0273-2289/97/63-65—0173$12.00

Simultaneous Enzymatic Synthesis of Gluconic Acid and Sorbitol

Production, Purification, and Application of Glucose-Fructose Oxidoreductase and Gluconolactonase

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ABSTRACT

With regard to the enzymatic synthesis of sorbitol and gluconic acid, a screening was carried out to identify promising producers of glucose-fructose oxidoreductase (GFOR) and gluconolactonase (GL). *Zymomonas mobilis* DSM 473 and *Rhodotorula rubra* DSM 70403 have been selected for the synthesis of GFOR and GL, respectively. Maximal enzyme production by these organisms has been achieved at D-glucose concentrations of 200 and 150 g/L, respectively. Both GFOR and GL were purified and characterized with respect to some of their catalytic properties. GL showed strict specificity for 1,5-(δ)-lactones and was activated by Mg^{2+} and Mn^{2+} ions. The potential use of soluble GFOR is limited by its inactivation during substrate conversion, and the effects of reaction temperature and pH on the "catalytic" stability of GFOR were evaluated. Exogenous adddition of auxiliary GL had no effect on oxidoreductase stability and did not improve productivities.

Index Entries: Glucose-fructose oxidoreductase; gluconolactonase; *Zymomonas mobilis; Rhodotorula rubra;* stability.

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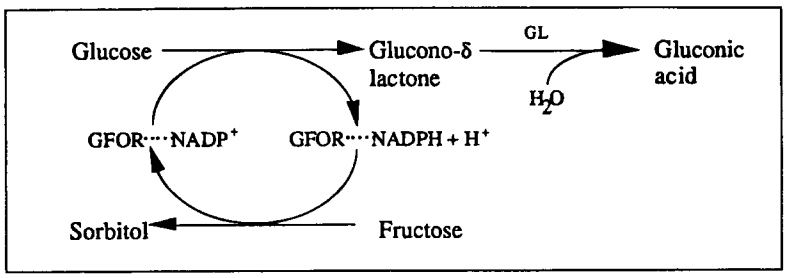
INTRODUCTION

Sorbitol and gluconic acid are bulk products with various applications in the food and pharmaceutical industry and have many other large scale uses in the chemical industry *(1,2)*. An enzymic route for the synthesis of these two compounds, which operates under mild conditions and avoids by-product formation, could be a true alternative to the classic production processes *(1,3)*. The ethanologenic bacterium *Zymomonas mobilis* was shown to synthesize sorbitol during growth on sucrose or on mixtures of fructose and glucose *(4)*. The enzyme responsible for sorbitol formation by *Zymomonas* is glucose-fructose oxidoreductase (GFOR) that simultaneously converts mixtures of glucose and fructose into, respectively, glucono-δ–lactone and sorbitol. Tightly protein-bound NADP(H) serves as the cofactor in the oxidoreduction reaction *(5)*. The simultaneous redox process shown in Scheme 1 is practically irreversible because gluconolactone is hydrolyzed to gluconic acid either in a spontaneous manner *(6)* or enzymatically by the action of gluconolactonase (GL) *(5,7–10)*. Possible and promising applications of GFOR for the production of sorbitol and gluconic acid have been proposed soon after the first identification of the enzyme. Both isolated GFOR and permeabilized cells of *Z. mobilis* have been employed in several studies aimed at the optimization of the enzymic substrate conversion in discontinuous and continuous mode of operation *(5,11–19)*. The principal advantages of GFOR over other enzyme/coenzyme systems for the production of gluconic acid and sorbitol are quite obvious: In contrast to other coupled enzyme systems, e.g., sorbitol-dehydrogenase/glucose-dehydrogenase or aldose-reductase/glucose-dehydrogenase *(20)*, GFOR is a self-regenerating redox-enzyme-system whose activity does not depend on the efficiency of a (second) regenerating reaction system *(21,22)*. Furthermore, the exogenous addition of the expensive and unstable NADP(H) is not necessary since the coenzyme in GFOR is bound to the protein in a nondissociable form. Consequently, the retention of the coenzyme in the reaction system during continuous operation employing specifically adapted techniques is not required, e.g., artificial enlargement of the coenzyme *(23)* or specific membrane technology *(21,24–26)*.

The aim of this work was threefold. First the identification of not yet reported organisms, *Zymomonas* sp. or others, that are capable of expressing significant and possibly higher activities of GFOR and/or GL than those previously reported was taken into consideration. Although cloning and overexpression of the gene encoding GFOR and GL from *Z. mobilis* was successful *(27)* and a six to nine fold increased expression of GFOR in the native host organism was described *(27,28)*, the identification of better producing wild type strains could be important. Second, the production, purification, and partial characterization of these enzymes was targeted. Finally, some aspects of the application of isolated GFOR with or without supplementation by GL for the synthesis of sorbitol and gluconic acid was studied.



