APPROVAL SHEET

Title of Dissertation: Quantitative *In Vitro* Microdialysis Sampling Coupled to HPAEC-PAD for the Monitoring of Mannan-Degrading Enzymes

Name of Candidate: Sarah Mae Bass
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Dissertation and Abstract Approved: 

Dr. William R. LaCourse
Professor, Department of Chemistry and Biochemistry

Date Approved: 9/12/17

ABSTRACT

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	Sarah M. Bass, Ph.D., 2017.
Directed By:	Professor, Dr. William R. LaCourse, Department of Chemistry and Biochemistry

In vitro microdialysis sampling coupled to high performance anion exchange chromatography and pulsed amperometric detection (MD-HPAEC-PAD) is a powerful tool for carbohydrate analysis in complex matrices. Microdialysis is a sampling technique that employs a semi-permeable membrane that achieves on-line sample cleanup and in situ sampling in both *in vivo* and *in vitro* applications. The selectivity of this sampling technique is due to the molecular weight cut-off of the probe, where freely diffusing species in a sample may be transported along a concentration gradient across the membrane into a continuous perfusion flow. The collected dialysate can be assayed to develop a dynamic concentration versus time profile of the system under study. Microdialysis sampling overcomes problems associated with traditional bioprocess monitoring by eliminating the need for sample removal, enzymatic reaction quenching, and filtration steps, while also minimizing sample handling and contamination issues associated with bioprocess sampling.

Improvement in the quantitative aspects of on-line microdialysis was accomplished using a computer-programmable pump designed for precision fluid delivery with flow rates in the nano- and microliter per minute range. A second pump

was added in-line to the setup to achieve on-line dilution, which compensates for long acquisition times associated with slow perfusate flow rates and dead volume prior to the injection valve. Technical improvements to the on-line *in vitro* MD-HPAEC-PAD setup were implemented to develop an assay for assessing the efficiencies of β-mannanase enzymes used in industrial processes.

A model digestion assay was developed to validate the technical improvements of *in vitro* microdialysis sampling as a quantitative assessment of β-mannanase enzymatic reactions. The development of an assay to measure the efficiency of β-mannanase enzymes will impact industrial applications by offering on-line sampling and sample cleanup when assessing manufacturing performance. This research provides a fingerprinting tool to accurately compare the breakdown of mannan by various β-mannanase enzymes. Overall, this research further extends microdialysis to be a powerful analytical tool for monitoring bioprocesses by allowing for on-line analysis and quantitative assessment of enzymatic efficiencies in complex samples.

QUANTITATIVE *IN VITRO* MICRODIALYSIS SAMPLING COUPLED ON-LINE TO HPAEC-PAD FOR ASSESSING MANNAN-DEGRADING ENZYMES

By

Sarah Mae Bass

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore County, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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Dedication

This dissertation is dedicated to my husband and kids for their unconditional love and support.

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There are many people I want to acknowledge for their assistance through this journey.

First, thank you to my research advisor Dr. William LaCourse for continually fighting for me, for believing in me, and for pushing me to do better.

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It has been a pleasure and inspiring to work with all my lab mates through the years. A special thank you to the MCAC for their technical expertise and assistance.

My parents and sisters inspire me in all areas of life because they never stop working hard. Faith, Mary and I would not be where we are without our parents' unconditional love and support. Thank you to my parents for believing in me and for giving me the opportunities to achieve my dreams. Thank you to my Bass family for their assistance and encouragement. And, thank you to my family that is no longer here - I know they would be proud of me.

Lastly, and certainly not least, thank you to my husband. I will never have the words to describe how grateful I am for his dedication, love, and the gifts he has bestowed upon me. Stephen, you are the best thing that has ever happened to me.

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1 Background and Significance: Monitoring of Carbohydrate-Based Bioprocesses using In Vitro Microdialysis Sampling

1.1 Introduction to Carbohydrate-Based Bioprocesses

1.1.1 Bioprocess Monitoring

Fermentation broths and enzymatic bioprocesses are used in the manufacturing of biotherapeutics and many other biological materials produced using recombinant genetic technology, as well as for the production of methanol and ethanol as alternative energy sources to fossil fuels, and for the manufacturing of alcoholic beverages.[1,2] Nature has developed very efficient, highly precise methods of controlling bioprocesses. The challenge is to transfer these principles into technically applicable and precise analytical systems that can be used for many industrial applications.[3] Bioprocess monitoring is generally performed by measuring physical and chemical parameters such as pH, temperature, nutrients and dissolved oxygen.[4, 5] But, carbon sources and metabolic by-products can impact the yield or quality of the desired products[1] and it is important to characterize the concentrations of these analytes in order to optimize manufacturing productivity.

Enzymatic bioprocesses and fermentation broths are complex mixtures of nutrients, waste products, cells, cell debris, and desired products.[1] In most bioprocesses the bioreactor is the most complex component that dictates the success of the overall process and, therefore, it is imperative to be able to control and optimize this component to ensure high product concentration and quality.[5] Process Analytical Chemistry (PAC) tools are used to measure parameters of bioprocesses through in- and

on-line analytical instrumentation. From PAC comes Process Analytical Technology (PAT), which is a series of tools designated by the Food and Drug Administration (FDA) specifically for the pharmaceutical manufacturing process.[6] The goals of these sets of tools are to improve understanding of processes to increase efficiencies, decrease costs through reducing production times, prevent waste by improving energy and material consumption, and facilitate continuous processing with special attention toward real-time monitoring.[6] Monitoring of physical and chemical parameters are used to provide a "big picture" of the entire process to achieve these goals. Figure 1.1 summarizes some of the various variables that affect bioprocesses.

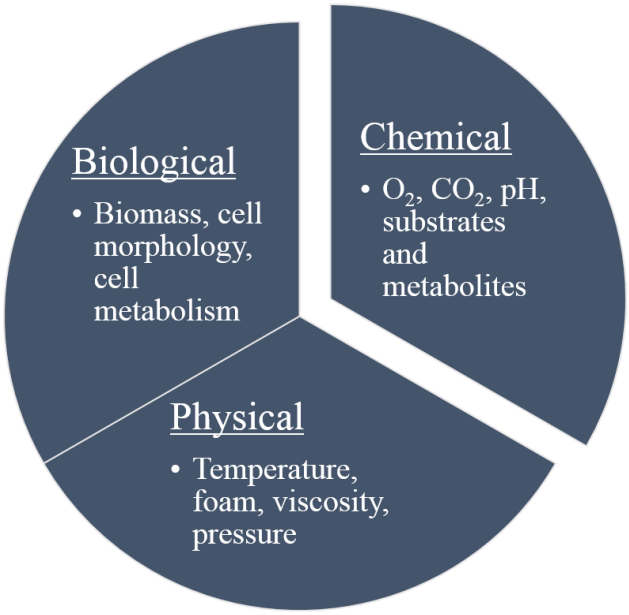


Figure 1.1 Biological, chemical, and physical variables of bioprocesses.

