REACTION KINETICS FOR STARCH HYDROLYSIS WITH GLUCOAMYLASE

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REACTION KINETICS FOR STARCH

HYDROLYSIS WITH GLUCOAMYLASE

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PREFACE

The research reported in this document was directed towards examining the kinetic relationships and factors important in the hydrolysis of soluble starches to glucose using the glucoamylase enzyme. Analytical methods developed are discussed along with actual kinetic data and corresponding mathematical relationships.

I would like to express my sincere appreciation to my major advisor, Dr. Bobby L. Clary, for the assistance and guidance he has provided throughout my doctoral program. Dr. Gerald H. Brusewitz, Dr. Don F. Kincannon, and Dr. Richard W. Whitney, members of my advisory committee, also offered important advice and assistance, and I extend my thanks to these gentlemen.

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CHAPTER I

INTRODUCTION

The machinery of living cells is made primarily of enzymes. Hundreds have been extracted, purified, and crystallized. Many others are recognized only by their catalytic action and have not yet been isolated in pure form. Basically, all enzymes are either simple or conjugated proteins (Gaudy, 1980), and exhibit a high degree of specificity in regard to the substrate on which they act.

While biological in nature and origin, enzymes possess many of the same general properties as inorganic catalysts (cobalt, platinum, etc.), i.e. they accelerate reaction rate and are entirely recoverable and usable after the reaction is complete. Because of this capacity for "recycle", relatively small concentrations of enzymes can be used to convert large amounts of substrate.

While enzymes have been used in food formulation and processing for many years, perhaps the greatest advancements have taken place in the last ten years. First, a number of new enzymes with potential for widespread application were recognized and separated. Perhaps the most notable of these were new amylases and invertases which had major impact on the beverage and sweetener industries. Previously, hydrolysis of grain starches to sugars was accomplished with enzymes naturally present in malting grains, etc. Unfortunately these enzymes left a considerable concentration of "limit dextrins", or unfermentable sugars, in

fermentation broths. With separation and concentration of alpha and glucoamylase enzymes, it was suddenly possible to artificially add the enzymes necessary to obtain a desired degree of saccharification. Production of glucoamylase (which allows complete hydrolysis of the starch molecule) was important to the recent interest in alcohol. This enzyme also made possible development of many low calorie beers, and production of corn starch fractions with various degrees of dextrinization (such as the Maltrin product line) for use as fillers, sweeteners, and other special-use ingredients.

Availability of the invertase enzyme led to development of high fructose corn syrups for use as beverage sweeteners. These sweeteners are used to provide sweet flavors with reduced calories. Discussion of this enzyme is covered in a later section.

Concurrently with commercialization of new enzyme preparations came developments in immobilization technology. In an immobilized process, the enzyme is made insoluble and retained in the solution. It is readily separable from the product or is retained in the reacting solution for reuse. This technology becomes more economically viable in direct proportion to enzyme cost. Thus, it is possible to perform some conversions with immobilized enzymes which previously were accomplished by other means, or not at all.

The enzymes and applications discussed are only a few of hundreds currently being used and researched in the food and chemical industry.

Unfortunately complete information on biological parameters affecting performance of enzyme systems is difficult to obtain. Enzyme function is undoubtedly researched by manufacturers, but information is

often unavailable to engineers who must design conversion processes and equipment.

Much research has been published on attack mechanisms and kinetics for the amylases. However, in many cases, the studies were too specific to be of practical use in design. In addition, process equipment and conditions may have a pronounced effect on enzyme activity. Finally, commercially available enzymes may actually contain several enzymes which work complementary. The kinetics of such "blends" are different from those of pure enzymes.

The problem has been particularly evident in the developing alcohol industry where even competent process engineers have had difficulty selecting and managing enzyme systems for specific applications. Information necessary to design an enzyme conversion system for special substrate and processing requirements must be developed based on a thorough understanding of enzyme kinetics, substrate to be used, and desired nature of final product. Such information can be used to optimize the conversion process by appropriate equipment design and process management. Effects of changing process conditions can be more accurately predicted.

There is much to be learned about enzymatic conversion of grain starch to basic sugars. Factors such as agitation rate, carbohydrate chain length, inhibitors, method of cooking, etc. are often overlooked in manufacturer's recommendations. Proper enzyme selection must be preceded by development of enzyme kinetics for enzymes and substrates to be used. It has been shown that many enzyme reactions can be expressed by the relationship:

$$V = \frac{V_m \cdot S}{K_m + S}$$

where: V = enzyme conversion velocity for a unit quantity of enzyme

S = substrate concentration at which velocity V is observed

V_m = maximum conversion velocity which can be obtained for a unit quantity of enzyme, and

K_m = conversion rate constant which is unique to the particular enzyme-substrate complex.

In order to accurately design an enzyme conversion system, one must evaluate V_m and K_m for all combinations of substrates and enzymes under consideration. The equation suggests that K_m and V_m are constant for all values of S. In fact, some researchers have reported different K_m values for high and low substrate concentrations. It is not clear whether K_m is actually different, or if experimental methods used in determining the values were inconsistent with the basic kinetic model and its underlying assumptions. To further complicate the problem, changes in pH and temperature affect V_m and K_m. Thus, V_m and K_m must also be determined for the range of pH, temperature, and substrate conditions which are used in the conversion process. For some enzyme-substrate combinations, the Michaelis-Menten relationship does not adequately describe reaction kinetics. Other mathematical models may be required for these situations.

Preliminary testing has shown that typical starch to glucose conversion velocities outside the fermenter are apparently too low to yield complete conversion within the 48 to 60 hours normally allocated for fermentation. Some acceleration of conversion must be taking place, since in practice fermentation can be completed within this time. If

such an acceleration in rate does occur, the magnitude of the increase and the factors involved should be investigated. Yeast cells may be supplying enzymes which convert starch to glucose, or co-factors may be present which act as catalysts.

One project (O'Neal, 1983) which has shown potential in cooking grain starch for subsequent enzyme conversion involves the use of extruders. In general, yields of sugar and alcohol from extrusion cooked products are consistently higher than with conventional batch cooking operations. However, in order to use the extrusion cooker successfully, advantage must be taken of lower liquid slurry temperature resulting from the process. It is thought that a slurry temperature of 100°F will be the highest necessary. Research at OSU (Downs and Clary, 1983) has shown that it is unnecessary to use alphaamylase to control viscosity during conversion of extruded starches. Thus, an enzyme system for this conversion process must be specially selected for low slurry temperatures and relatively long chain carbohydrates. This is a good example of the need for specific kinetic information on glucoamylase function.

CHAPTER II

REVIEW OF LITERATURE

Method of Operation

Much of the literature describing glucoamylase activity reports results for relatively low substrate concentration and long reaction times. The method of attack and progress of starch hydrolysis by these enzymes is well described.

A typical glucoamylase as described by Yang (1982) is capable of hydrolyzing both alpha 1-4 and alpha 1-6 glucosic linkages in amylose and amylopectin, thus achieving a complete breakdown of starch to glucose. The enzyme works by liberating individual glucose units beginning at a non-reducing end of the starch chain.

MacAllister (1979), Kerr (1951), and Pazur (1959) note that glucoamylase removes glucose molecules from starch rapidly up to a branch point. Hydrolysis then proceeds on the alpha 1-6 bond, but much more slowly. MacAllister also discusses several other enzymes with strictly debranching (alpha 1-6 specific) activity such as pullulanase and isoamylase.

Pazur (1959) presents an interesting discussion of the mechanism of attack. He discusses the possibility of various encounters of the enzyme with substrate, and conditions necessary in order that a particular encounter be effective in forming an active enzyme-substrate complex.

Similar information is presented by Metzler (1977) who describes the collision of particles in a solvent-enzyme system, and diffusion of the enzyme through the solution. Metzler suggests the "cage" effect of solvent molecules may result in as many as seven "bumps" or collisions between a substrate molecule and an enzyme molecule for each encounter. During this period of collision, both molecules are rotating about one or more axis, possibly bringing the molecules into a position necessary for substrate-enzyme complexing.

Appreciable quantities of free maltose and other short chain dextrins are not generated by activity of pure glucoamylase. This is substantiated by Corman (1948) and Lee (1976) for Aspergillus-niger and Rhizopus, two strains common in today's commercial glucoamylases. There may be varying amounts of maltose, trisaccarides and other short dextrins in solution depending upon the method of initial starch hydrolysis. Pazur and Ando (1959) point out that the presence of short chain fractions as intermediate products may indicate that the enzyme solution is not purely glucoamylase, but contains some alpha activity.

Lee (1976) notes that enzyme activity on acid hydrolyzed starches generally does not proceed as far as for substrates hydrolyized with alpha amylase. Acid treatment results in formation of some materials not susceptible to further hydrolysis by glucoamylase.

MacAllister (1979) discusses the formation of specialty products and sweep syrups by using combinations of alpha, beta, and glucoamylases. Combinations of these enzymes can be used to form products of varying DE (Dextrose Equivalent or chain length) for specific uses.

A typical high fructose corn syrup made from 95% glucose (the glucose formed from reaction of glucoamylases on corn starch) will

ultimately contain 42% fructose, 52% glucose, and 6% other saccharides. This is the practical limit for the reaction although it can be forced to 80% fructose by isomerization in the presence of various chemicals.

Equilibrium concentrations for glucose and its polymers are important where long reaction times lead to high final product concentrations. Lee (1976) and VanBeynum (1980) point out that enzyme reactions generally are reversible and under certain conditions free glucose may be reformed into maltose or other saccharides. VanBeynum suggests that if sufficient amounts of glucose are formed by alpha 1-4 splitting activity, formation of alpha 1-6 linkages will become significant. He observes that this may account for the well known transient peak in glucose concentration when dextrins are incubated for long periods of time with glucoamylases. This phenomena may help to explain why some researchers have observed erratic kinetics with high substrate concentration allowed to proceed near completion. VanBeynum reports that a 20% dry solid solution would have an equilibrium glucose concentration of near 90%.

Molecular Size

The relative sizes of starch and glucoamylase molecules may be important in the action of the enzyme. Determination of molecular weight for amylopectin is difficult, and actual size depends on the source of the starch. Greenwood (1976) reports the molecular weight for amylopectin as being in excess of 10^8, with amylose having a molecular weight in the neighborhood of several hundred thousand. According to Pazur (1965), the amylose and amylopectin molecules are somewhat smaller (by a factor of 10) than that suggested by Greenwood. Pazur suggests that the

relative size difference between amylopectin molecules and smaller enzyme and solvent molecules causes considerable hindrance to movement of enzyme molecules in the solution, apparently by a network formed by starch molecules. This is one of the few sources in the literature which suggests that decreasing reaction velocities may result from increasing substrate concentrations. Pazur (1965) notes that in typical reaction mixtures of starch and enzyme, several million substrate molecules may be in the immediate domain of one enzyme molecule. Solomon (1978) suggests that typical amylose molecules contain 500 or more glucose units while amylopectin may contain several thousand or more. According to Whitaker (1972), the amylose molecule size is 300-400 glucose units. Both Solomon (1978) and Pazur (1965) list the molecular weight of glucoamylase at approximately 97,000.

MacAllister (1979) describes a typical cereal starch amylopectin as consisting of short amylose-like chains of 12 to 50 glucose units with an average length of 20. Solomon (1978) also reports that branch points occur at intervals of approximately 20 to 30 glucose units in the amylopectin molecule.

Experimental Kinetics

It is difficult to find applicable kinetic information for commercially used glucoamylase preparations. Most studies used relatively long reaction times, pure enzyme and low substrate concentrations. Theoretically for monomolecular reaction, enzyme velocity may be expressed as a function of substrate concentration and rate constants using the Michaelis-Menten equation:

$$V = \frac{V_m \cdot S}{K_m + S}$$

where K_m is defined as the Michaelis-Menten constant and is the equilibrium constant for the enzyme and substrate (see Figure 1). V_m is the maximum velocity of the reaction.

Reed (1966), Weetall (1972), Humphrey (1974), Gaudy (1980), Levenspiel (1972), Metzler (1977), and Haldane (1965) have prepared good basic treatments of enzyme kinetics. Solomon (1978) discusses various enzyme reactor types and the appropriate selection for kinetic studies. Some analysis is made of basic equations describing performance for batch, plug flow, and continuously mixed reactors. These scientists also present methods for determining rate constants K_m and V_m from batch reactions. Methods for determining the constants are based on calculating initial velocities at various substrate concentrations.

Weetall (1972) has applied Michaelis-Menten kinetics to a study of reactor parameters required for continuous production of dextrose from 15-25 DE corn starch at 30% solids by weight. He observed considerable variation in rate as a function of time and background glucose, and was unsatisfied with the ability of the Michaelis-Menten relationship to predict velocity.

Reed (1966) and Gaudy (1980) point out that the two most common causes for a decrease in initial enzyme rate are depletion of substrate (modeled by the Michaelis-Menten equation) and inhibition. Reed (1966) also suggests that initial reaction velocity for a particular enzyme can be assumed constant if not more than 10-20% of the substrate has been transformed by the reaction. Several other researchers have found the velocity to be constant over a much longer period for higher substrate

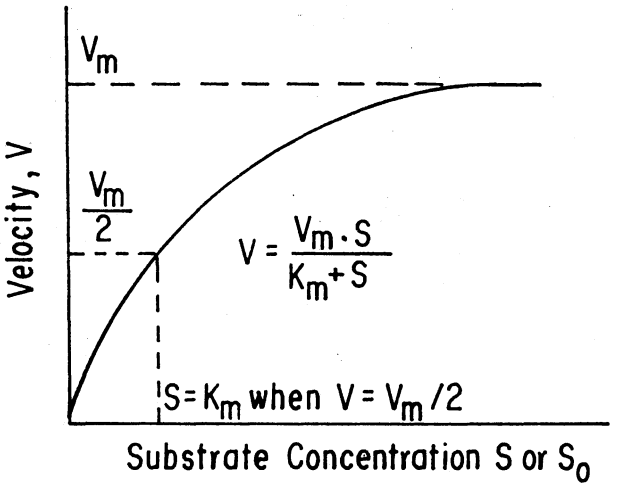


Figure 1. Michaelis-Menten Plot.

concentrations. This is consistent with Michaelis-Menten kinetics in the range where maximum velocity is controlling, providing enzyme activity is not being degraded or inhibited. Kerr (1951) and Solomon (1978) both report constant conversion velocities until approximately 60% of a starch substrate (1-20% solids) was depleted. The reduction of rate after this point may have been due to many factors including substrate depletion, reformation of polymeric units from free glucose or feedback inhibition.

A somewhat different kinetic approach was taken by Swanson (1978) who found that reaction rate data for hydrolysis of maltose by glucoamylase was reasonably well fit by the classical theory of simultaneous reaction and diffusion.

Solomon (1978) suggests that use of the Michaelis-Menten equation may not be adequate for all conditions. He points out that although inhibition should be considered, it is neglected in many kinetic studies. Solomon's concerns seem reasonable since the Michaelis-Menten equation predicts a change in velocity due only to disappearance of substrate. The equation may not be valid in cases where other phenomena are involved. Although the equation predicts nearly constant velocity above certain substrate concentrations, some researchers have reported varying rates at high concentrations. Attempts to describe such data using first order kinetics have not always been satisfactory.

Mannervik (1982) discussed general methods of experimentation and analysis for enzyme studies. Experimental design and data collection is outlined. Discrimination between rival models is discussed. Methods for determining and assessing experimental error are covered, and procedures for determining how well a proposed kinetic model fits

experimental data are suggested. A good discussion of Haldane's adjustment to the Michaelis-Menten relationship is presented.

Substrate and Product Inhibition

Haldane (1965) presents a complete treatment of enzyme structure, function and kinetics. He points out that inhibition by excessive substrate concentrations is fairly common. Haldane notes that when the product of an enzymatic reaction can be an inhibitor, it generally becomes significant when present in concentrations of the same magnitude as that of the substrate. A good discussion of the relationship between enzymes, substrates, products, the solvent is also given.

Haldane's enzyme treatment is broad, but thorough in discussion of the many types of inhibition and inhibitors which may play a part in enzyme reactions. This work is especially good with respect to potential inhibitory properties of substrates and products themselves, and for secondary substances which may act as inhibitors because of their similarity to the product and substrate. A similar discussion is offered for co-factors, activators, and substances which may actually improve the reaction velocity of a specific enzyme.

Kincannon (1977) has applied the Haldane adjustment to Michaelis-Menten kinetics in the study of a waste treatment problem. A satisfactory fit to experimental data was obtained with this method.

Rate Constant and Related Phenomena

Several researchers, including Underkaufler (1954) and Reed (1966) have researched the reaction velocity for glucoamylase. Reed (1966) reports an initial velocity for Rhizopus of 0.855 (expressed in moles

hydrolyzed per minute) for amylopectin and 0.783 for amylose. Reed's results clearly indicate an affinity of glucoamylase for substrates with long chain lengths. Initial reaction velocities for amylose are twice as fast as those for maltose. Reed points out that Dextrose Equivalent may not always be an adequate means of comparing chain lengths of various products. Dextrose Equivalent is a good indicator for dextrins produced from purely acid hydrolysis. However, if combinations of acid and enzyme hydrolysis have been used, Dextrose Equivalent may still be representative of reducing sugars, but not necessarily of the distribution of chain lengths.

Weetall (1972) has identified K_m and V_{max} for several substrates. Assays were performed at 60°C using enzyme thinned starch at 30% solids and pH 4.5 for twenty minutes. Starch used was 4% Lintner starch, or commercially purchased, 25 DE enzyme thinned cornstarch at 30% solids. Reaction was stopped by adjusting pH to 8-10 with sodium hydroxide. K_m values were determined at both high and low substrate concentrations. It is unclear how K_m values were obtained at high solid concentrations, or to what extent substrate concentrations were varied during the determination.

Gruesbeck and Rase (1972) performed their enzymatic studies with an incubation period of thirty minutes, and a solution of 35% starch at pH 4.3 and 60°C. The amount of glucoamylase used was rather large, 2.37×10^{-2} grams per milliliter. The reaction was allowed to proceed toward completion at 350 milligrams of glucose per milliliter. A plot of glucose produced versus time was generally non-linear throughout hydrolysis with perhaps a short linear range from 0 to 160 milligrams per milliliter. Significant quantities of free glucose were present during late

stages of the reaction. Glucoamylase activity was more rapid on amylopectin than on the unbranched amylose. Their suggestion is that the enzyme finds more points of attachment in the more numerous ends of the branched substrate.

