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Glucose Fermentation Pathway of *Thermoanaerobium brockii*

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Thermoanaerobium brockii was shown to catabolize glucose via the Embden-Meyerhof-Parnas pathway into ethanol, acetic acid, H_2-CO_2, and lactic acid. Radioactive tracer studies, employing specifically labeled [^{14}C]glucose, demonstrated significant fermentation of $^{14}CO_2$ from C-3 and C-4 of the substrate exclusively. All extracts contained sufficient levels of activity (expressed in micromoles per minute per milligram of protein at 40°C) to assign a catabolic role for the following enzymes: glucokinase, 0.40; fructose-1,6-diphosphate aldolase, 0.23; glyceraldehyde-3-phosphate dehydrogenase, 1.73; pyruvate kinase, 0.36; lactate dehydrogenase (fructose-1,6-diphosphate activated), 0.55; pyruvate dehydrogenase (coenzyme A acetylating), 0.53; hydrogenase, 3.3; phosphotransacetylase, 0.55; acetaldehyde dehydrogenase (coenzyme A acetylating), 0.15; ethanol dehydrogenase, 1.57; and acetate kinase, 1.50. All pyridine nucleotide-linked oxidoreductases examined were specific for nicotinamide adenine dinucleotide, except ethanol dehydrogenase which displayed both nicotinamide adenine dinucleotide- and nicotinamide adenine dinucleotide phosphate-linked activities. Fermentation product balances and cell growth yields supported the glucose catabolic pathway described. Representative balanced end product yields (in moles per mole of glucose fermented) were: ethanol, 0.94; L-lactate, 0.84; acetate, 0.20; CO_2, 1.31; and H_2, 0.50. Growth yields of 16.4 g of cells per mole of glucose were demonstrated. Both growth and end product yields varied significantly in accordance with the specific medium composition and incubation time.

By and large, obligate thermophiles have been examined in the past in order to understand the mechanisms for adaptation of life at high temperatures (3, 7, 12). Thermophilic bacteria appear to have a limited species composition, but also appear to possess the major nutritional categories (i.e., chemolithotroph, chemoorganotroph, photoorganotroph, etc.) and metabolize the same substrates as mesophiles (13, 23). As a consequence of growth at high temperature and unique macromolecular properties, thermophilic bacteria can possess high metabolic rates, physically (i.e., thermal resistance) and chemically stable enzymes, and higher end product-to-cell ratios than in metabolically similar mesophilic species (23). These features of thermophilic systems are of tremendous importance for the development of novel biotechnology (23).

Considerable effort has been invested in the bioconversion of renewable resources into fuels, chemical feedstocks (4, 6, 15, 18, 20), or both. Ethanol production by yeasts is limited to the metabolism of relatively expensive sugars (i.e., sucrose, glucose, etc.). Thus, anaerobic bacteria that actively ferment biopolymers (e.g., starch or cellulose) may offer advantages. Ethanol-producing thermophiles with high metabolic activities are especially interesting because they offer the potential of end product recovery during fermentation via culture heat-driven reduced pressure distillation.

Thermoanaerobium brockii has recently been isolated from volcanic features (25). This species actively ferments saccharides, including fermenting starch to ethanol. Like other described thermophilic anaerobic species (8, 9, 19), *T. brockii* fermentations (25) yield ethanol and additional end products (lactic acid, H_2-CO_2, and acetic acid). Understanding the catabolism of ethanol-producing saccharolytic thermophiles should provide useful insights for mutants and strain selection and optimal culture conditions for maximal end product formation. We report here initial studies on saccharide catabolism of this unique species.

MATERIALS AND METHODS

Chemicals and enzymes. All chemicals were reagent grade. Enzymes and coenzymes were obtained from Sigma Chemical Co. (St. Louis, Mo.), [1-^{14}C]-glucose, [6-^{14}C]glucose, [3,4-^{14}C]glucose, and [*U*-^{14}C]glucose were purchased from Amersham/Searle (Arlington Heights, Ill.). Purified *Clostridium pasteurianum* ferredoxin was a gift of L. Mortensen, Purdue University, West Lafayette, Ind. Methyl viologen (*N,N'*dimethyl-4,4'dipyridyldihydrochloride) was purchased from Serva (Heidelberg, West Germany). N-

and He gases were purchased from Matheson (Chicago, Ill.). Traces of O_2 were removed from gases by passage over heated copper filings.

