Abstract

Metabolic engineering and strain development rely on the analysis of enzymes and metabolites to guide the design of new experiments and strains. Many of the enzymes required for fermentation of hexose and pentose sugars to ethanol are assayed by monitoring the rate of disappearance or appearance of NADH. We have developed an automated kinetic assay method, which uses a Zymark SCICLONE Liquid Handling System, a Mitsubishi robotic arm, and a SpectraMax microplate reader from Molecular Devices. The method was tested by measuring the activities of enzymes required for pentose utilization, including xylose isomerase xylulokinase, and transaldolase, in cellular extracts prepared from *Zymomonas mobilis*. Results are comparable to those achieved using a manual method, but higher productivity and reduced variability can be achieved. The automated method is adaptable for measurement of any enzyme or metabolite that can be monitored by the oxidation of NADH or reduction of NAD.

Introduction

- Metabolic engineering of organisms and pathways requires knowledge of enzyme levels and metabolites (Fig. 1)
- Sometimes organisms engineered with similar genes have different performance patterns and metabolites and enzyme levels in these strains must be compared to determine why they perform differently. (ref)
- Key enzymes in the glycolytic and pentose utilization pathways can be assayed directly or by coupling to reactions that lead to the oxidation or reduction of NADH and NAD. The concentration of NADH can be monitored by the absorbance at 340 nm. Table 1
- Traditional enzyme assays are time consuming, labor intensive and require manual entry of data into an excel spread sheet for analysis.
  - Pipetting and mixing reagents and monitoring the reactions using a Beckman DU-640 spectrophotometer take 15 minutes for each set of 6 reactions.
  - Entry of the data into a spreadsheet requires additional time and can be a source of error.
  - The volume of each reaction is 500 ul (cost/reaction)
- A miniaturized and semi-automated method for assaying pentose utilization enzymes has been developed with several advantages over the traditional method.
  - 96 assays can be performed in 15 minutes
  - Less reagent is required per assay (200 vs 500 ul final assay volume) decreasing the cost per reaction.
  - Data is collected on a computer and can be imported directly into an excel spreadsheet increasing efficiency and avoiding the possibility of incorrect data entry.

Methods

Enzyme Assays

Each enzyme assay has three components that are combined to provide all the components for the reaction including a reaction mix with appropriate buffer, metal ions coupling enzymes and cofactors, substrate solution, and cell free extract containing the enzyme to assay. Table 1 shows the reagents used for assaying xylose isomerase, xylulokinase, and transaldolase. Reactions are prepared by combining a reaction mix and cell free extract and started by adding one or more substrates.

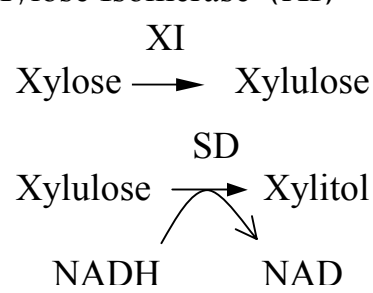
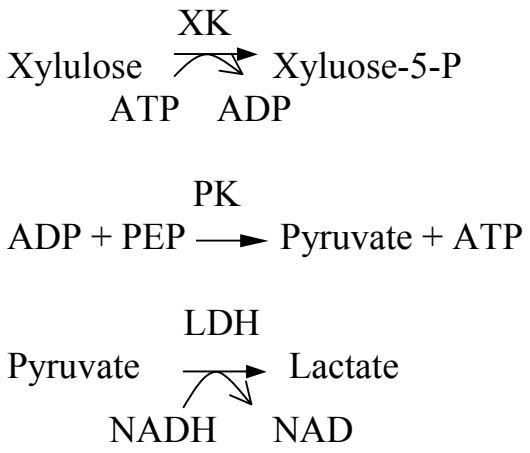
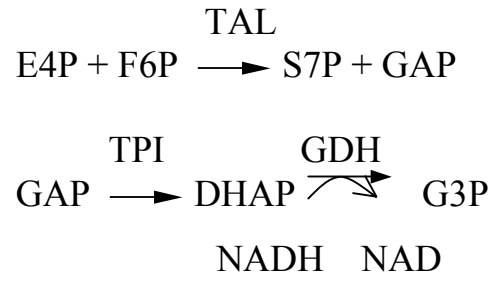
The reactions are monitored by measuring the absorbance at 340 nm for three minutes using a on a Beckman DU-640 spectrophotometer or a SpectraMax microplate reader from Molecular Devices. The rate of the reaction is recorded in an excel spread sheet as the change in absorbance over time and used to calculate specific activity of the enzymes.