Kerr (1950, 1951) also showed that glucoamylase typically exhibits a four times higher initial hydrolysis rate for amylopectin than for amylose. Kerr prepared amyloses of different molecular weights but in equal molar concentrations. The results indicate that for equal molar solutions, the amount of sugar formed per unit time was essentially the same though solids concentration varied over a wide range. Kerr concluded that the effective substrate concentration is the number of molecular terminals rather than the total number of glucoside bonds. This analysis was specific for amylose, and may not be adequate for comparing amylopectin starch of high D.E. versus one of low D.E. High D.E. substrates have more end points than low D.E. products. Nevertheless, activity on low D.E. product has been shown to be higher. Pazur's (1965) measurement of hydrolysis of maltose, nigerose, and isomaltose has shown the relative rates to be 30:3:1. Whitaker (1972) points out that split products of amylose or amylopectin continue to serve as substrates down to the level of maltose although K_m and V_{max} values are different. In discussing glucoamylase specifically, Whitaker lists K_m values for maltose of 1.6×10^{-3} molar; for amylose, 4.4×10^{-5} molar; and for amylopectin, 4.1×10^{-7} molar.

Pazur and Ando (1959) report that purified amyloglucosidase is capable of hydrolyzing maltose but has no transglucosylic activity. Most of Pazur's studies were done with low substrate concentrations and for time periods of one hour. When glucoamylase was incubated with

starch, 95% conversion was obtained. Conversion was 93% for pure amylose, and 98% for amylopectin.

Currently there is interest in identifying, separating, and culturing enzymes which can hydrolyze raw grain starch, thus eliminating the energy intensive and complex cooking or gelatinization step. Weller, Steinberg and Rodda (1984) have identified and separated an amylase fraction produced by growing Aspergillus awamore and A. niger on raw, ground corn. These Koji preparations were used to hydrolyze the starch of raw, ground, whole corn to sugars during simultaneous fermentation. These fermentations compared very favorably with control fermentations of conventionally prepared substrates. Other researchers are also pursuing this attractive alternative to conventional methods of hydrolysis. The work is in an early stage, and no definite kinetics have been reported for these enzyme preparations, so it is difficult to estimate their true catalytic power.

Fermentation Considerations

Lab research reported by Yang and Grow of Miles Laboratories (1982) is useful in relating basic enzyme kinetics to activity during fermentation processes for grain starches. Their work suggests that fermentation slows to whatever rate can be supported by conversion of dextrins to maltose and glucose.

Some data presented by Yang and Grow (1982) seems to indicate that rate of conversion of dextrins during fermentation is still closely tied to D.E. of the remaining soluble starch. In one particular test, as the percentage of glucose and maltose in the fermenter dropped, the rate of conversion of dextrins (DP3 and higher) increased.

Little information is available dealing with the ability of yeast cells to generate enzymes for hydrolysis of carbohydrates other than simple maltoses. Certain yeasts apparently do seem to have the capability of producing extra-cellular amylases of various types. Thus, it is conceivable that some yeasts are capable of hydrolyzing polysaccharides, especially alpha 1-4 linkages. This may help explain why enzyme rates outside the fermenter are different than those observed inside the fermenter.

CHAPTER III

OBJECTIVES

The following objectives were established for the research:

1. Develop simple, rapid, and inexpensive methods for determining reaction kinetics for glucoamylase.
2. For an enzyme-substrate system of gluco amylase and starch, determine the relationship between conversion velocity and:
   A. Dextrose Equivalent (carbohydrate chain length) of substrate,
   B. substrate concentration,
   C. potential cofactors or inhibitors such as glucose and alcohol which might be present in the substrate,
   D. contamination of substrate with fungicides such as Vitavax, and,
   E. agitation level for the reaction process.

Discussion of Objective 1

The problem of determining reaction rate kinetics for glucoamylase is essentially one of measuring in a test solution the change in free glucose produced by a measured amount of enzyme during a fixed reaction period. At first glance this appears to be a relatively simple problem since there are many methods available for glucose determination.

Typical methods for analysis of sugars include wet chemistry and titration, gas chromatography, infra-red analysis, and enzymatic conversion of glucose to another form such as peroxide which can be easily measured in direct proportion to the amount of glucose originally present in the sample. Relatively complex and expensive laboratory equipment is required for these tests, and it is desirable for laboratory personnel to be reasonably familiar with the chemistry and analysis involved. Normally, testing of a particular sample requires considerable preparation and calibration of analysis equipment.

A particular problem is deactivation of the enzyme after the prescribed reaction period. If determination of glucose cannot be made immediately, the enzyme must be deactivated or further enzymatic reaction will take place. Normally this is accomplished by pH adjustment or by heat deactivation. Either of these methods can result in problems with the analysis depending on which methods are used.

Recent development of the Yellow Springs Instrument Company Model 27 Glucose Analyzer has made it possible to rapidly and economically determine glucose in solution without the investment in equipment, time, and training required for other methods. While relatively well-proven as an analytical technique, little work has been published concerning use and limitations of the instrument as a tool in analysis of enzyme function and kinetics. Thus, a major objective (and a necessary prerequisite to further work) of the research was development of appropriate methods for using the YSI 27 in determination of reaction rate kinetics, especially in sample preparation.

Discussion of Objective 2

As pointed out in the literature review, the reaction rate for glucoamylase appears to be related to carbohydrate chain length, although the information reviewed is sketchy. Common methods of preparing grain starches for saccharification by glucoamylase involve either chemical or enzymatic hydrolysis of the basic crystalline structure of the starch molecule to dextrins of various chain lengths. This dextrinization is particularly critical in control of viscosity during cooking. Depending on method and degree of hydrolysis, extent of the dextrinization can vary considerably. It would be desirable to have a better idea of how reaction rate for glucoamylase is affected by substrate chain length.

Michaelis-Menten kinetics suggests that reaction rate is dependent on substrate concentration. It is usually assumed that most industrial starch conversion processes using enzymes take place at a substrate concentration high enough to insure operation of the process at maximum velocity. Nevertheless, this should be verified. High substrate concentrations may also produce inhibition to enzyme conversion (Haldane, 1965). This phenomena can be observed in rate versus concentration studies. The rate versus concentration studies should be extended to substrate concentrations of 1% or less, particularly for fermentation operations where low concentrations are reached during the final stages of fermentation.

Gaudy (1980) and Metzler (1977) both point out that certain common chemicals can be inhibitory to enzyme function either as direct inhibitors to the conversion catalysis, or as product feedback inhibitors. Thus, it is important to determine if any obvious product feedback

inhibition occurs with increasing glucose levels. As much as 80% of total starch conversion actually takes place during alcoholic fermentations, and the enzyme is exposed to increasing quantities of alcohol in the fermentation broth. It would be valuable to examine the possibility that alcohol may act as an inhibitor.

During the recent surge of interest in production of alcohol from grain starches, a number of non-standard feedstocks were considered as a means of reducing production cost. An example is out-of-date seed stocks no longer suitable for planting. Usually this grain has been treated with fungicides, and is unsuited for use in non-fuel production processes. A number of operators have experienced problems with this type of feedstock. A naturally arising question is whether fungicides are inhibitory to enzymes, or toxic to yeast cells.

Finally, the question of how much agitation is necessary during conversion and fermentation frequently arises. Certainly a high degree of agitation is required to control viscosity during high temperature hydrolysis of raw grain starch. However, the amount required for optimum enzyme function during the remainder of the conversion is not so easily determined. This problem was also considered in this research.

CHAPTER IV

THEORETICAL DEVELOPMENT OF ENZYME KINETICS

Enzyme Function

As pointed out earlier, enzymes are basically complex proteins with the ability to catalyze or accelerate specific biological reactions. Gaudy (1980) identifies six basic classes of enzymes depending on general function. The enzymes discussed in this research are hydrolase enzymes which promote the breakdown and splitting of various organic molecules.

Starch hydrolysis by a gluco-amylase begins with bonding of substrate and enzyme molecules. Alignment and positioning must be exactly right for complexing to take place. The excitation and strain caused by bonding is just sufficient to catalyze the reaction. Completion of the reaction leads to breaking of the complexing site and enzyme is freed for recycle. Gluco amylase possesses the special ability to sequentially hydrolyze glucose molecules in a dextrin chain, beginning at one end and moving along the chain liberating glucose units until complete hydrolysis is effected. Both alpha 1-4 and alpha 1-6 bonds can be broken. Many amylases require a co-factor such as calcium ion for optimum reaction, though this is more characteristic of alpha amylases than gluco amylases.

Enzymatic hydrolysis involves chemical reactions, and transfer of various groups from one point to another. Anything causing a change in ionization of transfer and binding sites, or conformation of the space structure will have a pronounced effect on the reaction. Both temperature and pH greatly affect the nature and velocity of the enzyme reaction. Normally, the relationship between pH and reaction velocity is a bell-shaped curve, with a relatively narrow range of pH values over which the enzyme is most active. In general, velocity increases with increased temperatures as predicted by the Arrhenius equation (Gaudy, 1980). However, proteins may be denatured by high temperature exposure, and so at some elevated temperature, increased reaction rate is counterbalanced by a decrease in activity due to denaturization. Denaturization, of course, causes a permanent loss of function. Low temperatures, on the other hand, may result in a virtual cessation of activity, but do not destroy functional ability of the enzyme. With an increase in temperature, the enzyme may resume it's role as biological catalyst.

Various forms of inhibition can also affect and influence enzyme function. Inhibition usually takes one of two forms. Competitive inhibitors are those that compete with the substrate for binding with the enzyme. Their configuration and geometry is similar to that of the substrate. Non-competitive inhibitors do not generally compete directly with the substrate. Such inhibitors may somehow restrict the enzyme from binding at all, or affect large areas of the substrate molecule. Certain heavy metals are often non-competitive inhibitors to enzyme reaction. Non-competitive inhibition is often irreversible. Many naturally occurring poisons are inhibitors of some enzyme catalyzed pathway.

The objectives outlined earlier address two potential types of inhibition. The first occurs when the product or substrate of an enzymatic reaction becomes an inhibitor. For gluco amylase the end product is free glucose, and the substrate is usually dextrins of various forms. The objective for feedback inhibition thus addresses the possibility that a buildup of glucose or high substrate concentration in the reacting medium could result in inhibition of conversion.

Alcohol and chemical fungicides may also appear in reacting solutions where gluco amylase is being used. The second part of the inhibition study dealt with investigation of potential inhibition by these two chemicals.

Enzyme Kinetics

The kinetic model development presented in this section is based on the assumption of mono-molecular kinetics. This assumption implies that the complete reaction sequence, from initial complexing to product formation and complex breakdown, is influenced only by the physics and biology of the two molecules themselves. It is not uncommon, however, for other factors and relationships to exert a significant influence on the reaction. As suggested above, for example, some catalytic reactions are themselves catalyzed by a co-factor such as calcium ion. Such co-factors often aid in the transfer of electrons and sub-groups during the reaction.

While it is important to consider the possibility of inhibition in any enzymatic conversion system, the development of basic enzyme reaction kinetics which follows assumes freedom from any inhibitory effects except substrate inhibition. Gaudy (1980), Metzler (1977), and Haldane

(1965) provide an excellent treatment of competitive and non-competitive inhibition.

Levenspiel (1972) presents a thorough treatment of the analysis of reaction rates for many types of systems. He suggests methods for determining a mathematical relationship which will best fit a particular reaction. He also presents methods for determining whether the selected model satisfactorily describes experimentally obtained data.

Levenspiel points out that for a reaction which has a shift in rate from low to high order as reactant concentration drops, reaction rate may be expressed as:

$$-r_A = \frac{k_1 C_A}{1+k_2 C_A} = -\frac{dC_a}{dt} \qquad (1)$$

where:

C_A = concentration of A (or product),

k_1 = forward rate constant in the reversible reaction, and

k_2 = rearward rate constant in the reversible reaction.

From equation (1), it can be seen that the reaction is of zero order with rate constant k_1/k_2 for high C_A, the reaction is of first order with rate constant k_1. Levenspiel continues by pointing out that the Michaelis-Menten equation is really a specialized form of this general relationship. In addition, this type of equation can be manipulated into a simple form for graphical testing. Two linear forms of the equation are:

$$-\frac{1}{r_A} = \frac{1}{k_1 C_A} + \frac{k_2}{k_1} \qquad (2)$$

and (multiply equation 2 by $\frac{k_1}{k_2}$ $(-r_a)$)

$$-r_A = \frac{K_1}{K_2} - \frac{1}{K_2}\frac{(-r_A)}{C_A} \qquad (3)$$

These forms are nearly identical to linearized forms of the Michaelis-Menten equation used for determination of rate constant K_m and maximum velocity constant V_M. These simple forms allow graphical testing of experimental results against the model.

Levenspiel discusses the method of initial rates where a series of tests is run with different initial substrate concentrations over a constant reaction period. Each run is extrapolated back to initial conditions to determine initial reaction rate. This method was used in this research and is also the basis for the analysis outlined by Gaudy (1980) and Metzler (1977) which is discussed further later.

Levenspiel notes that enzyme based reactions often must be treated differently than typical chemical reactions. In general, an enzyme reaction may be expressed by equation (4) below:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + P \qquad (4)$$

Where:

E = Enzyme unit

S = Substrate unit

P = Product

k_1 = Rate at which E and S form a stable complex which may lead to an enzyme catalyzed reaction

k_3 = Rate at which the enzyme-substrate complex ES, if activated, proceeds to make product P, and free enzyme for recycle

k_2 = Breakdown rate if ES is not activated, and

k_4 = Rate at which P and E may ES in a reverse reaction under certain conditions

Considerable quantities of intermediate, ES, may exist during the reaction. In many chemical processes, the amount of intermediate is small, and is often ignored. The need for consideration of this quantity in enzyme reactions led to the work by Michaelis and Menten.

Shortly after the reaction described by the equation (4) begins, the total amounts of E and ES vary only slightly, i.e., a steady state concentration is in effect and the rate of formation of complex ES is equal to its rate of breakdown. Equating the rate of formation to the rate of breakdown, we can express equation (4) in the form of:

$$k_1(E)(S) + k_4(E)(P) = k_2(ES) + k_3(ES),$$

which upon factoring gives

$$(E)\{k_1(S) + k_4(P)\} = (k_2 + k_3)(ES),$$

or

$$\frac{E}{ES} = \frac{k_2 + k_3}{k_1(S)+k_4(P)} \qquad (5)$$

In deriving the Michaelis-Menten equation from this general form, the back reaction, and hence k_4, is neglected since initially P is very small and thus is unlikely to result in any backward formation (this assumption requires that measurements of V for kinetic studies be made during the initial portion of the reaction when rate is constant). Equation (5) then reduces to:

$$\frac{E}{ES} = \frac{k_2 + k_3}{k_1(S)}$$

and k_1, k_2, and k_3 can be grouped together into one constant K_m.

The equation can then be expressed as:

$$\frac{E}{ES} = \frac{K_m}{S} \tag{6}$$

where K_m is the Michaelis-Menten constant.

Intuitively we can reason that maximum reaction velocity will occur when there is no free enzyme, i.e., the total enzyme available is tied up in complex ES. The maximum velocity is then:

$$V_m = k_3(E_T) \tag{7}$$

This relation is true only at high substrate concentrations. Otherwise the velocity is expressed as:

$$V_m = k_3(ES) \tag{8}$$

Furthermore, we know that free enzyme E must be related to the total amount E_T by:

$$E = E_T - ES$$

Substituting this expression into equation (6) we have

$$\frac{E_T - ES}{ES} = \frac{K_m}{S}$$

which upon rearranging gives:

$$\frac{E_T}{ES} = \frac{K_m}{S} + 1 \tag{9}$$

Relations (7) and (8) can be solved in terms of ES and E_T and substituted into (9) to give

$$\frac{V_m/k_3}{V/k_3} = \frac{K_m}{S} + 1$$

which after cancellation of like terms and rearrangement gives

$$V = \frac{V_m(S)}{K_m+S} \tag{10}$$

Equation (10) is the familiar Michaelis-Menten relationship for a rectangular hyperbola, with K_m as the shape constant. The individual backward and forward rate constants in the lumped form of K_m control the shape of the response curve of velocity to substrate concentration. The rectangular hyperbola is a common response curve for biological and chemical reactions even though the nature of the particular reactions may be completely different.

Low K_m values give a rapidly rising curve with a sharp break towards maximum velocity, while higher K_m values give a more gently rising and breaking curve. The value of the rate constant K_m is numerically equal to the substrate concentration at which observed velocity is one-half that of maximum velocity V_m.

Using the Michaelis-Menten relationship and typical K_m values for amylose starch (Reed, 1966), reaction velocities calculated for a range of starch concentrations from 1 to 20% were within 5% of the value for V_m. For common process reactions, the value of K_m is normally small in relation to the substrate concentration, and the maximum velocity is rapidly approached. Further work should be done to establish K_m values for commercial enzymes and various substrates when the process reaction kinetics are adequately described by a first order reaction.

Several points are worth considering when working with equation (10). The rate constant K_m is not necessarily indicative of the affinity of enzyme for its substrate since it includes the rate at which the complex breaks down to free enzyme and product. Care must be taken in interpreting K_m since it includes the individual rate constants for forward and backward reactions. If the value of K_m for a particular

enzyme-substrate system is known, it is possible to predict reaction velocity for any particular value of S providing the equation is an adequate model for the system. V is expressed as the velocity per unit amount of enzyme.

It is also important to note that K_m and V_m are affected by environmental conditions, particularly pH and temperature. Unfortunately, they are not affected to the same degree by a particular condition, and care must be exercised in extrapolating results obtained from a graphical analysis to other temperature and pH regions.

Determination of Rate Constants

Gaudy (1980) and Metzler (1977) present graphical methods for determining K_m and V_m from batch reactions where initial reaction velocity is measured over a range of substrate concentrations. Normally, data for plots of V versus S is not obtained by measuring slopes all along one particular batch curve, but by setting up a number of reaction vessels, each containing equal amounts of enzyme but varying concentrations of substrate. The reaction is allowed to run for only a short time, during which velocity is constant. This velocity is then plotted against substrate concentration.

The length of the initial portion of an enzyme reaction during which reaction rate is constant varies with concentration and type of substrate. Reed (1966) suggests that initial reaction velocity for a particular enzyme can be assumed constant if not more than 10-20% of the substrate has been transformed by the reaction. Several other researchers have found velocity to be constant over a much longer period. Kerr (1951) and Solomon (1978) both report constant conversion velocities until 60% of a starch substrate was exhausted.