Traditional bioreactor monitoring is performed at-line and involves manual removal of aliquots to produce representative samples of the bioprocess that are then transported for analysis. These samples require reaction quenching and filtration prior

to separation and detection. The delay in such procedures results in lost opportunities for discovering real-time process changes that could affect the safety, quality, and production cost efficiency.[3, 4, 6, 7] Additionally, these steps can result in contamination and alter the kinetics of the reaction, which could disturb manufacturing performance.[8] A sampling technique within a bioreactor that allows for cleanup without additional sample handing and preparation prior to its analysis would prove beneficial because it can provide real-time analysis of the dynamic bioprocess. On-line monitoring systems for bioprocesses are desirable, offering the advantages of quick feedback and reduced analysis time.

1.1.2 Carbohydrates

Carbohydrates are polymers that can be used in the production of renewable energy sources and as natural constituents or additives in food products. The past two decades have seen various novel types of carbohydrates introduced as new ingredients in food manufacturing, especially non-digestible oligosaccharides. Not only do carbohydrates contribute to flavor profile and physiochemical properties of foods and biotechnology products, but there is increasing documentation of their beneficial effect on health.[9] Generally, food-grade oligosaccharides are impure substances containing different degrees of polymerization. To use these polymers, it is necessary to characterize and profile them, which requires fast and efficient hydrolysis, sampling and sample clean-up, separation and detection steps. Enzymatic hydrolysis, as opposed to chemical methods, of carbohydrate polymers is preferred because it is environmentally friendly and selective. Carbohydrates (e.g., glucose, lactose, sucrose, maltose, etc.) are the major carbon sources essential for cell growth and product

synthesis in bioprocesses, and alcohols (e.g., ethanol, methanol, sugar alcohols, etc.), glycols (e.g., glycerol), and organic acids (e.g., acetate, lactate, formate, etc.) are by-products that often reduce product yields.[1]

1.1.3 Mannan Sugars and Mannanase Enzymes

The most abundant renewable natural biological resource is cellulose, a linear carbohydrate polymer consisting of D-anyhydroglucopyranose linked together by β-1,4-glycosidic bonds with various degrees of polymerization (DP) from 100 to 20,000.[10] Along with cellulose, hemicellulose is present in plant cell walls but, unlike cellulose, hemicellulose consists of shorter chains of DP 500 – 3,000 and can be branched. Mannan-type hellicelluloses are the major component of softwoods and are hydrolyzed to readily fermentable sugars mannose, galactose, and glucose. Mannan consists of mannose sugars linked by β-1,4-glycosidic bonds for its backbone, and can be linear or have glucose or galactose residues. Mannan-degrading enzymes are widespread, found in various bacteria and fungi that have potential in industrial applications.[11]

β-mannanase enzymes are endo-type enzymes responsible for cleaving the β-1,4-linked mannose backbone. Mannan and mannanase enzymes are finding increasing importance in biotechnology, and their applications are highlighted in Table 1-1. To date, the International Union of Pure and Applied Chemistry (IUPAC) has published a description of standard methods for measuring cellulase enzyme activities, but only an overview of the most common quantification methods for xylanases with a recommendation to establish a standard method.[12, 13] IUPAC states that since xylanases are the most studied enzymes for hemicelluloses they take priority over mannanases,

but that publication of standard procedures for mannanases should be taken up afterward.[13]

β-mannanase enzymes vary significantly in their abilities to hydrolyze mannans depending on their [enzyme] source.[14] The foundation of enzyme engineering is to directly correlate enzyme assays or screening approaches and the changes in enzyme function in a desired application.[10] Development of useful, predictive assays or screening procedures for mannanase prove difficult because of the complex nature of mannan substrate, whether from plant cell walls or within bioreactors.

Table 1-1 Summary of applications of mannanase enzymes in various industries.[15]

Industry	Application
Paper and pulp	Bleaching of softwood pulps – reduction of environmental pollution by facilitating lignin extraction without impairing pulp quality
Hydrolysis of coffee extract	Reducing viscosity of coffee extracts – inhibit gel formation during freeze drying of instant coffees and increasing reducing sugar content
Detergent (sanitization products, contact lens cleaner, dishwashing, health and beauty products)	Removal of mannan-containing stains – improve removal of mannan on fabrics, which has a "gluing" effect, as well as prevent transfer to other fabrics and improve brightness
Animal feed	Decrease intestinal viscosity from animal feeds – improves weight gain and feed conversion efficiency
Oil extraction of coconut meat	Eliminates problems of contamination and rancidity – improves quality of coconut oil while also minimizing need for oil refinement
Textile fiber processing	Scour and de-size cellulosic material – prepare materials for subsequent dying in garment manufacturing process
Degradation of thickening agents	Reduce viscosity of print paste – facilitate and improve cleaning of residual in processing equipment
Non-nutritional food additive	Improve gut flora – degrade mannans, which are resistant to digestive enzymes

1.2 Microdialysis Sampling

1.2.1 *In Vitro* Microdialysis Sampling Technique

Microdialysis is a sampling technique that employs a semi-permeable membrane that achieves on-line dilution[4], on-line clean-up[16], and *in situ* sampling in both *in vivo* and *in vitro* applications. Traditional areas of microdialysis application have mainly included *in vivo* work, neurochemical and pharmacokinetic studies, but the versatility of microdialysis sampling has recently extended its use into biotechnology.[7]

Microdialysis sampling is governed by diffusion; analytes are transported from areas of high concentration (e.g., bioreactor) to areas of low concentration through a semi-permeable membrane. The selectivity of the microdialysis sampling technique in a complex matrix is due to the molecular weight cut-off of the membrane (MWCO)[17], where freely diffusing species in the sample solution may be transported along a concentration gradient over a dialysis membrane into a continuous perfusion flow. This principle allows for the continuous removal of very small and representative samples which do not affect the volume of the bioreactor[18] and excludes large molecules such as proteins from the flowing perfusion fluid because of the MWCO of the membrane. The fluid continuously pumped into the microdialysis probe is termed the perfusate, and the fluid collected after sampling that contains the analyte(s) is known as the dialysate. Common microdialysis probe designs are illustrated in Figure 1.2.

