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Characterization of *Thermoanaerobacter* Glucose Isomerase in Relation to Saccharidase Synthesis and Development of Single-Step Processes for Sweetener Production

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Regulation of glucose isomerase synthesis was studied in *Thermoanaerobacter* strain B6A, which fermented a wide variety of carbohydrates including glucose, xylose, lactose, starch, and xylan. Glucogenic amylase activities and β-galactosidase were produced constitutively, whereas the synthesis of glucose isomerase was induced by either xylose or xylan. Production of these saccharidase activities was not significantly repressed by the presence of glucose or 2-deoxyglucose in the growth media. Glucose isomerase production was optimized by controlling the culture pH at 5.5 during xylose fermentation. The apparent temperature and pH optima for these cell-bound saccharidase activities were as follows: glucose isomerase, 80°C, pH 7.0 to 7.5; glucogenic amylase, 70°C, pH 5.0 to 5.5; and β-galactosidase, 60°C, pH 6.0 to 6.5. Glucose isomerase, glucogenic amylase, and β-galactosidase were produced in xylose-grown cells that were active and stable at 60 to 70°C and pH 6.0 to 6.5. Under single-step process conditions, these saccharidase activities in whole cells or cell extracts converted starch or lactose directly into fructose mixtures. A total of 96% of initial liquefied starch was converted into a 49:51 mixture of glucose and fructose, whereas 85% of initial lactose was converted into a 40:31:29 mixture of galactose, glucose, and fructose.

The production of sweetener from corn starch by microbial saccharidases is an important application of enzyme technology in the food industry (2, 7). The current process for high-fructose corn syrup production involves several separate enzymatic steps, including liquefaction by α-amylase, saccharification by glucoamylase, and isomerization by glucose isomerase. These steps require different reaction conditions (i.e., temperature, pH, and metal cofactors) (1, 3). Most industrial saccharidases used in sweetener production require high thermostability and low production costs and are produced by mesophilic microbes. Many studies have been made to prolong enzyme half-life in the reactor by using more-thermostable saccharidases from thermophilic organisms (12, 16, 26) or by using immobilization techniques (14, 25). Also, many efforts have been made in strain improvement for hyperproducers of saccharidases and in selection for the use of catabolite-resistant or constitutive mutants to lower enzyme production costs (21) (E. W. Hafner, U.S. patent 4,551,430, Nov. 1985).

Recently, biochemical properties and regulation of enzyme synthesis for highly thermostable amylolytic enzymes from *Clostridium thermohydrosulfuricum*, including glucogenic amylase, pullulanase, and α-glucosidase activities, have been reported (11–13, 17). *Thermoanaerobacter* strain B6A has these same enzyme activities (20). Although glucose isomerase and β-galactosidase have been detected in thermophilic microorganisms (6, 10, 22), little is known about the biochemical properties and regulation of these enzymes in thermoanaerobic bacteria. In this paper, we report on the regulation and general biochemical properties of glucose isomerase in relation to other saccharidase activities from *Thermoanaerobacter* strain B6A. Notably, growth conditions were discovered that enabled glucose isomerase to be produced in conjunction with glucogenic amylase and β-galactosidase activities. Thus, a single-step process was developed to produce fructose mixtures from starch or lactose with *Thermoanaerobacter* saccharidases.

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MATERIALS AND METHODS

Chemicals and gases. Medium components and all chemicals were reagent grade. Larchwood xylan (lot 113F-0003) was purchased from Sigma Chemical Co. (St. Louis, Mo.). The N_2-CO_2 (95:5) gas was obtained from Linde, Union Carbide Corp. (East Lansing, Mich.) and passed through heated copper columns to remove O_2 prior to use.

Organism. *Thermoanaerobacter* strain B6A isolated from a volcanic hot spring in Thermopolis, Wyo. (23), was obtained from Paul Weimer (USDA Dairy Forage Laboratory, Madison, Wis.) and was maintained by stringent anaerobic culture techniques (27) in CM5 medium (23) containing 0.5% xylan.

Culture conditions and growth measurement. Experimental cultures were grown at 60°C without shaking either in 125-ml serum bottles or in 26-ml anaerobic pressure tubes that contained 50 or 10 ml of TYE medium (27) supplemented with 1% carbon source and an N_2-CO_2 (95:5) gas headspace. Enzyme production time course studies during xylose fermentation were conducted in a Multigen fermentor (New Brunswick Scientific Co., Inc., Edison, N.J.) that contained 500 ml of TYE medium. The fermentor cultures were incubated at 60°C, gassed continuously with N_2-CO_2 (95:5), and mixed at 100 rpm. For the constant-pH experiments, 0.5 N NH_4OH was added during the fermentation by using a feeding pump which was connected to a pH controller. Cells used for crude enzyme extract preparations were cultured in a 14-liter New Brunswick fermentor containing 10 liters of TYE medium (pH 6.8)–1% xylose that was stirred at 60°C under a N_2-CO_2 (95:5) gas stream.