While rate constants K_m and V_m can be determined directly from plots of V versus S, linear forms of Equation (10) can be used which greatly simplify analysis of data for determination of constants. The two most popular forms are the Lineweaver-Burk:

$$\frac{1}{V} = \frac{K_m}{V(S)} + \frac{1}{V_m} \tag{11}$$

and the Eadie-Hofstee form:

$$V = V_m - \frac{K_m(V)}{S} \tag{12}$$

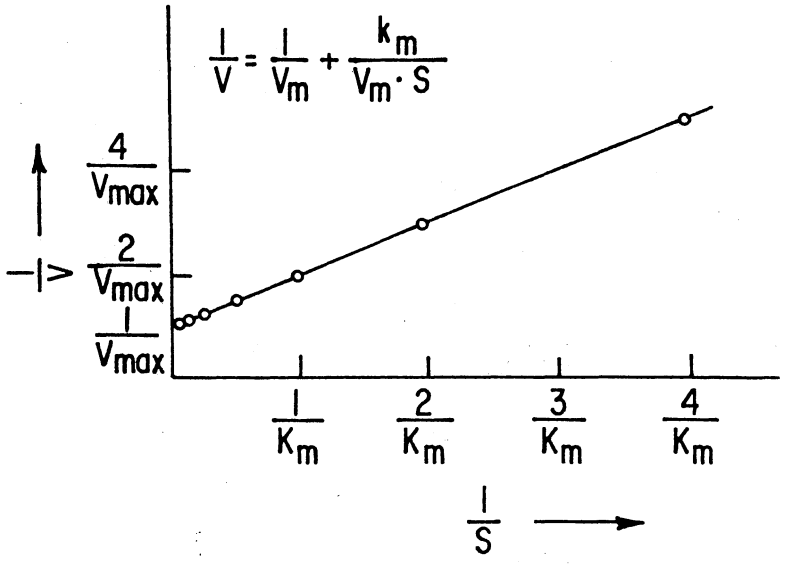
Rate constants can be easily determined from the graphical representations of these two equations shown in Figure 2. With either approach, slope and intercept are very dependent on data taken at low substrate concentrations (normally less than 1%). Intuitively, this should be so since V approaches V_m (a constant) at high substrate concentrations.

Haldane Adjustments for Substrate Inhibition

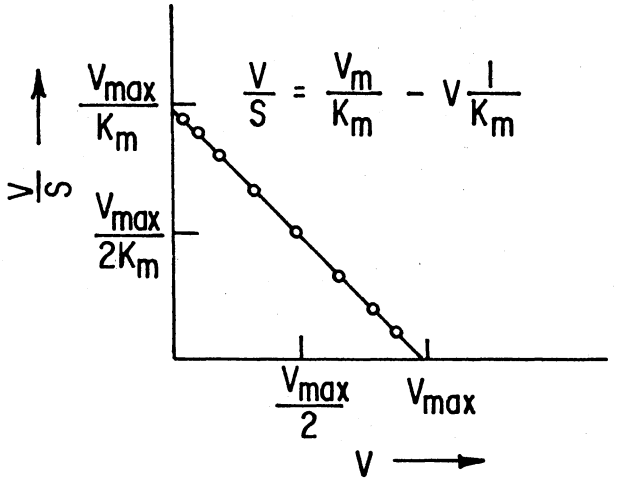
In some cases the substrate itself may act as an inhibitor to the conversion reaction. Haldane (1965) presents the following mathematical model to represent reactions with this characteristic:

$$V = \frac{V_m S}{K_m + S + S^2/K_I} \tag{13}$$

This relation is very similar to the Michealis-Menten equation, but includes a second constant (defined as K_I in this study) to account for substrate inhibition at higher substrate concentrations. The effect of the added term in Equation (13) is shown in Figure 3. Substrate inhibition causes conversion velocity to decline for increasing substrate concentrations rather than remain constant as predicted by Equation (10).



(A)



(B)

Figure 2. (A) Double reciprocal or Lineweaver-Burk plot of 1/V Vs. 1/S.
(B) The Eadie-Hofstee plot of V/S against V.

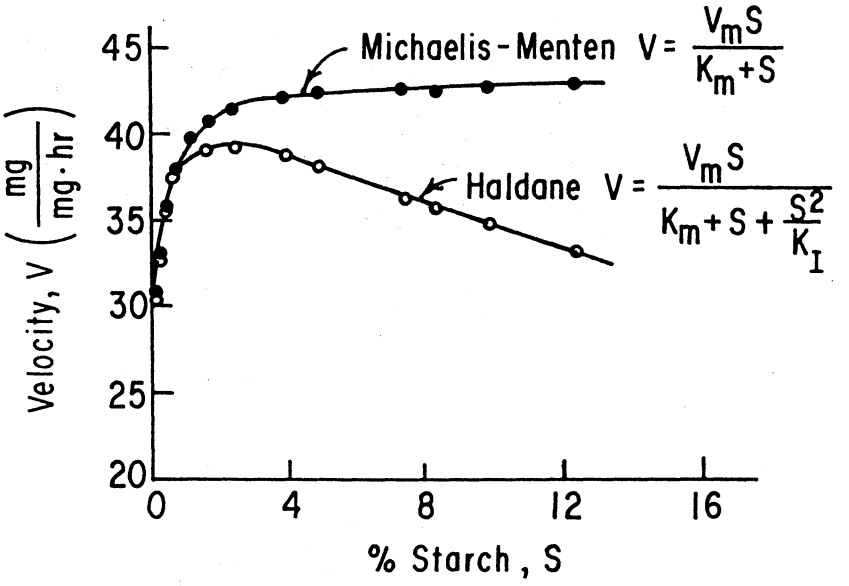


Figure 3. Michaelis-Menten Equation with Haldane Term.

Actually, K_I is a Michaelis constant for the inhibition reaction

$$ES + S \rightleftharpoons (ES)_2$$

which indicates that free substrate interacts with already complexed ES in an inhibitory manner. This reaction leads to a second form of ES rather than to product and free enzyme. For this type of substrate inhibition, Haldane suggests that a plot of velocity vs logarithm of substrate concentration should produce a bell-shaped curve.

Kincannon (1977) applied the Haldane equation to a waste treatment problem. K_m and V_m were obtained in the usual way using V and S data below maximum V values. Values for the Haldane constant were then determined by solving the Haldane equation for K_I using experimental data. An overall K_I was then computed as an average of individual K_I values. This method resulted in an acceptable fit to data which was not modeled satisfactorily by the Michaelis-Menten equation.

CHAPTER V

PROCEDURES

Selection of Substrate

During preliminary investigations, considerable difficulty was encountered in preparing whole corn substrates. A clarified substrate was obtainable, but only after repeated filtering, screening, and centrifuging of whole corn mashes. It was difficult to control the degree of mash dextrinization with the alpha-amylase enzyme used to reduce viscosity during cooking. Since conversion rate is significantly influenced by DE, it was important that initial DE of test substrates be both constant and known.

These problems, combined with the need to vary DE as a dependent variable in some tests led to a search for a standard substrate. Commercially available maltodextrins from American Maize Products Company and Grain Processing Corporation were chosen. Typical product data are shown in Appendix A. D.E. for these products ranged from 5 to 37. Solids concentrations up to 27.5% were achieved with no difficulty.

Enzyme Selection

The enzyme used was the "Gasolase" glucoamylase enzyme distributed by Bio-Con Industries. This enzyme was chosen because its use recommendations seemed most closely suited to laboratory work required by

research objectives. Typically, Gasolase enzyme is added to pre-liquified grain mashes after they have been cooled to 90°F and just before yeast is added for fermentation. Optimum pH is near 4.5.

Although the predominant enzyme in the Gasolase blend is glucoamylase, traces of other enzymes, particularly alphaamylase, are also present (See Appendix B). Gasolase is not appreciably different in function from other commercially available glucoamylases. Use recommendations for glucoamylases vary from one manufacturer to another. Miles, for example, suggests adding their saccharifying enzyme at 130-140°F for a two hour prefermentation saccharifying step. The primary reason for adding enzyme at this point is that conversion velocity rate is much higher than at fermentation temperatures. This initial saccharification step converts a portion of the dextrins to glucose which is then immediately available for uptake by yeast cells. Remaining dextrins are broken down in the fermenter.

Instrumentation for Determining Glucose

A Yellow Springs Instruments Model 27 Industrial Glucose Analyzer was used for glucose determination. The sensor of the Y.S.I. Model 27 uses a thin film of oxidase enzyme immobilized within a membrane. Samples injected into the instrument are oxidized to hydrogen peroxide which is then measured by electrochemical oxidation at a platinum anode. The assay is complete in 60 seconds and results displayed on a digital meter calibrated to known standards. An analog voltage proportional to substrate concentration is provided if recorder output is desired.

Particular advantages of this machine are the economy and speed with which glucose analysis can be made. Various other membranes are

available for determination of alcohol, sucrose, lactose, and even starch. The instrument is easy to use and has proven very reliable.

One factor must be considered when using the YSI 27. Glucose formed by conversion of dextrins is the beta form, which is also the substrate for the subsequent oxidase reaction. The instrument is calibrated to provide digital output based on equilibrium between alpha and beta forms. Thus, provisions must be made for assuring equilibrium, otherwise readings will be disproportionally high. In the laboratory, equilibrium can be assured by the use of high phosphate buffers which catalyze the rotation from beta to alpha. A short, high temperature holding period will also catalyze rotation. Several checks indicated that satisfactory rotation was obtained by heat exposure alone.

The output of the Y.S.I. 27 is linear between 0 and 500 mg/dl, and so it was necessary to dilute samples containing more than 500 mg/dl glucose. The Y.S.I. 27 calibration units of mg/dl (1 dl = 100 ml) will be used throughout this report

Laboratory Methods

Basic procedures for sample preparation and treatment, and determination of results are shown in Appendix C. They are adaptations of procedures and methods suggested by scientists at Yellow Springs Instruments Company and Seagrams and Son. The procedures have proven easy to use and provide good repeatability. The use of an acetate buffer at 4.63 is important, as this allows accurate control of reaction pH. For some samples, a high phosphate buffer was used in combination with a short holding period at high temperatures to insure equilibrium between alpha and beta forms of glucose. Deactivation of the enzyme was

accomplished by immersion in boiling water for six minutes as suggested by Seagrams and Son scientists.

Experiments for determining conversion velocity as affected by agitation were run in a New Brunswick chemostat where temperature and pH could be closely controlled while agitation levels were varied.

A standard enzyme solution was prepared fresh every 48 hours by adding 0.625 grams of dry Gasolase to 500 ml distilled water. In most cases, the amount of enzyme added to a particular sample was 1 ml enzyme solution per 10 ml test starch solution. Between tests the enzyme standard was refrigerated. Before each test, the enzyme solution was equilibrated at 32°C.

Temperature control was achieved by placing all samples in a constant temperature water bath with occasional agitation. Temperature was maintained at 32°C within 0.2°C. For each test series, control samples were prepared and treated identically to the enzyme replications. Automatic pipettes were used to insure rapid and repeatable volumetric transfers. All glassware used for measuring was calibrated and identified to insure consistency of measurements between tests. All starch samples and enzyme solutions were made from the same stock.

In determination of reaction velocity for various substrate concentrations, as many as 15 different concentrations were evaluated during a single day. In these cases, the samples were selected for treatment and analysis at random in order to minimize any systematic or additive errors. Each substrate level was also evaluated on at least three occasions.

Considerable effort was made to insure sterile conditions during testing. All glassware and laboratory tools were washed carefully,

cleaned with a special acidic cleansing solution. Between tests glassware was stored in an oven maintained at 100°C. Care was made to eliminate any cross contamination of samples during analysis, enzyme injection, and injection into the YSI 27. All operational procedures suggested by YSI for the Model 27 Glucose Analyzer were closely followed.

Determination of Reaction Time as a Function of Substrate Concentration

Data for determination of conversion velocity versus substrate concentration were obtained by measuring Initial enzyme velocities over a wide range of substrate concentrations. This method is consistent with procedures suggested by Gaudy (1980) and Metzler (1977). At high substrate concentrations, initial velocity is nearly V_m and will remain constant for a considerable reaction time. For low concentrations, however, the rate may be constant for only a short period. Thus, when performing a velocity evaluation, maximum allowable reaction time for any particular sample is dependent on substrate concentration. For samples with low concentrations, it was necessary to pre-determine maximum allowable reaction time by measuring the rate as often as possible until it became non-linear.

An example of such a test is shown in Figure 4, which shows conversion velocities measured for three different substrate concentrations over a one hour period. It is apparent that the maximum allowable reaction time for 0.25% starch concentration must be considerably less than for concentrations of 10% or higher. In fact, a reaction interval of nine minutes was chosen to insure being within a nearly linear range. For substrate concentrations of 1% or greater, longer reaction times

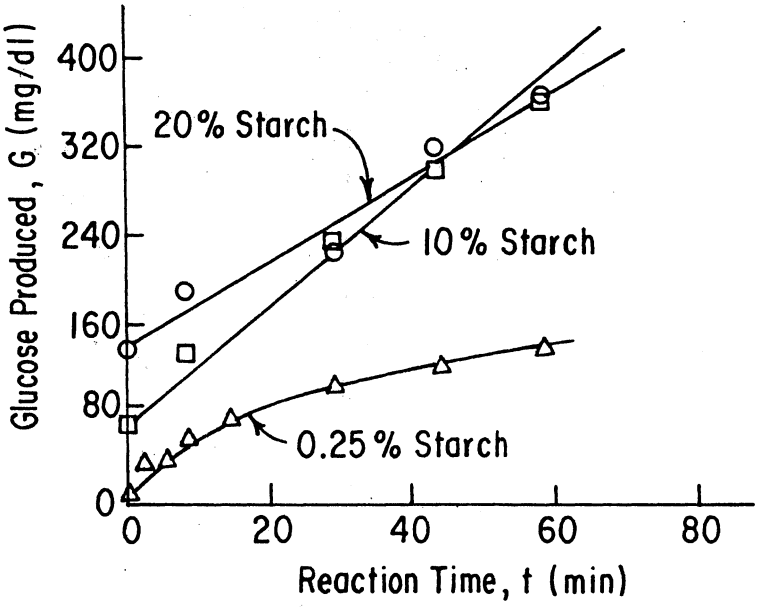


Figure 4. Effect of Incubation Period on Glucose Production.

could have been tolerated, but for the sake of consistency between samples, all were treated for nine minutes. The apparent reaction rate for the 10% substrate is higher than that for the 20% solution. This is in agreement with work previously reported by Downs (1983) and shows the negative effect of increasing substrate concentration on conversion rate.

Tests Performed

An outline of tests performed is presented in Appendix D. For test numbers 1, 2, 3, and 5, three enzyme treated reps were prepared at each treatment level. Two blanks containing only substrate were also prepared. The substrate for all tests except No. 1 was Lo-Dex 10 amylodextrin manufactured by American Maize Products. Lo-Dex 10 has an approximate Dextrose Equivalent of 10, and is fairly typical of a dextrinized starch which might be used in the production of alcohol. Grain Processing Corporation's line of Maltrin Products was used in test No. 5. Six different Maltrin products were used, with a Dextrose Equivalence range from 5 to 36. Except for tests 4 and 6, reaction times were kept equal at 60 minutes.

CHAPTER VI

RESULTS

Conversion Rate as Affected by Agitation and Glucose Concentration

The effects of background glucose concentration and agitation level on conversion rate are shown in Figures 5 and 6. Figure 5 indicates that conversion rate for background glucose concentrations from 0-160 mg/dl is relatively constant. Varying agitation rate did not have a noticeable effect on conversion rate. In fact, Figure 6 clearly shows that conversion rate is virtually constant for three different agitation levels. The three rpm for which data is presented represent respectively: very gentle agitation, moderate agitation, and violent agitation.

The possibility that glucose itself may act as a feedback inhibitor, particularly during fermentations, seems to be discounted by the evidence in Figure 5. Free glucose during a typical starch conversion and fermentation does not normally exceed 50 mg/dl (1 dl = 100 ml), thus, there would be little reason to expect decrease in conversion as a result of background glucose concentration. Similar tests using whole corn mashes have shown that conversion rate is constant for glucose concentrations up to 500 mg/dl. This was not surprising, as other researchers have noted that conversion rate for starch and glucoamylase remains relatively constant for high background glucose concentrations.

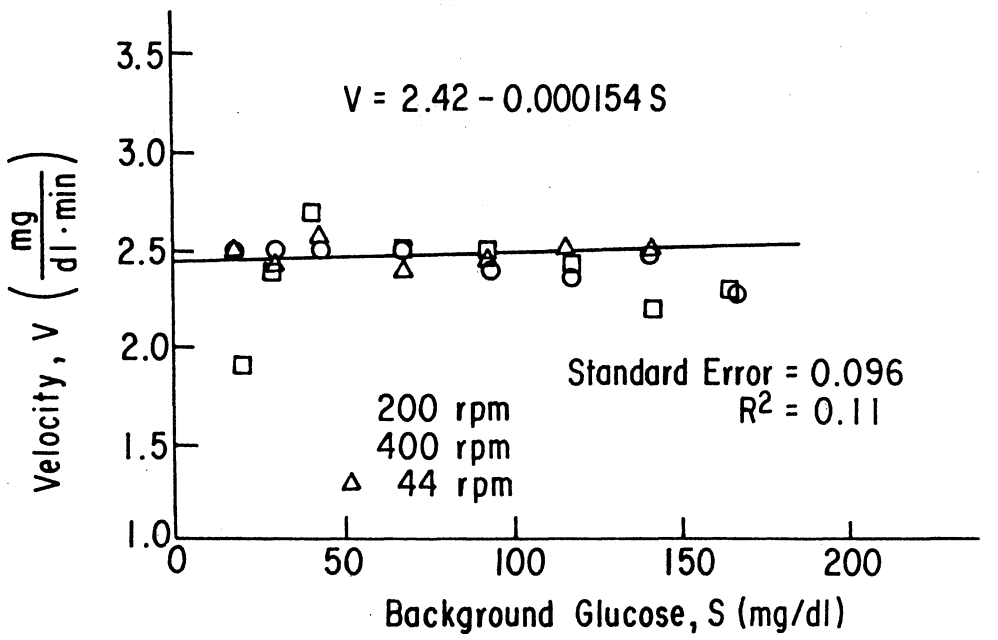


Figure 5. Conversion Rate Vs Glucose Concentration.

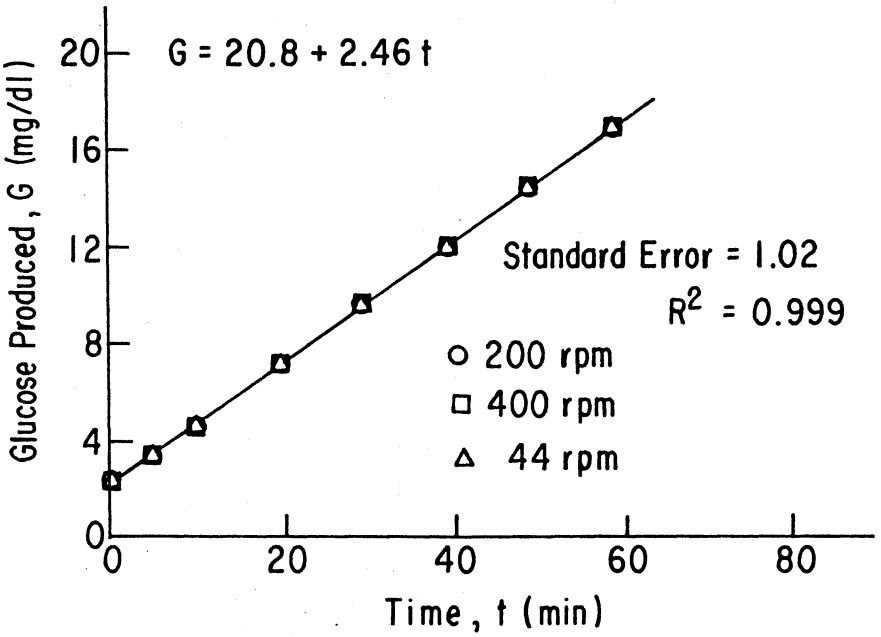


Figure 6. Glucose Production at Different Agitation Levels.