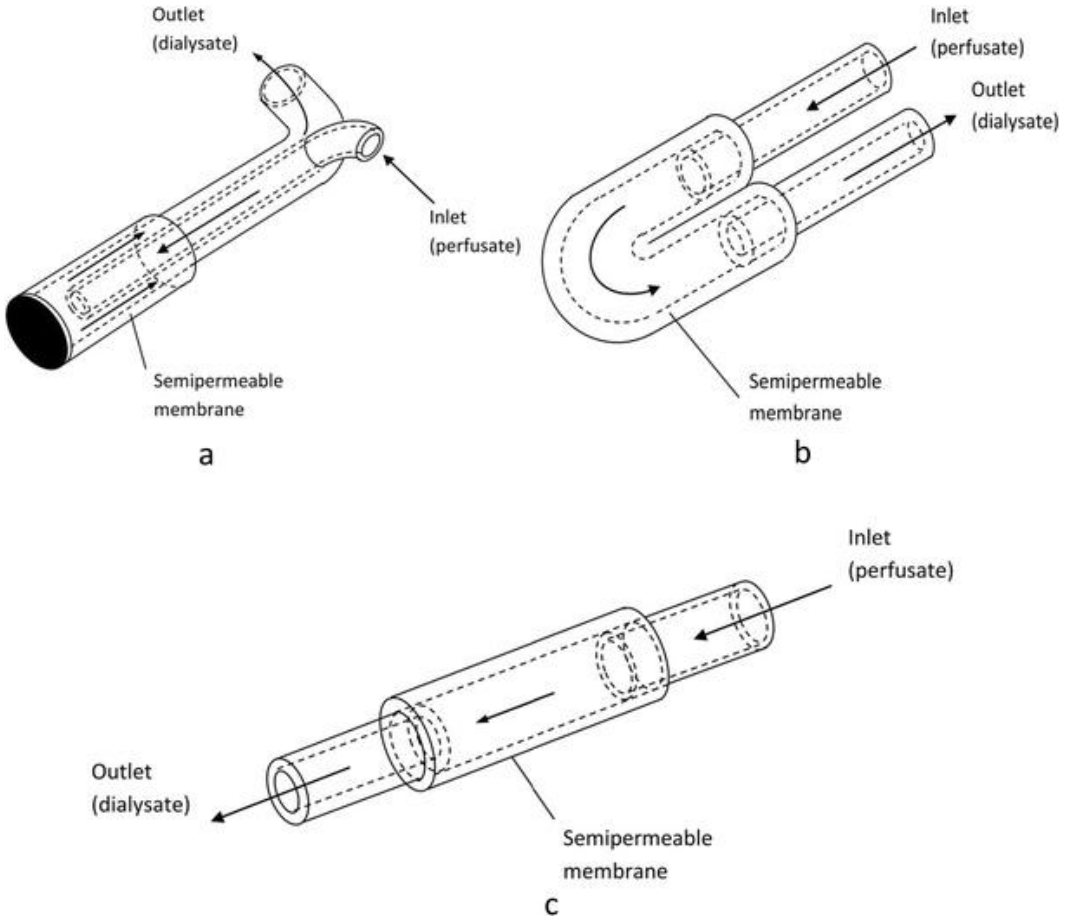


Figure 1.2 Schematic illustration of common microdialysis probe designs: (a) concentric, (b) loop, and (c) linear microdialysis probes.[19]

1.2.2 Calibration Techniques for Quantitative Microdialysis Sampling

Microdialysis was initially developed for the sampling of small molecules in the brain in 1972 by Delgado *et al*.[20] and popularized by Ungerstedt and Pycock in 1974[21]. In 1990, Bungay and Morrison demonstrated that data acquired after microdialysis sampling can be treated quantitatively.[22] The quantitative evaluation of microdialysis in bioprocesses can be described by the extraction fraction (EF), defined in Equation (1)[22], where C_{out}, C_{in}, and C_{medium} are the analyte concentrations in the dialysate, perfusion fluid, and bioreactor, respectively, and Q_d is the perfusion rate.

$$EF=\frac{C_{out}-C_{in}}{C_{medium}-C_{in}}=1-e^{\left(-\frac{1}{Q_d(R_d+R_m+R_{medium})}\right)}$$

Equation 1

Microdialysis recovery, also known as EF, is governed by the resistance of the analyte transport through the medium exterior of the probe (R_{medium}), the membrane (R_m) and the dialysate (R_d). R_d and R_m are dependent on the properties of the membrane, such as composition, pore size, membrane length, and area.[23] Generally, in microdialysis experiments the analyte is absent from the perfusate and R_{medium} is zero in a well-stirred solution for bioprocess monitoring, simplifying Equation (1) to Equation (2)[22]; EF is typically expressed as a relative recovery (RR) (Equation (3)). Figure 1.3 shows the relationship between RR and flow rate.

