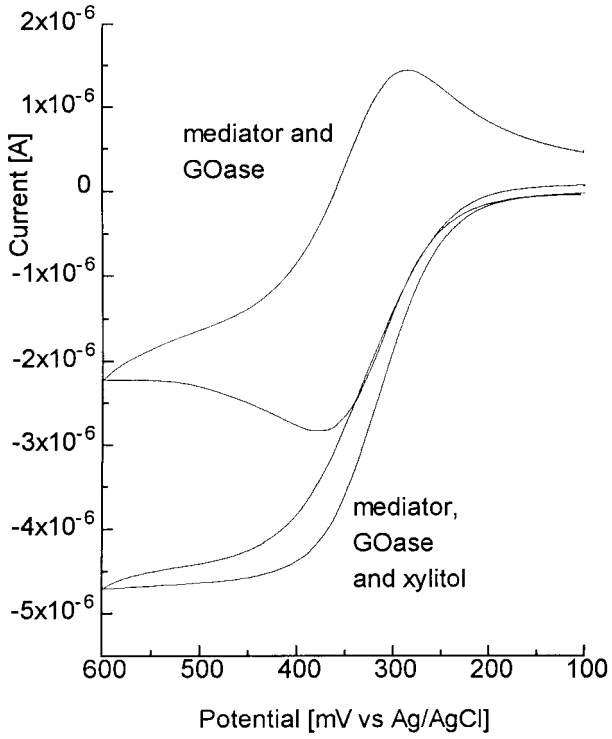


Figure 3. Catalytic current obtained with the mediator poly(polyethyleneglycol(3400)-co-1,1′-bis-hydroxymethyl-ferrocene): 20 U GOase in 628 µl of carbonate buffer (50 mM, pH = 10.8) (= 31.8 U/mL), 15,4 mg (6.82 mM) mediator (calculated for $[fc\text{-}PEG3400]_1$), addition of 100 mg (1 M) xylitol.).

versus Ag/AgCl], 1,1′-ferrocene dimethanol [$E \approx 275$ mV versus Ag/AgCl], hydroxymethylferrocene [$E \approx 230$ mV versus Ag/AgCl] and *N*-ferrocenylmethyl-*N*,*N*,*N*-trimethylammonium tetrafluoroborate[15] [$E \approx 445$ mV versus Ag/AgCl]. It was not necessary to maintain anaerobic conditions, because at pH 10.8 molecular oxygen is not able to regenerate GOase. Accordingly, no product formation could be observed within 8 days with the active immobilized enzyme at this pH value. The cyclovoltammetric experiments showed that the charge of the mediator has a greater effect on the catalytic current than the redox potential. Mediators which are neutral in their reduced form showed the highest catalytic currents. However, in most cases the low solubility of the ferrocene mediator and not the low catalytic current per mole was the limiting factor. The highest absolute catalytic current was obtained with the mediator poly(polyethyleneglycol(3400)-co-1,1′-bis-hydroxymethyl-ferrocene) (Fig. 3), but this mediator deactivated the immobilized enzyme rapidly under electrolysis conditions. Therefore, we used the second best mediator, α,ω-bis-methylferrocene-polyethyleneglycol(20 000), for our continuous electrochemical regeneration of immobilized GOase.

Other mediators, like the hydride acceptors *N*-methyl-1,10-phenanthroline-5,6-dionium-tetrafluoroborate and tris-(1,10-phenanthroline-5,6-dion)-ruthenium(II)-perchlorate,[16] proved to be less effective than the ferrocene mediators. Because of its comparatively high positive potential, the mediator iron(II)-tris-(3,4,7,8-tetramethyl-1,10-phenanthroline)-sulfate[17] [$E \approx 660$ mV versus Ag/AgCl] showed an indirect electrochemical oxidation of xylitol already without addition of GOase, which was indicated by a high catalytic current in the absence of the enzyme. The iron mediators potassium hexacyanoferrate [$E \approx 215$ mV versus Ag/AgCl] and potassium tetracyano-(1,10)-phenanthroline-ferrate [$E \approx 340$ mV versus Ag/AgCl], which have been used for the electrochemical redox activation of GOase before,[18] showed no catalytic activity with GOase and xylitol. With potassium tetracyano-(1,10)-phenanthroline-ferrate a strong increase in the oxidation current could be observed upon addition of GOase in absence of substrate.

The continuous electrochemical regeneration of GOase was performed in a circular flow fixed-bed reactor consisting of (1) an FPLC-column filled with immobilized GOase, (2) an electrochemical flow-through cell for the anodic reoxidation of the mediator and (3) an HPLC-pump for establishing a continuous flow.