Cell growth in media containing soluble substrates was determined by measuring the optical density of the culture broth at 660 nm. When xylan was present in the culture medium, ethanol concentration in culture supernatant was used to measure growth. Ethanol was measured in acidified samples by gas chromatography by using a flame ionization detector, with N_2 as the carrier gas, and by methods described elsewhere (11). Experimental ethanol production was related to growth by standard optical density versus ethanol concentration plots determined with xylose medium.

Enzyme preparations and assays. For the preparation of the washed cells and culture supernatants, cultures were harvested during late exponential growth phase by centrifugation at 8,000 × *g* for 15 min. The supernatant was decanted, and the cells were washed with double-distilled water and suspended in the appropriate amount of double-distilled water.

Cell extract was prepared by using fermentation-grown cells recovered at the exponential phase (11 h) with a Pellicon cell harvester (Millipore Corp., Bedford, Mass.) and washed with double-distilled water. Wet cell paste (1 g) was suspended either in 50 ml of 20 mM sodium phosphate buffer (pH 7.0) containing 10 mM $MgCl_2$ for the glucose isomerase preparation, in 50 ml of 100 mM sodium acetate buffer (pH 5.5) for the glucogenic amylase preparation, or in 50 ml of 100 mM sodium phosphate buffer (pH 6.0) for the β-galactosidase preparation. The cells were disrupted by passage through a French pressure cell (American Instrument Co., Inc., Silver Spring, Md.) at 20,000 lb/in^2, and the supernatant was collected after centrifugation at 15,000 × *g* for 30 min at 4°C. Protein concentration was determined by the assay method of Lowry et al. (15). Xylanase activities were assayed by measuring the rate of reducing-sugar formation from xylan, whereas amylase was reported as glucose or reducing sugars produced from starch or pullulan (when indicated). The reaction mixture contained 1% substrate in 0.1 M sodium acetate buffer at pH 6.0, 5.5, or 5.0 with pullulan, soluble starch, or xylan as the respective substrates. After a 30-min incubation at 65°C, the reaction mixtures were boiled in a steam bath for 5 min to stop the reaction. The samples were centrifuged, and the amounts of reducing sugars were quantified by the dinitrosalicylic acid method (18). Alternatively, glucose in the supernatant was determined by using either a glucose analyzer (model 27) (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio) or a Sigma enzymatic glucose diagnostic kit. One unit of activity is defined as the amount of enzyme which released 1 μmol of reducing sugar per min under the assay conditions described above, with glucose as a standard for amylase activity and with xylose as a standard for xylanase activity.

Glucose isomerase activity was measured by incubating a reaction mixture that contained 0.8 M glucose (in 0.1 M sodium phosphate or 0.1 M MOPS [morpholinepropanesulfonic acid] buffer [pH 7.0]), 10 mM $MgSO_4$, 1 mM $CoCl_2$, and the enzyme. After incubation at 65°C for 30 min, the amount of fructose formed was estimated by the cysteine carbazole sulfuric acid method (8). β-Galactosidase activity was assayed by measuring the amount of phenoxide ion liberated from *ortho*-nitrophenyl-β-D-galactopyranoside at 420 nm after an equal volume of 1 M Na_2CO_3 was added to the reaction mixture. The reaction mixture contained 10 mM KCl, 1 mM $MgSO_4$, 5 mM 2-mercaptoethanol, and 2.7 mM *ortho*-nitrophenyl-β-D-galactopyranoside with the enzyme in 100 mM sodium phosphate buffer (pH 6.0) (19) and was incubated at 60°C for 20 min.

One unit of amylase, glucose isomerase, or β-galactosidase activity is defined as the amount of enzyme required to produce 1 μmol of glucose, fructose, or phenoxide ion per min, respectively, under the assay conditions described above. All the enzyme activities were determined at points at which product formation was linear with time.

TABLE 1. Cellular location of saccharidase activities in *Thermoanaerobacter* strain B6A[a]

Saccharidase	Carbon substrate	Final growth (OD_{660})[b]	Activity (U/ml)	
			Supernatant	Washed cell suspension[c]
Glucose isomerase	Xylose	1.15	0.00	0.21
β-Galactosidase	Lactose	0.98	0.00	0.14
Amylase	Starch	1.07	0.04	0.19
	Pullulan	1.07	0.73	0.43
Xylanase[d]	Xylan	0.80	0.47	0.18

[a] Cultures were grown in TYE medium with 1.0% carbon substrate at 60°C.
[b] OD_{660}, Optical density at 660 nm.
[c] Cells were suspended in 1/10 culture volume of double-distilled water, and activity was converted relative to the original culture volume.
[d] Because of the interference of xylan optical absorbance, cell growth was calculated from ethanol production in relation to a standard growth curve.