$$EF=\frac{C_{out}}{C_{medium}}=1-e^{\left(-\frac{1}{Q_d(R_d+R_m)}\right)}$$

Equation 2

$$RR=100*\frac{C_{out}}{C_{medium}}$$

Equation 3

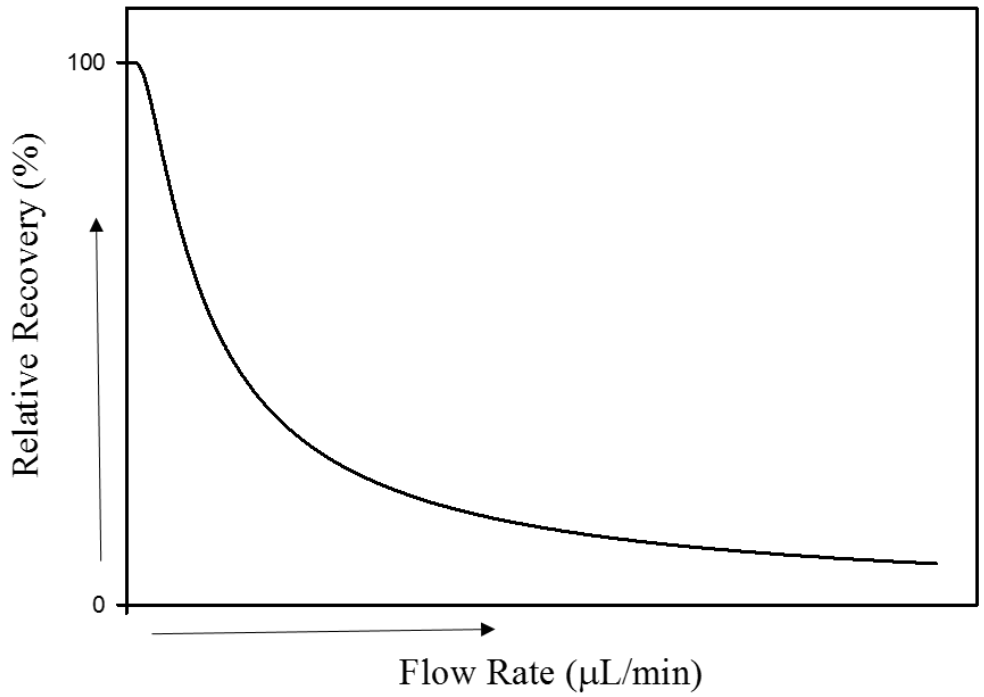


Figure 1.3 A general plot of the relationship between relative recovery and flow rate for microdialysis.

In most cases where microdialysis sampling is applied to bioprocesses, an initial decrease in EF may be observed that can be associated with fouling of the membrane due to analyte-membrane interaction. Such interactions result in an increased analyte resistance within the vicinity of the membrane without necessarily passing through the dialysis layer and thus with time, this is reflected as a decrease in EF. The subsequent stabilization in the EF is associated with a uniform surface coverage of the membrane that resists further analyte-membrane interaction. In order to account for this, the Andrade effects factor, A, as described by Torto *et al*. is shown by Equation (4).[8] The outer and inner radius of the microdialysis membrane are described by r_o and $r_{i;}$ the effective dialysis length is described by L, the diffusion coefficient through the

membrane is D_m, and the aqueous phase volume of the membrane is described by ϕ_m in Equation 4.

$$R_m = \frac{\ln\left(\frac{r_o}{r_i}\right)}{2\pi L D_m \emptyset_m} + A$$

Equation 4

This equation describes the membrane's resistance to diffusion in sampling from enzymatic bioprocesses where the analyte-membrane interaction is significant.[8] From the theoretical treatment of the microdialysis process, high recoveries will be achieved at slow perfusion rates. While employing slow perfusion rates introduces the possibility of sample loss due to evaporation, recovery may be increased by employing a high effective dialysis length (EDL) especially if the probe is to be used to sample from large areas, such as in a bioprocess. Microdialysis is a well-established technique for the on-line monitoring of substances both *in vivo* and *in vitro* and has proven to be an ideal sampling technique, causing minimal perturbation to physiological processes.[16,22]

1.2.3 Microdialysis Sampling of Bioprocesses

Microdialysis sampling reduces analysis time and offers the benefits of eliminating enzymatic reaction quenching and filtration steps. Additionally, sample loss is not an issue when microdialysis is employed since no sample is removed from the bioreactor. This sampling technique can sample from complex matrices without depleting the enzymes and without perturbing the reaction.[8] Microdialysis sampling

does not disturb the kinetics of the reaction under study[24], allows for continuous dynamic time profile changes in a biological matrix without affecting the environment of the bioreactor[25], and can be coupled to an analytical system in an on-line setup[26]. Importantly, this technique minimizes sample handling and contamination issues associated with bioprocess sampling.[27]

1.3 Enzyme Kinetic Characterization

Enzymes are specialized proteins that catalyze metabolic reactions, often transforming organic compounds into large amounts of energy. For example, the cellular metabolism of glucose produces 30 – 38 equivalents of ATP, the energy currency of the cell, and the combustion of glucose produces 2870 kJ of energy as heat[28] (Figure 1.4). Carbohydrates are energy-rich compounds that are essential to biological functions of plants and animals. Transferring the complexity, selectivity and efficiency of biological processes is the basis of biotechnology.

Combustion of glucose: $C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ O_2 + 2870$ kJ energy

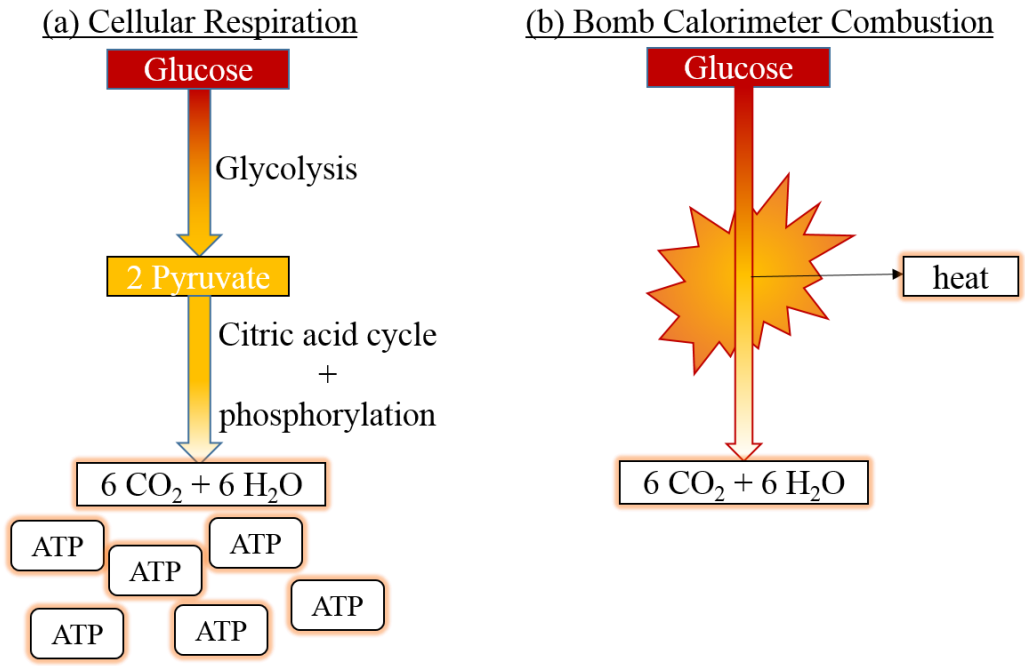


Figure 1.4 Glucose as an energy source. (a) 30 – 38 equivalents of ATP (energy currency of cells) are produced during the metabolism of glucose. This process takes place first with the conversion of one glucose to two pyruvates via ten enzyme-catalyzed reactions of glycolysis. Then, pyruvate is further broken down to carbon dioxide and water via nine reactions of the citric acid cycle coupled to oxidative phosphorylation. (b) The combustion of glucose in a bomb calorimeter results in an explosive release of energy as heat.[28]