Conversion Rate for Various Substrate Concentrations

Figure 7 shows initial reaction velocities for substrate concentrations from 3 to 18% (solids concentration). The decreasing velocities at higher substrate concentrations were unexpected although several researchers have noted that certain enzyme - substrate systems exhibit a declining rate for high substrate concentrations. This phenomena leads to consideration of the Haldane "adjustment" to standard enzyme kinetics. For a typical whole corn mash, conversion rate did appear to remain essentially constant with substrate concentrations, as shown in Figure 8. However, results were considerably more erratic and undependable at high substrate concentrations because of increasing viscosity and difficulty in preparing samples.

Using a typical K_m value for amylose (Reed, 1966), the Michaelis-Menten equation enzyme reaction velocities were calculated using various starch concentrations of amylose (see Table I). Conversion rate quickly approaches V_{max} for substrate concentrations greater than 1%. For most process reactions, K_m is normally small in relation to substrate concentrations, and V_{max} is rapidly approached. Further work should be done to establish K_m values for commercial enzymes and various substrates when kinetics can be adequately described by the Michaelis-Menten relation with appropriate modifications. For K_m values appreciably different from those reported by Reed (1966), the substrate concentration at which V will depart significantly from V_{max} may be increased.

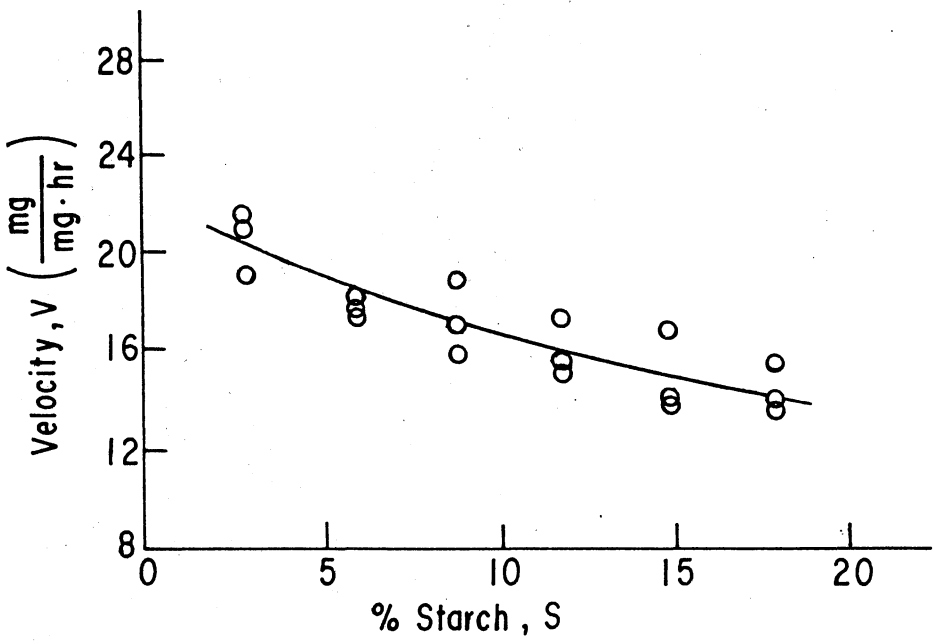


Figure 7. Initial Conversion Rate Vs Substrate Concentration.

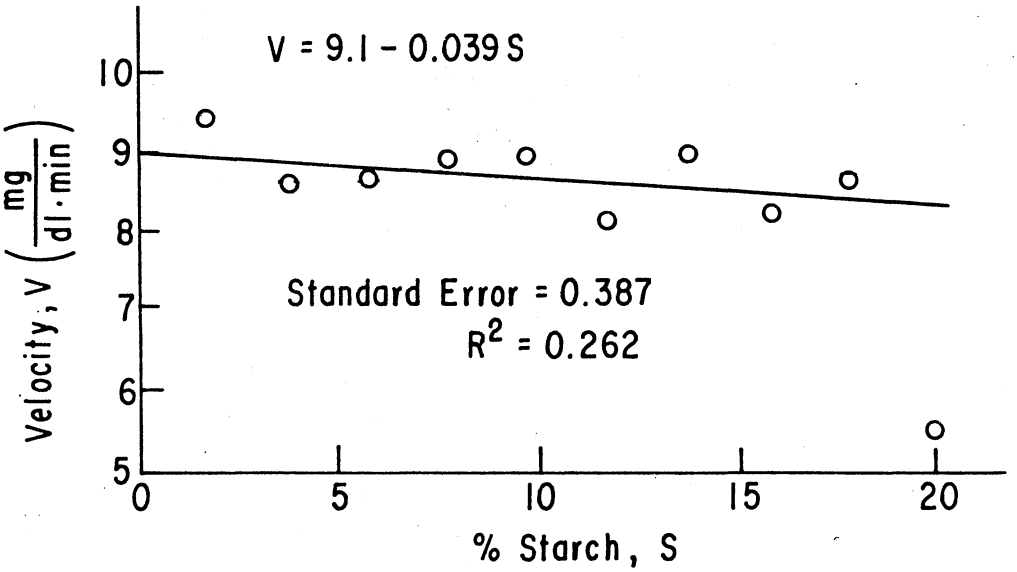


Figure 8. Conversion Rate Vs Substrate Concentration (Whole Corn Mash).

Table I

Enzyme Velocity Vs Substrate Concentration

% Starch	Enzyme Velocity, V
20	0.998 V_m
10	0.996 V_m
5	0.983 V_m
1	0.962 V_m
0.5	0.926 V_m

$K_m = 0.5$ g/l

For substrate concentrations less than 1%, velocity is most likely controlled by substrate depletion, and hence accurately described by the Michaelis-Menten equation. For concentrations much greater than 1%, the velocity should approach V_{max}. However, other factors such as inhibition, changes in mass transfer and movement between substrate and enzyme, etc. may affect V in this range. Thus, the simple Michaelis-Menten relation may not be adequate at high substrate concentrations. Figure 7 certainly suggests that other factors are involved at substrate concentrations greater than 6%.

While the results shown in Figure 7 definitely show a decrease in conversion velocity with increasing substrate concentration, it is clear that additional information was needed for lower substrate concentrations. Neither V_m or the shape of the reaction curve leading to V_m can be determined from data in Figure 7. The need to develop further information in the lower substrate concentration range led to further tests described later in this section.

During early testing, it was felt that the reduction in velocity shown in Figure 7 was probably related to buildup of free glucose in the system. However, Figures 5 and 6 show this to be unlikely for glucose concentrations of less than 200 mg/dl. Maximum background glucose concentrations observed during rate versus substrate concentration tests was 137 mg/dl for a 25% solution, well below levels shown in Figures 5 and 6. No other explanation for the decrease in rate is immediately obvious. Perhaps the most plausible explanation which can be offered is based on information presented by Pazur (1965) who points out that the size of a starch molecule is considerably larger than that of a glucoamylase molecule. For high substrate concentrations, this relative size difference can result in considerable resistance to free movement of enzyme molecules within the solution. Pazur and Metzler (1977) describe the kinetics of encounter between substrate and enzyme molecules. Many "bumps" between molecules may occur before a productive contact leading to complexing of the two molecules. According to Metzler, relative rotation between the two molecules is important in an ultimately successful alignment. These analyses suggest that favorable contact dynamics may be suppressed at high substrate concentrations.

Conversion Rate as Affected by Dextrose Equivalent of Substrate

Figure 9 shows the effect of dextrose equivalence on conversion rate for Gasolase. A pronounced decrease in conversion rate occurs as dextrose equivalent of the substrate increases. This was anticipated; Reed (1966) reports that glucoamylases exhibit definitely higher conversion velocities for substrates with increasing chain length. There

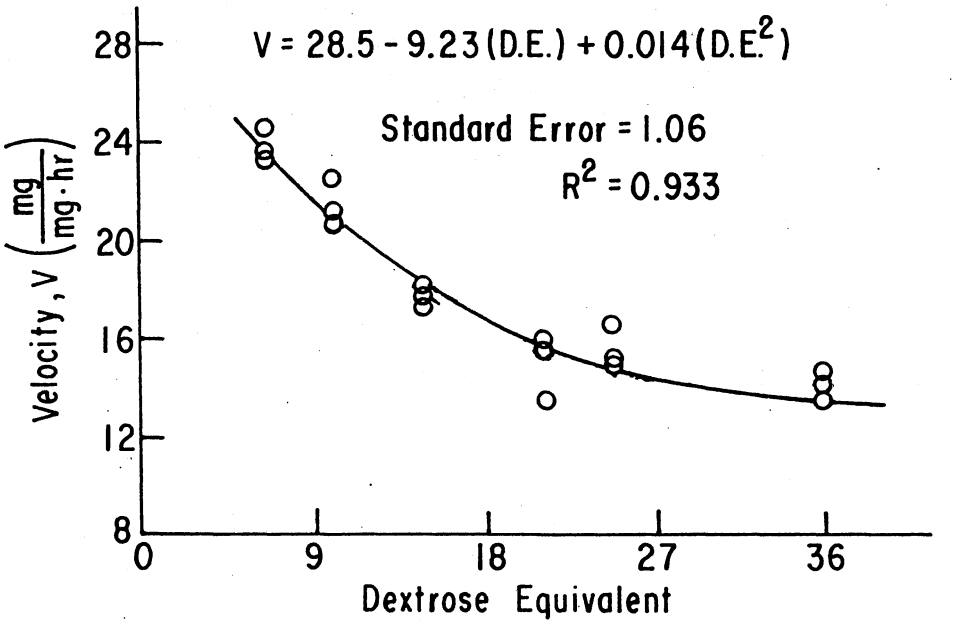


Figure 9. Conversion Rate Vs Dextrose Equivalent (Maltrin Products).

is a relatively rapid drop in conversion rate as dextrose equivalence increases to around 20. It is interesting to note that most enzyme companies suggest that their glucoamylases be used with a preliquified grain slurry with a dextrose equivalence of approximately 10 to 14. The data in Figure 9 suggest that higher conversion rates would be obtained utilizing substrates with lower Dextrose Equivalent.

In practice, degree of dextrinization is often dictated by viscosity limitations for the process. However, in some cases, it might be worthwhile to process with a lower D.E. if viscosity is not a serious problem. As pointed out earlier, the glucoamylase enzyme results in complete breakdown of a dextrin once attached, and chains of intermediate length are not produced in significant quantities. Thus, it is likely that an advantage in conversion rate due to longer substrate chain length will continue throughout conversion.

No specific explanation for this phenomena was discovered in the literature. Dextrose equivalence can loosely be used as an indicator of the number of short chain saccharide units in the slurry. A high D.E., therefore, implies a high concentration of maltose, trisaccharides, tetrasaccharides, and so on with corresponding reductions in the number of long-chain molecules. Once an enzyme is attached to the non-reducing end of a starch molecule, it proceeds along the chain at a relatively uniform rate, liberating glucose units. The reduction in rate for shorter chain lengths may result from transfer of enzyme groups from fully hydrolized chains to new chains. Obviously the time associated with transfer of the enzymne is non-productive in terms of liberating new glucose units. Thus, if the mash has a high concentration of short chain saccharides, it is reasonable to assume that the glucoamylase unit

will require more time transferring between chains, leaving less time for hydrolysis. This analysis does not apply to alpha-amylases which randomly hydrolize alpha 1-4 bonds and disassociate themselves after each action.

The more constant conversion rate for D.E. from 20 to 36 is perhaps explained by manufacturers' data on distribution of molecule lengths in Maltrin products. For products with D.E. 5 - D.E. 15, the percentage of high molecular weight dextrins decreases very rapidly. However, for maltrin products M200, 250, and 365, the percentage of long-chain dextrins is similar. There is still some shift of short chain dextrins toward maltose and trisaccharide units which may further decrease reaction time.

Although the research deals primarily with use of artificially prepared substrates, several tests were run using the same experimental methods on mash samples pulled from the batch cooker in the pilot plant at OSU. The average conversion rate for these mashes was 21.4 mg/mg-hr which compares favorably with rates obtained in this portion of the study. Data in Figure 9 suggest that a rate of 21 mg/mg-hr should occur at a D.E. around 12, very close to the D.E. expected using Miles' process and Taka-Therm liquifying enzyme.

It would be interesting to compare conversion rates for extrusion cooked substances since no liquifying enzyme is required in this process. It has been reported that dry extrusion of cereal grains results in approximately 20% dextrinization of carbohydrates. However, it is unlikely that this dextrinization results in short chain carbohydrates, thus extrusion cooked substances may be largely long chain starch molecules. The conversion rate for such substrates may be higher than for

enzyme or acid treated substances. Preliminary work indicates that conversion rates are, in fact, higher for extrusion cooked products, about the same as for Maltrin products with a D.E. of 5. Further research needs to be done in this area.

Conversion Rate as Affected by Percent Alcohol in the Substrate

It is widely recognized that high levels of alcohol during fermentation can inhibit yeast activity. For this reason, the conversion rate of enzymes as affected by varying levels of alcohol in the substrate such as might be encountered during fermentation was investigated. Alcohol concentrations were varied from 0 to 12% by volume. Results are shown in Figure 10.

For unexplained reasons, measured conversion rates increased with increases in alcohol percentages. A rather uniform rate of 19.0 mg/mg-hr was observed for alcohol concentrations from 3 to 12% by volume. In contrast, the average rate for controls was about 16 mg/mg-hr. Transition to the higher rate occurred between 0 to 3% alcohol. The data shown in Figure 10 was determined on two different days, possibly causing additional scatter, particularly for control samples. Despite this scatter, there is clearly an increase in velocity resulting from alcohol in the substrate. Velocity values observed for control samples agree well with those observed for the same initial substrate concentrations shown in Figure 7.

The relationship between alcohol content and increase in rate has not been reported in the literature. In fact, most references on enzymology suggest that alcohol might tend towards being an inhibitor for

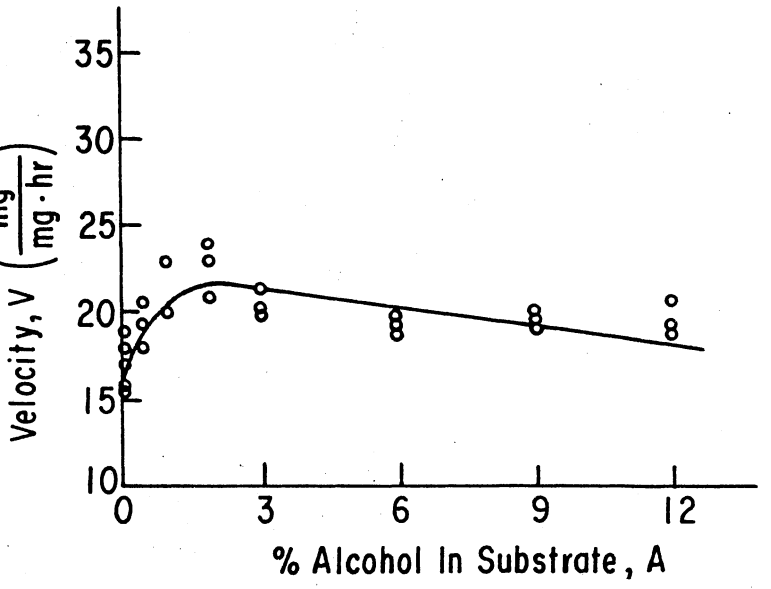


Figure 10. Initial Conversion Rate for 6% Lo-Dex 10 Substrates Containing Various Concentrations of Ethanol.

most processes. It is possible that the alcohol tends to act somewhat as a solvent, helping to improve contact dynamics between the enzyme and the substrate molecules and granules. This should be an interesting area for further work.

Conversion Rate as Affected by Vitavax (A Fungicide)in Substrate

The search for low-cost substrates for conversion to alcohol has led many plant operators to consider use of out-of-date or damaged seed grains. In many cases, these grains have been treated with fungicides such as Kaptan or Vitavax. Such chemicals may have adverse effects on both yeast activity and extracellular conversion enzymes. Figure 11 shows the effect of Vitavax, a common fungicide, on the conversion rate of Gasolase. The amount of Vitavax added varied between 0 and 200% of normal. Results show no affect in rate up to 200% of normal addition. Vitavax as an extracellular enzyme inhibitor was not a problem. It seems reasonable to expect that similar chemicals would also cause no problems.