Scheme 1. Simultaneous conversion of glucose and fructose to sorbitol and gluconic acid by glucose-fructose oxidoreductase (GFOR) and gluconolactonase (GL)-an enzyme that catalyzes the hydrolysis of glucono-δ-lactone. The cofactor NADP(H) tightly bound to GFOR is indicated.

MATERIALS AND METHODS

Chemicals

Chemicals were reagent grade and purchased from Sigma (Deisenhofen, Germany) unless otherwise stated. The gel material for enzyme purification and electrophoresis was from Pharmacia (Uppsala, Sweden).

Screening

All micro-organisms were obtained from the German type culture collection DSM (Braunschweig, Germany) or the Centraalbureau voor Schimmelcultures (Baarn, The Netherlands) and were cultivated at 30°C according to the instructions of the suppliers using the following media components in g/L: *Zymomonas* (glucose [20.0], yeast extract [10.0], casein peptone [10.0]; pH 6.0, 60 rpm, 48 h, sealed serum flasks); *Pseudomonas* (casein peptone [5.0], meat extract [3.0], pH 7.0, 24 h, 100 rpm); *Gluconobacter, Acetobacter, Alteromonas* (glucose [25.0], yeast extract [5.0], casein peptone [3.0], pH 7.0, 48 h, 100 rpm); yeasts (peptone from soybean [3.0, Fluka], malt extract [30.0], pH 5.6, 24 h, 100 rpm). All aerobes were grown in baffled 250-mL Erlenmeyer flasks. Cells were harvested by centrifugation at 15,000*g* and 4°C for 20 min, washed twice, diluted 1:3 in 10 m*M* MES-buffer pH 6.4 and disrupted three times in a French pressure Mini cell (American Scientific Company, Silver Spring, MD) at 1200 psi. Debris and intact cells were removed by ultracentrifugation at 100,000g for 20 min (L8-70; Beckman, Fullerton, CA). The clear supernatants (cell extracts) were analyzed for enzyme activities and total protein as described in Enzyme Assays.

Production of GFOR and GL

Z. mobilis (DSM 473) was grown in shaken flask culture (serum bottles) for 48 h at 60 rpm on a reciprocal water-bath incubator (Infors HT; Infors, Bottmingen, Switzerland). The medium (pH 6.0) contained in g/L,

yeast extract (5.0), KH_2PO_4 (0.5), $M_gSO_4 \times 7H_2O$ (0.02), $Fe(NH_4)_2(SO_4)_2 \times 6H_2O$ (0.02), biotin (0.001), calcium panthotenate (0.002), and carbohydrate component as indicated. *R. rubra* (DSM 70403) was cultivated at 30°C and 100 rpm for 24 h in complex medium or mineral medium (pH 5.6). The complex medium contained in g/L: glucose (20–200), malt extract (30), soja peptone (3). The mineral medium contained in g/L: glucose (20–200), KH_2PO_4 (0.88), K_2HPO_4 (0.13), NaCl (0.10), $(NH_4)_2SO_4$ (1.00), $M_gSO_4 \times 7H_2O$ (0.5), $CaCl_2 \times 2H_2O$ (0.1), biotin (0.001), 1 mL trace element solution ($Fe(NH_4)_2(SO_4)_2 \times 6H_2O$ [20 mg], $CuSO_4 \times 5\ H_2O$ [4 mg], KI [10 mg], $MnCl_2 \times 4H_2O$ [30 mg], $Na_2MoO_4 \times H_2O$ [25 mg], $ZnCl_2$ [20 mg]).

Alternatively, *Z. mobilis* was cultivated, without aeration, at 30°C and 150 rpm in a 20-L MBR fermentor with a working volume of 18 L (MBR, Wetzikon, Switzerland) fitted with pH and oxygen electrodes. 14 L of medium (with 200 g/L glucose) were inoculated with 1.4 L of preculture grown on the same medium for approx 50 h at 60 rpm. A constant pH value of 6.0 was achieved by the addition of 3*M* potassium hydroxide. *R. rubra* was grown in the same bioreactor system at 30°C, 300 rpm, a controlled pH of 5.0 and 0.5 vvm aeration rate using the specified complex medium containing 150 g/L glucose. Antifoam (polypropylenglycol) was added as required. Samples were periodically taken throughout the cultivations and for subsequent measurements centrifuged (3800–11,000*g*, 20–40 min, 4°C), washed and treated as described for the Screening Section.

Purification of GFOR and GL

Harvested cells of *R. rubra* were resuspended (1:1 w/v) in 10 m*M* MES buffer pH 6.4 containing 5 m*M* dithiothreitol (DTT) and disrupted at constant 4°C in a continuously operated Dyno Mill (Bachofen, Switzerland) using an average residence time of 7 min (glass beads of 0.5 mm diameter). Cells of *Z. mobilis* were two-fold diluted in 50 m*M* MES, pH 6.4 and subjected to a three-time disintegration in a 20K French pressure cell at 1200 psi. Following ultracentrifugation (30,000–100,000*g* for 40 min, 4°C) the supernatant was recovered and stored at –20°C until used for further purification.

