Revised from biofuel Enzyme Kit by Biorad (Catalog Number: 166-5035 EDU)

Although the early combustion engines were designed to run on ethanol, the high price of ethanol and inexpensive fossil fuels such as petroleum pushed off the ethanol aside and the petroleum becomes the major player in fuel arena. But supplies of fossil fuels are limited and the impact of their combustion on the environment is already badly felt. So, ethanol is once again being sought as sustainable energy source without harming the environment. Generally the alternative sustainable energy sources are hunted on the banner of biofuels, which refers to a group of fuels that are produced from a biological source that was recently living, in contrast to fossil fuels that were created from biological sources long dead. Current biofuel technologies tend to fall four main categories- cellulosic ethanol, ethanol production from sugar and starch sources and biodiesels. Currently, much of the ethanol used in the fuel industry is a result of conversion of starch, such as that found in kernels, to sugars for fermentation to ethanol. Conversion of starches to sugar has two primary drawbacks. First, it takes away a food source from people and livestock. Second, a lot of the corn plant goes to waste, since the only polysaccharide that is converted to ethanol is the starch of the corn kernel. Converting the waste products of food plants or plants that are not used as a food or feed source into fuel is a potential method to produce fuel for sustainability. For these reasons current research into the production of ethanol as fuel source is concentrating on the use of cellulose rather than starches. However, converting corn stalks, husks, grasses, or other non-food plant products to ethanol does require some petroleum. But the current research shows promising data of converting cellulose to ethanol. Experts calculate that it takes 1 gallon of gas to produce 5 gallons of cellulose-derived ethanol. Ideally, three steps are required to produce ethanol from cellulose: Pretreatment, Enzymatic hydrolysis and Microbial fermentation, which should be optimized to produce the largest amount of ethanol with the least amount of energy and cost input. In the pretreatment the plant material is first processed mechanically, as well as with acids or enzymes and heat to remove lignin. Lignin is a highly complex, aromatic macromolecule found in high quantities in secondary cell walls of fibrous and woody plant tissue in close association with cellulose, which inhibits the enzymatic activity of cellulase, which is needed in enzymatic hydrolysis step. Once the lignin is removed, the cellulose is more exposed and can be more readily broken down into glucose by cellulase. The biofuel industry uses cellulases to convert the cellulose in plant cell walls to sugars, such as glucose. The sugar can then be converted to ethanol by microbial fermentation. There are three different types of cellulase is being used to convert glucose from cellulose. Endocellulases- these enzymes break down the internal bonds of the long chains of glucose molecules that form cellulose. Exocellulases- these enzymes break the covalent linkages between the glucose units of cellulose that are on the end of the cellulose molecules, releasing cellobiose. Cellobiases: these enzymes break down the cellobiose left behind as a result of the work of the first two enzymes.





**One way that ethanol can be produced from plant matter is by completing the following three tasks:**

**1. Pretreatment: Removal of non-cellulose biomolecules such as lignin**

**2. Enzymatic hydrolysis: Cellulases hydrolyze cellulose to produce six carbon sugars**

**and enzymes can be added to hydrolyze hemicellulose to five carbon sugars**

**3. Microbial fermentation: Converts sugar products into ethanol**

In this laboratory experiment, you will be studying cellobiase. Cellobiase is involved in the last step of the process of breaking down cellulose, to glucose. This is a natural process that is used by many fungi as well as bacteria (some present in termite guts, others in the stomachs of ruminants and also in compost piles) to produce glucose as a food source. This natural process is exploited to produce biofuel. This is why the scientific study of the cellobiase is crucial to make it more active and viable during the different condition used to convert cellobiose to glucose. However, Solutions of cellobiose (substrate) and glucose (product) are clear, which makes it difficult to quantitatively detect the product (glucose) or disappearance of substrate (cellubiose) to know about enzyme kinetics. The advent of a simple colorimetric assay using an artificial substrate, p-nitrophenyl glucopyranoside, can be used to detect enzymatic activity of cellobiase. The substrate p-nitrophenyl glucopyranoside is composed of a beta glucose covalently linked to a molecule of nitrophenol. When the bond connecting these two molecules is cleaved with the help of cellobiase, the p-nitrophenol is released. To stop the activity of the enzyme and to create a colored product, the reaction mixture is added to a basic solution. When the p-nitrophenol is placed in a basic solution, the hydroxyl group on the nitrophenol loses an H+ to the OH– of the base, which changes the bonding within the phenolic ring, so that the molecule will absorb violet light (and reflect yellow light). This makes the solution yellow, which can be detected visually by comparing the deepness of the yellow color to a set of standards of known concentration of p-nitrophenol or by using a spectrophotometer to produce more accurate, quantitative results.

It is also possible to detect any effects of pH, temperature, substrate concentration or enzyme concentration have on the initial rate of reaction by using different pH condition, temperature, substrate and enzyme concentration respectively.