Within 3 weeks of electrolysis, a turnover of 7.4 mM xylitol (Fig. 4) with an activity-time-yield of 85 µg per unit GOase per day, and a current yield of 34.6% could be achieved. This results in a total turnover number (TTN) for GOase of 208 720 (6.7 cycles/min). After 21 days, the current was interrupted and the enzymatic reaction stopped. When the current flow was started again (25 days), the product formation started again. But shortly afterwards, due to a lack of sterility, the immobilized enzyme column was infected by microorganisms resulting in some irreproduciblity of the product determination. To evaluate our experiments, we compared the results of the electrochemical regeneration with the results of the aerobic regeneration with and without addition of catalase (Table 1).

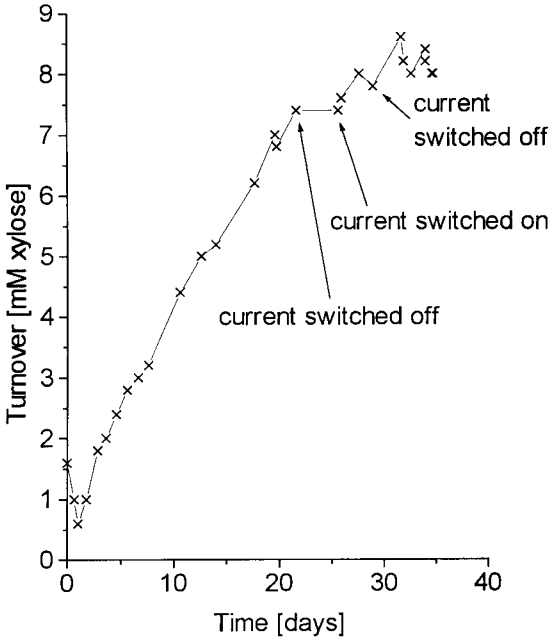


Figure 4. Turnover-current diagram of the electrolysis.

After the electrolysis, the pH had decreased to pH 9, and 50% of the enzyme was still active. During electrolysis, the activity of the mediator gradually decreased, due to the known tendency of the ferrocenium ion to loose the iron by precipitation of iron(III) hydroxide in the strongly basic solution (Fig. 5).

Discussion

The synthetically valuable enzyme GOase can be reactivated by an anaerobic indirect electrochemical method applying polyethyleneglycol-modified ferrocene as a redox catalyst in buffer systems which do not contain amino functions. The amino functions seem to undergo complexation of the copper ion within the active site of the enzyme. In contrast to the aerobic process with a pH-optimum of 6.5–7.3, the indirect electrochemical process has a pH-optimum of 10.8 in sodium carbonate

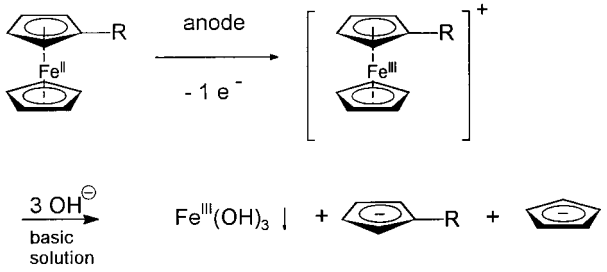


Figure 5. Decomposition of the oxidized ferrocene mediators in basic solution.

buffer. This is presumably due to a different enzyme mechanism. In the presence of oxygen, one important step in the catalytic cycle is the transfer of a proton from Tyr-495 to the intermediately formed superoxide radical anion which is a strong base.[19–22] Because of the absence of oxygen in the electrochemical process, a higher pH value seems to be necessary to induce this deprotonation. The total turnover numbers for the electrochemical regeneration of GOase and the aerobic regeneration in presence of catalase are almost the same. Compared to the aerobic regeneration without catalase, the electrochemical regeneration is more effective by a factor of 3 (Table 1). The activity-time-yield of the electrochemical regeneration compared to the aerobic regeneration with native GOase and catalase is approximately 50%, referred to the immobilized GOase activity. This shows that the electrochemical regeneration of immobilized GOase is approximately half as fast as the aerobic regeneration of native GOase.

After the electrolysis, 50% of the original enzyme activity could be recovered. However, during the electrolysis, the pH decreased to pH 9, at which the enzyme is not very stable under aerobic conditions. At lower pH aerobic regeneration of the enzyme and the concomitant development of the enzyme inhibitor hydrogen peroxide gradually begins. With better pH control during the electrolysis we should be able to improve the enzyme stability under electrolysis conditions even further. In a continuous process, this problem will not occur because the buffer is renewed continuously.