All purification steps were caried out at room temperature except $(NH_4)_2SO_4$ precipitations at 4°C. Column chromatography was performed on a FPLC system (Pharmacia) using detection of eluting protein at 280 nm. Gel-filtration was accomplished using either Sephadex G-25 coarse (2.5 × 10 cm, 10 mL/min) or prefilled PD-10 and NAP 5 desalting columns. Concentration was carried out by cross-flow (10 kD Mini-Ultrasette; Filtron, Nothborough, MA) or dead-end ultrafiltration (Amicon-stirred 50-mL cell equipped with 10 kD membrane) as well as by using 10 kD Centricon tubes (Millipore, Eschborn, Germany). Hydrophobic interaction chromatography was carried out on Phenylsepharose fast flow (25 mL) equilibrated with 30% $(NH_4)_2SO_4$ pH 6.4 and eluting protein with a 5% step gradient (30–0%). In case of GL, 5 m*M* DTT was added to all buffers. Cation exchange

chromatography of GFOR was carried out on a 1-mL column of Mono S HR 5/5 equilibrated with 10 m*M* MES, pH 6.4. Elution was accomplished at 0.5 mL/min using a linear gradient of 1*M* NaCl (0–150 m*M* in 60 min). Anion exchange chromatography of GL was performed on Mono Q HR 5/5 equilibrated with 20 m*M* piperazine, 1 m*M* DTT, 0.1% Tween, pH 6.0, and elution was at 0.5 mL/min with a linear gradient of 50–350 m*M* NaCl.

Enzyme Assays

GFOR activity was quantitated in a coupled assay together with *R. rubra* gluconolactonase by measuring the decolorization of a 0.29 m*M* *p*-nitro-phenol solution at 405 nm and 25°C. 400 m*M* Glucose and 800 m*M* fructose in 10 m*M* MES buffer pH 6.4 were used as substrates. It was proven (cf Results section) that 6–8 U/mL of lactonase are needed to avoid underestimation of GFOR activity because of rate-limitations in the coupled reaction (lactone hydrolysis). The activity of gluconolactonase was determined by the same method using 4 m*M* glucono-δ-lactone as a substrate. One unit of activity refers to 1 µmol gluconic acid formed per minute by the action of either GFOR/gluconolactonase or gluconolactonase alone. All rates are corrected for nonenzymic hydrolysis of gluconolactone in the absence of lactonase and, at 25°C, an equilibrium constant of 0.185 ± 0.05 of D-glucono-δ-lactone/D-gluconic acid is assumed *(6)*.

The pH and temperature optima of GFOR and GL were determined in discontinuous assays. In case of GFOR, but not GL, the reaction was terminated after 10–15 min by heating (10 min, ≈ 100°C) prior to employing HPLC analysis of product formation or substrate consumption (less than 15% conversion). Samples of GL-catalyzed conversions were analyzed immediately to reduce spontaneous hydrolysis of the lactone substrate. The substrate concentrations were 400 m*M* glucose/800 m*M* fructose (GFOR) and 100 m*M* glucono-δ-lactone (GL). The effect of various compounds on the stability and activity of both enzymes was tested in standard assays as indicated.

Simultaneous Substrate Conversions by GFOR/GL

Conversion of mixtures of fructose and glucose was carried out at the temperature indicated using varying activities of GFOR with or without supplementation by GL. GFOR was employed in varying degrees of purity whereas GL was used after ammonium sulfate precipitation (cf Results section). Incubation was in 100 m*M* potassium phosphate buffer at a constant pH value between 5.5–7.3 (maintained by the controlled addition of alkali). The reaction progress was monitored by reading either the volume or the weight of the consumed alkali. Samples (100–250 µL) were taken from the reaction mixture at times indicated. Approximately 100 µL were heat-inactivated (10 min; boiling water bath) and used for HPLC analysis (cf Analytical). The rest of the original sample was gel-filtered (NAP 5

Table 1
Screening for Microbial Producers of Glucose-Fructose Oxidoreductase (GFOR) and Gluconolactonase (GL)

Organism	Strain	GFOR (U/mg)	GL (U/mg)	Wet biomass (g/L)
Z. mobilis	DSM 424	0.06 (0.08)*	n.d.	5.2
Z. mobilis	DSM 473	0.30 (1.51)*	0.81	7.8
Z. mobilis	DSM 762	n.d. (n.d.)	0.06	1.5
Z. mobilis	DSM 3580	0.32 (2.04)*	0.60	4.7
P. acidovorans	DSM 50251	n.d.	1.20	10.1
P. aeruginosa	DSM 1707	n.d.	1.62	5.6
P. fluorescens	DSM 50090	n.d.	2.42	4.8
P. chlororaphis	DSM 50083	n.d.	0.94	9.9
R. rubra	DSM 70403	n.d.	8.18	10.0
R. rubra	DSM 70404	n.d.	3.15	7.1
R. gracilis	CBS 6681	n.d.	2.70	8.5

*Values in parenthesis are measured in the presence of 8 U/mL gluconolactonase; n.d., not detectable.

colums) to remove substrates and products, and used for quantitation of residual GFOR activity.