Single-step conversion of starch or lactose to fructose. Starch (i.e., maltodextrin [dextrose equivalence, or D.E., 10] and soluble starch) or lactose, in 0.1 M sodium phosphate buffer containing 10 mM $MgSO_4$ and 1 mM $CoCl_2$, was incubated with cell extracts prepared from xylose-grown cells at various temperatures (60 to 70°C) and pHs (6.0 to 6.8). Samples were taken during time course experiments performed with 50-ml serum vials containing 5 ml of reaction mixture that were sealed with rubber bungs and shaken at 100 rpm in a New Brunswick water bath shaker. Samples withdrawn from the reaction mixture were boiled in a steam bath for 5 min and centrifuged before sugar analysis. Quan-

TABLE 2. Effect of growth substrate on saccharidase synthesis in *Thermoanaerobacter* strain B6A

Growth substrate[a]	Final growth (OD_{660})[b]	Sp act (U/mg of cell protein)[c]		
		Glucose isomerase	Glucogenic amylase	β-Galactosidase
Starch	1.36	0.00	0.61	0.41
Lactose	0.83	0.00	0.54	0.46
Maltose	0.75	0.00	0.43	0.38
Cellobiose	1.23	0.00	0.59	0.44
Glucose	1.60	0.00	0.42	0.31
Xylose	1.47	0.62	0.60	0.47
Xylan	0.68	0.39	0.58	0.33
Xylose + glucose	1.46	0.36	0.48	0.34
Starch + glucose	1.24	0.00	0.46	0.35
Lactose + glucose	1.00	0.00	0.50	0.40
Xylose + 2-deoxy-Glc	0.80	0.40	0.38	0.33
Starch + 2-deoxy-Glc	0.95	0.00	0.48	0.42
Lactose + 2-deoxy-Glc	0.46	0.00	0.30	0.33
Glucose + 2-deoxy-Glc	0.98	0.00	0.33	0.25
2-Deoxy-Glc	0.11	ND	ND	ND
None	0.10	ND	ND	ND

[a] Cultures were grown on TYE medium containing 0.5% main substrate with or without 0.3% supplementing glucose or 0.1% 2-deoxyglucose (2-deoxy-Glc).
[b] Optical density was measured at the early stationary growth phase. OD_{660}, Optical density at 660 nm.
[c] ND, Not determined.

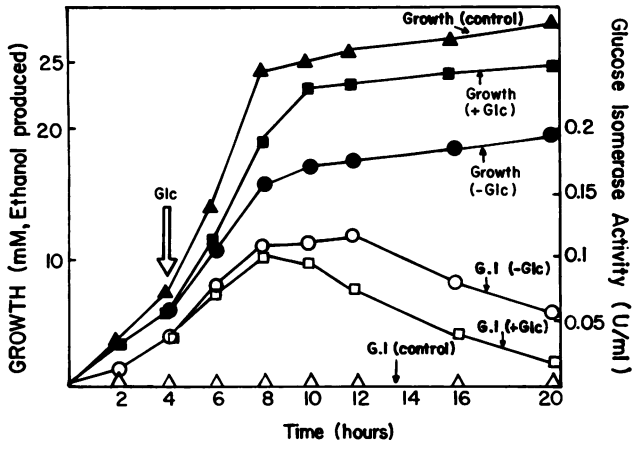

FIG. 1. Effect of glucose (Glc) addition on glucose isomerase (G.I) synthesis during xylan fermentation. Cultures were grown on CM5 medium with 0.5% xylan at 60°C, and 0.3% glucose was added during the middle of exponential growth phase (▽). Ethanol concentration in culture broth was measured as a growth indicator, and 25 mM ethanol represents an optimal density of 1.0 at 660 nm in a standard growth curve. Closed symbols represent culture growth, and open symbols represent enzyme activity. Culture conditions indicated by symbols are as follows: triangles, glucose only without xylan (control); circles, xylan only without glucose addition; squares, xylan with glucose addition.

titative and qualitative analyses were performed by high-pressure liquid chromatography, as described previously (20). Aminex HPX-87C and HPX-87P columns (Bio-Rad Laboratories, Richmond, Calif.) were used for analysis of starch and lactose conversion, respectively. The columns were maintained at 85°C, and the sugars were eluted with water. Glucose, fructose, galactose, lactose, or maltose (1% [wt/vol]) was used as a standard for determination of each saccharide concentration in the samples.

RESULTS

Locations and types of saccharidase activities. *Thermoanaerobacter* spp. ferment starch and hemicellulose but not cellulose (22a, 24). In preliminary experiments, halos appeared around colonies grown either on Remazol Brilliant Blue-xylan agar plates or starch agar plates stained with iodine, indicating that *Thermoanaerobacter* strain B6A produced amylase and xylanase activities.