Table 1. Comparison of electrochemical and aerobic GOase regeneration

	Electrolysis	Aerobic GOase reaction *without* catalase	Aerobic GOase reaction *with* catalase
Amount of GOase (immobilized or native)	30.2 U (immob.); ≙149.6 U (native)	62.5 U (native)	62.5 U (native)
Concentration of xylitol	200 mM	200 mM	200 mM
pH of buffer	pH 10.8	pH 7.0	pH 7.0
Type and amount of buffer solution	50 mM sodium carbonate, 50 mL	50 mM phosphate, 20 mL	50 mM phosphate, 20 mL
Concentration of mediator	1 mM	–	–
Turnover	7.42 mM	13.4 mM	40.4 mM
Reaction time	31 187 min	6 975 min	15 457 min
Total turnover number of GOase	208 720 (6.7/min)	72 896 (10.5/min)	219 776 (14.2/min)
Total turnover number of mediator	15 (0.7/day)	–	–
Current yield	34.6%	–	–
Activity-time yield (with respect to immob. or native enzyme)	85 μg /($U_{imm.}$·day) 17.2 μg /($U_{nat.}$·day)	133 μg /(U·d)	181 μg /(U·d)
Activity-time yield (% of aerobic regeneration with catalase)	47 $\%_{imm.}$ 9.5 $\%_{nat.}$	74%	100%

GOase has three possible redox states: the reduced, semi and oxidized form. The semi and oxidized forms are in equilibrium, which is reached after 3 h in aqueous solution. Because of this equilibrium, only 5% of the total enzyme is in the active oxidized form. The 95% in the semi form is not directly accessible for the normal aerobic regeneration.[23]

Potassium hexacyanoferrate and potassium tetracyano-(1,10)-phenanthroline-ferrate are mediators only for the interconversion of the semi and the oxidized form of GOase. The electrochemical activation of GOase by the oxidized form of these mediators, described earlier,[18] can be explained by the conversion of the 95% of the enzyme in the semi-form into the oxidized form of GOase. With our ferrocene mediator, a one-electron transfer agent, we are able to oxidize both the reduced and the semi form of GOase.

As the oxidized forms of the ferrocene mediators do not show very good long-term stability under the basic electrolysis conditions (see above) we are currently trying to develop alternative mediator systems which are stable in both relevant oxidation states in basic solution, do not oxidize the substrate directly, and oxidize the semi and reduced form of GOase to the oxidized form.

Conclusion

GOase can be utilized in a continuous indirect anaerobic electrochemical regeneration process for synthetic purposes at a pH value of 10.8, in the same way as it has been used before in aerobic reactions. The turnover is limited due to the equilibrium of the GOase reaction.[3]

The results presented in this paper open the path for a modified electrochemical enzyme membrane reactor for immobilized GOase. In such a reactor the whole range of substrates for the synthetically interesting enzyme GOase could be oxidized.

Experimental

Buffer solutions. Phosphate buffers were prepared by adding a NaH_2PO_4 solution of the desired molarity to a Na_2HPO_4 solution of the same molarity until the desired pH value is reached. In some cases, the desired pH values were adjusted by addition of HCl or NaOH. Sodium carbonate buffers were prepared by adjusting the pH of a $NaHCO_3$ solution of the desired molarity with NaOH.

Immobilization procedure.[24,25] 2.5 g of DELOXAN® and 5 mL of phosphate buffer (0.1 M; pH = 7), which contained 2.5% glutaric dialdehyde (221.2 μL of a 50% aqueous solution) were shaken in a 100 mL Erlenmeyer flask for 2 h (150 rpm) at room temperature. A reddish colour showed the formation of the Schiff's base. The solution was removed by suction filtration, and the modified DELOXAN® was washed three times with 10 mL of phosphate buffer (0.1 M; pH = 7) before it was immersed in 2500 μL of phosphate buffer (0.1 M; pH = 7), which contained the amount of GOase that was to be immobilized (e.g. 16 U), and shaken for another 3 h (150 rpm) at room temperature. Finally the solution was removed by suction filtration, and the DELOXAN-GOase was washed with 5 mL of a solution of 1 M NaCl in phosphate buffer (0.1 M; pH = 7). The immobilized enzyme was best stored under carbonate buffer (50 mM; pH = 10.8) at 4°C. Immobilization under carbonate buffer (50 mM; pH = 10.8) containing 1 M D-galactose instead of phosphate buffer (0.1 M; pH = 7) resulted in even higher activity yields, but the immobilized enzyme proved to be less stable under electrolysis conditions.