Enzyme kinetics are used to assess the role of enzymes in catalysis, and the importance of assessing enzyme kinetics cannot be overstated. Characterizing enzymes allows for control and manipulation of metabolic events (e.g., bioprocesses), and is useful in drug design. Enzyme kinetics characterization leads to better understanding of complex carbohydrate substrates, such as the branching and linkages, and how to harness the unique abilities of enzymes, such as those from extremophiles. Enzyme kinetics are characterized in multiple ways and are summarized in Figure 1.5. Since enzymes are sensitive to many factors, the conditions of the assay used for kinetic

characterization must be well-defined and specified to ensure reproducibility between samples and laboratories.

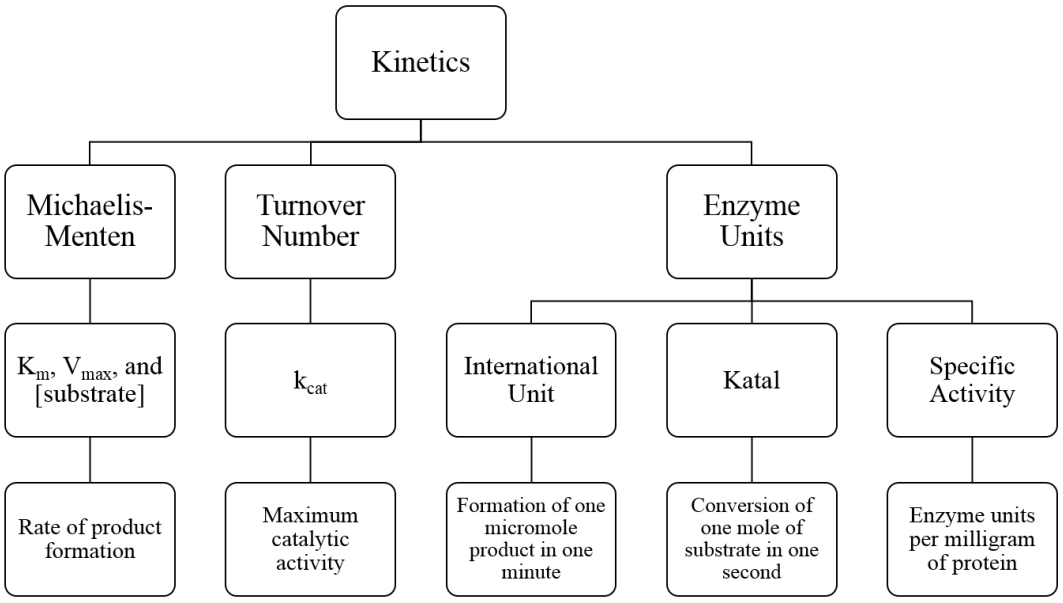


Figure 1.5 An overview of enzyme kinetic characterization parameters.

The Michaelis-Menten equation relates the initial rate of product formation (υ) to: substrate concentration [S], the maximum theoretical rate at infinite substrate concentration V_{max}, and the Michaelis constant K_m, which is the substrate concentration at one half of V_{max}. Figure 1.6 summarizes the Michaelis-Menten equation and the traditional hyperbolic plot relating reaction rate to substrate concentration to derive the kinetic parameters. There are many enzymes that do not obey this simplified formula of enzyme kinetics which relies on several assumptions including: the reaction involves only one substrate, the breakdown of the enzyme-substrate complex to form enzyme and product is irreversible, the substrate concentration is greater than the total enzyme

plus enzyme-substrate complex concentration, and all other factors (i.e., pH, temperature, ionic strength) that influence the rate of the reaction are constant.[28]

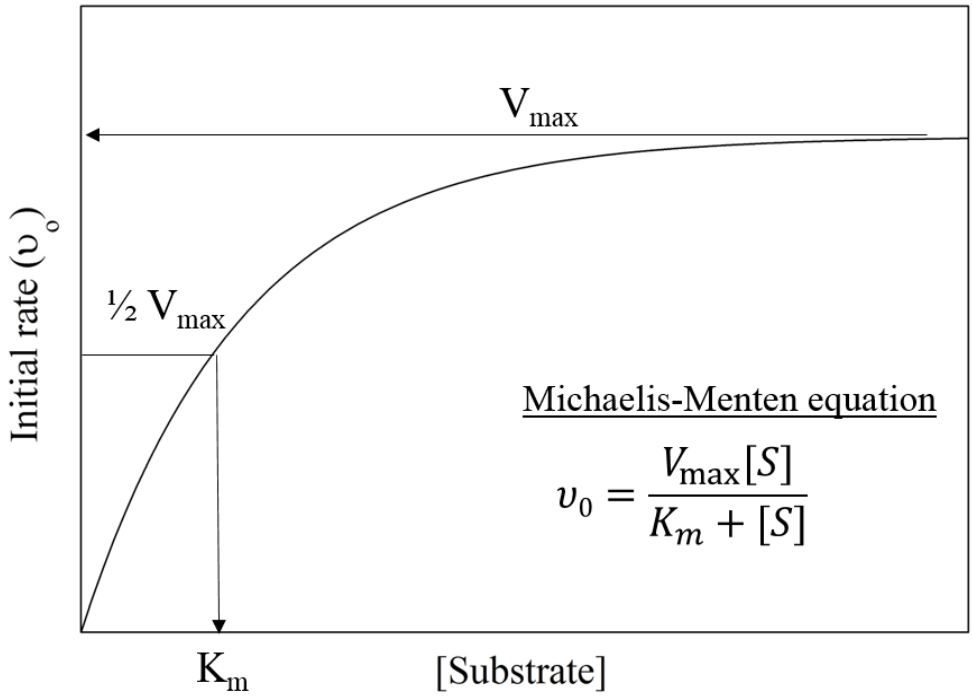


Figure 1.6 The relationship of the Michaelis-Menten equation to the plot of the initial rates of an enzymatic reaction relative to varying substrate concentrations. The Michaelis-Menten kinetic parameters K_m and V_{max} can be extrapolated by various linear transformations of the hyperbolic plot.