Experiments were initiated to determine the kinds of saccharidases obtained and their cellular locations when *Thermoanaerobacter* strain B6A was grown on different saccharides as carbon and energy sources. *Thermoanaerobacter* strain B6A produced glucose isomerase and β-galactosidase, as well as glucogenic amylase and xylanase activities (Table 1). Glucogenic amylase activity was extracellular and cell bound when starch or pullulan was used as a substrate. It was not possible to discern whether the glucogenic amylase represented a mixture of α-amylase and glucoamylase or of amylopullulanase and α-glucosidase by the assays used. However, about 70 to 80% of xylanase activity was excreted into the medium, and the remaining activity was cell-associated in xylan-grown cultures. On the other hand, glucose isomerase and β-galactosidase activities were totally cell bound.

Regulation of glucose isomerase production. Experiments were conducted to determine the regulation of glucose

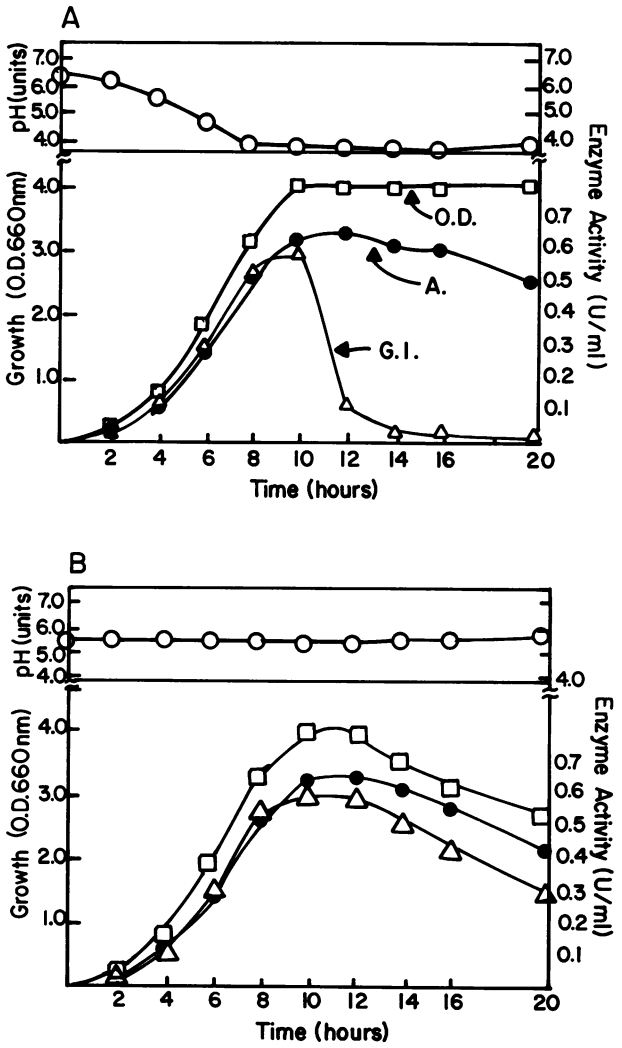

FIG. 2. Effect of pH control on saccharidase production during xylose fermentation. Cultures were grown in 500 ml of TYE medium containing 2% xylose in a Multigen fermentor at 60°C without pH control (A) or with pH controlled at 5.5 by feeding with 0.5 N NH_4OH (B). G.I., Glucose isomerase; A., amylase; O.D., optical density.

isomerase synthesis in relation to amylase and β-galactosidase production. Glucose isomerase was produced only when either xylose or xylan was present as an inducer in the culture medium (Table 2). Notably, glucogenic amylase and β-galactosidase were produced constitutively on the wide range of growth substrates tested. The specific activities of glucogenic amylases from starch-grown cultures and of β-galactosidase from lactose-grown cultures were equal to those obtained from xylose-grown cultures. The synthesis of glucogenic amylase and glucose isomerase was partially expressed by the presence of 0.1% 2-deoxyglucose or 0.3% glucose in culture media containing 0.5% starch, xylose, or lactose, whereas the synthesis of β-galactosidase was not subjected to catabolite repression.

Enzyme de novo synthesis by xylose induction was confirmed by comparison of sodium dodecyl sulfate electrophoresis gels of cell extract total proteins from either xylose- or glucose-grown *Thermoanaerobacter* cells. The low fold purification of glucose isomerase from *Thermoanaerobacter*