Galactose oxidase assay. (a) Native GOase:[26] 2200 μL of a solution of 110 mg (2 mM) 2,2′-azino-bis-(3-ethylbenzothiazolin-6-sulfonic acid) diammonium salt (ABTS) in 100 mL phosphate buffer (0.1 M; pH = 6) were introduced into a 1 cm cuvette and saturated for 1 min with oxygen by a stream of oxygen bubbles using a syringe needle. After addition of 1000 μL of a 1 M aqueous D-galactose solution and 10 μL of a 50 U/mL peroxidase solution [0.5 U] and after 2 min of temperature equilibration in a UV spectrophotometer 50 μL of a GOase solution—containing approximately 0.25 U GOase per mL—were added to the assay mixture, and the absorption at λ = 405 nm was recorded for 3 min. From the increase of absorption the GOase activity of the solution could be calculated by standard procedures[27] ($\varepsilon_{ABTS,\,405\,nm} = 36.8\ L/(mmol)\cdot cm$). As ABTS is a single-electron-donor and GOase a two-electron-acceptor a factor 1/2 had to be regarded in the calculation of the enzyme activity. The activity per microliter was determined by using the following equation:

$$\text{activity}\left[\frac{U}{\mu L}\right] = 0.8859 \bullet \Delta A/\Delta t$$

(ΔA = absorption increase; Δt = time in minutes).

(b) Immobilized GOase: Instead of 50 μL of GOase solution an exact amount of DELOXAN-GOase (2–3 mg) was immersed in the assay solution and stirred with a cuvette stirrer during the assay, which lasted 20 min. From the absorption increase of the linear part of the absorption curve the activity of the amount of DELOXAN-GOase used in the assay could be calculated according to the following equation:

$$\text{activity [U/weight]} = 0.04361 \bullet \Delta A/\Delta t$$

(weight = weight of immobilized enzyme in g;

ΔA = absorption increase; Δt = time in minutes).

Cyclic voltammetry experiments. A BAS 100 B/W (BAS Inc., West Lafayette, Indiana, USA) digitally controlled electrochemical analyzer was used together with a glassy carbon working electrode (diameter: 3 mm), a Pt counter electrode and a Ag/AgCl reference electrode. The scan rate was 4 mV/s.

Electrolysis. 200 mM (1.52 g) of xylitol (substrate) and 1 mM (1 g) of α,ω-bis-methylferroene-polyethyleneglycol(20000) were dissolved in 50 mL of carbonate buffer (50 mM; pH 10.8). The electrodes used were a sigraflex® cylinder (carbon foil) working electrode (37 cm^2), a Pt counter electrode and a Ag pseudoreference electrode. The potential applied was 500–540 mV versus Ag, using a flow of 1 mL / min through the FPLC-column filled with 10 g of DELOXAN DAP III (30 U GOase).

Turnover determination. The turnover was determined by gas chromatography of the silylated compounds. This was performed by drying 15 μL of the electrolysis solution at 10^{-3} mbar and room temperature for 20 min, adding 2 mL of a silylation mixture containing 1-trimethylsilylimidazole, trimethylchlorsilane and ethylacetate (1:0.2:9)[28] to the residue, and refluxing the mixture for approximately 5 s. After cooling to room temperature, 1 mL of *n*-hexane and 1 mL of distilled water were added, and the mixture was shaken vigorously for 10 s. After phase separation the upper (organic) phase was filtered through a dry hydrophilic 0.2 μm cellulose membrane filter to remove traces of water before the solution could be injected into the GC. With *n*-tetradecane as external standard a calibration curve for the signal of silylated β-L-xylopyranose was recorded. For low turnover—up to 10%—no external standard was necessary, because xylitol could be used as internal standard. (Hewlett–Packard HP 5890 Series II Gas Chromatograph; HP-1 capillary column: 12 m, 100% dimethylpolysiloxane, inner diameter 0.25 cm, film thickness 0.33 μm; air pressure 195 kPa; hydrogen pressure 150 kPa; nitrogen pressure 310 kPa; inlet 200°C; FID detector 250°C; temperature program: 120°C (1 min), 10°C/min, 173°C (4 min isothermic separation), 60°C/min, 280°C (8 min); retention times: *n*-tetradecane (3.02 min), silylated α-L-xylopyranose (6.24 min), silylated xylitol (6.44 min), silylated μ-L-xylopyranose (6.91 min)).