Other Considerations

For the experiments discussed in this section, average conversion rate for starch to glucose was near 20 $\frac{\text{mg glucose}}{\text{hr}\cdot\text{mg Gasolase}}$ (all conversion rates, V, will be reported as $\frac{\text{mg glucose}}{\text{hr}\cdot\text{mg Gasolase}}$ unless otherwise noted. These units will be shown as mg/mg-hr hereafter) during the one hour reaction period. Actual rates varied from one situation to another depending upon starch concentrations, dextrose equivalence, etc., but rate was repeatable given similar situations. As mentioned earlier, a sample of whole cooked corn was filtered and treated in the same manner

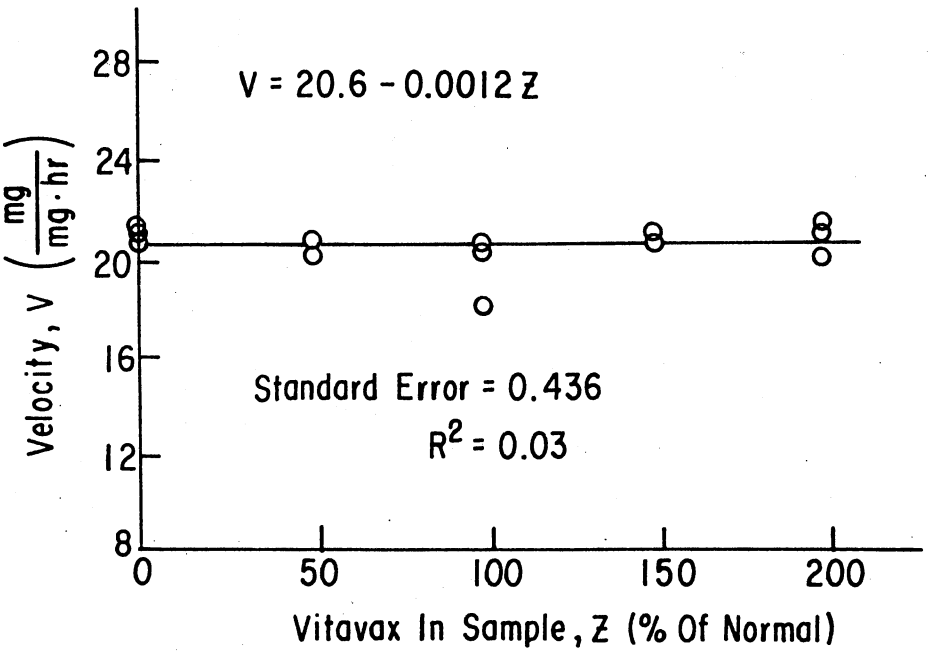


Figure 11. Initial Conversion Rate Vs Vivatax %.

as processed starch. This sample also exhibited a conversion rate of 20 to 21 mg/mg-hr. All rates were measured at a pH of 4.63 and a temperature of 32°C. In another test, the New Brunswick chemostat was used to evaluate conversion velocity during continuous flow. Feed rate of Lo-Dex 10 into the reactor was varied to give different detention times and substrate concentrations. Enzyme was metered with the flow of Lo-Dex to maintain a constant rate of addition. Results are shown in Table II. Reaction velocity varied from 7.7 mg/mg-hr at a substrate concentration of 2386 mg/l to 21.2 mg/mg-hr at a concentration of 7190 mg/l, or 6.7% starch. This rate is similar to rates observed in the batch tests discussed above. The reduction in rate with further exposure of enzyme to substrate may be due to substrate depletion, or tied in some way to length of reaction.

Table II
Conversion Velocity Vs Retention Time

t(hrs)	S(mg/l)	V(mg/mg-hr)
1.18	7,190	21.2
2.5	6,100	13.8
5.4	4,500	9.1
8.8	2,390	7.7

Many enzyme manufacturers recommend a separate conversion step at higher temperature. Such conversion steps normally last from one to two hours and can be accomplished either in batch or continuous equipment.

The rationale is that conversion at high temperature allows some liquified starch to be converted to dextrose which is then available for immediate use, favoring rapid development of a strong yeast colony. A test using Gasolase at 54°C with other conditions maintained as in previous experiments yielded a conversion rate of 114 mg/mg-hr, roughly five times the rate at 32°C. With such an increase, it would be possible to condense the amount of saccharification taking place normally in ten hours into a two hour span. With no acceleration in rate it is obvious that conversion at approximately 20 mg/mg-hr will fall short of converting high starch concentrations (20%) to glucose within the normally allotted 48-hour period. According to recommendations by Bio-Con (1980, 1979, 1977), approximately one pound of Gasolase would be required to saccharify one ton of 72% starch corn. If the actual conversion rate was a constant 20 mg/mg-hr, approximately 1.35 pounds of enzyme would be required to saccharify the entire amount of starch in 48 hours. At enzyme rates suggested by Bio-Con, saccharification time would be approximately 65 hours. It is generally agreed that fermentations can be pursued to completion within 48 hours. This has been observed in our lab, using manufacturers' recommendations. Thus, some factor causing acceleration of conversion rate must be taking place inside the fermenter. Two possible explanations have been previously addressed: potential increase in conversion rate due to development of alcohol in the fermenter, and the potential increase in rate as substrate concentration is reduced. Also, as fermentation progresses, D.E. should drop as short chain molecules are converted to glucose. The ratio of short chain to long chain molecules should then remain relatively low until near the end of fermentation. Yeast cells themselves are

capable of reducing maltose to glucose. This could be particularly significant since it would relieve some of the load for hydrolysis of maltose from the glucoamylase units, allowing operation under more favorable conditions.

A good argument is whether or not a prefermentation conversion step is justified. Certainly conversion at high temperature exceeds that at fermentation temperature. Initial concentrations of glucose should insure a rapid start in the fermenter. However, as pointed out in the review of literature, very rapid yeast growth and high yeast populations may lead to production of unusually high concentrations of glycerol. Additionally, in the presence of high concentrations of glucose, the mechanism by which yeasts can break down maltose may be inactivated. Although higher quantities of dextrose may be available, total fermentable sugars may not actually increase because of deactivation of maltose splitting capabilities. This is not substantiated in the literature, and further work needs to be done. Additionally, there is some reason to believe that unusually high concentrations of glucose can adversely affect activity of certain metabolic pathways in yeasts essential to production of alcohol.

Although it has been shown that preliminary saccharification does lead to rapid initial fermentation, many researchers report that the conversion of dextrins to glucose becomes a limiting factor near 30 hours into the fermentation, so there may be no real advantage in preceding fermentation with prolonged, high-temperature conversion.

Analysis of Experimental Reaction Rate Data

Reaction velocities for substrate concentrations from 3% to 18% were presented in Figure 7. However, this data was not sufficient for

development and testing of kinetic models describing the overall relation between substrate concentration and reaction velocity. Standard relationships such as the Michaelis-Menten equation and adjustments require information on velocity for substrate concentrations near the expected value of K_m. Graphical methods for determining rate constants V_m and K_m, place more weight on velocities at small substrate concentrations than those at concentrations where V approaches V_m. This can easily be seen in Figure 2a where the X-axis plots the inverse of the substrate concentration. Values taken only at high substrate concentrations tend to congregate near the Y-axis, yielding no decent indication of the slope of the line.

In order to provide the range of substrate concentrations necessary for a good analysis, substrate concentrations ranging from 0.25% to 30% solids by weight were prepared and evaluated. Even for this range of concentrations, considerable lumping of the high substrate concentration data occurred (see Figures 12 and 13).

Data for this portion of the experiment was produced during experiments conducted during different days, though all samples were treated identically except for designed comparisons. A more complete listing of experimental procedures is given in Appendix C and D and earlier sections.

Deactivated Studies

Raw values of observed velocities and substrate concentrations for these tests are tabulated in Appendix 5. Substrate concentrations were varied from 0.25% to 27.5%. As discussed earlier reaction period of nine minutes was selected to insure obtaining velocities in the proper

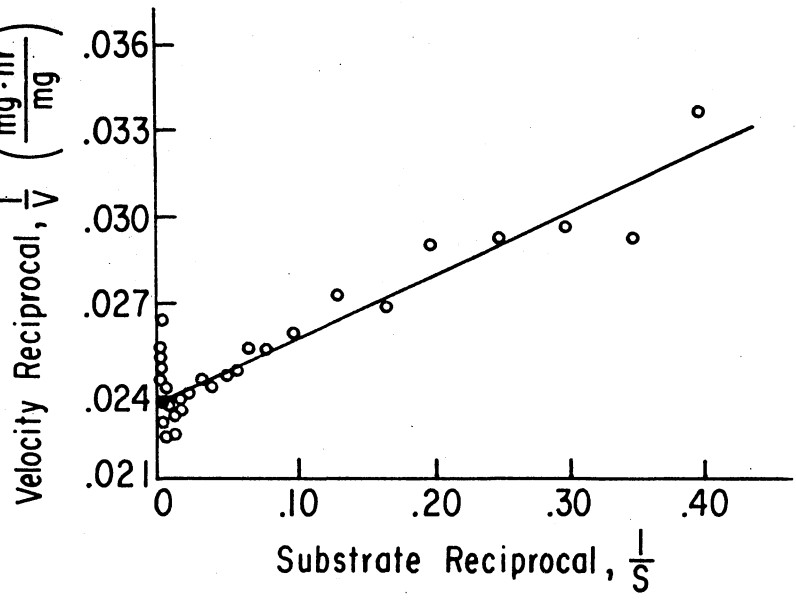


Figure 12. Lineweaver-Burk Plot for Determination of Experimental V_m and K_m.

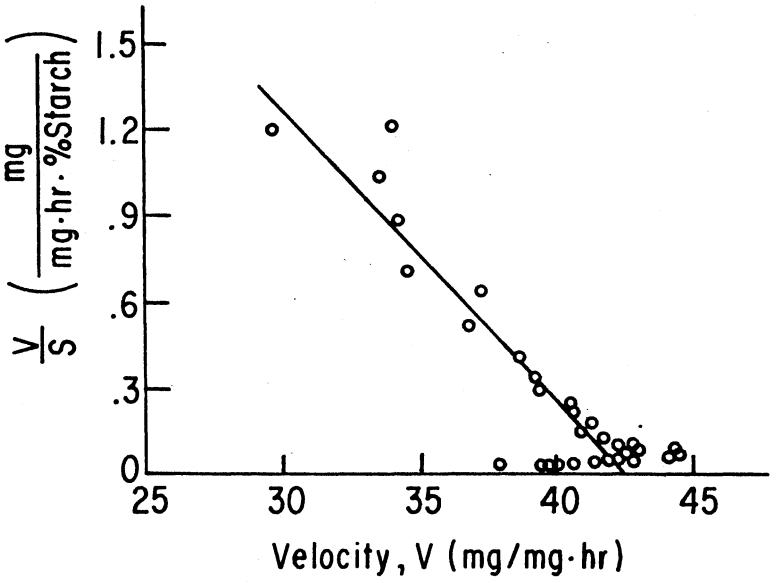


Figure 13. Hofstee Plot for Determination of Experimental V_m and K_m.

portion of the curve, while still allowing the reaction to proceed long enough for measurable amounts of glucose to be formed.

Calculation of Deactivated K_m and V_m

Conversion velocity is plotted versus substrate concentration in Figure 14. While the data exhibits the general form expected for a first-order reaction, there is a decline in velocity with increasing substrate concentration after peak velocity is reached.

The raw data was plotted in linearized form using the Lineweaver-Burk and Hofstee plots shown in Figures 15 and 16. Values of V for high substrate concentrations were eliminated from this phase of the analysis. This was considered a reasonable approach for three reasons.

1. The Michaelis-Menten analysis is most sensitive to data taken at low substrate concentrations (in the neighborhood of K_m). The analysis becomes somewhat confused for the case where V does not approach some maximum value.
2. It was anticipated that a Haldane term would be required in the model to account for the decline in velocity at high substrate concentrations. This implies that a different mechanism is controlling the reaction in this range, characterized by a different constant K_I as noted in an earlier section. In this case Kincannon (1977) suggests determining K_m and V_m using data for S less than that where maximum velocity is obtained. As is often the case with experimental biological data, good judgement must be used in selecting data to be used for analysis.

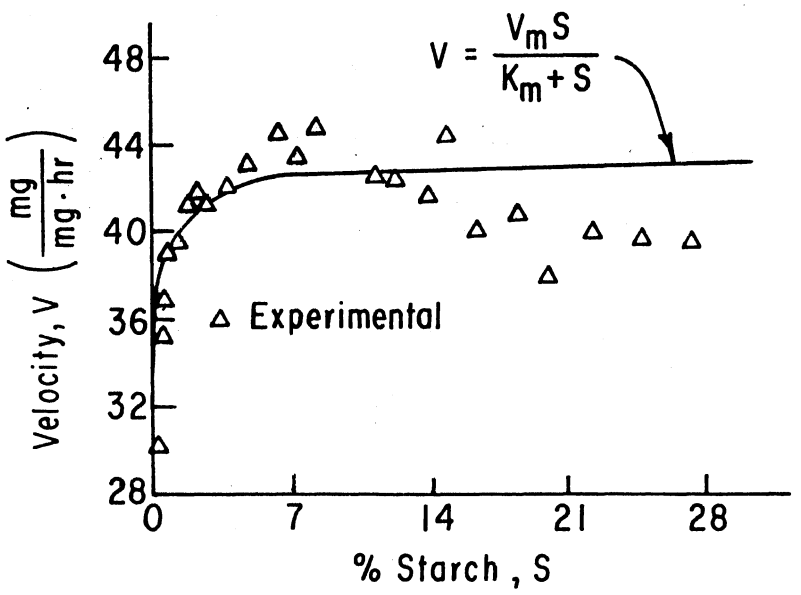


Figure 14. Experimental and Michaelis-Menten Predicted Reaction Velocities for Various Substrate Concentrations.

$$\frac{1}{V} = 0.024 + 0.0022\ (\frac{1}{S})$$

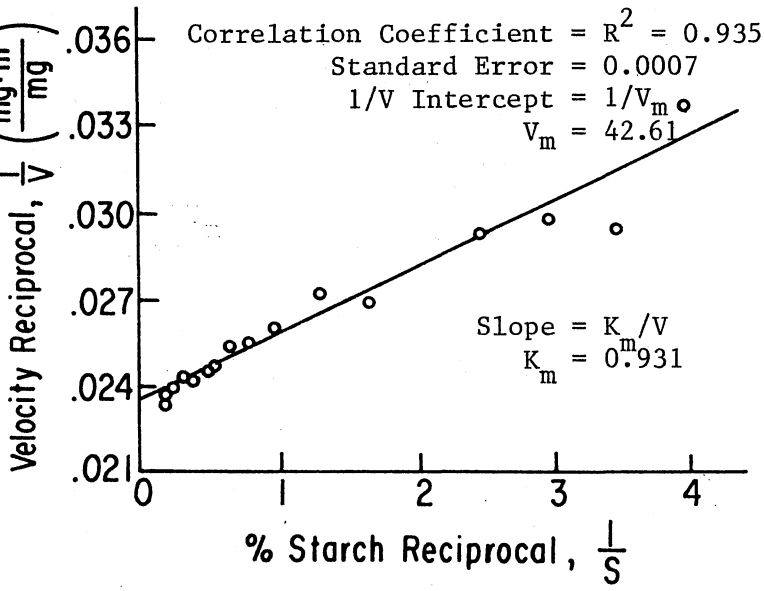


Figure 15. Adjusted Lineweaver-Burk Plot.

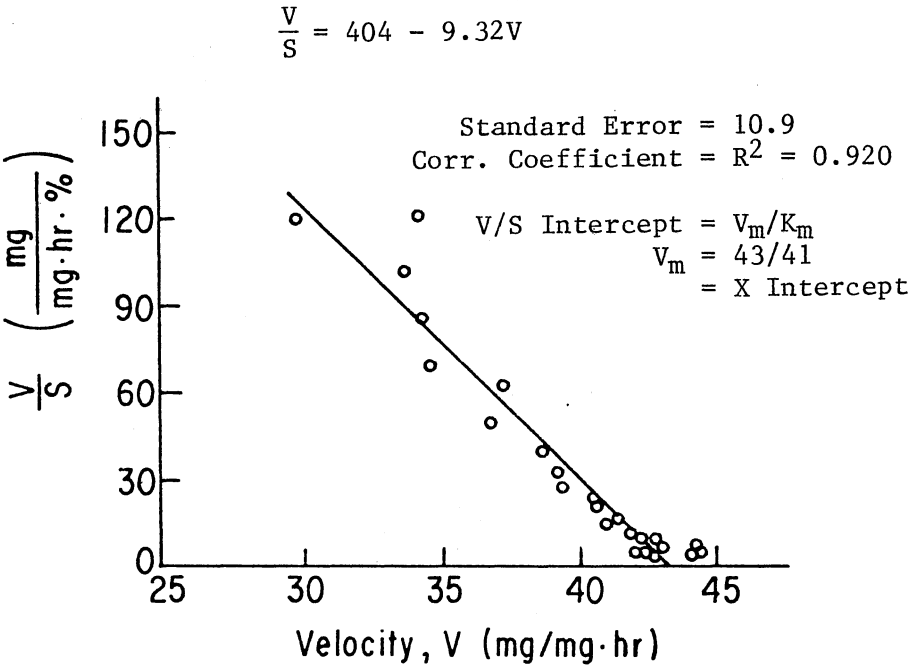


Figure 16. Adjusted Hofstee Plot.

3. Even when the Haldane modification to the Michaelis-Menten analysis is used, it is important that methods for determination of all constants be consistent with those commonly used in enzyme rate studies. Determination of K_m and V_m is very important to the analysis, regardless of what form the final equation may take. These constants result from a practical consideration of what is taking place in the reaction, and are determined from accepted analytical methods. At the least, they describe the initial portion of the reaction up to the point where V_m maximum velocity was measured.

Both the Lineweaver-Burk and the Hofstee plot give similar values for K_m and V_m. The average K_m is 0.1002% and the average V_m is 43.01 mg/mg-hr. These constants were substituted in the Michaelis-Menten equation, and values of V predicted for substrate concentration from 0.25% to 27.5% (the same range as for experimental data). These results are also plotted in Figure 14. The model predicts the experimental results with reasonable accuracy, particularly for substrate concentrations of less than 5%. However, the model does not accurately predict observed peak velocity, or the gradual decline with increasing concentration after peak.

It is not surprising that first order kinetics is not entirely adequate for describing the data in Figure 14. As pointed out earlier, the Michaelis-Menten equation is based on mono-molecular interaction between enzyme and substrate, and on the premise that reduction in velocity is due only to substrate depletion. In regions of high substrate concentration, or where there is interaction or inhibition between molecules other than pure substrate and enzyme, the analysis fails.

Reactions where there is apparent inhibition at high substrate concentrations are often modeled with the Haldane adjustment to the Michaelis-Menten equation:

$$V = \frac{V_m S}{K_m + S + S^2/K_I} \tag{11}$$

A third term is added to the denominator of the equation containing an dimensionless inhibition constant K_I. Addition of this term causes predicted values of V to decline with further increase in substrate concentration after V_m is reached. It should be noted that the Haldane equation still relies on K_m and V_m predicted using Michaelis-Menten analysis. Using a value of $K_I = 300$ (determined by trial and error), the Haldane equation provides an excellent prediction of V for S less than 5% and greater than 14%. However, it underpredicts V considerably between S=7% and S=14% as shown in Figure 17.