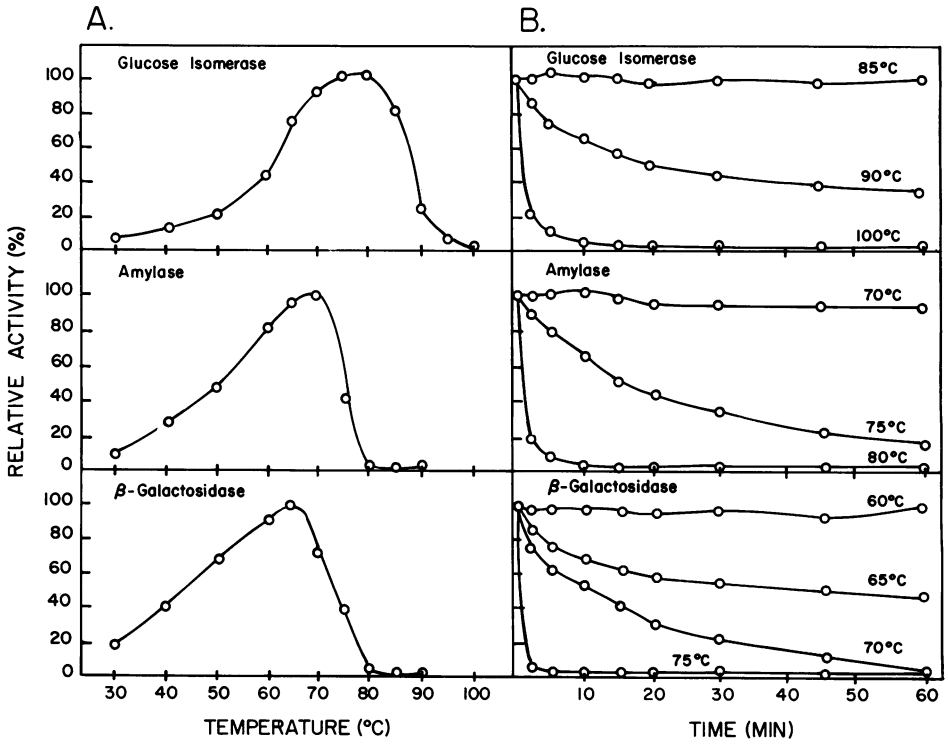

FIG. 3. Comparison of temperature optima for activities (A) and stabilities (B) of glucose isomerase, amylase, and β-galactosidase. Enzymes were assayed with cell extract from xylose-grown cells. A 100% activity value corresponds to 0.60, 0.58, and 0.46 U/mg for glucose isomerase, amylase, and β-galactosidase, respectively. Cell extracts in 50 mM sodium phosphate buffer (pH 7.0), 100 mM sodium acetate buffer (pH 5.5), and 100 mM sodium phosphate buffer (pH 6.0) for glucose isomerase, amylase, and β-galactosidase, respectively, were preincubated at the indicated temperatures, prior to the assay for residual enzyme activities.

strain B6A indicated that the enzyme is the major protein in xylose-grown cells (approximately 10% of cell extract total protein; C. Lee and J. G. Zeikus, Biochem. J., in press). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of xylose-grown cell extract total protein demonstrated an extra band corresponding to the subunit molecular mass (50 kilodaltons) of the purified enzyme, whereas the glucose-grown cell extract lacks the band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

In order to assess the mechanism of glucose isomerase synthesis, experiments were performed in which glucose was added during xylan fermentation time courses and glucose isomerase activity was periodically assayed. Because of the turbidity of xylan, cell growth was monitored by measuring the concentration of ethanol in the culture broth. Ethanol was one of the major end products of *Thermoanaerobacter* fermentations (23), and its exponential production during growth is proportional to cell density as measured by A_{660} during xylose fermentation. Synthesis of glucose isomerase during exponential growth was not totally catabolite repressed by glucose (Fig. 1), while xylanase production completely ceased after glucose addition during exponential growth on xylan (data not shown). In addition, these results indicated that synthesis of glucose isomerase is induced by xylan, since the culture containing only glucose (control) as a growth substrate did not produce glucose isomerase during glucose fermentation.

Figure 2 compares glucose isomerase and amylase activity levels during xylose fermentations with (Fig. 2A) and without (Fig. 2B) a controlled pH of 5.5. Syntheses of both enzyme activities were tightly growth coupled under either condition. Glucose isomerase activity, however, decreased rapidly in the stationary-phase cultures that were not maintained at pH 5.5. Amylase and β-galactosidase (data not shown) were quite stable throughout the stationary phase. Consequently, pH control at 5.5 was used in xylose fermentations to obtain *Thermoanaerobacter* enzyme preparations that contained active levels of amylase, glucose isomerase, and β-galactosidase.

Physicochemical properties of saccharidases. The temperature and pH activity and stability profiles for glucose isomerase were compared with those for the β-galactosidase and the amylase activities in *Thermoanaerobacter* extracts prepared from washed whole cells grown on xylose. Glucose isomerase, amylase, and β-galactosidase displayed apparent temperature optima for activity at between 75 and 80°C, at 70°C, and at 65°C, respectively (Fig. 3A). The effect of temperature on the stability of these enzyme activities is shown in Fig. 3B. Cell extracts for enzyme assays were preincubated prior to measurement of the residual activities in 100 mM sodium phosphate buffer (pH 7.0) containing 10 mM $MgSO_4$ and 1 mM $CoCl_2$ for glucose isomerase, in 100 mM sodium acetate buffer (pH 5.5) for amylase, and in 100 mM sodium acetate buffer (pH 6.0) for β-galactosidase. Under these conditions, glucose isomerase, amylase, and β-galactosidase were stable for up to 60 min at 85, 70, and 60°C, respectively.