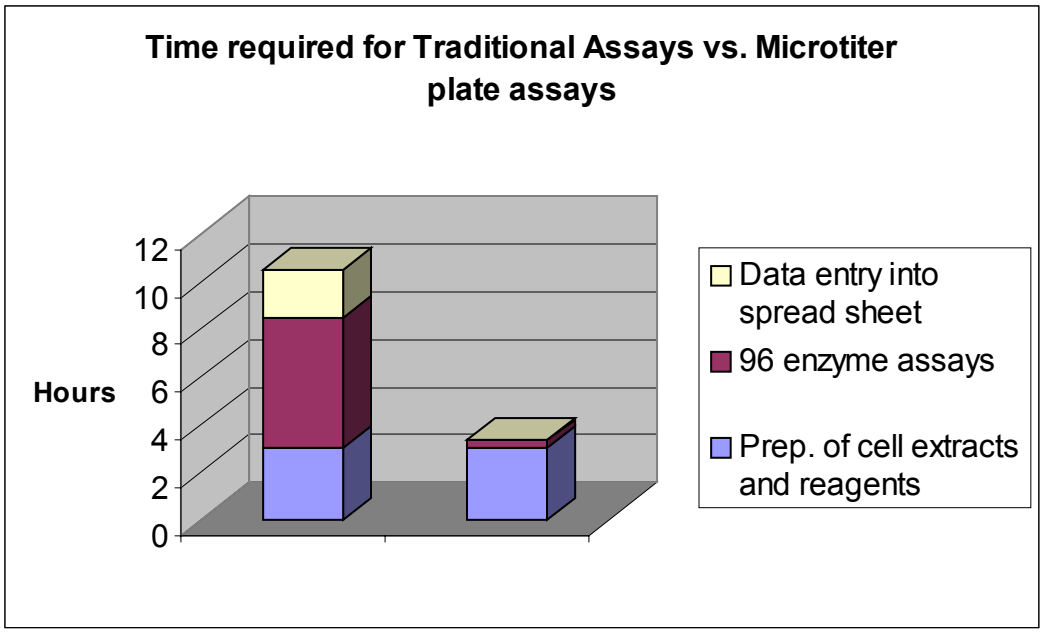
One unit of activity is equal to the conversion of 1 umol of substrate to 1 umol of product per minute and is proportional to the amount of NADH used or produced. Activity is calculated as the slope of the change of absorbance vs time divided by the extinction coefficient for NADH multiplied by the dilution factor. Specific activity is equal to the activity divided by the concentration of protein in the cell extract. For assays performed in the microplate, activity was divided by the pathlength of 0.55cm. Protein was measured using the BCA reagents from Pierce.

Growth of cells and preparation of cell free extracts.

