Enzymatic Digestion of Polysaccharides

Part II: Optimization of Polymer Digestion and Glucose Production in Microplates

Paul Held, Laboratory Manager, Applications Dept, BioTek Instruments, Inc., Winooski, VT

Hydrolysis of carbohydrate polymers such as cellulose, xylan and starch is used to produce fermentable sugars for bio-ethanol production. The exogenous digestion of these polymers on an industrial scale can be cost prohibitive without optimization of the enzyme reaction conditions. We have previously shown the ability to quantitate these enzymes; here we describe the optimization of some of the reaction conditions for the enzymes that catalyze the digestive hydrolysis of polysaccharide polymers into monomeric constituents.

Key Words:

Biofuel

Lignocellulosic

Cellulose

Xylan

Starch

pH

Ethanol

Agilent Technologies, Inc.
100 Tigan St., PO Box 998
Highland Industrial Park,
Winooski, Vermont 05404 USA

Phone: 800-227-9770
Email: bio.customercare@agilent.com
www.agilent.com

Introduction

Digestion of biomass material and its fermentation represents the best path forward for bio-ethanol production. While starch based feedstocks have been used extensively, it is believed that lignocellulosic energy sources offer a better long-term prospect as a source for fermentable sugars. Lignocellulosic feedstocks, such as wood chips, corn stover or switchgrasses do not directly compete with food sources for land or consumption. Regardless of the source of feed stock, current production costs make them unpalatable as a fuel. The International Energy Agency (IEA) has estimated that the cost of producing and retailing diesel and ethanol from cellulose to be $1.10 per liter of gasoline-equivalent, while ethanol from corn or sugar cane costs $0.62-0.75 compared to $0.54 for gasoline [1].

The use of lignocellulosic material to produce ethanol presents a number of challenges relative to the use of starch containing sources such as corn, which can be digested with amylases. Cellulose molecules are actually crystalline in nature with very little water hydration. Plant material is a complex structure that contains a number of polysaccharide polymers. Primary and secondary walls contain cellulose, hemicellulose and pectin, albeit in different proportions. The secondary walls of woody tissue and grasses are composed predominantly of cellulose, lignin, and hemicellulose. The cellulose fibrils are embedded in a network of hemicellulose and lignin. Cross-linking of this network is believed to result in the elimination of water from the wall and the formation of a hydrophobic composite that limits accessibility of hydrolytic enzymes and is a major contributor to the structural characteristics of secondary walls. Xylan, which accounts for up to 30% of the mass of the secondary walls in wood and grasses, contributes to the recalcitrance of these walls to enzymatic degradation.

In order to improve the ethanol yield from these polymers, scientists have turned to the use of specific enzymes from a number of different sources. Using a combination of in vitro digestion, in conjunction with in vivo genetic manipulation of yeast and bacteria strains, the digestion efficiency to fermentable sugars cannot only be increased, but the ability to ferment monosaccharides other than glucose can be improved.

Amylase enzymes catalyze the breakdown of starch into sugars. Starch is a mixture of amylose and amylopectin polysaccharide polymers. Amylose consists of long strands of glucose sugars linked by α-(1→4) glycosidic bonds, while amylopectin has not only α-(1→4) glycosidic bonds, but also α-(1→6) glycosidic branches (Figure 1). Depending on the source, starch is usually 20-25% amylase and 75-80% amylopectin. α-Amylase acts randomly along the starch chain hydrolyzing the α-(1→4) glycosidic bonds to produce a combination of maltotriose, maltose and limit dextrins. The typical optimal pH for α-amylase is 6.7-7.0. β-Amylase, which works from the non-reducing end of the polymer, hydrolyzes the second α-(1→4) glycosidic bond to produce the two-glucose sugar maltose. γ-Amylase also works from the non reducing end of amylase to cleave α-(1→4) glycosidic bonds to produce glucose, it will also cleave α-(1→6) linkages found in pectin.
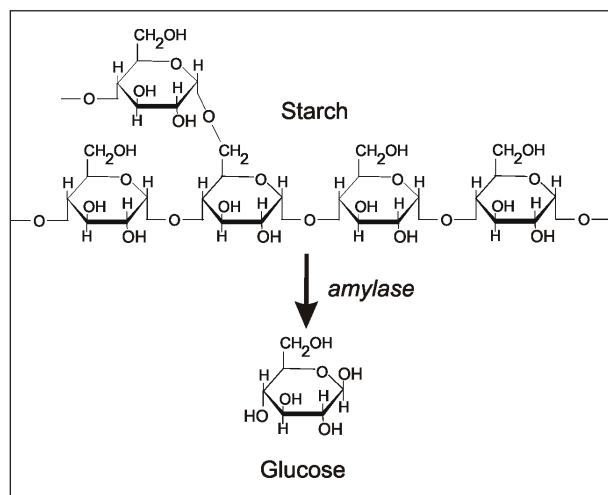
Figure 1. Structure of starch polysaccharide and its digestion to glucose.