Organism and growth conditions. *T. brockii* neotype strain HTD4 was cultured as previously described (25).

Basal medium (LPBM) contained per liter of distilled water: NH_4Cl, 1.0 g; $MgCl_2 \cdot 6H_2O$, 0.2 g; KH_2PO_4, 0.30 g; $Na_2HPO_4 \cdot 7H_2O$, 2.0 g; trace mineral solution, 9 ml; 2.5% $FeSO_4$, 0.03 ml; 0.2% resazurin, 1 ml; vitamin solution, 5 ml; and 20 ml of 2.5% Na_2S was added before autoclaving. Trace mineral solution contained per liter of distilled water: 12.8 g of nitrilotriacetic acid neutralized to pH 6.5 with KOH; $FeSO_4 \cdot 7H_2O$, 0.1 g; $MnCl_2 \cdot 4H_2O$, 0.1 g; $CoCl_2 \cdot 6H_2O$, 0.17 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $ZnCl_2$, 0.1 g; $CuCl_2$, 0.02 g; H_3BO_3, 0.01 g; $NaMoO_4 \cdot 2H_2O$, 0.01 g; NaCl, 1.0; and Na_2SeO_3, 0.02 g. The culture medium gas phase contained N_2. The pH of the medium was 7.2 after autoclaving. Buffered basal medium contained LPBM and an additional 20 mM potassium phosphate buffer of the same pH. Basal media used were supplemented by 0.05 or 0.1% yeast extract and 0.15 to 0.5% glucose as indicated in the text. Cells used for growth and metabolism studies were cultured in 24-ml anaerobic tubes (18 by 142 mm) from Bellco (Vineland, N.J.) that contained 10 ml of medium and were sealed with no. 1 neoprene stoppers. Test tube cultures were incubated at 65°C without shaking. Cells used for analysis of enzyme activities were grown in 14-liter New Brunswick Microferm fermentors that contained 12 liters of TYEG medium (i.e., LPBM supplemented with 0.3% yeast extract, 0.5% glucose, and 1% tryptone). Fermentor cultures were maintained at 65°C with constant stirring (100 rpm) and continuous N_2 gassing (50 cm^3/min). Cells were harvested in the late exponential growth phase and collected by centrifugation at 35,000 × *g* in a Sorvall RC-5 centrifuge (Du Pont Instruments) equipped with a KSB continuous flow system.

Metabolic characterization. All growth and metabolic experiments employed duplicate or more anaerobic culture tubes, and individual experiments were duplicated or triplicated. Growth was determined by measuring the increase in turbidity at 540 nm or by cell dry weight. Optical density was quantified directly by insertion of the anaerobic culture tubes into a Spectronic 20 (Bausch & Lomb, Inc., Rochester, N.Y.) spectrophotometer. Cell dry weight was determined by filtration of the fermentation broth through a 0.45-μm filter (Millipore Corp., Bedford, Mass.) and followed by drying overnight at 65°C to a constant weight. Glucose was determined with Statzyme reagent (Worthington, Chicago, Ill.). Fermentation products formed during growth were quantified by gas chromatography and by enzymatic analysis. Gases were analyzed with the procedures described by Nelson and Zeikus (14). Organic alcohols and acids were determined as described by Zeikus et al. (25). L-Lactic acid was determined with a standard enzyme assay (1).

Cells were grown in TYEG or buffered LPBM medium for the radioactive tracer determination of the positions of glucose metabolized to CO_2. [^{14}C]glucose was diluted in 3% glucose and autoclaved separately before the addition to test tube cultures. The specific activity of $^{14}CO_2$ produced during growth was determined by the gas chromatograph-gas proportional counting procedures previously described (14).

