The goal of this experiment is to determine the metabolic potential of microbes growing on defined quantities of glucose as a primary energy source by measuring growth yields and fermentation products. The general objective will be to compare the growth yields and end products formed in *Escherichia coli* utilizing primarily the Embden-Meyerhof pathway (1) and *Zymomonas mobilis* utilizing primarily the Entner-Douderoff pathway (1-5) for the catabolism of glucose. The relationships between growth yield and ATP formation (12-14) will provide evidence for the utilization of a particular pathway. A recombinant strain of *E. coli*, (14,15) containing the pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adh*) genes from *Z. mobilis* has been engineered to produce ethanol as the primary fermentation product that allows the regeneration of NAD from NADH formed from the oxidative reactions in the fermentative pathway. This strain has served as the basis for the development of E. coli KO11 and other strains in current use for commercial production of ethanol from lignocellulosic biomass (18). A strain of *Enterobacter asburiae*, JDR-1, which efficiently ferments all the carbohydrate components in acid hydrolysates of woody biomass, has been similarly engineered to produce the ethanologenic strain, *E. asburiae* E-1 (19, 20). The parent and ethanologenic strains of *E. coli* and *E. asburiae* will be compared to *Z. mobilis* with respect to metabolic potential. Specific objectives will include 1) comparison of growth rates and yields of *E. coli* B, *E. coli* KO11, *E. asburiae* JDR-1, *E. asburiae* E1, and *Z. mobilis* grown with glucose as a carbon source, 2) quantification of fermentation products formed 3) determination of the levels of specific enzymes as indicators of the fermentative pathways used, and 4) definition of fermentation pathways for $^{13}$C-1- and $^{13}$C-6-labeled glucose. Basic techniques will involve turbidity measurements for microbial growth, colorimetric and spectrophotometric assays, and high performance liquid chromatography (HPLC) for quantifying fermentation products, and $^{13}$C-NMR spectrometry to follow $^{13}$C distributions in fermentation products. Students should work in groups of two.

**PROCEDURES**

**Inoculation and estimation of growth.** To 16 x 150 mm screw cap culture tubes, add 2.5 ml of appropriate minimal medium at 2 x final concentration (Appendix 1), and autoclave. Make additions according to the following protocol:

<table>
<thead>
<tr>
<th>TUBE</th>
<th>ORGANISM</th>
<th>GLUCOSE STOCK (M) 2.5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>E. coli</em> B (or <em>E. asburiae</em> JDR-1)</td>
<td>0.10</td>
</tr>
<tr>
<td>2</td>
<td><em>E. coli</em> B (or <em>E. asburiae</em> JDR-1)</td>
<td>0.20</td>
</tr>
</tbody>
</table>
Additions of sterile glucose will be followed by inoculation with 0.2 ml of exponential stock cultures of the appropriate organism. Tubes will be sealed with caps and incubated at 30 C without shaking.

For the turbidimetric assay of growth, mix each tube to provide a uniform suspension, and determine light scattering (as optical density) with a spectrophotometer at 600 nm. A blank containing uninoculated medium should be used. Measurements should be made every 2-8 hr to follow the progress of culture and establish the time for reaching stationary phase.

Upon reaching stationary phase, each culture should be transferred to 4 x 1.8 ml microfuge tubes, centrifuged for 10 min room temperature, and the medium transferred to clean tubes for analysis of glucose, pH and fermentation products. For pH measurements, dilute 0.4 ml of the spent medium (supernate) into 3.6 ml deionized water (DW) in a suitable vessel and determine the pH with a combination probe.

The pellets should be washed (and combined) 2 x with distilled water by suspension and centrifugation. Each pellet should then be thoroughly suspended in 1.0 ml 1.0 N NaOH, transferred to a glass tube and heated for 10 min at 95 C (hot water bath) with frequent mixing on a vortex mixer. After cooling, add 1.0 N HCl, mix, and assay for protein by the Bicinchoninic Acid (BCA) method (ref 10, Appendix 2).