A modified form of the Haldane equation shown above was obtained which provides a much more satisfactory prediction tool:

$$V = \frac{V_m(1+K_m)S}{K_m+S+\dfrac{(1+S)K_I}{V_m}} \tag{12}$$

This equation is essentially the same as the basic Haldane equation, but includes an adjustment to the numerator to allow more accurate prediction of V near V_m. Also, the third term in the denominator has been modified so that the "inhibition" constant is now a power term, and V_m has replaced K_I in the denominator. A more thorough discussion of these adjustments, and methods used for determining equation (15) will be discussed later. Using a K_I value of 1.65, and other constants as previously determined, values of V predicted by the above

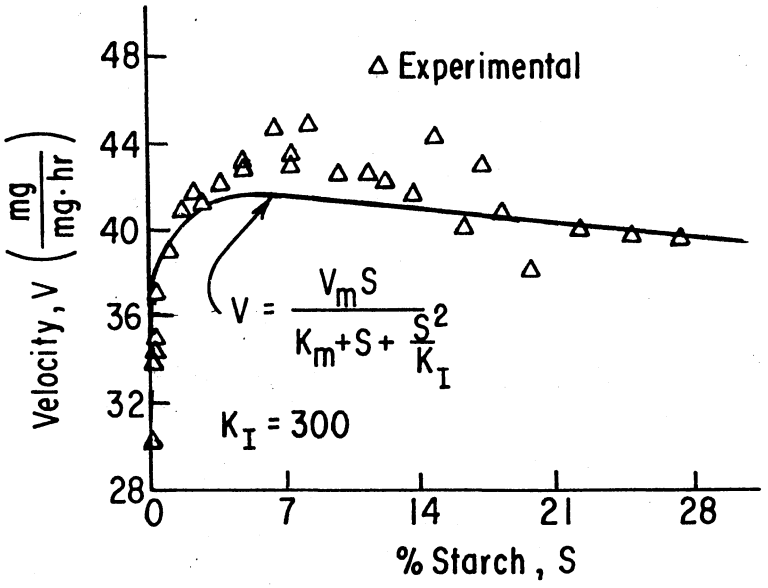


Figure 17. Haldane Equation for Heat Deactivated Data.

equation are shown in Figure 18. This model is a reasonably good predictor over the entire substrate range although it estimates the initial portion of the curve with slightly higher slope, and somewhat underestimates values of V near V_m. Curve fit statistics for equations (14) and (15) are shown in Table III.

Finally, the data was analyzed to see if additional manipulation and curve fitting could provide a better fit to experimental data. After many tries, a satisfactory fit was obtained after transforming both S and V by taking natural logarithms and fitting resulting data with a second order polynomial. Transformed data, along with the polynomial equation determined by linear regression, is shown in Figure 19. While this method does provide a reasonably good fit over the entire data range, it is lacking in terms of any meaningful physical or biological interpretation. The modified Haldane equation incorporates several terms which are determined by standard methods, and have significant physical meaning to the enzyme scientist. For this reason, the polynomial fit to transformed data is interesting, but without much use as an interpretive tool. It is interesting to note that the curve is generally bell shaped. Haldane (1965) points out that in cases where substrate inhibition is a factor, the shape of curve plotted with data transformed using logarithms does, in fact, produce a bell-shaped curve.

Other Considerations for Heat Treated Samples

The velocities plotted in Figure 14 are roughly twice those reported earlier in this section. A thorough check of procedures and calculations revealed no errors. In earlier work, velocity was determined at ten minute intervals staring at t=0 for Lo-Dex as a basis for

TABLE III

STATISTICAL DATA FOR KINETIC MODELS

Model	Figure #	(intercept) A	(slope) B	R^2	SE	Average error	Average % error
$V = \frac{V_m S}{K_m + S + \frac{S^2}{K_I}}$	Figure 17	-3.1205	1.0952	0.8666	1.2684	1.041	2.647
$V = \frac{V_m(1 + K_m)S}{K_m + S + \frac{(1+S)^{K_I}}{V_m}}$	Figure 13	-0.2588	1.0048	0.8237	1.4587	1.159	2.91
$V = \frac{V_m S}{K_m + S + \frac{S^2}{K_I}}$	Figure 24	-6.4542	1.2345	0.9501	1.2896	1.245	4.469
$V = \frac{(V_m + K_I)S}{S + K_m + \frac{S^2}{V_m}}$	Figure 25	-6.4491	1.1864	0.9498	1.2942	1.315	4.736
$V = \frac{(V_m + K_I)S}{K_m + S + \frac{S^{K_I}}{V_m}}$	Figure 26	-0.0392	0.9842	0.9478	1.3194	1.137	4.214
$V = \frac{(V_m + K_I)S}{K_m + S + \frac{S-K_m}{V_m}^{K_I}}$	Figure 27	1.9123	0.9219	0.9443	1.3622	1.126	4.204

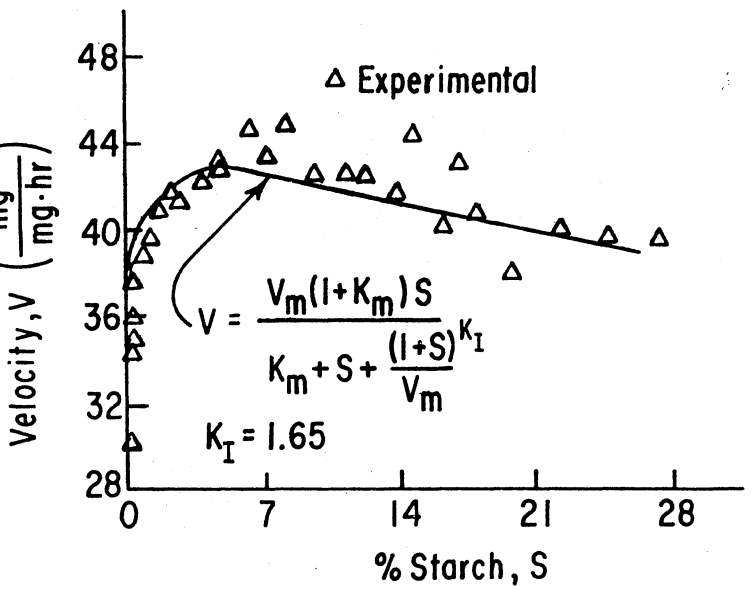


Figure 18. Adjusted Haldane Equation.

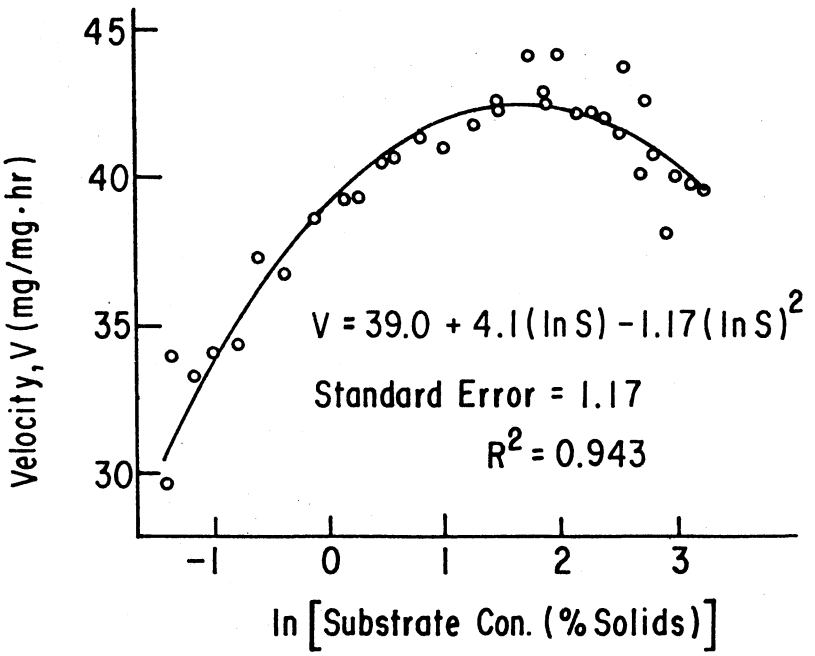


Figure 19. Second Order Polynomial Fit of Transformed Data.

establishing one hour as an acceptable reaction interval for substrates with 3% solids or greater. Plots of this data certainly appeared generally to be linear for more than one hour. Furthermore, a one hour incubation period was similar to that used by other researchers under similar conditions. However, closer inspection of the data seemed to indicate that conversion velocity during the first 10 minutes of the reaction was somewhat higher than when determined over a one hour period. A change in reaction rate would have gone undetected if it occurred during the first 10 minutes because data taken over the next 50 to 60 minutes obscures the single high point at 10 minutes.

This problem is illustrated in Figure 4 where three substrate solutions were evaluated at various times over a one hour period. The length of the linear portion of the curves is dependent on substrate concentration, and slopes are somewhat different. Even though data for the two higher concentrations appears to be essentially linear, it is revealing to compare values obtained at 9 minutes and at 60 minutes. At 9 minutes, velocity values for both concentrations would be near 40, while at 60 minutes, the values would be in the twenties.

It is not completely clear whether the difference in velocities obtained at 9 and 60 minutes is actually due to an accelerated rate during the first few minutes of reaction, or because of experimental error. Methods used in the analysis proved dependable and yielded consistent results provided as test conditions (in terms of pH, temperature, reaction time, etc.) were maintained.

Distortion of Data Because of Heat Deactivation

The most likely explanation for the difference in velocities determined at nine minutes and at 60 minutes is experimental bias. After careful review of methods and procedures it was concluded that a possible source of such bias was heat-deactivation of the enzyme prior to evaluation in the YSI 27. Although samples were small (10-15 ml), there was still a brief period of time during which enzymes were brought from incubation temperature (32°C) to deactivation temperature (60°C or higher). Enzyme activity is temperature related; thus conversion rate is accelerated during temperature elevation. When the incubation period is 60 minutes, extra glucose produced by deactivation is not significant in comparison to the total produced. Also, the increase is constant between samples, so there is no other distortion to the data. On the other hand, when exposure time is very short, as in the nine minute studies, glucose produced during deactivation may be a significant part of the total. The data in Figure 4 seems to support this explanation.

Deactivation by pH Adjustment

Another way of suspending enzyme activity is to adjust pH out of the active range for the enzyme. On the basic side, pH adjustment to 11 or higher is sufficient to stop activity, while a reduction in pH to around 2.5 would also halt the reaction. However, reduction of pH to 2.5 was thought to be lower than desired because of potential hydrolysis of remaining dextrins and starch molecules.

A test was set up to compare observed rates using both heat and pH adjustment for enzyme deactivation. Heat deactivated samples were

treated as for previous samples. Upon completion of the incubation period, the pH adjusted samples were treated with sodium hydroxide to raise the pH to 11.5. Just prior to analysis, pH was adjusted back to 4.5 with phosphoric acid. All samples were assayed in the YSI 27. Other experimental factors between samples were kept constant. Substrate concentration was 6%.

Results of this test are shown in Figure 20. It is clear that for short reaction periods heat deactivated samples resulted in a higher apparent conversion rate. After about 3 minutes, the apparent rate is nearly equal for the two methods. Remembering that total glucose produced during a particular time interval would be the integral under the curve for each method, it is easily seen that heat deactivation can significantly distort the apparent conversion velocity when the incubation period is short. Obviously if Figure 20 is carried out to 60 minutes, the initial difference in the two curves is not particularly influential in determination of rate.

Unfortunately, deactivation using acid-base adjustment is more complex than heat deactivation, and requires more effort and attention during the experiment. Additionally, during subsequent tests, this method occasionally resulted in erratic performance of the YSI 27. Stability of the instrument can be disturbed if salt levels in samples are high. Salts produced by neutralization of acid with bases may have produced the stability problems. Thus, although it was felt that a major part of the problem of difference in rates had been identified, a solution was still needed. It should be noted, however, that heat-deactivation is still a viable method for incubation periods near 60 minutes.

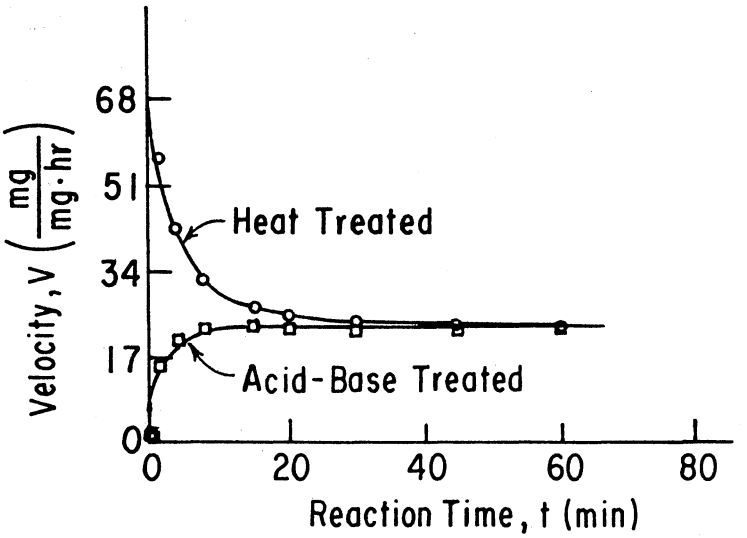


Figure 20. Effect of Deactivated Method on Conversion Rate.

Injection of Samples Directly into the YSI 27 Without Deactivation

A second method investigated was that of direct injection of incubating samples into the YSI without deactivation. This had not been considered before because of the belief that at least two or three measurements for each sample should be made with the YSI. Obviously if the enzyme is not deactivated, repeated measurements of an active sample will result in increasing readings. However, over the course of the study, it was observed that there was seldom more than 2-3% variation between multiple readings taken for one sample, suggesting that one reading would be satisfactory. Of course, another way of obtaining duplicate readings (and perhaps more appropriate anyway) is simply to provide duplicate sample though it is somewhat more difficult to isolate the source of any variation this way (whether due to sample treatment, or variation in the instrument).

For remaining tests, procedures were modified to allow direct injection of incubating samples into the YSI 27. Preliminary testing showed this would not be a problem, provided good timing and preparation was observed. In the sample chamber of the YSI, the sample is exposed to the immobilized enzyme for 40 seconds. Results are displayed digitally, and the instrument is ready for recycle. The clear cycle, required before another sample can be injected, takes 20 seconds. Thus, it was a relatively simple matter to plan so that time between samples was allowed to clear the machine for the next reading. The incubation period for each sample was taken as the time between addition of enzyme, and display of results on the machine face. Samples were retained in the constant temperature bath until just prior to injection into the YSI.

Clearing of the machine between cycles was planned so that the clear cycle was completed just prior to injection of the next sample, thus ensuring proper and consistent zeroing between samples.

While these methods require more attention to lab practice and planning, they worked well, and eliminated need for enzyme deactivation.

Non-deactivated Tests

Using methods for glucose determination in reacting samples outlined in the previous section, another evaluation of V versus S was made. The range of substrate concentrations was 0.25% to 30%. Raw values of V and S are tabularized in Appendix E, and plotted in Figure 21. Peak velocity is somewhat less than for previous tests and a steep decline in rate occurs with increasing substrate concentration. The decline in rate is much more pronounced than for heat-deactivated data.

Linearized forms of the Michaelis-Menten relationship were again used for determination of K_m and V_m. As in previous analysis, some of the high concentration velocities were eliminated from the determination of constants K_m and V_m. The adjusted data plotted in both Lineweaver-Burk and Hofstee form is shown in Figure 22 and 23. Both methods give similar values for K_m and V_m, the average being K_m = 0.1077% and V_m = 37.12 mg/mg-hr.

It is interesting to note that the average K_m of 0.1077% for this case is nearly identical to the average K_m for the heat deactivated studies (0.1002%). At first glance this may seem contradictory since there is a considerable difference in maximum velocity, V_m. However, as pointed out in the review of literature, K_m is actually a shape constant which determines the initial slope of the plot of V vs S, and how

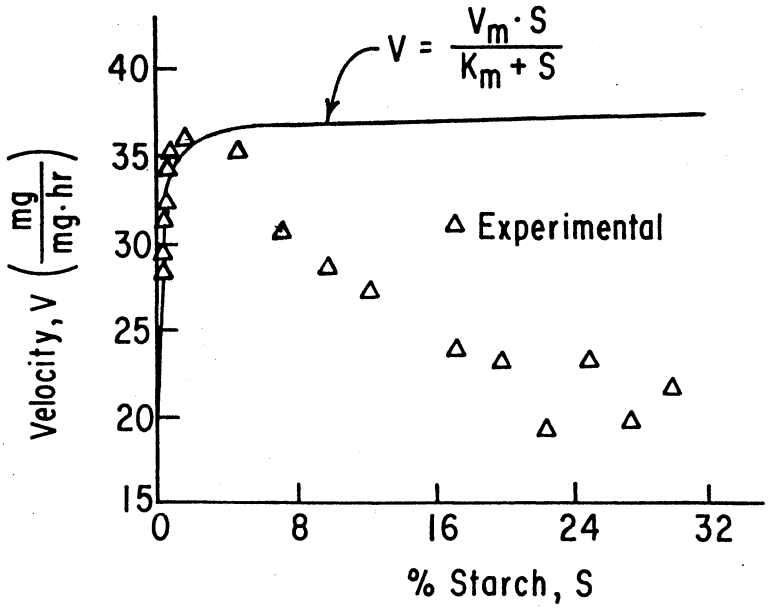


Figure 21. Experimental and Michaelis-Menten Predicted Velocities for Non-Deactivated Test

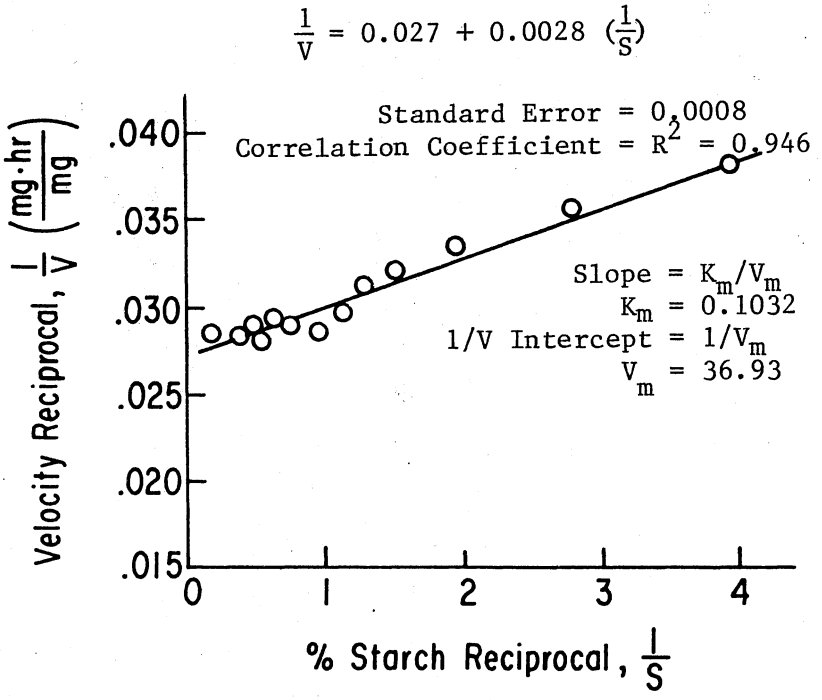


Figure 22. Adjusted Lineweaver-Burk Plot (Non-Deactivated Data).