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References and Notes

1. Ögel, Z. B.; Brayford, D.; McPherson, M. J. *Mycol. Res.* **1994**, *98*, 474–480.
2. Galactose oxidase, E.C. 1.1.3.9; *Enzyme Handbook*; Springer: Berlin, **1996**; pp. 1–6.
3. Root, R. L.; Durrwachter, J. R.; Wong, C.-H. *J. Am. Chem. Soc.* **1985**, *107*, 2997–2999.
4. Drueckhammer, D. G. *Synthesis* **1991**, *7*, 499–525.
5. Mazur, A. W. *ACS Symp. Ser.* **1991**, *466*, 99–110.
6. Witczak, Z. J.; Mielguij, R. *Synlett* **1996**, 108–110.
7. Mazur, A. W.; Hiler, II, G. D. *J. Org. Chem.* **1997**, *62*, 4471–4475.
8. Brielbeck, B.; Frede, M.; Steckhan, E. *Biocatalysis* **1994**, *10*, 49–64.
9. Frede, M.; Steckhan, E. *Tetrahedron Lett.* **1991**, *32*, 5063–5066.
10. Steckhan, E. *Top. Curr. Chem.* **1994**, *170*, 83–111.
11. Steckhan, E.; Frede, M.; Herrmann, S.; Ruppert, R.; Spika, E.; Dietz, E. *Dechema-Monographien* **1992**, *125*, 723–752.
12. Dicks, J. M.; Aston, W. J.; Davis, G.; Turner, A. P. F. *Anal. Chim. Acta* **1986**, *182*, 103–112.
13. Cooper, J. A. D.; Smith, W.; Bacila, M.; Medina, H. *J. Biol. Chem.* **1959**, *234*, 445–448.
14. Trademarks of Degussa AG, Hanau, Germany.
15. Krämer, L. Ph.D. Thesis, Bonn 1997: 199 mg (1.35 mmol) of trimethyloxoniumtetra-fluoroborate were suspended under argon atmosphere in 50 mL of absolute dichloromethane. To this suspension a solution of 218 mg (0.90 mmol) *N,N*-dimethylaminomethylferrocene in 50 mL of absolute dichloromethane was added dropwise, and the reaction mixture was stirred overnight. On addition of 400 mL of diethylether 246mg (0.71 mmol) of product precipitated (79%, $t_m = 168$–172°C).
16. Hilt, G.; Jarbawi, T.; Heineman, W. R.; Steckhan, E. *Chem. Eur. J.* **1997**, *3*, 79–88.
17. Komoschinski, J. Ph.D. Thesis, Bonn 1989: 3 equivalents of 3,4,7,8-tetramethyl-1,10-phenanthroline were dissolved in a solution of 10% methanol in water. To the boiling solution 1 equivalent of iron(II)sulfate heptahydrate was added. After evaporation of the solvent no further purification of the complex was necessary.
18. Johnson, J. M.; Halsall, H. B.; Heineman, W. R. *Biochemistry* **1985**, *24*, 1579–1585.
19. Wachter, R. M.; Montague-Smith, M. P.; Branchaud, B. P. *J. Am. Chem. Soc.* **1997**, *119*, 7743–7749.
20. Wachter, R. M.; Branchaud, B. P. *J. Am. Chem. Soc.* **1996**, *118*, 2782–2789.
21. Wachter, R. M.; Branchaud, B. P. *Biochem* **1996**, *35*, 14425–14435.
22. Branchaud, B. P.; Montague-Smith, M. P.; Kosman, D. J.; McLaren, F. R. *J. Am. Chem. Soc.* **1993**, *115*, 798–800.
23. Borman, C. D.; Saysell, C. G.; Sykes, A. G. *J. Biol. Inorg. Chem.* **1997**, *2*, 480–487.
24. Personal communication with Degussa AG, Hanau, Germany; Olsson, B.; Ögren, L. *Anal. Chim. Acta* **1983**, *145*, 87–99.
25. Petersson, B. A.; Hansen, E. H.; Ruzicka, J. *Anal. Lett.* **1986**, *19*, 649–665.
26. Assay procedure TV 32 700, October 1993, Boehringer Mannheim GmbH, Germany.
27. Bergmeyer H. U. *Methods of Enzymatic Analysis*, VCH: Weinheim, 1984 (3rd ed.), Vol. 6, pp. 682–693.
28. Rizzo, A. F.; Saari, L.; Lindfors, E. *J. Chromatogr.* **1986**, *368*, 381–386.