Cellulose is a linear chain of several hundred to ten thousand β-(1→4) linked D-glucose units. Unlike starch this polymer has no branching (Figure 2). The chain structure forms a stiff extended conformation that results in extensive hydrogen bonding with adjacent chains. The result is the formation of microfibrils with high tensile strength and poor water solubility.
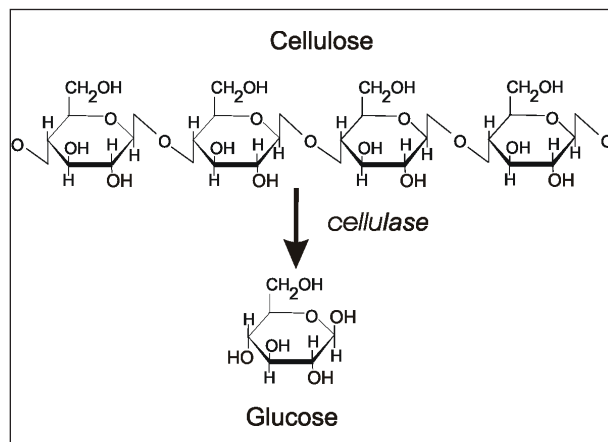

Figure 2. Structure of cellulose and its digestion to glucose.

Cellulase enzyme catalyzes the hydrolysis of cellulose into glucose (Figure 2). Cellulase enzymes are produced primarily by fungi, bacteria, and protozoans. Termites and ruminant herbivores digest cellulose through the action of symbiotic bacteria located in their intestines and ruminating chambers respectively. Cellulases are a mixture of slightly different enzymes that include endo-1,4-β-glucanase enzymes, which attack internal bonds, exo-1,4-β-glucanase enzymes, which cleave two to four units from the ends of cellulose strands and cellobiase, which cleaves the disaccharide cellobiose into two glucose moieties.

Xylanase enzymes digest xylan polymers, which are a major constituent of the hemicellulose, into xylose. Xylans are heteropolymers consisting primarily of the pentose sugars xylose and arabinose. The main backbone of xylan consists of β-(1→4) linked xylose residues (Figure 3) with side chains branching from the 2 and 3 position carbons also. Xylose is a five carbon monosaccharide that can form either furanose (5-member) or pyranose (6-member) ring structures.
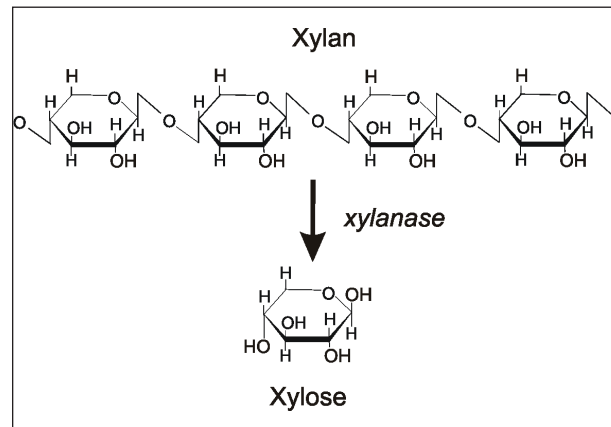

Figure 3. Structure of Xylan and its digestion to xylose.