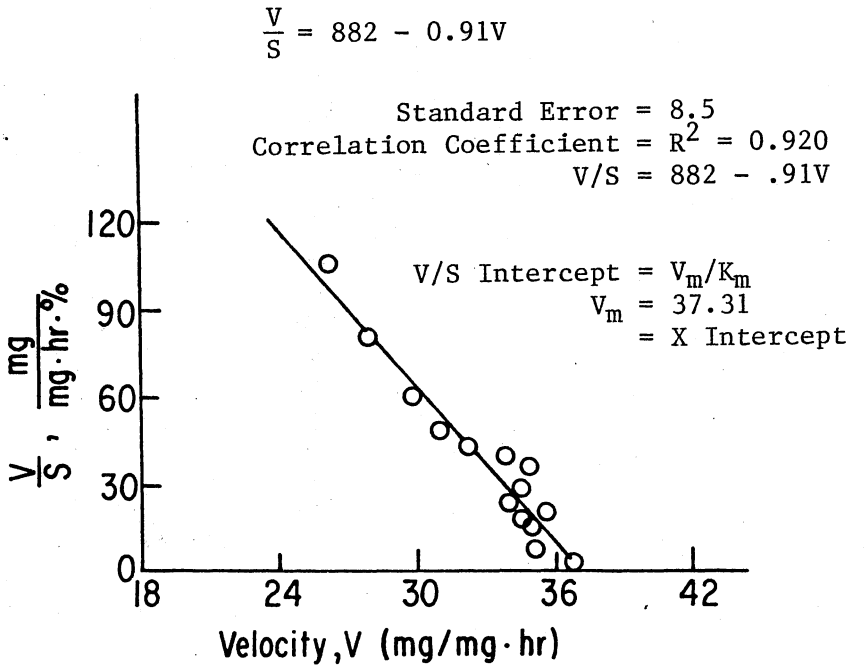


Figure 23. Adjusted Hofstee Plot (Non-Deactivated Data).

sharply the curve breaks towards V_m. Since K_m is numerically equal to the substrate concentration at which $V = V_m/2$, this provides substantiating evidence that heat deactivation causes distortion of observed enzyme velocities. The theoretical development for K_m reviewed earlier suggests that small changes in reaction rate due to increased temperature should not affect the value of K_m. The maximum velocity changes, not the substrate concentration at which V_m occurs, indicating the value of such a constant in determining kinetic models. The value of V_m is certainly of interest, but the scientist is likely to learn more about the function of particular enzyme system from the value of K_m. It is also more likely to be comparable to other systems with similar properties and conditions.

The two rate constants were substituted into the Michaelis-Menten equation, and values of V predicted over the range of substrate concentrations used in the experiment. Predicted values are shown along with raw data in Figure 21. Again, the initial portion of the experiment is modeled reasonably well by the standard equation. However, for substrate concentrations larger than about 4%, the equation is completely inadequate.

The standard Haldane adjustment to the Michaelis-Menten equation was evaluated next. Best fit was obtained with a dimensionless K_I value approximately equal to the maximum velocity V_m. No explanation can be offered for this. While there is a chance that the similarity of the two values is pure coincidence for this case, it may well be that there is some biological or physical explanation. A plot of the standard Haldane model

$$V = \frac{V_m S}{K_m + S + S^2/K_I} \tag{15}$$

using the previously determined K_m and V_m, and with $K_I = V_m$, is shown in Figure 24 along with experimental data. The basic Haldane equation provides a surprisingly good fit as a first try. However, it underpredicts observed values of V near V_m by a considerable amount.

Underprediction is caused because the Haldane term becomes too large before the equation can predict the largest velocity values observed experimentally. This suggests further modification, either to the numerator so that V_m plus some additional quantity is multiplied by the substrate concentration, or to the Haldane term itself, so that it develops magnitude more slowly. However, it is important to maintain the general form of the basic Haldane equation so that the constants K_m, V_m, and K_I have physical and biological meaning. A number of different modifications were investigated, several of which offered improvement. In all cases, the number of new constants or terms was limited to one so that the new model was no more complicated than the basic Haldane equation. The best three of these are discussed below.

Adjust V_m. The K_I in the denominator of the standard Haldane equation was replaced with V_m as discussed above, and added to V_m before multiplication by S. Using V_m in place of K_I in the denominator permits the equation to be kept in the general form, while allowing K_I to be used to adjust V_m to fit a particular situation.

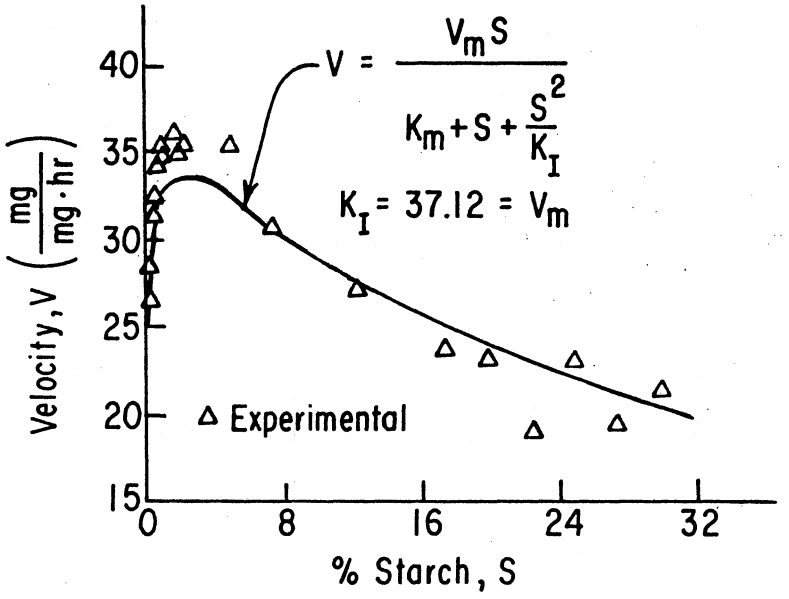


Figure 24. Haldane Equation for Non-Deactivated Tests.

$$V = \frac{(V_m + K_I)\ S}{K_m + S + S^2/V_m} \tag{16}$$

This equation provided the best fit at a K_I value of 1.5, and is plotted with experimental data in Figure 25. Here K_I has units of V, mg/mg-hr. Large values of V are predicted reasonably well, but the curve does not decline rapidly enough to stay with experimental data at high substrate concentrations.

Adjust Both V_m and Haldane Term. One method of effecting a more rapid decline for the curve in the high substrate concentration region is to increase the exponent of the Haldane term. Noting that the optimum value of K_I in the previous model were between 1.5 to 2.5, K_I was also substituted as the exponent for the Haldane term. This allowed adjustment of both terms with a single constant K_I.

$$V = \frac{(V_m + K_I)\ S}{K_m + S + S^{K_I}/V_m} \tag{17}$$

This equation provided the best fit with a K_I of 2.05. A plot of experimental versus predicted values is shown in Figure 26. This model provides a definite improvement, although it does to predict a little high for large substrate concentrations. The addition of K_I as the exponent to the Haldane term adds considerable flexibility to the equation. Small variations in K_I will provide considerable changes in slope without affecting the adjustment of V_m.

It must be noted that the form of equation (17) is not dimensionally correct. The constant K_I appears in both the numerator and denominator, but not with terms which are dimensionally equal. Thus, equation (17) is empirically correct only for the units of V and S used in this

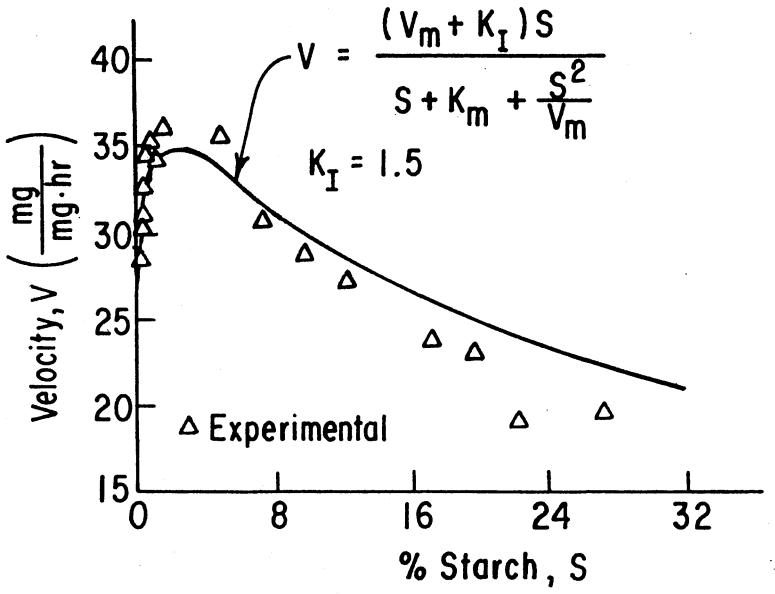


Figure 25. Haldane Equation Adjusted by V_m.

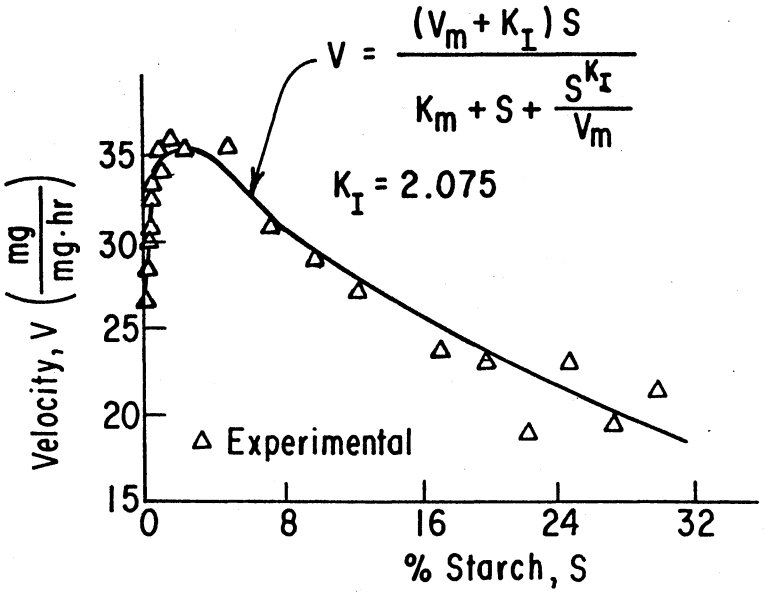


Figure 26. Haldane Equation Adjusted by V_m and Exponent.

report. Another choice of units might result in a single value of K_I being inadequate. However, this does not present a particularly difficult problem, since two separate constants could be determined easily enough. The constant in the exponent of the denominator would then be dimensionless, and the constant in the numerator would have the same units as V. The constant in the exponent is by far the more powerful factor, and would be chosen first, with the choice of the constant in the numerator then being one of fine tuning. This discussion also applies to equation (18) discussed in the next section. For the sake of simplicity and continuity of discussion, only one K_I term will be considered for these models.

Adjustment to S in Haldane Term. Perhaps the best fit to experimental data was obtained with equation (18).

$$V = \frac{(V_m + K_I)\ S}{K_m + S + \frac{(S-K_m)^{K_I}}{V_m}} \qquad (18)$$

Essentially, equation (18) is identical to equation (17), except that S in the Haldane term has been modified by subtracting K_m, and taking the absolute value. This adjustment helps the model predict high values of V by reducing the divisor in the region of K_m. However, since K_m is very small, the adjustment is significant only when S is very small, and is not a factor when S is large. Thus, the ability of the model to control slope at high substrate concentrations is not compromised. Velocities predicted with this equation are compared against experimental data in Figure 27.

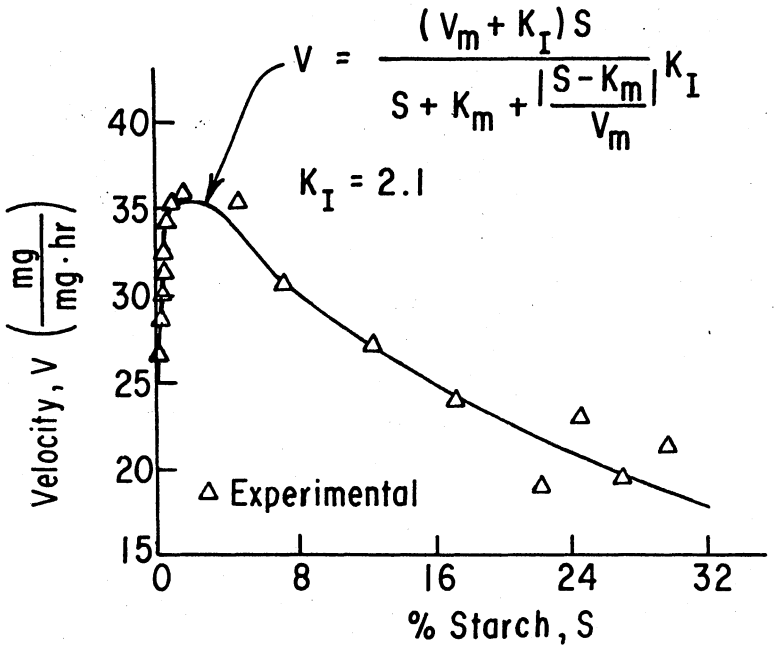


Figure 27. Haldane Equation Adjusted by S.

Other Adjustments. An adjusted Haldane model was developed for the heat-deactivated data discussed in the previous section. This equation

$$V = \frac{V_m (1+K_m) S}{K_m + S + \frac{(1+S)^{K_I}}{V_m}} \qquad (15)$$

is in similar form, but with adjustment to both V_m and the Haldane term. The ability to vary the degree of slope control is retained by keeping K_I as the exponent in the Haldane term. Although smaller than previous K_I values used to adjust V_m, K_m can be used to accomplish the same purpose by use of a V_m multiplier equal to K_m plus one. Similarly, the Haldane term is adjusted to be less sensitive to very small values of S by adding one to S before raising to the K_I power. Dimensional continuity is retained with a dimensionless K_I. Although equation (15) did not provide an acceptable fit for non-deactivated data, it is discussed here as an example of how terms of the Michaelis-Menten-Haldane equation may be adjusted to fit specific situations

Selection of Appropriate Model

The models and results discussed in this section have been specifically developed for enzymes, substrates, and conditions of this test. Because of variability inherent in both enzymes and substrates, it is likely that each particular situation will demand its own model and constants if velocity is to be accurately predicted. However, the foregoing discussion has shown that many different situations can be modeled with the Michaelis-Menten-Haldane relationships, with some changes to the individual terms. The physical and biological meaning and interpretation of the equations are not changed. They are retained in standard form, with only one new constant to be evaluated.

CHAPTER VII

CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER STUDY

Conclusions

Conclusions from this research refer specifically to one enzyme-substrate system but may be expected to generally apply to other similar enzyme-substrate systems.

1. For processed starch products used in this work, starch to glucose conversion rates for Gasolase reached a maximum at a concentration of 5-8%, then decreased with further increases in substrate concentration.
2. The first order relation predicted by the Michaelis-Menten equation provides a reasonable prediction of velocity at low substrate concentrations (less than 4%). At higher concentrations substrate inhibition disrupts the kinetics. The reaction is a Haldane type, where substrate inhibition becomes a factor at increasing substrate concentrations.
3. The Haldane adjustment to the Michaelis-Menten relation provides the best approach to modeling experimental data. In its basic form, this incorporates a third term in the denominator which is dependent on S and makes use of an inhibition constant K_I. This term controls slope of the V vs S curve and can be adjusted to reflect the amount of inhibition taking

place in a specific reaction. Haldane equations were developed along with statistics describing their ability to predict conversion velocity.

4. Without modification, the Haldane relationship consistently underpredicts the highest observed values of V. The equation can be modified to allow for more accurate prediction of both high values of V and variations in slope in the declining portion of the curve. Adjustments are chosen so that the basic biological and physical meaning of the Michaelis-Menten-Haldane equation are retained. Standard methods for determining K_m and V_m are still used, and K_I is determined by trial and error.

   In this way, it is possible to use only the Michaelis-Menten equation if the substrate concentration range is less than that where significant inhibition occurs. For higher concentrations, the Haldane term and other modifications may be added as needed. In either case, constants K_m and V_m are determined in the same manner.

5. Data representing the reaction after V_m is reached can be ignored in determination of K_m and V_m provided the use of K_I in the Haldane equation can provide enough flexibility to model the remainder of the curve.

6. In the standard Haldane equation, it appears that for Gasolase and processed starch, the value of K_I is nearly the same as that of V_m. This may be simply due to coincidence, or there may be some physical or biological explanation which can not be offered at this time. For this particular case the

similarity between K_I and V_m allowed greater flexibility by allowing K_I to be assigned another function in the equation.

7. When performing kinetic evaluations, care must be taken to insure that a proper selection of incubation period is chosen. Velocity must be measured only during the initial linear portion of response. The length of the linear period is closely related to substrate concentration, and declines with decreasing substrate concentration. Generally, this will not be a problem unless the incubation period is very short. For short reaction periods it is likely that use of heat for enzyme deactivation will result in distortion. Since the additional glucose produced due to enzyme acceleration is small, extending the length of the reaction to 30 minutes or more was sufficient for this research. The value of K_m determined from data analysis was essentially unchanged whether heat deactivation or direct injection was used. The distortion due to heat treatment does not change the shape of the curve, only the maximum velocity.

8. Where reaction periods of less than 30 minutes were used, it was possible to directly inject reacting samples into the YSI 27 for analysis. The instrument proved reliable and stable, and multiple readings for any particular sample were not required. The time of reaction was taken as the time between injection of enzyme into the sample, and display of the digital reading on the YSI 27.

9. Conversion rate for Gasolase and processed starch decreased as Dextrose Equivalent of the substrate increased. This was most likely due to a more effective use of time since the enzyme must move from one dextrin to another more often for short average chain length.
10. Conversion rate was higher for a 6% substrate containing small amounts of alcohol than for the same substrate containing no alcohol. Alcohol apparently acts as a co-factor or accelerator in some way, perhaps making it easier for the enzyme to attach to substrate, or to move through the matrix of solvent and substrate molecules.
11. Conversion rate was not affected by Vitavax, a typical fungicide.
12. Conversion rate was relatively unaffected by agitation level.
13. Background glucose did not inhibit conversion rate for concentrations from 0 to 200 mg/dl.
14. An engineer or scientist determining enzyme kinetics for the purpose of process design should select test and evaluation conditions as close as possible to anticipated operating conditions, particularly at high substrate concentrations. Similarly, when reviewing the literature, it is important that the process designer understand how differences between test conditions may affect reported results.

Recommendations for Further Study

The scope of the research was purposely limited to one enzyme-substrate system. The complexities of determining enzyme kinetics while

maintaining an understanding of the many factors involved required this limitation. A number of intriguing questions and research possibilities have been identified. Some apply directly to the specific enzyme and substrates involved in this study. Others will require application of the information and methods generated to entirely new areas.