Preparation of cell extracts. Anaerobic conditions were maintained throughout the entire procedure, and all manipulations were performed under a helium atmosphere at 4°C. Cells (2 g, wet weight) were placed in a 15-ml Corex tube that contained 8 ml of 25 mM Tris-hydrochloride (pH 7.4) with 3M dithiothreitol (DTT) and 5 μg of DNAse. After thorough mixing, the suspension was passed through a French pressure cell at 48,300 kg/m^2. The lysate was collected in a centrifuge tube, sealed with a flanged rubber bung, and centrifuged at 10,000 × *g* for 30 min. The supernatant was removed with a glass syringe and injected into glass vials that contained helium gas and were sealed with soft rubber stoppers. Extracts were used immediately or stored at −20°C. The protein content of extracts was determined by the method of Bradford (2) by using Bio-Rad Laboratories, (Rockville Centre, N.Y.) reagents.

In experiments designed to assay ethanol dehydrogenase during the growth phase, cell suspensions were withdrawn from cultures at various time intervals, placed into shell vials (Scientific Products, catalog no. B7810-4X), sealed with rubber stoppers, and treated by shaking for 30 min with toluene (2%, vol/vol). The toluene-treated cell suspensions were used directly in enzyme assays.

Enzyme assays. All assays were performed at 40°C (unless specified in the text) under anaerobic conditions as described by Zeikus et al. (24). All activities were measured by modifications of standard assay methods (1, 24). Determinations of specific activities were made in a range where linearity with protein concentration was established. A unit of enzyme activity represents the amount of enzyme catalyzing the conversion of 1 μmol of substrate per min into specific products. The following describes the concentration of components in the reaction mixtures (1-ml total volume) used for analysis of specific enzymes: glucokinase (EC 2.7.1.2), 0.1 M Tris-hydrochloride (pH 7.5), 60 mM $MgCl_2$, 1 mM DTT, 0.5 mM NADP, 2 mM ATP, 15 mM glucose, and 2.0 U of glucose phosphate dehydrogenase; glucose phosphomutase (EC 2.7.5.1), 0.1 M Tris-hydrochloride (pH 7.5), 60 mM $MgCl_2$, 1 mM DTT, 0.5 mM NADP, 5 mM glucose-1-phosphate, 0.01 mM glucose-1,6-diphosphate, and 2.0 U of glucose-6-phosphate dehydrogenase; glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 0.1 M Tris-hydrochloride (pH 7.5), 2.5 mM $MnCl_2$ or 6 mM $MgCl_2$, 2 mM glucose-6-phosphate, 1 mM DTT, and 1.0 mM NAD(P); gluconate-6-phosphate dehydrogenase (EC 1.1.1.43), as described above but with gluconate-6-phosphate replacing glucose-6-phosphate; FDP aldolase (EC 4.1.2.13), 0.05 M Tris-hydrochloride (pH 7.5), 0.1 mM cysteine-hydrochloride, 0.1 M potassium acetate, 2 mM FDP, 0.7 mM $CoCl_2$, 0.25 mM NADH, 20 U of triosephosphate isomerase, and 2.0 U of glycerol-3-phosphate dehydrogenase; pyruvate kinase (EC 2.7.1.40), 0.1 M Tris-hydrochloride (pH 7.5), 5 mM ADP, 1 mM DTT, 10 mM KCl, 15 mM $MgCl_2$, 0.5 mM phosphoenol pyruvate, 0.25 mM NADH, and 10 U of lactate dehydrogenase; glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), 0.1 mM Tricine-hydro-