The kinetic efficiency of an enzyme can also be described by its turnover number k_{cat}, a measure of the maximum catalytic activity. K_{cat} is defined as the number of substrate molecules converted to product per enzyme molecule per unit time at enzyme saturation. K_{cat} can be calculated from the Michaelis-Menten reaction for initial rate if the total enzyme concentration is known. Then, k_{cat} equals the ratio of V_{max} to the total enzyme concentration: $V_{max}/[E_T]$. Under *in vivo* conditions substrate concentration is rarely saturating and the active sites of enzymes are often unoccupied. Since K_m is inversely proportional to the enzyme affinity for substrate and k_{cat} is

directly proportional kinetic efficiency of the enzyme, the ratio of the two together (k_{cat}/K_m) provides the catalytic efficiency of an enzyme operating on substrate concentration that is below saturation amount. However, in many situations, the actual molar amount of enzyme is unknown and complicates the determination of these parameters.

When the enzyme concentration is unknown, or is not completely purified from its cellular source, observed enzyme activity is used to characterize its kinetic ability. Because enzymes are dependent on their environments, it is imperative that all conditions be controlled in enzyme activity assays. These factors must be reported to ensure reproducibility and allow for comparison between samples and laboratories. The International Commission on Enzymes defines One International Unit of enzyme as the amount that produces one micromole of product in one minute. Other definitions of activity are katal and specific activity. One katal is the amount of enzyme that converts one mole of substrate to product in one second. Specific activity is the enzyme units per milligram of protein; the specific activity of enzymes increases during purification.

There are two classifications for assays measuring enzyme activity: stopped and continuous. In both cases, the amount of product formed, or substrate depleted, is monitored over the course of the reaction. A stopped assay measures the amount of product that is formed at fixed time intervals. This type of assay requires stopping the reaction by protein denaturing with strong acid, strong base, heating, cooling, or adding some other chemical inhibitor or complexing agent. The time intervals must be chosen so that the rate of product formation is linear. A continuous assay monitors the progress of the reaction as it occurs. This type of assay is convenient since results are monitored

in real-time and, therefore, any deviation from the initial rate is immediately identified. Not all enzymes have an assay method that permits the continuous observation of the reaction, but those that do rely on observation of physical parameters such as fluorescence, viscosity, changes in absorbance, etc.[29]

The work of this dissertation uses microdialysis coupled to high performance anion exchange chromatography and pulsed amperometric detection (MD-HPAEC-PAD) for the assessment of mannanase enzyme activity. The key features of employing microdialysis for enzyme kinetic characterization are that it allows for continuous sampling of a bioprocess without requiring reaction quenching at timed intervals and reduces the need for sample clean-up. By coupling microdialysis to HPAEC-PAD the formation of product(s), and sometimes substrate(s), can be observed in real-time. This dissertation lays the groundwork toward the development of a standard continuous mannanase enzyme kinetic assay, while also providing a fingerprint of specific enzyme activity and the substrate breakdown pattern. MD-HPAEC-PAD is a powerful analytical tool for monitoring carbohydrate-based bioprocesses.

1.4 Carbohydrate Analysis

HPAEC-PAD delivers high resolution separations and sensitive detection of a wide variety of carbohydrates including monosaccharides, sialic acids, sugar phosphates, and oligosaccharides, without analyte derivatization.

1.4.1 Separation of Carbohydrates

The pKa values of some common monosaccharides lie between 12.0 – 14.0 (Table 1-2). At neutral pH, most carbohydrates are not anionic, but under alkaline conditions carbohydrates are oxyanions. Therefore, separations of carbohydrates are possible using an alkaline mobile phase with an anion exchange column. Retention of oligosaccharides increases with decreasing pKa, and the retention of larger carbohydrates depends on their size and conformation. This separation technique only requires up to two mobile phase components: hydroxide and acetate. Isocratic hydroxide is suitable for separating smaller saccharides, whereas hydroxide and acetate can be used to separate larger carbohydrates.

Table 1-2 pKa values of some common sugars in water at 25 °C.

Carbohydrate	pKa
Fructose	12.03
Mannose	12.08
Xylose	12.15
Glucose	12.28
Galactose	12.39
Dulcitol	13.43
Sorbitol	13.60
α-Methyl glucoside	13.71

1.4.2 Detection of Carbohydrates

The use of basic conditions to achieve carbohydrate separations marries well with PAD, which is a direct detection technique that requires high pH. In the early

1990's, a three-pulse waveform was recommended for carbohydrate detection (Figure 1.7 (top)). This triple-pulse waveform directly detects carbohydrates by the electrical current produced when they are oxidized at the surface of a gold electrode. However, the products of this oxidation foul the surface of the electrode. To clean the electrode surface, the potential is raised higher than the detection potential, which oxidizes the gold electrode surface and desorbs the carbohydrate oxidation products. Then, the potential is decreased below the detection potential, which reduces the gold oxide on the electrode surface back to gold. This three-step waveform pulses back to the original detection potential and the cycle repeats. With the use of high potential oxidative cleaning, the working electrode is prone to wear and a gradual decrease in sensitivity. In the later 1990's, a new waveform was developed to improve the triple-pulse waveform. A fourth potential step was added where reductive cleaning begins with a negative potential, rather than a positive one (Figure 1.7 (bottom)). This fourth pulse minimizes the gold oxide formation and improves the long-term stability of the working electrode. In addition to minimizing electrode wear, this quadruple-pulse waveform produces consistent long term peak area response (long term reproducibility) and avoids electroactive interferences, such as from amino acids. Finally, the quadruple-pulse waveform is half of the length of time thereby doubling the data collection rate, a great benefit when coupled to chromatography to produce sharp elution peaks. HPAEC-PAD is selective and specific; it is a well-established analytical technique, and plays a significant role in carbohydrate analysis.