Determine Conversion Rates of Gluco-Amylase and Extruded Starch Products

Extrusion cooked products can be hydrolyzed directly by gluco-amylase without pre-liquification with alpha-amylase. Gelling or retrograding is not a problem with typical concentrations of extruded starch products, and control of viscosity is not as critical to stirring. As pointed out in the discussion of results, the gluco amylase enzyme acts more rapidly on substrates with low D.E. than those with high D.E. While some mechanical damage to the starch molecule occurs during dry extrusion, it should not result in significant quantities of short chain molecules. Thus, it is anticipated that reaction rates for extruded products should be more rapid than for conventionally processed starch. This should be verified for different extruded substrates and processing conditions.

In addition to determination of reaction kinetics, analytical techniques used in this research can be used for investigation of the effect of extrusion on the starch molecule. The nature and amount of starch damage which takes place during extrusion needs to be quantified. Glucose concentration can be economically and quickly determined with the YSI 27, and a number of different enzymes may be used to hydrolyze starch and intermediates sequentially to glucose, thus yielding information on the effect of extrusion on the starch molecule.

The Effect of Substrate Concentration on Conversion Velocity

This relationship has been reasonably well defined for the enzyme-substrate system used in this research, but there is still uncertainty about the nature of the reaction for high substrate concentrations. The reaction appears to be a Haldane type, and is modeled reasonably well by a form of the Haldane equation. However, very little has been learned about the nature of the inhibition. Further work on this problem would be valuable to understanding of the entire reaction.

Effect of Relative Size Difference Between Substrate and Enzyme

The only explanation which can be offered for the decline in conversion velocity at high substrate concentrations is that interference to motion and favorable contact dynamics occurs between the enzyme and the matrix of starch molecules. Further work should help clarify the nature of this phenomena. For example, amylose molecules of varying chain length may be used, thus keeping the number of attachment points constant while varying substrate concentration and molecule size. Attention should be given to determining proper experimental protocol for this analysis.

Effect of Fermentation

It appears that enzyme conversion rate is accelerated during fermentation. This may be due to a biological "pulling" of the reaction in the direction of glucose formation. It may also be due to production by yeast cells of extracellular enzymes which assist in reduction of

various dextrins to glucose. Again, combinations of special starch products and enzymes can be used to gather knowledge of the process in the fermenter. The variety of special starch products now available makes it possible to design a particular substrate for almost any need. Alternatively, an existing substrate can be modified with a special additive which will allow a particular measurement to be made.

Also important during fermentation is the effect of D.E. on conversion rate. It would be interesting to measure D.E. of the substrate at various times throughout fermentation to see what changes may take place. Such information would be useful in understanding the role of gluco amylases during fermentation.

Effect of Alcohol in Improving Conversion Rate

The apparent accelerating effect of alcohol on conversion of dextrins to glucose is certainly worth some additional effort. If alcohol does, in fact, act in some way as a co-factor or solvent which improves the ability of the enzyme to attack the starch molecule, the phenomena could be of some importance. Answers to these questions may already exist in the literature, though no mention was found in sources reviewed. A thorough literature review for this specific problem, followed by contact with enzyme manufacturers should preceed additional laboratory work.

Other Substrates and Process Conditions

It is again worth emphasizing that results reported in this research apply specifically to the gluco amylase enzyme manufactured by Bio-Con acting on processed starch substrates as described in the

Appendices. Nevertheless, it is certainly reasonable to expect that the experimental methods and procedures can be extended to other work. Also, the experimental relationships developed based on Michaelis-Menten-Haldane equations are consistent with accepted principles of enzyme kinetics, and should have application in general form to other problems similar in nature.

A natural extension of this work would be kinetic studies for alpha amylases. Alpha amylases are heavily used in starch processing and beverage industries for preliminary liquification and viscosity control. Specific blends of enzymes containing alpha amylases, beta amylases, gluco amylases and other enzymes can be combined to produce a particular type of product. Kinetic study of such blends would be worthwhile.

Study of Haldane Effect

The results show the Haldane effect to be very important for gluco-amylases and starch substrates. Further work should be done in this area to determine if the Haldane effect may apply to other starch hydrolyzing amylases such as alpha amylase, and to similar substrates. Noting the accelerating effect of alcohol, it would be interesting to see if addition of alcohol to substrates can counteract the Haldane effect to some degree.

Direct Use of YSI 27 for Enzyme Kinetic Studies

The YSI 27 immobilized enzyme glucose analyzer proved reliable and well suited to this study. Some additional refinement of experimental procedures with the instrument are possible. During the clearing cycle,

a neutral buffer is circulated in the sample chamber for cleansing purposes before the next injection. One possibility for rapid determination of rates at a specific substrate and enzyme concentration would be to replace the buffer with the substrate in question (neutral pH would be required), and then inject an enzyme standard. Since the instrument assays for a specific time period, repeated samples could be quickly run. Variations in the enzyme standard could also be quickly evaluated with this technique.

The need for determining background glucose in a particular sample is also eliminated with this technique since the instrument is zeroed prior to each injection, and any reading obtained is thus due to production of new glucose. Also, the instrument samples for only 40 seconds, so concern about operating during the initial linear portion of the reaction is not so critical.

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APPENDICES

APPENDIX A

Typical Carbohydrate Profile
MALTRIN

	MALTODEXTRINS					CORN SYRUP SOLIDS			
	M040 D.E. 5	M050 D.E. 5	M100 M500 D.E. 10	M150 M550 D.E. 15	M155 D.E. 15	M200 M600 D.E. 20	M250 D.E. 25	M255 D.E. 25	M365 D.E. 36
DP1									
Average	0.0	0.5	0.5	0.7	0.9	2.3	8.3	2.4	6.7
Range	0.0-0.3	0.1-0.8	0.3-0.8	0.6-10	0.6-1.2	2.0-2.5	8.2-8.4	2.4-2.4	6.5-6.8
DP2									
Average	0.3	0.5	2.7	4.5	4.8	7.9	7.3	8.1	27.8
Range	0.1-0.5	0.2-0.7	2.3-2.9	3.9-4.8	4.7-4.9	7.7-8.1	7.2-7.4	7.8-8.4	24.7-30.9
DP3									
Average	0.6	0.7	4.3	6.6	6.9	9.6	7.0	9.5	15.4
Range	0.3-0.9	0.4-0.9	4.2-5.0	5.9-7.1	6.7-7.1	9.2-10.0	6.5-7.4	8.8-10.2	13.0-17.7
DP4									
Average	0.6	0.8	3.7	5.3	5.3	6.2	6.3	6.0	9.2
Range	0.3-0.9	0.5-1.1	3.3-4.1	4.7-5.5	5.1-5.4	5.4-6.9	5.3-7.2	5.1-6.9	9.1-9.3
DP5									
Average	0.6	0.8	3.1	4.4	4.4	5.5	5.4	5.7	4.2
Range	0.3-0.9	0.5-1.1	2.7-3.4	3.9-4.7	4.2-4.6	4.5-6.5	4.2-6.5	4.5-6.9	3.9-4.4
DP6									
Average	1.0	0.7	5.7	8.6	8.8	12.7	4.3	13.7	2.5
Range	0.5-1.5	0.4-1.0	5.1-7.0	7.3-9.7	8.3-9.2	11.0-14.3	2.9-5.6	12.2-15.1	1.4-3.5
DP7									
Average	1.2	0.6	7.1	9.8	9.5	9.8	3.3	9.6	2.6
Range	0.6-1.8	0.3-0.9	6.5-8.3	8.8-10.9	9.3-9.8	8.2-11.4	1.8-4.8	8.6-10.5	1.0-4.1
DP8									
Average	0.8	0.5	4.5	4.9	4.5	2.5	2.9	1.2	1.5
Range	0.4-1.2	0.2-0.8	4.2-5.3	4.6-5.4	4.3-4.6	1.5-3.4	1.0-4.6	0.5-1.8	0.5-2.5
DP9									
Average	0.5	0.4	3.1	2.9	4.6	0.2	2.1	0.8	1.3
Range	0.2-0.8	0.1-0.7	2.8-3.6	2.7-3.1	2.0-4.6	0.2-0.2	0.3-3.8	0.2-1.3	0.3-2.2
DP10									
Average	0.3	0.1	1.6	0.3	1.4	0.1	2.2	0.7	1.1
Range	0.1-0.5	0.0-0.2	0.6-2.9	0.1-0.7	0.4-1.8	0.1-0.1	1.0-3.3	0.1-1.2	0.1-2.0
Above DP10									
Average	94.1	94.4	64.4	52.0	48.9	43.2	51.0	42.3	27.7
Range	91.2-97.5	91.8-97.3	56.8-68.1	48.0-56.2	47.9-54.1	37.5-49.4	41.0-61.6	35.3-49.8	22.0-34.1

APPENDIX B

DESCRIPTION OF THE GLUCOAMYLASE "GASOLASE"

The following description of "Gasolase" enzyme is extracted directly from Biocon (U.S.) INC. product literature (Biocon, 1981).

Gasolase

Gasolase is a carefully selected blend of enzymes derived mainly from Aspergillus niger and Rhizopus niveus. All of the enzymes incorporated in Gasolase have been approved by the FDA and BIBRA as suitable for ethanol production.

Gasolase is a sophisticated wide range enzyme system that is more than just a simple spectrum amyloglucosidase from Aspergillus niger. Gasolase incorporates alphaamylase, amyloglucosidase, protease, beta glucanase, cellulase, hemicellulase, pectinase, pantothenic acid, biotin, and a number of other essential vitamins and enzymes combined to give a complete system capable of dealing with the wide range of alcohol production conditions normally encountered in the ethanol production industry. Because of the spread of enzyme activities found in Gasolase, it is available only in powder form, as the stability characteristics of enzymes in liquid form are often limited.

APPENDIX C

LABORATORY PROCEDURES

Determination of Glucoamylase Activity Using the Y.S.I. Glucose Analyzer

MATERIALS

Yellow Spring Glucose Analyzer - Y.S.I. #27
Dextrose kit
Temperature controlled water bath, 32°C ±.2°C
Timer or stopwatch
pH meter
Test tubes & caps (30 ml), holder and agitator
Analytical balance
Glassware
Temperature controlled water bath, 100°C ±1°C

REAGENTS

1. Phosphate buffer

   Dissolve 30 grams of Na_2HPO_4, and 30 g of N_2HPO_4 in 1 liter of distilled water. Store in fridge.

2. Acetic-Acid, Sodium acetate buffer - pH 4.6

3. Starch

   Starch solutions were made based on % dry solids, wet basis. (Maltrin and Lo-Dex products.) Solutions should be refrigerated between tests and should have a maximum shelf life of 24 hours. Samples with high solids concentrations should be well mixed. All samples should be agitated prior to analysis or other activity.

4. Enzyme

   Dissolve 0.625 gms gasolase in 500 ml distilled H_2O. Mix well before use, and keep refrigerated. Prepare weekly.

DETERMINATION

1. Using a volumetric pipette, dispense 10 ml of buffered starch solution into a test tube.

2. Cap the tube and place in the 32°C chamber water bath for a length of time sufficient to achieve thermal equilibrium.

3. Using a fast flow pipette or other rapid dispensing volumetric measuring device, dispense 1 ml of diluted enzyme into the tubes and start timer. Immediately mix the contents of the tube and replace in the water bath.

4. Blanks containing only substrate should be included for determination of background glucose.

5. Incubate for the required period of time.

6. Using a volumetric pipette, add 10 ml of phosphate buffer to the tube. Mix well, and immediately immerse the tube in boiling water for 5-6 minutes.

7. Remove from boiling water and cool to room temperature.

8. Determine the amount of glucose present using the glucose analyzer. Follow the instructions in the Y.S.I. Model 27, instruction manual. Mix the test tube well before filling the syringepet.

9. Total time of reaction is taken from addition of enzyme to immersion in boiling water (or to injection in Y.S.I. #27 if no heat treatment used).

NOTE

1. Test tubes should be only partially immersed in boiling water. A water level 2.5 cm above the contents of the tube is sufficient.

2. Addition of the phosphate buffer in Step 6 may be omitted if previous testing has established that heat exposure is satisfactory to insure equilibrium between the alpha and beta forms of glucose.

3. When no heat deactivation of the enzyme is used, the time of reaction is measured from addition of enzyme to the sample, to injection of the sample into the YSI Model 27 for glucose determination. For this analysis, it is important that sample temperature be maintained as close as possible to 32°C up to injection. Duplicate injections of one sample into the YSI will not be possible with this method as the enzyme will continue producing glucose while the initial analysis is being performed.

CAUTION

Do not place rubber or glass stoppered test tubes into boiling water.

CALCULATIONS

Units

The activity or conversion rates reported in this paper may be calculated from the following expression (some modification may be required depending on the particular test and procedures being used.

$$V = \frac{YSI_f(VS_f) - YSI_i(VS_i) \quad \frac{mg\ (ml)}{dl} \quad \frac{(dl)}{100ml}}{t\ (hrs) \quad \frac{625\ mg}{500\ ml} \quad (1\ ml\ added)}$$

$$V = \frac{YSI_f(VS_f) - YSI_i(VS_i)}{125\ t} \quad \frac{mg\ glucose\ produced}{mg\ enzyme - hr}$$

where:

YSI_i = initial YSI reading (background) - mg/dl

YSI_f = final YSI reading (at end of t) - mg/dl

VS_i = initial sample volume (volume when YSI_i is made) - ml

VS_f = final sample volume (volume when YSI_f is made) - ml

t = incubation period - hrs

APPENDIX D

Enzyme Tests

Test #1

Starch solutions from 3 to 18% solids were prepared in increments of 3%. American Maize Products' Lo-Dex 10 soluble starch substrate was prepared in Fisher Certified acetate buffer (pH 4.63). Substrate samples and enzyme solutions were prepared according to procedures outlined in the methods section. Two blanks and three enzyme treated reps were processed at each solids concentration. Enzyme activity was determined as total change in glucose during a one hour incubation period. Enzyme deactivation was accomplished by heat treatment. Phosphate buffer was added to insure glucose equilibrium.

Test #2

Five starch solutions containing 10% Lo-Dex 10 in acetate buffer were prepared. Distilled 190 proof grain alcohol was added to achieve alcohol concentrations of 0%, 3%, 9%, and 12% by volume. Standard enzyme solutions were used to assay conversion velocity for samples during a 60 minute incubation period. Two blanks (no enzyme added) and three enzyme treated reps were tested for each level of alcohol. Analytical methods were as discussed in Test #1.

Test #3

Five different levels of fungicide were added to a standard 10% solutions of Lo-Dex 10 in acetate buffer. In addition to the control, levels of 50%, 100%, 150%, and 200% of normal addition rates were evaluated. Normal rates were determined from manufacturer's recommendations for seed corn. The incubation period was 60 minutes. Sample preparation and analysis was the same as covered in Test #2.

Test #4

Test #4 involved determination of reaction rate at several agitation rates. A New Brunswick chemostat was used to insure that test conditions could be maintained constant while agitation rate was varied. The substrate solution was 6% Lo-Dex 10 in acetate buffer. Temperature and pH was continuously monitored and controlled according to preparation and treatment procedures outlined previously. Duration of the test was 60 minutes (with one or two observations over an extended period) with samples taken every 10 minutes. Agitation was determined as agitator shaft rpm. Evaluation was done at 0, 44, 200, and 400 rpm. The three levels of agitation corresponded to gentle, moderate, and violent agitation. Analytical methods were identical to those previously discussed.

Test #5

Test #5 was run to determine rate of glucose production for 10% starch solutions with six different Dextrose Equivalents (carbohydrate chain length). Maltrin Products with approximate Dextrose Equivalents of 5, 10, 15, 20, 25, and 36 were used. Further information on these substrates is contained in Appendix A. These samples were processed and analyzed with standard enzyme solutions and methods.

Test #6

The purpose of Test #6 was to develop V vs S data necessary to determine enzyme reaction kinetic constants K_m and V_m. Conversion velocity was determined over a range of substrate concentrations from 0.25% to 30% solids in solution. In general, procedures used for these tests were similar to those used in previous tests. However, several differences are worth noting:

1. In order for assumptions underlying Michaelis-Menten kinetics to be correct, velocities must be measured during the initial portion of the reaction, while conversion rate is constant. Thus, it was necessary to perform screening to determine maximum acceptable incubation period for the range of substrates to be considered. Graphically, this is depicted in Figure 4. For these tests, an incubation period of 9 minutes was chosen.
2. Each test at a particular substrate concentration was replicated two times, with one background or blank.
3. At high substrate concentration, solutions were relatively viscous, and care was needed to insure that measurement and sample injection were not affected.
4. Whenever possible, each days testing involved enough samples to be representative of the entire substrate concentration range tested, 0.25% to 30%. Within this sample "subset", the preparation and testing of samples was performed at random.

5. Phosphate buffer was not added to these samples. In previous testing, a number of evaluations were run comparing rates obtained for samples treated with and without phosphate buffer. No difference in final values was observed, and addition of phosphate buffer was deleted.
6. Some of the evaluations for Test #6 were done with heat deactivation of enzymes, and some with no deactivation. This is discussed in the results section.

Incubation time was taken as nine minutes from the point of enzyme addition to sample injection into the YSI 27. The machine performs its analysis over a fixed time interval, and enzyme concentration was constant. Each sample was thus treated alike in terms of reaction length.

Experience with the machine obviated the need for duplicated injections for individual samples, and the problem of replicated samples was treated by preparing additional samples at each of the substrate concentrations to be evaluated.

APPENDIX E

RAW DATA FOR DETERMINATION OF K_m, V_m, AND K_I

	Heat Deactivated		Non-Deactivated	
	S(% solids)	V(mg/mg-hr)	S(% solids)	V(mg/mg-hr)
1	.25	29.92	.25	26.4
2	.285	34.29	.35	28.16
3	.333	33.76	.5	29.92
4	.4	34.4	.65	31.09
5	.5	34.67	.75	32.26
6	.6	37.39	.85	34.02
7	.75	36.85	1	35.2
8	1	38.67	1.25	34.61
9	1.25	39.31	1.5	34.02
10	1.5	39.41	1.75	35.78
11	1.75	40.64	2	34.61
12	2	40.75	2.5	35.2
13	2.5	41.49	5	35.2
14	3	41.07	7.5	30.50
15	4	41.92	10	28.74
16	5	42.88	12.5	26.98
17	5	42.4	15	36.96
18	6.5	44.48	17.5	23.46
19	7.5	43.2	20	22.88
20	7.5	42.67	22.5	18.77
21	8.5	44.64	25	22.88
22	10	42.29	27.5	19.36
23	10	42.35	30	21.12
24	11.5	42.29		
25	12.5	42.08		
26	14	41.44		
27	15	44.21		
28	16.5	40		
29	17.5	42.77		
30	18.5	40.69		
31	20	37.87		
32	22.5	39.84		
33	25	39.57		
34	27.5	39.36		

VITA 2

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Doctor of Philosophy

Thesis: REACTION KINETICS FOR STARCH HYDROLYSIS WITH GLUCOAMYLASE

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