chloride (pH 8.1), 5 mM potassium phosphate, 20 mM neutralized sodium arsenate, 2 mM FDP, 2 mM DTT, 1 mM NAD, and 1.0 U aldolase; pyruvate dehydrogenase (coenzyme A [CoA] acetylating) (EC 1.2.7.1), 0.1 M Tris-acetate (pH 7.8), 5 mM pyruvate, 0.1 mM CoA, 7 mM sodium arsenate, 2 mM methyl viologen; lactate dehydrogenase (FDP activated) (EC 1.1.2.3), 0.1 M imidazole-hydrochloride buffer (pH 6.2), 0.25 mM NADH, 10 mM pyruvate, 1 mM FDP; acetaldehyde dehydrogenase (CoA acetylating, EC 1.2.1.10), 0.1 M Tris-hydrochloride (pH 7.2), 1 mM DTT, 0.1 mM CoA, 7 mM sodium arsenate, 0.5 mM NAD, 10 mM acetaldehyde, and 0.5 U of phosphotransacetylase; hydrogenase (EC 1.12.1.1), 0.1 M Tris-acetate (pH 7.8), 2 mM methyl viologen, 2 mM DTT and 1 atmosphere (ca. 100 KPa) of H_2 gas; phosphotransacetylase (EC 2.3.1.8), 0.1 M Tris-acetate (pH 7.8), 5 mM pyruvate, 2 mM methyl viologen, and 0.1 mM CoA (7 mM sodium arsenate was added after initial pyruvate dehydrogenase activity stops); acetate kinase (EC 2.7.2.1), 0.1 M Tris-hydrochloride (pH 7.2), 3 mM $MgCl_2$, 2 mM glucose, 0.5 mM NADP, 1 U each of hexokinase and glucose-6-phosphate dehydrogenase, 1 mM ADP, and 4 mM acetylphosphate; myokinase (EC 2.7.4.3), as described above but with acetylphosphate omitted; ethanol dehydrogenase (EC 1.1.1.12), 0.1 M Tris-hydrochloride (pH 7.8), 2 mM DTT, 1 mM NAD(P)H, and 5 mM acetaldehyde.

Pyridine nucleotide oxidation or reduction reactions were measured at 334 mM (ε_{334} = 6.10 mM^{-1} cm^{-1}) with an Eppendorf recording spectrophotometer. Methyl viologen reduction was measured spectrophotometrically at 578 mM (ε_{578} = 9.78 mM^{-1} cm^{-1}).

RESULTS

Glucose fermentation products. Typical end product formation kinetics during glucose fermentation by *T. brockii* are shown in Fig. 1. Ethanol, L-lactate, acetate, hydrogen, and carbon dioxide production paralleled growth and glucose consumption. L-Lactate but not D-lactate was produced because similar values for lactic acid were obtained by both gas chromatography and the L-lactate-specific enzyme assay. Other products, such as butyrate, succinate, formate, or other alcohols, were not detected with these analytical procedures. The same fermentation product ratios were formed at 65 or 45°C.

A fermentation balance typical for glucose metabolism by *T. brockii* and determined after 9 h of growth is shown in Table 1. Yeast extract was included in the media at final concentrations of 0.05 or 0.10%. This was sufficient to support growth (25) and allowed reasonable carbon recovery from glucose fermentation. Product formation continued after measurable increase in growth had ceased (10 h). At the end of 20 h of incubation in 0.05% yeast extract medium, 211 μmol of ethanol, 170 μmol of L-lactate, and 25 μmol of acetate were formed.

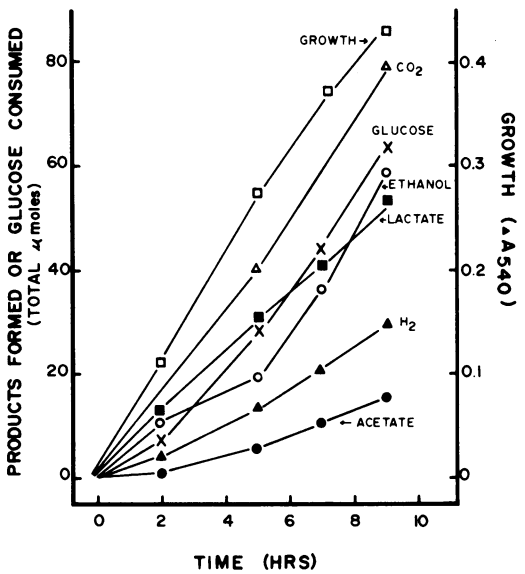

FIG. 1. *The relation of end product formation to growth of T. brockii on glucose. Anaerobic culture tubes were incubated at 65°C and contained LPBM medium supplemented with 0.05% yeast extract and 0.35% glucose. Results are given as total micromoles formed per tube at the indicated times.*