Figure 4 illustrates the dependence of saccharidase activities on pH. Glucose isomerase displayed a broad pH range for activity, from 5.5 to 9.0, with an apparent pH optimum of 7.0 to 7.5. During the enzyme assay at a high pH, chemical isomerization of glucose to fructose by alkali caused a high

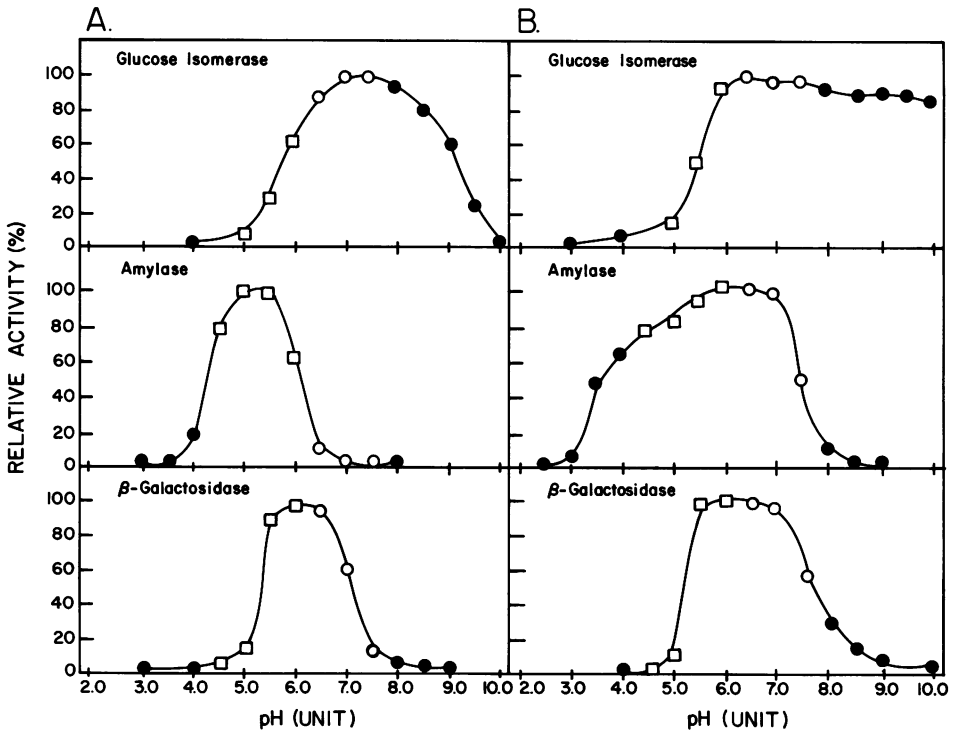

FIG. 4. Comparison of pH optima for activities (A) and stabilities (B) of glucose isomerase, amylase, and β-galactosidase. Enzyme activities were assayed with cell extracts in 100 mM glycylglycine hydrochloride (●), sodium acetate (□), and sodium phosphate (○) buffers. Residual activity (B) was measured after treatment at 60°C for 1 h.

background reading, and the amount of product formed by enzymatic isomerization was calculated by subtracting the control value from the total experimental value. The amylase and β-galactosidase activities displayed a narrower pH range for activity than the glucose isomerase did, and the apparent pH optima were between 5.0 and 5.5 and 6.0 and 6.5, respectively. The stability of these enzymes in relation to pH was examined by measuring residual activities after incubation for 1 h at 60°C at different pH values (Fig. 4B). Under these assay conditions, glucose isomerase, amylase, and β-galactosidase activities were stable at pHs 6.0 to 7.5, 5.5 to 7.0, and 5.5 to 7.0, respectively. All the saccharidase activities were stable, and they displayed at least 60% of maximal activity at pH 6.0.

Single-step conversion of starch, maltodextrin, or lactose into fructose syrups. The feasibility of using *Thermoanaerobacter* saccharidases in a single-step enzymatic process to produce fructose syrups from liquefied starch or whey lactose was examined by using a crude extract of xylose-grown *Thermoanaerobacter* cells which contained environmentally compatible saccharidases. Figure 5 depicts a typical time course for production of monosaccharides from starch (maltodextran [D.E., 10] and soluble starch were used in this study) or lactose by using a single-step process with *Thermoanaerobacter* saccharidases at 60°C. More than 90% of the starch or lactose was hydrolyzed into glucose mixtures within 4 h, while the isomerization of glucose into fructose approached equilibrium by the end of the incubation period (20 to 48 h). An unknown oligosaccharide, putatively allolactose, was produced at the early stages of lactose conversion and was slowly degraded into monomers. Allolactose is a reversion product formed by β-galactosidase activity.