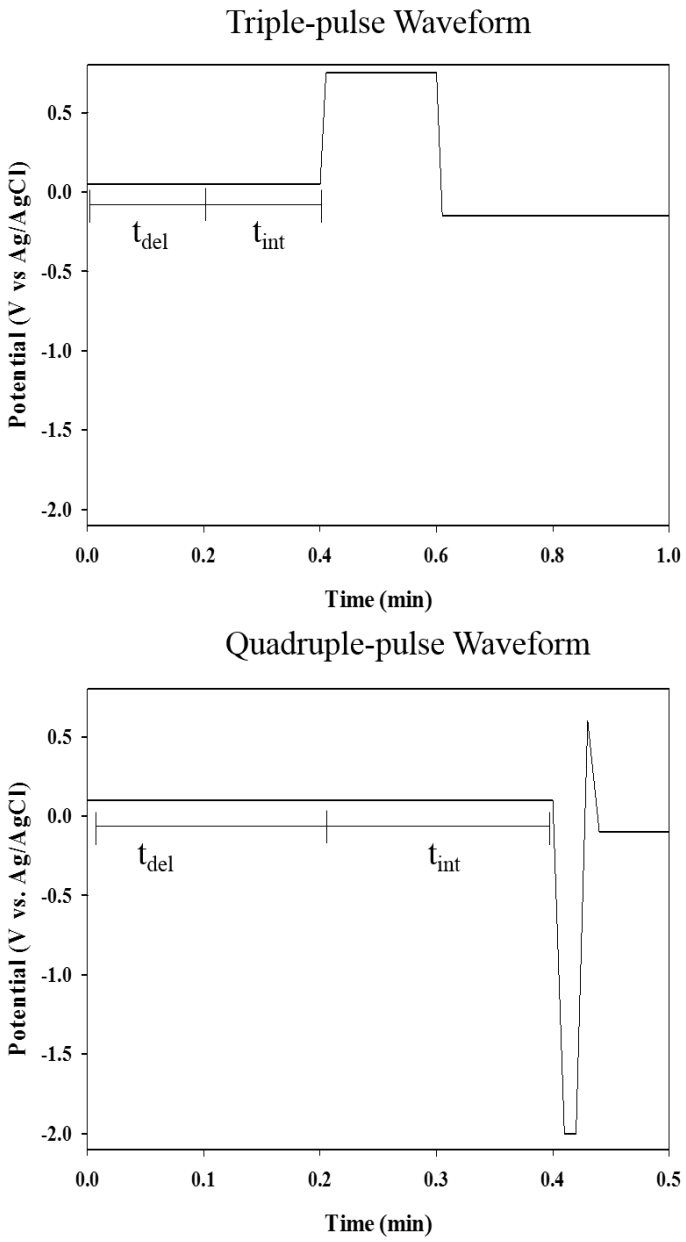


Figure 1.7 Diagrams of pulsed waveforms for electrochemical detection of carbohydrates. (Top) Diagram of the triple-pulse waveform sequence for carbohydrate detection. After a 200 ms delay time, the current is integrated over a period of 200 ms and followed up with gold oxide formation and subsequent gold oxide reduction to clean the working electrode surface. (Bottom) Diagram of the quadruple-pulse waveform sequence for carbohydrate detection, long considered the standard waveform. After a 200 ms delay time, the current is integrated for 200 ms, followed by a quick reductive cleaning potential pulse. The potential is increased to enable gold oxide formation and subsequently lowered to reduce the gold oxide and clean the working electrode surface.

1.4.3 On-line Microdialysis Sampling Coupled to Ion Chromatography: Considerations in instrumental design

Ion chromatography (IC) has undergone a rapid expansion since its development in the 1970's.[30] Today IC is the leading analytical method for determining ionic constituents of all kinds in any kind of sample matrix. The impressive characteristics of IC include: determination of many ions simultaneously in a relatively short time, high selectivity, large dynamic working range, high sensitivity and low use of samples and reagents. In most IC applications, despite the use of ultra-pure water, there are often difficulties and limitations to the incompatibility of the sample with the IC system. An overview of these limitations is outlined in Table 1-3. Because of these limitations, sample preparation is often required and can be considered the bottleneck of IC applications. Sample preparation techniques introduce risk into the overall analytical method because it can result in contamination, loss of analytes, and time consumption from the additional workload with respect to the total analysis time. Membrane separation is one technique that is coupled to IC to overcome traditional issues associated with sample preparation.[30]

Table 1-3 Difficulties of sample analysis for ion chromatography. These issues are a function of incompatibility of the sample with the IC system.[30]

Limitations of sample analysis for Ion Chromatography
1. Particulates (e.g. undissolved minerals, dust, cells, cell debris) and high-molecular weight components (e.g. proteins, lipids, surfactants) require removal.
2. Water immiscible solvents and non-aqueous samples can't be processed directly.
3. Solid and semi-solid samples require extraction or decomposition.
4. Gaseous analytes that require conversion to ionic state in liquid phase.

In analytical chemistry, and IC specifically, the use of membrane separation is still limited. There are a wide variety of membrane compositions and structures available, allowing users to customize their application based upon their analytes of interest. Microdialysis was traditionally founded in neuro-physiological science and pharmacokinetic and drug discovery studies. Since the mid 1970's there have been over 14,000 papers published with its use. Only more recently has there been increasing interest in microdialysis for process monitoring and environmental analysis. Most research demonstrating the utility of microdialysis hyphenated techniques has occurred over the last two decades. The applications generally include the determination of alkaline earth metals and carbohydrates in biological matrices.[30]

On-line connection of microdialysis to chromatography comes with its own difficulties. Most work has focused only on relative changes of analytes over time, rather than quantification and calibration. Most notably, the transfer of microdialysate can be exceptionally long with the use of low perfusate flow rates (<1 to 10 μL/min), connecting tubing between the microdialysis probe and injection loop, and the injection loop volume. However, these issues can be overcome. One way to overcome these issues is the use of linear probes and low perfusion rates to enable the achievement of concentration equilibrium. By setting the experimental conditions to allow equilibrium to be established between the outer environment (i.e., stirred sample solution) and the continuously flowing perfusate, calibration can be omitted and there is no sample dilution.[30]

1.4.4 *In vitro* Microdialysis Sampling of Carbohydrate-Based Bioprocesses

Initial *in vitro* microdialysis sampling studies highlighted the ability of the technique to monitor small-scale bioprocesses over an extended period (32 h), and to follow an enzymatic reaction occurring at a different pH than the detection pH.[17, 31] The utility of microdialysis sampling for sample clean-up was illustrated by coupling on-line to systems such as a biosensor to monitor glucose in fermentation broths[32], and to HPLC with refractive index detection to monitor hydrolysis products of pulp hydrolysates and liquors from various enzyme sources[2]. Since then, *in vitro* microdialysis sampling has been applied to characterize substrates[2, 33-35], enzymes[34, 36, 37], unknown carbohydrates in complex matrices[34], and as a screening tool for polysaccharide characterization[23, 35]. Table 1-4 summarizes the use of *in vitro* microdialysis sampling of carbohydrate-based bioprocesses. In many of these experiments, quantitative data was not pursued and the actual concentrations of analytes present in the bioprocess were undetermined.