TABLE 1. *Glucose fermentation products of T. brockii*

Product	Amt produced[a]	
	0.05% Yeast extract	0.1% Yeast extract
Ethanol	60	51
L-Lactate	54	120
Acetic acid	13	14
CO_2	84	63
H_2	32	11
Glucose degraded	64	79
O/R index	1.11	1.11
C_1/C_2	1.15	0.97
C-recovery	1.02	1.16

[a] Results are given as total micromoles of product formed or glucose consumed per tube after 9 h of incubation. Anaerobic culture tubes contained 10 ml of buffered LPBM medium with excess glucose (0.35%) and were incubated at 65°C.

Growth yield on glucose. Growth yields on glucose were determined in batch cultures that contained either buffered LPBM medium with 0.05% yeast extract or complex TYEG medium. A culture optical density (absorbance at 540 nm [A_{540}]) of 1.0 corresponded to 2.75 mg of cells (dry weight) per 10 ml of culture. Growth yields of 16.4 and 12.1 g of cells/mol of glucose consumed were obtained after 7 h on basal and

complex media. Growth yields were somewhat higher on TYEG medium in the time range of 5 to 7 h (15.1 g/mol of glucose) than that of the entire 7-h fermentation period (12.1 g/mol of glucose). Parallel analysis of growth and glucose consumption in TYEG medium suggested that some other carbon source(s) were used before glucose metabolism.

Metabolism of glucose to pyruvate. To assess the glycolytic pathway employed by *T. brockii*, we initiated radioactive tracer studies with specifically labeled [^{14}C]glucose. The results of experiments for tracing the formation of $^{14}CO_2$ originating from various positions in glucose are summarized in Table 2. Determinations were made after growth in culture tubes that initially contained equal specific radioactivity per glucose. The specific radioactivity for CO_2 was 2.5 to 3.0 times higher when the label resided in positions 3 and 4 of glucose as compared to uniformly labeled glucose. No significant $^{14}CO_2$ was obtained from label at position 1 or 6 of glucose.

Cell extracts contained high activities (Table 3) of the following glycolytic enzymes: glucokinase, FDP, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate kinase. Neither glucose phosphomutase, glucose-6-phosphate dehydrogenase, nor gluconate-6-phosphate dehydrogenase was detected with either NADP or NAD as electron acceptors.

Enzyme activities associated with conversion of pyruvate to end products. An active NAD-linked lactate dehydrogenase that was activated by low concentrations of FDP, was present in cell extracts (Table 3). The enzyme functioned only in the direction of pyruvate reduction and required NAD as coenzyme. The enzyme was specific for L- and not D-lactate as determined by the reduction of 3-acetyl NAD in the presence of L-lactate and FDP.

TABLE 2. *$^{14}CO_2$ formation by T. brockii grown on differentially labeled [^{14}C]glucose*[a]

Position of ^{14}C in glucose	Sp act (dpm/μmol of CO_2)
1-^{14}C	200
6-^{14}C	150
3,4-^{14}C	6,500
U-^{14}C	2,500

[a] *T. brockii* was grown in either LPBM-0.05% yeast extract medium or TYEG medium that contained 0.15% glucose. The specific activity of the glucose was initially adjusted to 14,000 dpm/μmol in all experiments. After 10 h of growth at 65°C the specific activity of CO_2 in the gas phase was determined. CO_2 levels obtained from background growth in the absence of added glucose were subtracted, as well as the zero-time levels of radioactive CO_2. Results represent the average of triplicate experiments that employed both media.