Optimization of these enzymes in a laboratory or industrial setting requires experimentation with a number of different variables. The ability to measure multiple samples rapidly in small volumes offers significant advantages in terms of time, expense and effort. The use of microplates is one way that large numbers of samples can be handled with minimal amounts of reagents and time. Here we describe the use of microplates to assess physical assay characteristics, such as pH, temperature and enzyme concentration, of some of the enzymes that catalyze the hydrolysis of plant polysaccharides.

Materials and Methods

EnzCheck Ultra Amylase assay (cat # E33651), Amplex® Red Glucose assay (cat# A13261), and EnzCheck Ultra Xylanase assay (cat# E33650) kits as well as the fluorescent cellulase substrate (cat# E33953) were obtained from Life Technologies. Carboxymethyl cellulose (cat # 419273), xylanase from *Thermomyces lanuginosus* (cat# X2753), from *Trichoderma longibrachiatum* (cat # X2629), cellulase from *Aspergillus niger* (cat# C1184), α-amylase from *Aspergillus oryzae* (cat# 10065) from *Bacillus licheniformis* (cat# A4551) and *Bacillus subtilis* (Cat#10069) were purchased from Sigma-Aldrich. Corning 96-well solid black half area plates (cat# 3694) were used for all experiments.

Amylase Substrate Assay

Amylase activity was measured using an EnzCheck Ultra Amylase assay kit. The assay was performed as described previously [2]. For pH titration experiments, enzyme dilutions were made using d-H$_2$O as the diluent in 25 μL. Reaction pH was maintained using 25 μL of 100 mM phosphate buffer at different pH levels and reactions were initiated with the addition of 50 μL of working substrate solution. In order to correct for the influence of pH on the fluorescent signal data was normalized using a fluorescence reference supplied by the assay kit at each pH. The effect of temperature was measured by running separate assays at defined temperature settings using the Synergy™ H4 Hybrid Multi-Mode Microplate Reader to control temperature.

Xylanase Substrate Assay

Xylanase activity was measured using an EnzCheck Ultra Xylanase assay kit as described previously [2]. With pH titration experiments, enzyme dilutions were made using d-H$_2$O as the diluent in 25 μL. Reaction pH was maintained using 25 μL of 100 mM phosphate buffer at different pH levels and reactions were initiated with the addition of 50 μL of working substrate solution. Data was normalized using a fluorescence reference supplied by the assay kit at each pH. The effect of temperature was measured by running separate assays at defined temperatures using the Synergy H4 Hybrid Multi-Mode Microplate Reader to maintain temperature.

Cellulase Substrate Assay

Cellulase activity was also measured directly using an EnzCheck Cellulase substrate as described previously [2]. In experiments using pH titrations, enzyme dilutions were made using d-H$_2$O as the diluent in 25 μL. Reaction pH was maintained using 25 μL of 100 mM phosphate buffer at different pH levels and reactions were initiated with the addition of 50 μL of working substrate solution. Correction for the influence of pH on fluorescent signal data was made by normalization each well of the initial unreacted read to a common value. Subsequent data was normalized using the same factor and expressed as the change in signal of the initial unreacted fluorescence. Fluorescence for all experiments was measured kinetically with a Synergy H4 Hybrid Multi-Mode Microplate Reader. Excitation was set to 360 nm and emission wavelength at 460 nm.

Results

In order to demonstrate the utility of microplates and microplate readers, three different commonly used enzymes responsible for the digestion of polysaccharides used as feed stocks for ethanol production were investigated for activity under a number of environmental conditions.

Amylase

Amylase enzyme from B. subtilis was found to be resistant to high temperatures. Enzyme temperatures from 35° to 65° C resulted in very similar enzyme titration curves when the temperature was varied. Interestingly, temperatures very near ambient (25° C) had the greatest reduction in enzyme activity (Figure 4) when assessed using the fluorescent amylase substrate.
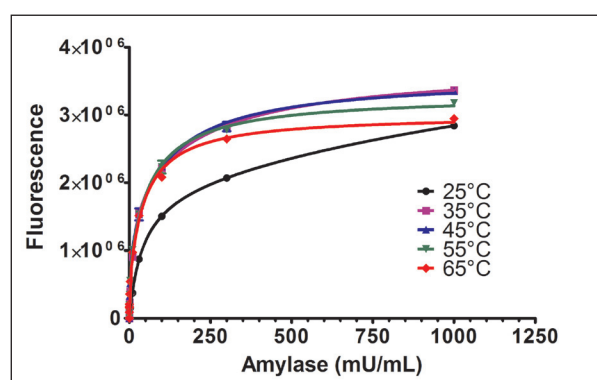

Figure 4. Amylase isolated from *B. subtilis* activity at different temperatures.