Zymomonas mobilis strain C25 was grown at 30^{o}C to late log phase (OD_{600} of 1.0) in rich medium containing 10 g/l yeast extract 2 g/l KH_2PO_4 and 20 g/l glucose added separately after autoclaving. Cells were collected and washed once with sonication buffer (10 mM Tris, pH 7.6, 10 mM $MgCl_2$, 1 mM DTT) and stored at -80°C prior to analysis. Cell-free extracts were prepared by suspending cells to OD_{600} of 10 in sonication buffer. A 0.5 ml cell suspension was sonicated in two; 45 second bursts with the Branson model 450 sonifier (Branson ultrasonics, Danbury, CT, USA) set on 62% duty cycle and an output control of 2. The sonicated samples were then centrifuged at 14,000 rpm and 4°C for 60 minutes using a Beckman GS-15R centrifuge. The resulting supernatant was used as cell-free extract.

Assay Components

Reactions	Assay components	References
Xylose Isomerase (XI)  	0.256 mM NADH 10 mM $MgSO_4$ 1 mM triethanolamine pH 7.5 1U/ml SD *50 mM xylose*	
Xylulokinase (XK)  	0.2 mM NADH 2.0 mM $MgCl_2 \cdot 6H_2O$ 2.0 mM ATP (no ADP) 0.2 mM PEP 50 mM Tris·HCl, pH 7.5 5 U/ml pyruvate kinase (PK) 5 U/ml (LDH) *8.5 mM D-xylulose*	
Transaldolase (TAL)  	0.38 mM NADH 87 mM triethanolamine (pH 8.5) + 17 mM EDTA 2.0 U/ml GDH 20 U/ml TPI *33 mM F 6 P* *1.2 mM E 4 P*	


Time required for Traditional Assays vs. Microtiter plate assays
Hours
12
10
8
6
4
2
0
Data entry into spread sheet
96 enzyme assays
Prep. of cell extracts and reagents

Comparison of Assay methods

Assay	Activity	St. Dev.	%CV	Number assayed
XI Spectrophotometer	0.15	0.01	3	4
XI Microplate	0.12	0.02	16	15
XI Robot	0.1	0.02	12	16
XK Spectrophotometer	0.26	0.01	4	5
XK Microplate	0.23	0.02	9	16
XK Robot	0.22	0.02	11	16
TAL Spectrophotometer	0.78	0.09	9	10
TAL Microplate	0.71	0.06	9	15
TAL Robot	0.68	0.06	9	15

Applications of Automated Assays

1. Compare enzyme activities and metabolites in cells grown on different substrates and at different stages of growth.

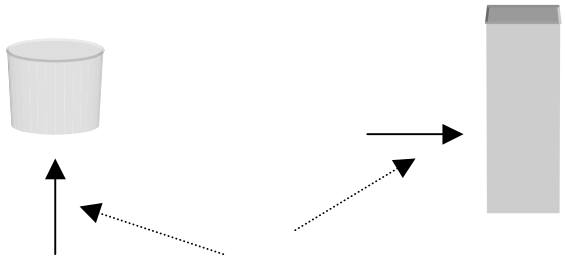
2. Measure activities in newly engineered strains of yeast and bacteria

3. Collect data to facilitate metabolic engineering and metabolic modeling projects.

4. Improve performance of specific enzymes with directed evolution.

Discussion

- Miniaturized microplate assays have several advantages over cuvette assays performed in the spectrophotometer.
  - Multiple assays can be performed simultaneously.
  - Smaller assay volume decreases the cost of each assay.
  - Data is automatically recorded by the Softmax Pro (Molecular Biosystems) software and can be exported directly into an Excel spreadsheet.
- The Microplate assays are more variable than the cuvette based assays which maybe due to the difference in the light path in the two assays.



  - The cuvette has a path length of 1 cm. The pathlength in the cuvette is dependent upon the volume of the reaction and more sensitive to pipetting errors.
  - The light passes through the liquid air interface. Bubbles and meniscus formation will affect the absorbance reading.

- The microplate assays compare favorably with the spectrophotometric method to assay enzymes required for pentose utilization.
- The method is adaptable for assaying any reaction that measures the appearance or disappearance of NADH or NADPH.
- The assays can be adapted to measure enzyme levels in cell extracts from any organism including *Saccharomyces*.