Determination of glucose utilization. Samples of complete uninoculated medium and medium obtained from each culture (after removal of cells) will be assayed for total carbohydrate by the method of Dubois et al (6). To 18 x 150 mm test tubes add 0.10 ml or 0.50 ml of each sample (diluted to 100 fold with water) and make to 2.0 ml with DW. Add 0.1 ml 80% phenol, mix, followed by 5.0 ml concentrated H$_2$SO$_4$. The H$_2$SO$_4$ should be added rapidly with a wide bore 10 ml pipet directly into the sample to generate maximum heating (Use protective clothing and eye shields). Upon cooling, the absorbance should be determined at 480 nm, using a blank with water as sample. A standard curve for glucose should be prepared ranging from 0.1 to 1.0 μmol of glucose.

In addition to the nonspecific colorimetric assay for glucose, the utilization of glucose will be quantitatively determined by HPLC along with the determination of fermentation products.

Analysis of fermentation products. Fermentation products, including lactate, acetate, formate, succinate, and ethanol will be quantified using HPLC. Samples of media (after centrifugation) will be filtered through 0.2 μm (BioRad Aminex HP-87H, H$^+$ form, ref. 7) pre-equilibrated with 0.009 N H$_2$SO$_4$ at 70 C. The column should be preceded by a guard column (BioRad carbohydrate cartridge). The injection and elution may be carried out by a Waters
U6K manual injector, a 730 data module, and a M45 A pump. Alternatively, samples may be resolved following delivery with a 710B WISP automated injector and chromatography controlled with Waters 610 solvent delivery system at flow rate of 0.5 ml/min. Products will be detected by differential refractometry with a Waters 2410 RI detector. Data analysis may be performed with Waters Millennium Software, using a Waters HPLC system. Standards of each compound to be detected will be run to determine retention times and quantify levels of each compound.

Determination of enzyme activities in cell-free extracts. Glucose-6-phosphate dehydrogenase (G-6-P dehase) occurs in E. coli as the enzyme which introduces G-6-P to the hexose monophosphate pathway and shows a preference for NADP as cofactor. An analogous activity occurs in Z. mobilis for the Entner-Doudoroff pathway and will utilize either NAD or NADP (3). Enzymes unique to the Embden-Meyerhof (E-M) pathway include phosphogluconate isomerase (PGI) and fructose-1, 6-diphosphate aldolase while enzymes unique to the E-D include 6-phosphogluconate dehydratase) (PGA dehydratase) and 2-Keto-3-deoxy-6-phosphogluconate aldolase (KDPG aldolase). To evaluate the potential of each pathway in each organism, extracts may be assayed for G-6-P dehase, PGI, and PGA dehydratase as described by Stephenson et al. for the assay of these activities in Azotobacter biejerinckii (9). The G-6-P dehydrogenase should be assayed with NAD and NADP separately to determine the specificity of the enzyme for each pyridine nucleotide. Shaking cultures (aerobic) at mid-log phase of E. coli and Z. mobilis will be harvested by centrifugation at 5000 x g and washed with 10 volumes of cold distilled water and 10 volumes of cold 0.025 M sodium-potassium phosphate (NaKP) buffer, pH 7.4. The cells are then suspended in 5 volumes of NaKP buffer and passed through a French Pressure Cell 2 times at 13,000 lb/in². The homogenate is centrifuged at 10,000 x g for 20 min at 0 C to remove unbroken cells. The supernate is centrifuged at 100,000 x g for 90 min to provide a soluble enzyme preparation (100S) and a particulate fraction (100P). The 100S fraction will be assayed for the soluble enzymes of glucose metabolism. The 100 P fraction may be assayed for membrane bound cytochromes. A flow diagram outlining the approach is given in Appendix 3.

For the present experiment, only the G-6-P dehydrogenase (G6PDH, NAD- and NADP-specific) and the phosphoglucone isomerase (PGI) activities will be determined for metabolic comparisons of the different bacteria. Cytochromes will not be determined.