Analytical

Glucose, fructose, sorbitol, and gluconic acid in reaction samples were base-line separated by HPLC using an Aminex HPX 87C (7.8 × 300 mm) ion exchange column (BioRad, Hercules, CA) at 85°C with 10 m*M* calcium nitrate as eluent. The flow rate was 0.7 mL/min, and detection was accomplished by refraction index. Gluconolactone was determined using a LiChrosorb NH_2 column (Merck, Darmstadt, Germany) with 77% acetonitrile/23% 10 m*M* potassium phosphate pH 5.5 as eluent (1.5 mL/min, 30°C). Protein was determined according to Bradford *(29)* using bovine serum albumin as standard.

RESULTS

Screening

Among 25 micro-organisms selected because their genera such as *Acetobacter* sp., *Gluconobacter* sp., and *Pseudomonas* sp. show some pheno- or genotypic similarity to *Zymomonas (4)*, none were capable of producing detectable activities of GFOR. This result further supports the assumption that GFOR is an enzyme unique to *Z. mobilis (4)*. The specific activities synthesized by different strains of *Z. mobilis* differed widely (Table 1), and the addition of exogenous GL activities (6–8 U/mL assay) was essential to

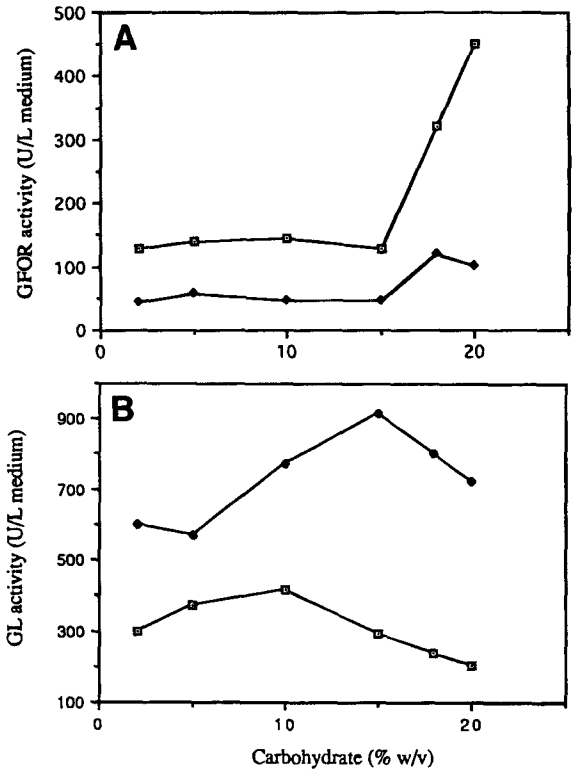


Fig. 1. **(A)** Production of GFOR by Z. *mobilis* DSM 473 using D-glucose (□) or D-fructose (◆) as a sole carbon source (30°C, 60 rpm, 48 h, microaerobically-sealed serum bottles). **(B)** Production of GL by *R. rubra* DSM 70403 using D-glucose as a carbon source in mineral (◆) medium and complex (□) medium (30 °C, 100 rpm, 24 h, baffled flasks).

avoid underestimation of actual GFOR activity especially when comparison of different strains had to be made. Since GL seemed to be an important enzyme at least from the analytical point of view, in addition to GFOR, the amount of GL produced by various organisms was assayed. Several bacteria (*Pseudomonas* sp.), but especially yeasts of *Rhodotorula* sp. were found to produce much higher activities than *Zymomonas*. In other yeast strains such as *Candida* sp. or *Pichia* sp. the volumetric and specific GL activities were low or even not detecable. From these preliminary screening studies Z. *mobilis* DSM 473 and *Rhodotorula rubra* DSM 70403 were selected for more detailed studies pertaining to GFOR and GL production, respectively. Z. *mobilis* DSM 473 was preferred over Z. *mobilis* DSM 3580 because of the better biomass formation of the former organism (cf. Table 1).

Enzyme Production

The effect of the type of the carbon source and its concentration on biomass formation and enzyme production by Z. *mobilis* and *R. rubra* was studied in shaken flask experiments, and results are shown in Figs. 1A and 1B.