A closer examination of the data indicates that maximal enzyme activity is near 35° C. When the fluorescence produced by 300 mU/mL of amylase enzyme at different temperatures is plotted, the signal is maximal at 35° C. Signal is markedly greater at 35° C than what is observed at 25° C, while temperatures above 35° C show a slight decrease in activity (Figure 5).
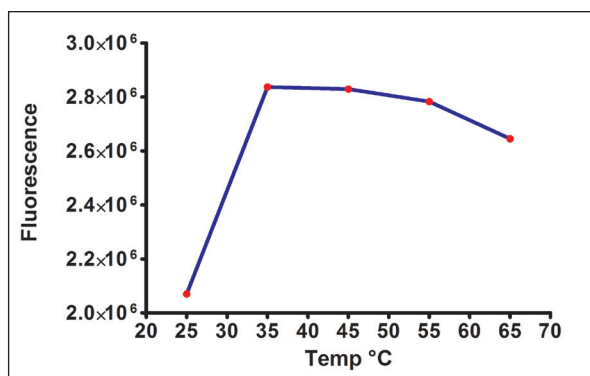

Figure 5. Fluorescence produced by 300mU/mL of amylase enzyme isolated from *B. subtilis*. The fluorescence was plotted after 30 minute incubation from assays run at different temperatures. Data represent the mean of 4 determinations.

The effect of pH activity was tested on amylase enzyme isolated from three different sources. *B. subtilis*, also known as hay bacillus or grass bacillus is a Gram-positive, catalase-positive bacterium commonly found in soil. *B. lichenformis* is a Gram-positive thermophilic bacterium found in soil as well as on bird feathers, which it is able to degrade. *Aspergillus oryzae* is a filamentous fungus commonly used to digest rice and other grains. As demonstrated in Figure 6, enzymes from these different sources have markedly different responses to differing pH levels. In order to correct for differences in fluorescent signal as a result of pH levels rather than enzyme digestion, equal amounts of enzyme (100 mU/mL) were incubated with the substrate at 25° C for 10 minutes. The background fluorescence from an unreacted sample (no enzyme) at that pH raw was first subtracted and then the blanked data normalized by a known fluorescence reference at the same pH. Under this analysis one observes that *B. lichenformis* is unaffected by pH in regards to enzyme activity, while *Aspergillus oryzae* enzyme has markedly higher activity at lower pH and virtually no activity above a pH of 9.0. *B. subtilis*, while demonstrating activity at all pH levels tested, has maximal activity at a pH of 6.0 (Figure 6).
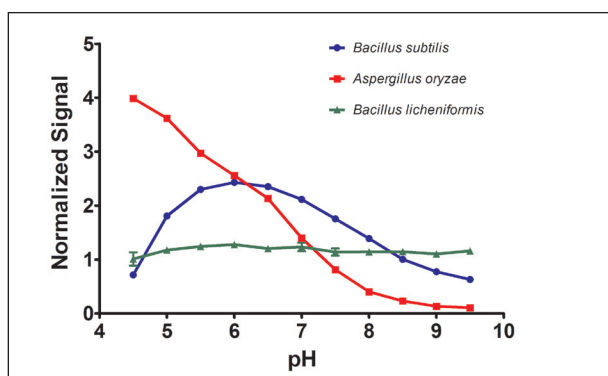

Figure 6. Effect of pH on amylase enzyme activity. Amylase enzyme isolated from different organisms were compared for their activity at different pH levels.

Xylanase

Xylanase enzyme isolated from the fungi *Thermomyces lanuginosus* was found to be very temperature stable. Using the Synergy™ H4 to incubate assay plates from 25° - 65° C, xylanase activity was measured after a 15 minute incubation. Enzyme concentration titration curves indicate that higher temperatures result in slightly more hydrolysis than ambient temperatures (Figure 7). Despite incubation at 65° C, enzyme activity demonstrated a 6.5% increase in signal as compared to activity incubated at 25° C.