G-6-P dehase is measured directly by monitoring the reduction of NADP or NAD at 340 nm in a 3.0 ml volume containing the following: 5.0 mM NADP or NAD, 0.3 ml; 30.0 mM G-6-P, 0.5 ml; 100 mM Tris-HCl buffer, pH 7.9, 2.1 ml; 100S fraction, 0.1 ml. Initiate the reaction with addition of G-6-P.

PGI is measured by the rate of formation of G-6-P from fructose-6- phosphate (F-6-P) by coupling this reaction to G-6-P dehase oxidation to 6-phosphogluconate and concomitant reduction of NADP in a 3.16 ml volume containing the following: 100 mM MgCl₂, 0.2 ml; 10 mM NADP, 0.2 ml; 100 mM F-6-P, 0.3 ml; 1 mg/ml G-6-P dehase, 300-400 i.u./mg, 0.01 ml; Tris-HCl buffer, pH 7.9, 2.4 ml; 100S, 0.05 ml. Initiate the reaction with the extract after checking the amount of G-6-P in the F-6-P added.
Measurement of NAD and NADP reductions should be made with a recording spectrophotometer in 1.0 cm cuvettes. The moles of nicotinamide nucleotide reduced can be quantified on the basis that both NADPH and NADH have molar absorbivities at 340 nm of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at pH 7 for a 1 cm light path.

The continuous kinetic spectrophotometric assays provide a basis for estimating average velocities for time periods that represent maximum velocities (Vmax), identified as a linear relationship for product formed versus time (dP/dt). Specific activities may be determined as velocity (umol product/min) per mg protein after measuring the concentration of protein in each sample assayed by the BCA method (10). These measurements are required for the comparisons of enzyme levels in crude extracts that accurately reflect the relative activities in the cells, essentially correcting for variations in cell disruption.

Analysis of carbon flow by NMR. The use of glucose labeled with $^{13}$C in specific carbons results in the labeling of intermediates and end products that are predictive of the pathway by which these substrates were metabolized. The kinetic evaluation of the use of a particular substrate provides insight into the sequence of events which occur. Cultures of E. coli B, E. coli KO11, and Z. mobilis will be exposed to $^{13}$C-1-glucose or $^{13}$C-6-glucose, and the distribution of label in specific carbons in different fermentation products will be determined by $^{13}$C-NMR. (17). The outline for this experiment is given in Appendix 4

TREATMENT OF DATA

In a section under the heading "PROCEDURES", steps for each procedure should be identified and presented in outline form. Included should be standard curves for assays (e.g. coloimetric assay of glucose and protein, relationship or peak area and concentration for compound), identifying conditions (i.e. instrument used, wavelength for spectrophotometric assays, temperature of incubation, and analyses, etc.), and specific comments which qualify any use of the procedure.

The "RESULTS" section should include: 1) Table 1: growth data for Z. mobilis and E. coli under anaerobic and aerobic conditions, to include turbidity, pH of medium, glucose utilization, and cell protein yield. Figure 1: HPLC profiles of fermentation products accumulated by Z. mobilis and E. coli parent, mutant, and recombinant lines. 2) Table 2: fermentation products as determined by HPLC, indicating moles/ml, total moles and moles formed per mole of glucose consumed. 3) kinetic profiles of NAD- and NADP-linked glucose-6-phosphate dehydrogenase and phosphoglucone isomerase for Z. mobilis (Fig. 3a), E. coli B (Fig. 3b), and E. coli KO11 (Fig. 3c) grown under aerobic conditions. 4) Table 3: Enzyme levels in Z. mobilis and E. coli presented as umoles products per min per mg protein. 5) Fig. 5 $^{13}$C NMR profiles of bacterial cultures.

In a section under heading "DISCUSSION" indicate the following: 1) confirmation of expected pathway utilized by each organism based upon fermentation products, enzyme levels, and $^{13}$C-labeling distributions determined by $^{13}$C-NMR. 2) the relationships of growth yield
and expected ATP formation, relative to the pathway proposed from data above 3) the genetic and biochemical basis of the high amounts of ethanol produced by E. coli KO11 compared to the other bacterial cultures.

REFERENCES