TABLE 3. *Catabolic enzymes of T. brockii grown on glucose*[a]

Enzyme	Substrates[b]	Sp act at 40°C (U/mg of protein)
Glucokinase	Glucose, ATP	0.40
Fructose-1,6-diphosphate aldolase	FDP	0.23
Glyceraldehyde-3-phosphate dehydrogenase	Glyceraldehyde-3-phosphate, NAD	1.73
Pyruvate kinase	Phosphoenolpyruvate, ADP	0.36
Lactate dehydrogenase (fructose-1,6-diphosphate activated)	Pyruvate, NADH	0.55
Pyruvate dehydrogenase (CoA acetylating)	Pyruvate, CoA, MV	0.53
Hydrogenase	H_2, MV	3.30
Phosphotransacetylase	Acetyl-CoA, arsenate	0.55
Acetaldehyde dehydrogenase (CoA acetylating)	Acetaldehyde, NAD, CoA	0.15
Ethanol dehydrogenase (NAD)	Acetaldehyde, NADH	0.48
Ethanol dehydrogenase (NADP)	Acetaldehyde, NADPH	1.57
Acetate kinase	Acetyl P, ADP	1.50

[a] The assay conditions are described in the text.
[b] Abbreviations: MV, methyl viologen; acetyl P, acetylphosphate.

The effect of FDP concentration on lactate dehydrogenase activity at different pH values is shown in Fig. 2. The affinity of the activator FDP for the enzyme depended largely on the pH, whereas the maximal activity was almost unchanged in the pH range of 6.2 to 7.8. Half maximal activation was attained with imidazole-hydrochloride buffer at pH's of 6.2, 7.0, and 7.8, and 5×10^{-6} M, 2.2×10^{-5} M, and 2×10^{-3} M FDP, respectively.

The fermentation studies above indicated that CO_2 formation corresponded to the sum of the C_2 products (i.e., ethanol and acetate). Catabolic activity levels of pyruvate dehydrogenase (CoA acetylating) were demonstrated by using methyl viologen as an electron carrier (Table 3). This activity required CoA with a stoichiometry of 1 mol of CoA per 2 mol of methyl viologen reduced. A similar CoA requiring reaction but with low activity (0.03 U/mg) was observed by coupling pyruvate oxidation to the reduction of NAD. This activity was enhanced to 0.2 to 0.3

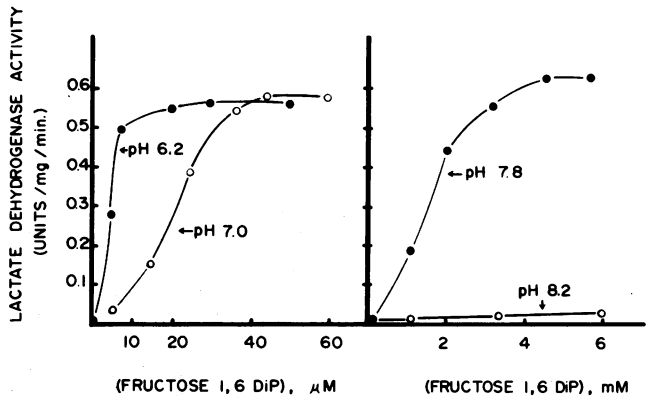

FIG. 2. *The relation of pH and fructose-1,6-diphosphate concentration to lactate dehydrogenase activity of T. brockii. Conditions: Imidazol-hydrochloride buffer, 100 mM; NADH, 0.25 mM; pyruvate, 10 mM; FDP, as indicated; pH as indicated; cell extract, 5 μl (0.05 mg of protein); total volume, 1 ml; gas phase, helium; 40°C.*

U/mg by the addition of ferredoxin (0.1 mg/ml) from *Clostridium pasteurianum*. Cell extracts also contained catabolic amounts of hydrogenase, phosphotransacetylase, and acetate kinase (Table 3). Phosphotransacetylase was estimated by using the CoA dependence of pyruvate dehydrogenase. CoA was regenerated from acetyl CoA by endogenous phosphotransacetylase when either phosphate or arsenate was added to the reaction mixture. Acetate kinase activity in direction of acetate formation from acetyl phosphate and ADP was measured in spite of the presence of myokinase activity (0.25 U/mg), which interfered with the assay.