Table 3 compares the influences of specific reaction condition changes on the final saccharide product ratio achieved during single-step conversion of starch and lactose by *Thermoanaerobacter* saccharidases. Although equivalent results were obtained from maltodextrin and soluble starch at a 5% (wt/vol) initial concentration, an attempt to use a high concentration of soluble starch had failed because of its high viscosity at 65°C. During enzymatic starch conversion, a higher sweetener concentration ratio for fructose to glucose and a higher yield of starch conversion into monosaccharides were achieved at 70 than at 60°C. The highest sweetener conversion (51:49, fructose to glucose) was achieved at pH 6.0 and 70°C from 5% (wt/vol) maltodextrin, with a final conversion yield of 96%. Enzymatic hydrolysis of 5% lactose at pH 6.8 versus 6.4 lowered the total conversion yield of lactose but did not affect the final product ratio between galactose, glucose, and fructose. Enzymatic hydrolysis of 20% lactose at 65 versus 60°C lowered both the lactose conversion yield and the galactose concentration in the final products.

DISCUSSION

To our best knowledge, these findings represent the first reported studies on the general physicochemical properties and regulation of glucose isomerase from a thermophilic microorganism. Although thermophilicity is required in industrial glucose isomerase, thermostable enzymes produced by mesophilic bacteria have been examined as principal industrial sources (4). Furthermore, these studies represent the first demonstration that a saccharidase mixture produced by a single microorganism can be used to directly process starch or lactose into a fructose sweetener. Several previous

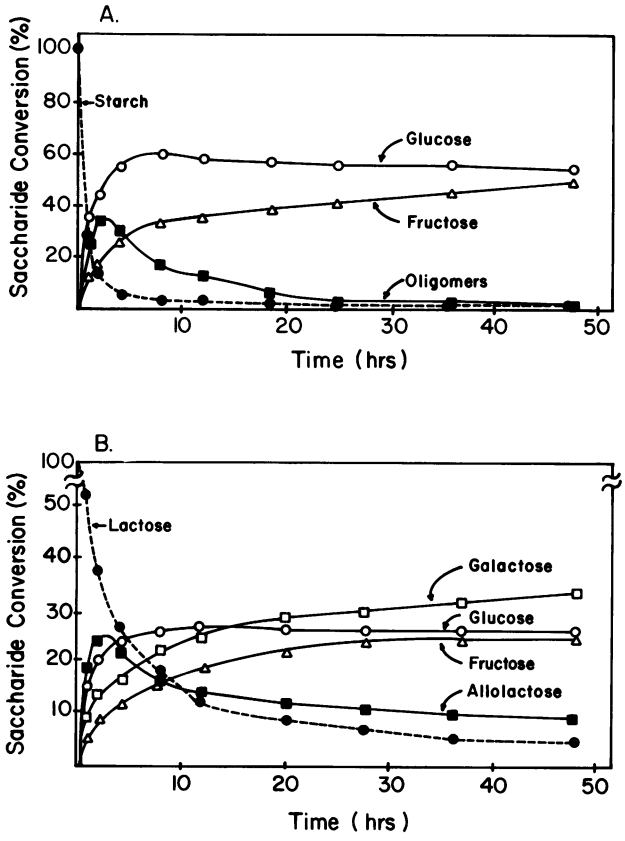

FIG. 5. Single-step conversion of starch (A) and lactose (B) into a fructose mixture by a *Thermoanaerobacter* enzyme preparation. A 5% solution of maltodextrin (A) and a 5% solution of lactose (B) were incubated with cell extracts at 60°C in 100 mM sodium phosphate buffer at pH 6.0 and 6.5, respectively. The oligomers curve (A) indicates saccharides that were smaller than maltodextrin (D.E., 10) as determined from the high-pressure liquid chromatogram. The major component of the oligomers was maltose.

studies have demonstrated that glucoamylase and glucose isomerase mixtures from different microbes can process oligodextrins into fructose syrups with marginal success, because of environmental incompatibilities (e.g., optimal pH and thermostability) between the enzymes (P. Guillaume and P. Walon, U.S. patent 4,009,074, Feb. 1977; R. O. Horwath and R. M. Irbe, U.S. patent 4,605,619, Aug. 1986).

The constitutive and catabolite repression-resistant synthesis of amylase activities in *Thermoanaerobacter* strain B6A is distinctive from the inducible and glucose catabolite-repressed synthesis of amylase activities reported for *Clostridium thermosulfurogenes* and *C. thermohydrosulfuricum* (11–13). However, the mechanism of or reasons for the partial catabolite repression by glucose of synthesis of glucose isomerase and amylase in *Thermoanaerobacter* strain B6A are not presently understood. Thermostable β-galactosidase in aerobic *Thermus* species has been previously reported, and enzyme synthesis is regulated by induction and glucose catabolite repression (22). On the other hand, the β-galactosidase of *Thermoanaerobacter* strain B6A is constitutive and non-catabolite repressible.