The volumetric GFOR activities during growth on glucose were approx four fold higher than those obtained with fructose as a carbon source. The optimal glucose concentration with regard to biomass formation and enzyme production is different. The highest GFOR activities of approximately 450 U/L were obtained at 20% glucose (Fig. 1A) that is a sugar concentration where reduced growth of the organism was already observed (4.7 g/L). Maximal biomass yield of approx 8 g/L found between 100–150 g/L glucose. The use of fructose alone or equimolar mixtures of fructose and glucose reduced the biomass yields by approx 15–25%. Growth of *R. rubra* and concomitant GL production was monitored using glucose as a sole carbon source in concentrations up to 200 g/L. The optimal sugar concentrations for enzyme formation on mineral medium or on complex medium are distinct (100 and 150 g/L, respectively) and decrease when glucose is further increased beyond these concentrations (Fig. 1B).

In fermentor cultivations of *Z. mobilis* (200 g/L glucose) the formation of GFOR and of GL was found to be growth-associated and the production of these enzymes occured simultaneously (not shown). The stationary growth-phase was reached after 25–30 h cultivation time coinciding with depletion of glucose. After 30–35 h, the volumetric activity of GFOR started to decrease rapidly so that the time of cell harvest was important. In discontinuous experiments, a wet cell concentration of 17 g/L and enzyme activities of 800 U GFOR/L and 450 U GL/L were obtained. Fermentor cultivations of *R. rubra* on complex medium supplemented with 150 g/L glucose yielded approx 45 g/L wet biomass and 1200–1400 U GL/L medium after 20 h of growth. Enzyme production is growth-associated and, like in the case of GFOR, GL activities started to decrease by 10–20% when incubation was continued for further 5–10 h after the maximal activity had been attained.

Enzyme Purification

Since the addition of crude *R. rubra* GL was found to inhibit GFOR activity to some extent, a minimal purification of GL was required for its use in analysis and conversion studies. Table 2 presents a summary of the purification strategy and the results obtained. The fractions of GL achieved by the procedure outlined in Table 2 were added to a crude GFOR preparation, and their effect on the apparent oxidoreductase activity was determined. Ammonium sulfate precipitation of GL (45% saturation with a 90% saturated solution) followed by resuspension in 10 m*M* MES pH 6.4 was found to cause removal of all components inhibitory to GFOR. Consequently, for assaying GFOR and for supplementing the oxidoreductase during substrate conversions, this preparation of GL was useful after gel-filtration followed by four to five fold concentration (ultrafiltration). The Mono Q preparation was taken for the subsequent characterization of GL, although the isolation of the enzyme in homoge-

Table 2
Partial Purification of Guconolactonase from 70 mL Crude *R. rubra* Extract

	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield %	Purification factor (-fold)
Crude cell extract	1191	561	2.1	100	1.0
Ammonium sulfate (45%)	1406	483	2.9	118	1.4
Phenylsepharose fast flow	842	51	16.4	71	7.8
Mono Q	754	4.3	175.3	63	83.5

Table 3
Purification of Glucose-Fructose Oxidoreductase
from 50 mL crude extract of *Z. mobilis*

	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield %	Purification factor (-fold)
Crude cell extract	2750	561	2.2	100	1.0
Ammonium sulfate (30%)	2842	483	3.3	≈100	1.4
Phenylsepharose fast flow	2236	51	20	81	7.8
Gel-filtration, ultrafiltration	1015		20-30	37	7.8 - 9.8
Mono S	563	2.3	245-310	20	114

neous form has not been fully accomplished by the ion exchange step (not shown).

GFOR was purified to apparent homogeneity by a similar procedure, and the results are shown in Table 3. The final cation exchange chromatographic step on Mono S yielded pure GFOR (SDS PAGE; not shown) although the specific activity of the resulting preparation could vary between 245–310 U/mg probably because of some inactivation of the enzyme during chromatography. The moderate yield of homogeneous enzyme is mainly a result of the concentration and desalting steps and the concomitant loss of enzyme activity.

Enzyme Characterization

GFOR and GL were characterized with regard to pH- and temperature-activity profiles, stability and specificity. The pH optimum of GFOR is between 6.5–7.0 whereas the pH dependence of GL catalysis could not be

Table 4
Activation and Inhibition of Glucose-fructose Oxidoreductase and Gluconolactonase

GFOR activity			GL activity		
Compound	Concentration	Effect % *	Compound	Concentration	Effect % *
$NADP^+$	0.5; 2.5 mM	71; 40	$MnCl_2$	2.0 mM	156
NAD^+	0.5; 2.0 mM	93; 75	$MgCl_2$	2; 3; 5 mM	166; 181; 182
Tween 20	0.1 %	148	Tween 20	0.1 %	113
Triton X-100	0.1 %	146	Triton X-100	0.1 %	114
Sucrose	25 %	123	$MgAc_2$	2.0 mM	167
Trehalose	25 %	120	Trehalose	25 %	113
Glycerol	25 %	120	Glycerol	25 %	105
DMSO[a]	25 %	130	$CaAc_2$	0.5 mM	130
DTT[b]	5 mM	105	DTT	5 mM	≈100