An NAD-linked, CoA-acylating acetaldehyde dehydrogenase was present in cell extracts. This activity was only demonstrated when the enzyme was protected by DTT and NAD before the addition of acetaldehyde. The reaction absolutely required the presence of CoA. The depletion of CoA terminated the reaction after an equivalent amount of NADH was formed. Addition of arsenate (10 mM) restored the maximal reaction rate by its effect on the endogenous phosphotransacetylase. These properties resembled those of acetaldehyde-CoA-acylating dehydrogenase described for *Escherichia coli* (16).

Both NADP- and NAD-linked ethanol dehydrogenase activities were detected in cell extracts (Table 3). The NAD-linked ethanol dehydrogenase, but not the NADP-linked activity, was very oxygen sensitive. An experiment was designed to determine whether the NADP-linked activity was present in the early, as well as late, stage of growth. Cell suspensions of the early, middle, and late logarithmic growth phase or of the early stationary growth phase were treated by shaking with toluene and were then assayed for NADP-linked alcohol dehydrogenase. Similar specific activities were observed at each of the growth stages. These activities corresponded to about 25% of that obtained in cell extracts.

The enzyme activities described above that did not require the use of temperature-labile enzyme components in the reaction mixture displayed considerably high activities when examined at 60°C.

DISCUSSION

These data suggest a glucose fermentative pathway for *T. brockii* (Fig. 3). Glucose is activated by glucokinase and metabolized to pyruvate by enzymes of the Embden-Meyerhof-Parnas pathway. The [^{14}C]glucose tracer experiments and undetectable levels of glucose-6-phosphate and gluconate-6-phosphate dehydrogenase activity exclude operation of the hexose monophosphate and Entner-Douderoff pathways in catabolism. End products are formed from pyruvate (i) to L-lactate via a FDP requiring L-lactate dehydrogenase; (ii) to acetate, H_2 and CO_2, via an atypical clostridial phosphoroclostic reaction; and (iii) to ethanol via NAD- and NADP-linked acetyl-CoA and acetaldehyde oxidoreductases. The phosphoroclastic reaction of *T. brockii* appears atypical because only low amounts of H_2 are formed from pyruvate.

Product analysis gave typically (in moles per mole of glucose fermented): ethanol, 0.94; L-lactate, 0.84; acetate, 0.20; CO_2, 1.31; and H_2, 0.50. These values depended to some extent on the level of yeast extract in the medium and possibly also on other growth conditions. Relative levels of H_2 could significantly decrease and those of lactate could significantly increase, with a small increase of the yeast extract content in the medium. No other products were detected. Fermen-

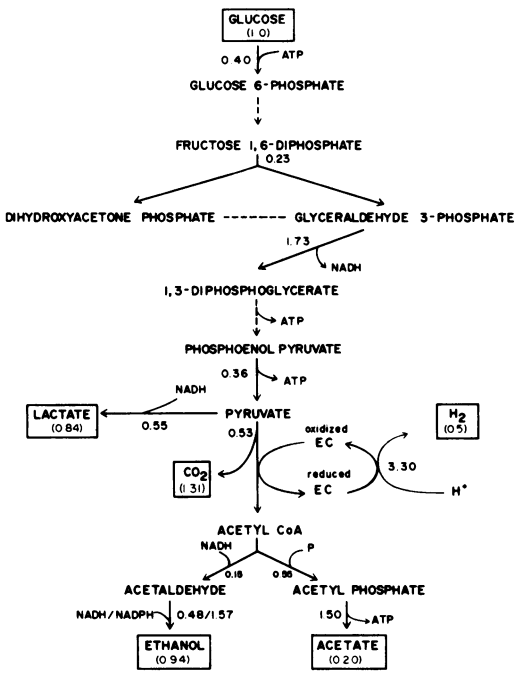

FIG. 3. *Glucose catabolism pathway in T. brockii. Glucose and end product values represent total moles. Specific enzymatic reaction values represent micromoles of product formed per minute per milligram of cell extract protein at 40°C.*

tation analysis indicated that nearly 100% of the glucose metabolized was recovered in these products, the O/R index was balanced, and the amount of CO_2 produced equaled the sum of acetate and ethanol. No significant change in product pattern was observed in old versus young cultures or cultures grown at optimal or minimal temperatures. These results are in line with the nonsporulating nature of this organism (25). Changes in product pattern in response to sporulation or growth temperature changes, as well as induction of new enzymes, have been observed in sporulating organisms (9, 10).