The effect of pH levels on *Thermomyces lanuginosus* xylanase activity was tested from a pH of 5.0 to 9.5. As demonstrated in Figure 8, the enzyme works best in an acidic pH. Enzyme reactions (100 mU/mL) carried out at a pH of 9.5 had less than 25% of the fluorescence of reactions at a pH of 5.0 after 30 minute incubation.

Note that the effect of pH on the fluorescence of the reacted substrate must be accounted for when comparing results at different pH levels. In these experiments, equivalent concentrations of a reference standard were used to normalize data prior to plotting.
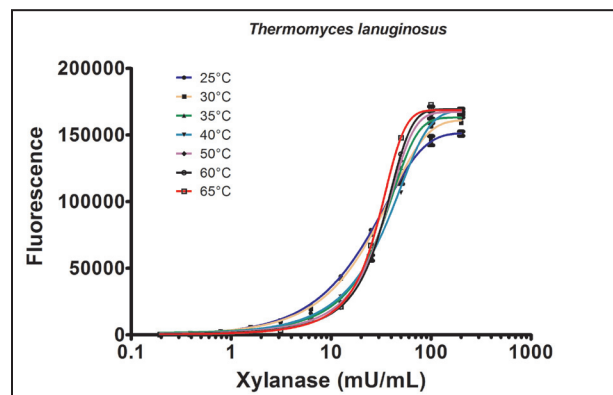

Figure 7. Effect of temperature titration on *Thermomyces lanuginosus* xylanase activity.

Enzyme titrations performed at different pH levels corroborate the fixed concentration data. With increasing pH levels less enzyme activity is observed.
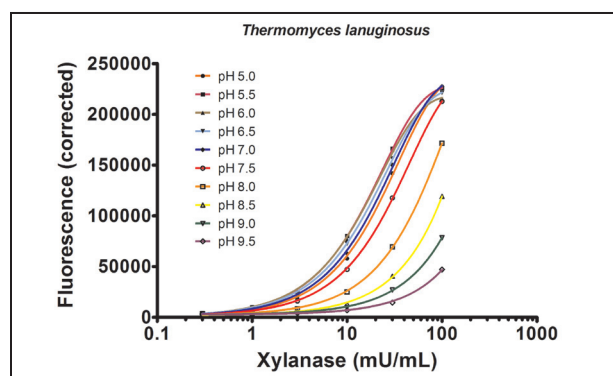

Figure 8. Effect of pH titration on *Thermomyces lanuginosus* xylanase activity.

By examining the average rate of reaction (Mean V) from at a fixed concentration of (10 mU/mL) a clearer picture of the effect of pH on enzyme activity is elucidated. As demonstrated in Figure 9, enzyme velocity peaks at a pH of 5.5; further increases in pH levels results in a gradual decline in activity to almost no activity at pH levels above 9.0.
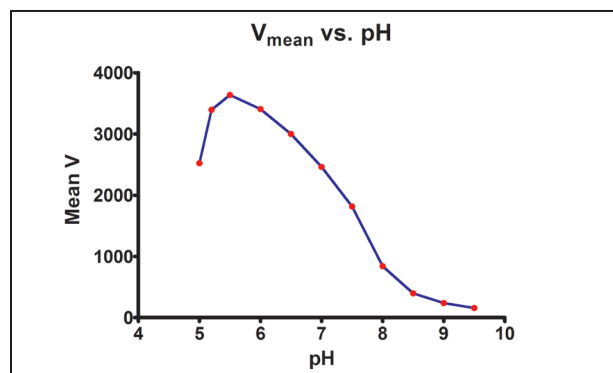

Figure 9. Effect of pH on reaction rate of xylanase enzyme isolated from *Thermomyces lanuginosus*.