*Based on standard assay at 25°C in the absence of additives; [a]dimethylsulfoxide; [b]dithiothreitol; Ac, acetate.

accurately quantitated because of increasing spontaneous hydrolysis of the lactone in alkaline regions, at least with the experimental assay lacking pH control that was employed throughout this study. The temperature optima of GFOR and GL were found to be 40°C and 32°C, respectively. The temperature effect on the GL-mediated hydrolysis probably also includes some minor pH effect because during the incubation period of 10 min a decrease in the initial pH of 6.5 to 5.5–6.0 was observed.

Tests of the substrate spectrum of GL revealed that the enzyme has a strict specificity for δ-lactones (D-glucono-[100%], D-cellobiono-[13%]; 4 m*M*) whereas all γ-lactones (D-glucono, D-glucurono-, D-galactono, D- and L-gulono-, L-arabono, D-ribono-; 4 m*M*) were not hydrolyzed. The only substrate accepted by GFOR in the reduction half-reaction is fructose that could not be replaced by other ketoses such as D-ribulose, D-xylulose, D-psicose, lactulose or L-sorbose (800 m*M*). Some activity was seen with 2-deoxy D-glucose (16% of the activity with the parent compound D-glucose; 400 m*M*). The apparent Michaelis constants of GFOR for fructose and glucose were determined as 400 m*M* and 20 m*M*, respectively.

The activity of GFOR and GL was probed in dependence of various additives (Table 4). Divalent cations especially Mn^{2+} and Mg^{2+} activated GL by more than 50%. The corresponding anion appears to be less important. GFOR was inhibited by free coenzyme $NAD(P)^+$ and activated by some nonionic detergents (Tween, Triton). For stability of GL at 25°C and 4°C (half lives 5 and >35 d, respectively), the addition of 0.1% Tween 20 and 5 m*M* DTT was required. NaCl (100 m*M*), K-gluconate (4 m*M*) or glycerol (25%) had slight additional stabilizing effects.

Table 5
First-order Deactivation Constants (k) of Glucose-Fructose Oxidoreductase at 25°C

	k (h^{-1}) pH 5.5	k (h^{-1}) pH 6.4	k (h^{-1}) pH 7.3
Buffer	0.008	0.005	0.062
1 M Glucose	0.020	0.043	0.081
1 M Fructose	0.054	0.061	0.057

Stability of GFOR

The process-relevant stability of GFOR was studied with regard to two aspects considered especially important. The identification of a minimal required level of purity of the enzyme and the operational stability of this GFOR preparation during substrate conversion. The activity of various enzyme fractions differing in their specific activity according to Table 3 was monitored at 25°C and 45°C over a relevant time period in the absence of substrates or with one substrate added at a 1*M* concentration. The stability of GFOR in crude extracts of *Zymomonas* and that of the pure oxidoreductase were comparable, with a half-life of approx 55 h at 45°C and with no loss of enzyme activity at 25°C for a period of at least 2 wk. Since GFOR is an oligomeric protein that might dissociate upon dilution, its stability was assayed in dependence of the total enzyme or protein concentration (BSA added). In the concentration range of 50 µg/mL to 1 mg/mL, no effect on the stability of GFOR was observed using either crude or partially purified enzyme (20 U/mg). Substrates and products did not improve the stability of GFOR to any significant extent. In contrast, fructose and especially glucose decreased the stability of GFOR as did alkaline incubation conditions. Assuming first-order kinetics of enzyme denaturation, inactivation constants were calculated from plots of *Ln* (activity) vs incubation time and are summarized in Table 5. It is noteworthy that the destabilization by fructose showed no pH dependence whereas that by glucose did. Purification of GFOR to whatever extent did not change these stability characteristics significantly, and conversion studies were thus carried out with a crude *Zymomonas* enzyme preparation.