Growth yields (*Y*s) on glucose varied according to specific growth conditions between 15.1 and 16.4 g of cells per mol consumed. Based on end product analysis and fermentation pathways described above, a value of 2.2 mol of ATP per mol of glucose catabolized was obtained. An approximate value for Y_{ATP} is 6.8 to 7.5 g of cells per mol of ATP, which is in the range reported for glucose fermentation by a wide variety of anaerobic bacteria (17). However, the Y_{ATP} value obtained was lower than that generally accepted (10.5 g/mol of ATP). This may reflect not providing excess growth factors required for anabolism, the high maintenance energy requirements for growth at 65°C or both. On the basis of the data presented here, there is no reason to assume ATP synthesis by an electron transport mechanism in addition to the substrate level phosphorylations described.

The catabolism of *T. brockii* was not typical of documented saccharolytic clostridia in features such as relatively low H_2 production, high lactic acid levels, and absence of butyrate as a fermentation product. The pyruvate dehydrogenase activity in *T. brockii* involves a low potential electron acceptor similar in nature to *C. pasteurianum* ferredoxin. The addition of *C. pasteurianum* ferredoxin to *T. brockii* extracted activated electron transport reactions from pyruvate to reduction of NADs. FDP requiring lactic dehydrogenase has not been reported in clostridia, but has been observed in some lactic acid bacteria (22). A NADP-linked ethanol dehydrogenase was demonstrated in *C. thermosaccharolyticum* and *Leuconostoc mesenteroides* (5, 9). NADP-linked ethanol dehydrogenase was induced, together with NADP-linked glucose-6-phosphate and gluconate-6-phosphate dehydrogenases, in sporulating cultures of *C. thermosaccharolyticum*. These events have been accompanied by changes in the product pattern and accumulation of ethanol (9). The NADP-linked ethanol dehydrogenase in *T. brockii* was present during all growth stages, and no change in product pattern was observed during cultivation. Thus, *T. brockii* is distinct from *C. thermosaccharolyticum* both at the enzyme level and on the basis of cellular and growth features (25). The role of the NAD- and NADP-linked alcohol dehydrogenase has not been demonstrated. Until enzyme purification is attained, it remains unclear whether one or more distinct alcohol dehydrogenases are involved in formation of ethanol. It is possible that these activities are involved with hydrogenations that control the levels of the reduced pyridine adenine nucleotides in the cell.

It will be of interest to examine the regulation of end product formation in *T. brockii*. This organism contains a regulatory lactic dehydrogenase similar to that of lactic acid bacteria which contain pyruvate-formate lyase rather than pyruvate dehydrogenase (21, 22). The control of end product formation from pyruvate in clostridia is ascribed to properties of ferredoxin-NAD(P) oxidoreductases (11).

Ethanol yields during saccharide fermentations by *T. brockii*, as well as by other promising thermophiles (e.g., *C. thermocellum* or *C. thermohydrosulfuricum*), are limited by the production of other end products and cell toxicity to high ethanol concentrations (>1%). The data presented here suggest that ethanol production yields (i.e., the substrate-to-product ratio) with

T. brockii can be improved by altering the normal electron flow during saccharide fermentation from lactate formation into ethanol production. This may be accomplished by selection for lactate dehydrogenaseless mutants or by manipulation of specific cultural parameters that influence the regulatory properties of lactate dehydrogenase. In regard to the latter proposal, factors that lower the intracellular level or influence the activity of FDP will enhance ethanol production. Therefore, utilization of slow substrate feed rates or slowly metabolized substrates (e.g., starch in lieu of glucose) and maintenance of neutral to alkaline pH should enhance ethanol yields. The selection of alcohol-tolerant strains is of prime importance for further increasing the final concentration of ethanol produced by *T. brockii*.

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