As expected of enzymes from thermophiles, the apparent optimum temperatures for glucose isomerase (75 to 80°C), glucogenic amylases (70°C), and β-galactosidase (65°C) in crude extracts were relatively high. The reported temperature optima of glucose isomerases from other microorganisms vary with enzyme sources, ranging from 45 to 90°C (5). The temperature optima of glucogenic amylase activities reported for *C. thermohydrosulfuricum* (R. O. Horwath and R. M. Irbe, U.S. patent 4,605,619, Aug. 1986) were similar to those for *Thermoanaerobacter* spp. The temperature optima of β-galactosidases range from 35 to the high of 80°C reported for *Thermus* strains (6, 9). Analysis of *Thermoanaerobacter* saccharidase thermostabilities showed that more than 95% of glucose isomerase, glucogenic amylases, and β-galactosidase was retained after 60 min of incubation at 85, 70, and 60°C, respectively.

TABLE 3. Effect of reaction conditions on final monosaccharide product ratio during a starch or lactose conversion process with a *Thermoanaerobacter* enzyme preparation[a]

Substrate	Concn (% [wt/vol])	pH	Temp (°C)	Conversion yield (%)[b]	Final product ratio[c]
Liquefied starch (maltodextrin [D.E., 10])	5	6.0	60	92	53:47
	5	6.0	65	93	51:49
	5	6.0	70	96	49:51
	20	6.0	60	84	58:42
	20	6.0	70	91	51:49
Soluble starch	5	6.0	60	90	55:45
Lactose	5	6.4	60	85	40:31:29
	5	6.8	60	81	40:31:29
	5	6.8	65	76	35:33:32
	20	6.4	60	75	36:33:31

[a] Cell extract (5 and 10 mg/ml of reaction volume) prepared from xylose-grown cells was added into the reaction mixture containing 5 and 20% substrate, respectively.
[b] Total amount of monosaccharides hydrolyzed from the substrate.
[c] Relative ratio between monosaccharides was measured after 48 h of incubation under the given reaction conditions. Ratios are of glucose to fructose with the two starch substrates and of galactose to glucose to fructose with the lactose substrate.

The pH optima for glucose isomerase, glucoamylase, and β-galactosidase are generally between 7.0 and 8.5, 4.5 and 5.5, and 4.5 and 7.5, respectively (5, 9, 12). Although the apparent pH optima for *Thermoanaerobacter* glucose isomerase, β-galactosidase, and glucogenic amylase activities fall in these ranges, all three enzymes were stable and active at pH 6.0 to 6.5 and 60°C. Among the saccharidases studied, glucose isomerase displayed the least acid stability and activity. In a separate report (Lee and Zeikus, in press), we purified the *Thermoanaerobacter* glucose isomerase to homogeneity and showed that the 200,000-M_w tetramer has pH and thermal properties identical to those reported here. Therefore, the rapid destruction of glucose isomerase activity in the stationary growth phase during xylose fermentation without pH control is presumably due to enzyme instability at pH values lower than 5.0.

The differences in pH optima and temperature stabilities of these saccharidases have been a major problem in demonstrating the feasibility of a single-step process for conversion of starch or lactose to fructose mixtures. The present study indicates that saccharidases simultaneously produced by *Thermoanaerobacter* strain B6A are environmentally compatible at pH 6.0 and 60°C and can be used coordinately in a single-step conversion process for production of fructose sweetener from starch or milk-derived substrates.

The final ratios between glucose and fructose during the single-step conversion process from starch at various temperatures were very similar to the theoretical values at

equilibrium of the glucose isomerization reaction (1, 14). The maximum percentage of starch conversion from liquefied starch during this process was also very similar to the values (94 to 96% of glucose converted) obtained after starch saccharification in the multistep commercial processes (1). The *Thermoanaerobacter* saccharidase preparation could be operated at 70°C to achieve higher equilibrium concentrations of fructose and faster reaction rates than those obtained in industrial processes. If the process is refined, a single-step starch hydrolysis process at a high temperature (>60°C) and a low pH (<pH 6.0) would lower costs for producing high-fructose sweetener from corn starch. The *Thermoanaerobacter* saccharidases provide a first-stage feasibility model for development of such a process. Use of *Thermoanaerobacter* saccharidases can also directly enhance sweetness in milk-containing products by converting lactose to fructose during the manufacturing process. Hydrolysis of lactose in these products can potentially solve the digestion problems of lactase-deficient adults, improve the solubility during ice cream and yogurt production, and require less additive sugar in dairy products. Further studies on long-term stability of the immobilized enzymes and its food safety are required before any commercial value for *Thermoanaerobacter* saccharidases can be proposed.

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