Enzymic Synthesis of Sorbitol and Gluconic Acid

The use of GFOR for the synthesis of sorbitol and gluconic acid appears to be promising because the enzyme is capable of fully converting extremely concentrated sugar solutions (3*M* glucose, 3*M* fructose) whereas substrate inhibition is absent and product inhibition moderate *(12,18,19)*. However, in contrast to results achieved with "immobilized" GFOR, i.e., with the enzyme embedded into the matrix of permeabilized cells of

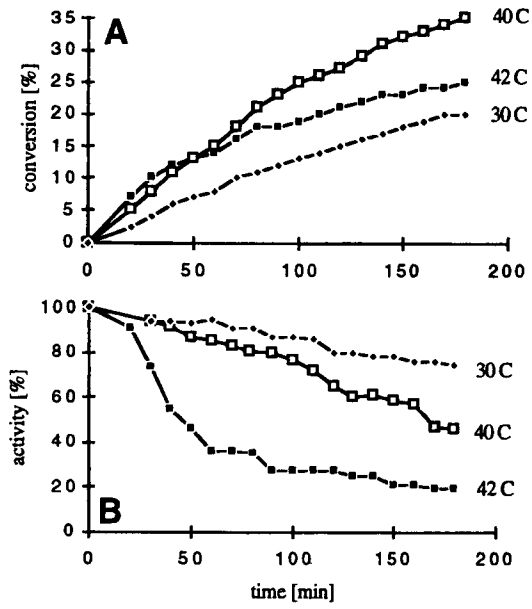


Fig. 2. Time-course of substrate conversion and residual GFOR activity in dependence of the reaction temperature. The conditions were: 1*M* fructose, 1*M* glucose, 100 m*M* P_i, pH 6.4 titrated with 1*M* KOH, 20 U/mL gluconolactonase from *R. rubra*. **(A)** Substrate conversion, **(B)** enzyme activity.

Zymomonas, the isolated enzyme is unstable during substrate conversion. The half-life of GFOR in buffer at 25–40°C is in the range of at least several days whereas substantial enzyme activity (30–40%) is lost within a few hours when the incubations are carried out in the presence of both substrates (Fig. 2A). Considering the time-courses for substrate conversion, an operational temperature optimum is difficult to define because inactivation occurs even at a reaction temperature of 30°C (Fig. 2B). However, GFOR loses activity at comparable rates at 30 and 35°C whereas substrate conversion is significantly higher at the latter temperature. All conversion/time profiles obtained at reaction temperatures above 35°C showed downward curvature indicating progressing loss of enzyme activity. The rapid inactivation of soluble GFOR is in marked contrast with the reported excellent process-stability of cell-bound GFOR at 39°C *(15)*. Destabilization by either fructose and glucose (cf Table 5) does not reflect this inactivation of the isolated enzyme. We have recently shown that the addition of 5–10 m*M* dithiothreitol as a protectant of sulfhydryl groups could stabilize GFOR to some extent *(18,19)*. The alkaline component employed for the titration of gluconic acid produced during reaction was also found to be important. Tris base could decrease but not completely abolish the rate of enzyme inactivation (Nidetzky and Fürlinger, unpublished results). Another important variable is the pH of the reaction mixture which is maintained by the addition of alkali during product formation (cf Table 5). At 30°C, a variation of

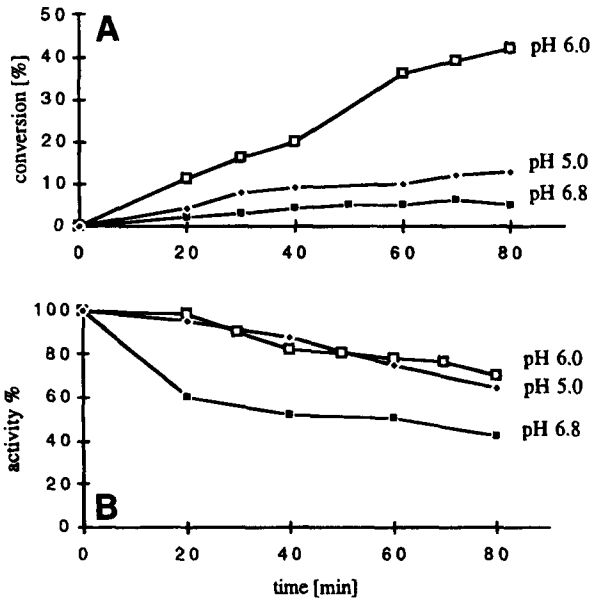


Fig. 3. Time-course of substrate conversion and residual GFOR activity at a reaction temperature of 40°C in dependence of the reaction pH. The conditions were: 1*M* fructose, 1*M* glucose, 100 m*M* P_i, pH 5.5–6.8 titrated with 1*M* KOH, 20 U/mL gluconolactonase from *R. rubra*. **(A)** Substrate conversion, **(B)** enzyme activity.

the controlled pH value in the range of 5.5–6.8 had only minor influence on the reaction rate reflecting the pH/activity profile of the enzyme and did not affect the stability of GFOR. At 40°C, however, the pH is extremely important as demonstrated by the data in Figs. 3A and B. When pH values above 7.0 were set, fast inactivation of GFOR was observed at all reaction temperatures applied. The ionic strength of the buffer system (0; 0.1; 1.0 M Pi) was found not to be a determinant of enzyme stability and activity.