Cellulase

Using a fluorescent substrate, cellulase activity can be measured directly. The direct assay has similar detection limits as the glucose determination, but does not require an overnight digestion. Using the direct substrate assay, the effect of pH on *Aspergillus niger* cellulase enzyme activity was investigated. As seen in Figure 10, *Aspergillus niger* cellulase exhibits significant activity in acidic pH levels. Enzyme activity is maximal from pH 4.6 to pH 6.0. Enzyme activity diminishes above pH 6.0 to virtually no activity above pH 8.0.
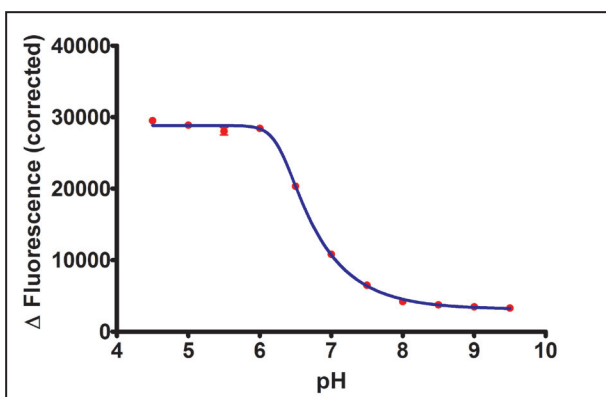

Figure 10. Effect of pH Titration of Aspergillus niger cellulase Activity.

Discussion

These data demonstrate the utility of microplate readers to perform classic enzymology experiments on critical enzymes necessary for the digestion of polysaccharide feed stock into fermentable monosaccharides for the production of ethanol. Depending on the initial source of material different enzymes are necessary. Grain based feed stock rich in starch requires amylase enzymatic activity, while plant and wood material needs cellulase and xylanase activity to break down the cellulose and xylan material contained in the plant fibers. Regardless of the enzymatic requirements it is important that the conditions for maximal enzymatic activity be optimized. Because microplates provide the ability to test large numbers of samples they are an ideal tool to test multiple experimental conditions.

The use of cellulosic second-generation material to generate fermentable sugars has some marked advantages over first generation starch based grains. Cellulosic materials do not compete directly with food stocks for resources such as tillable land, fertilizers and water. In addition cellulosic material can be obtained from sources often considered to be waste. However the use of cellulosic materials also provides a number of challenges.

Cellulose is notoriously hard to break down. Its linear chains of glucose moieties closely pack with one another forming hydrogen bonds between chains resulting in a crystalline material that is insoluble in water and many organic solvents. It can be chemically digested with a combination of high temperature and concentrated acids [3]. However, this technology is currently unfeasible from both an economic and hazardous waste standpoint. Towards that end, efforts have focused on ways to reduce the amount of acid used as a treatment by using a combination of dilute acids in conjunction with enzymatic hydrolysis. Enzymes that can be shown to work well in high temperatures in an acidic environment would provide a distinct advantage over those that are labile under the same conditions. Likewise the xylanase enzymes that work effectively under similar conditions are desirable as xylan is a significant component of most cellulosic feed stocks.

The hydrolysis of xylan results in the pentose sugars xylose and arabinose which are not fermented by wild strains of the yeast *Saccharomyces cerevisiea*. To address this limitation several different strains have been genetically modified in regards to xylanase and arabinose transport proteins and pentose fermentation pathways [4].

The use of ethanol as a means to replace a portion of the transportation fuel used in the United States has had legislative support for a number of years. Ethanol as a biofuel is very appealing for a number of reasons. Ethanol is currently used as an additive to gasoline in the US to replace MBTE as a means to increase gasoline oxygen content. Besides being ecologically more palatable, ethanol also increases the octane of gasoline. Because it is currently being used in large quantities the infrastructure to deliver the fuel already exists.

References

1. Fairley, P. (2011) Next Generation Biofuels, Nature 474:S2-S5.

2. Held, P. BioTek Application note "Enzymatic Digestion of Polysaccharides; Part I" www.biotek.com .

3. Xiang, Q. Lee, Y.Y., Pettersson, P.O., and Torget, R.W. (2003) Heterogeneous Aspects of Acid Hydrolysis of α-Cellulose, Applied Biochemistry and Biotechnology 107:505-514.

4. Jeffries, T.W. (2004) Metabolic Engineering for Improved Fermentation of Pentoses by Yeasts, Appl. Microbiol Biotechnol. 63:495-509.

　　　　　　　　　　　　　　　　AN022812_07, Rev. 02/28/12