Effect of Gluconolactonase

GL is useful as an auxiliary enzyme for assaying GFOR activity (cf Table 1) and, in vivo, it accelerates the hydrolysis of gluconolactone produced by GFOR. It is tempting to speculate that the presence of GL could have a beneficial effect on GFOR activity and, even more important, on its stability during substrate conversion in vitro. However, no such effect was observed when reactions were carried out in the presence or absence of GL and the resulting time-courses of product formation and inactivation of GFOR were compared. In a temperature range of 25–40°C, neither the stability of GFOR nor the space time yield of reaction was improved (when essentially complete substrate conversion was taken into consideration). For an efficient product formation in discontinuous reactions, the addition of exogenous GL is thus not required.

DISCUSSION

The results of the present work led to the identification of two previously uncharacterized strains, *Z. mobilis* DSM 473 and *R. rubra* DSM 70403, which are useful for the production of glucose-fructose oxidoreductase and gluconolactonase, respectively. According to the screening carried out here, the hypothesis of GFOR being an enzyme unique to *Z. mobilis* was corroborated *(4)*. Not even traces of this oxidoreductase activity were found in any other species studied. Recently it became clear that GFOR has a very specialized physiological role in *Zymomonas* as it enables the organism to grow in concentrated sugar solutions while sorbitol (produced by GFOR) counteracts negative osmotic effects *(30)*. Synthesis of GFOR by *Z. mobilis* DSM 473 showed a qualitatively similar dependence on the type and concentration of the carbon source as reported for *Z. mobilis* ATCC 29191 *(5)*. The specific GFOR activities were comparable in both strains of *Z. mobilis*. The biomass formation by *Z. mobilis* DSM 473 was, however, two fold higher than those reported earlier *(5)*. An alternative procedure for isolating GFOR in homogeneous form, based on hydrophobic and ion exchange chromatographies, was established, albeit the yields of pure enzyme need to be further improved. The results of a subsequent characterization of GFOR are in good accordance with the results of Zachariou & Scopes *(5)*. When incubated in buffer with or without one of its substrates, GFOR exhibits stability characteristics typical for a mesophilic enzyme. The extreme instability of the oxidoreductase during its catalytic action remains still enigmatic, but was substantiated by analysing enzyme inactivation in dependence of the reaction temperature and pH. At least at present, the loss of GFOR activity during product synthesis certainly limits the applicability of the soluble enzyme.

R. rubra was identified as an outstanding natural producer of the auxiliary enzyme gluconolactonase. Partial purification of this enzyme was accomplished throughout this study that allowed characterization of the enzyme as well as its use for analytical purposes. The strict specificity of *R. rubra* GL for δ-lactones agrees with earlier reports on a gluconolactonase from *Saccharomyces cerevisiae* and *Pseudomonas fluorescens (8,10)*, but distinguishes the new enzyme from other lactonases apparently hydrolyzing both δ- and γ-lactones *(9)*. The activation by divalent cations is in accordance with data of metal analyses for a mammalian gluconolactonase indicating the presence of these ions bound to the native protein *(7)*.

In the enzymatic synthesis of sorbitol and gluconic acid by GFOR, the presence of GL could be important. One tentative beneficial role of GL acting in combination with GFOR might be the removal of product inhibition because of the accelerated hydrolysis of the gluconolactone product in the presence of GL. However, no evidence was found in the course of the present study that would support any effect of GL during substrate conversions (either present in crude extracts of *Zymomonas* or added exogenously

from a partially purified *R. rubra* preparation). We could detect no influence on the attainable space time yield and on GFOR stability.

In some cases the presence of GL may be even undesirable. During continuous conversions (where a certain substrate conversion has to be achieved) the average residence time in an enzyme reactor must be adjusted in accordance with the volumetric activity of the biocatalyst employed. One particular advantage of the homogeneous enzyme reactor is that high concentration of the biocatalyst can be used to guarantee product synthesis at efficient reaction rates. When taking into account that the formation of gluconic acid requires titration with alkali that in turn remains as a possible candidate responsible for some inactivation of GFOR, it might be desirable to carry out the enzymic conversions at a (dilution) rate that is significantly faster than that of the subsequent spontaneous hydrolysis of the formed glucono-δ-lactone. The first-order rate constants (h^{-1}) of the spontaneous decomposition of the 1,5-lactone at 25°C are 3.17 and 1.08 at pH 6.40 and 5.95, respectively *(6)*. These rate constants correspond to half-lives of 0.21 and 0.64 h. Considering volumetric activities of 5,000–10,000 U GFOR per-L, average residence times of 0.5 h or less seem to be feasible even when concentrated sugar solutions are to be converted. Under such conditions certainly the absence and not the presence of GL activity would be desirable and important.

ACKNOWLEDGMENTS

Part of this work was supported by the Jubiläumsfonds der Österreichischen Nationalbank, Grant 5089 to B. N.

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