Table 1-4 Summary of processes where microdialysis sampling was employed for characterization.

Target Compound(s)	Bioprocess	Method	Reference
Hydrolysate manno-oligosaccharides	Characterization of hydrolysis of ivory nut mannan + endomannanase (*A. niger*)	HPAEC-PED	Torto *et al.* (1995)[31] Torto *et al.* (1996)[17]
Glucose	Monitoring of *E. coli* fermentations with a biosensor	MD-glucose oxidase biosensor	Palmisano *et al.* (1997)[32]
Glucose, mannose, galactose, and ethanol	On-line monitoring of lignocellulosic hydrolysates by ATCC 96581 (*S. cervisiae*) and bakers' yeast	MD-HPLC-RI	Palmqvist *et al.* (1998)[2]
Hydrolysates up to DP17	Profiling wood hydrolysate using an automated system with desalting	MD-HPAEC-MS	Torto *et al.* (1998)[33, 38]
Glucose and galactose	Characterization of lactose hydrolysis first order rate constant	HPAEC-PAD	Zook *et al.* (1998)[39]
Glucose, gluconolactone and hydrogen peroxide	Characterization of glucose oxidase reaction to determine Michaelis-Menten enzyme kinetic parameters	HPAEC-PAD	Zook *et al.* (1998)[40]
Lactate and pyruvate	Determining impact of oxygen depletion on carbohydrate metabolism in mammalian cell cultures	MD-LC-UV	Wu *et al.* (2001)[36]
Debranched starch hydrolysates up to DP 80	Characterization and fingerprinting of various starches	HPAEC-PAD	Nilsson *et al.* (2001)[23]
Hydrolysate oligosaccharides	Characterization of bagasse	MD-HPAEC-PAD/MS	Rumbold *et al.* (2002)[34]
Hydrolysate oligosaccharides	Characterization of polysaccharide linkages and branching in legume seeds	MD-HPAEC-ESI-MS	Okatch *et al.* (2003)[35]
Maltose, glucose, and hydrolysate intermediates	Characterization of novel enzymes	MD-HPAEC-PAD	Nilsson *et al.* (2006)[37]

1.4.5 Quantitative *In Vitro* Microdialysis Sampling of Bioprocesses

In addition to offering sampling and clean-up for bioprocess monitoring, microdialysis has great value in the quantitative characterization of enzymes. For

example, microdialysis sampling has been applied for the characterization of the glucose oxidase reaction.[40] Also, using a novel microdialysis cell, standard exponential decay curves (Figure 1.8) were used to determine the observed rate constants of the enzymatic digestion of lactose in milk.[39]

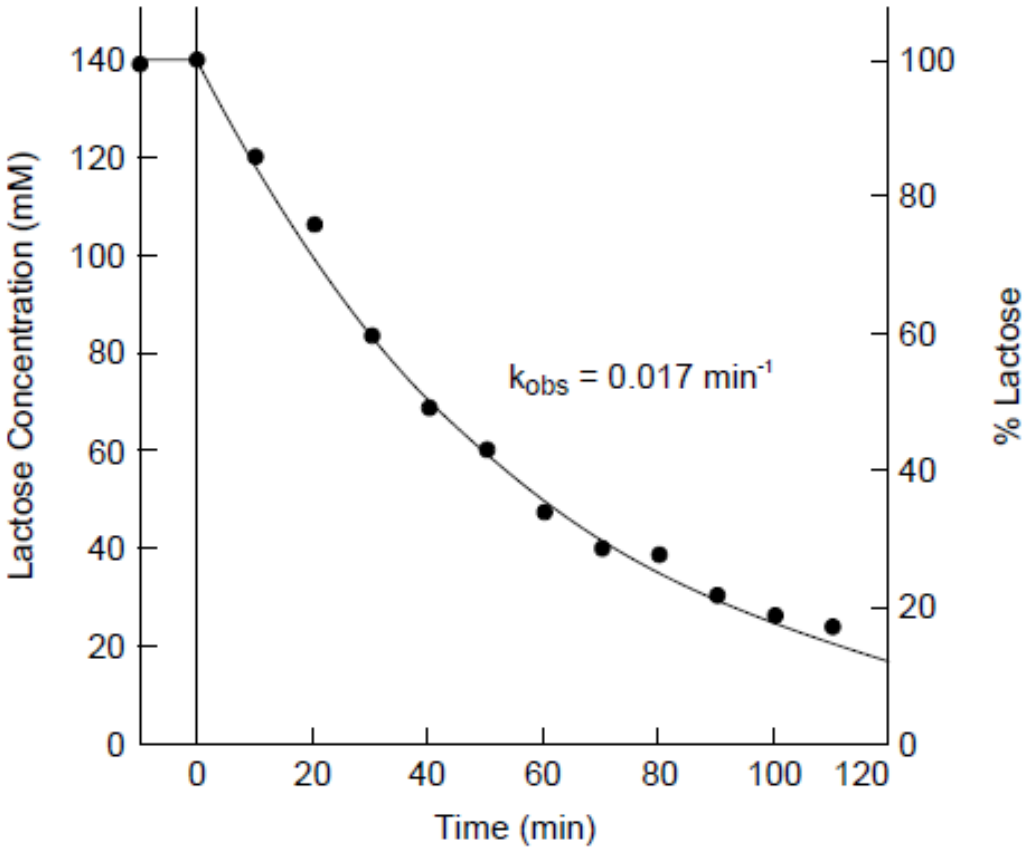


Figure 1.8 First order rate constant plot of lactase reaction of lactose by Lactaid® in whole milk.[39]

Microdialysis coupled to HPLC-UV was used to obtain the kinetic parameters of β-glucosidase, employing an internal standard for increased accuracy in quantitation. The observed enzymatic kinetic parameters were confirmed by comparing to standard spectrophotometric assays and literature values.[41] (Figure 1.9) The importance of this work was highlighted by the technique's ability to monitor the hydrolysis of salicin to

saligenin, where a K_m value for salicin was reported for the first time. All of this work illustrates that in addition to the benefits of sampling and sample clean-up, microdialysis has great value in the quantitative determination of enzyme kinetic parameters.

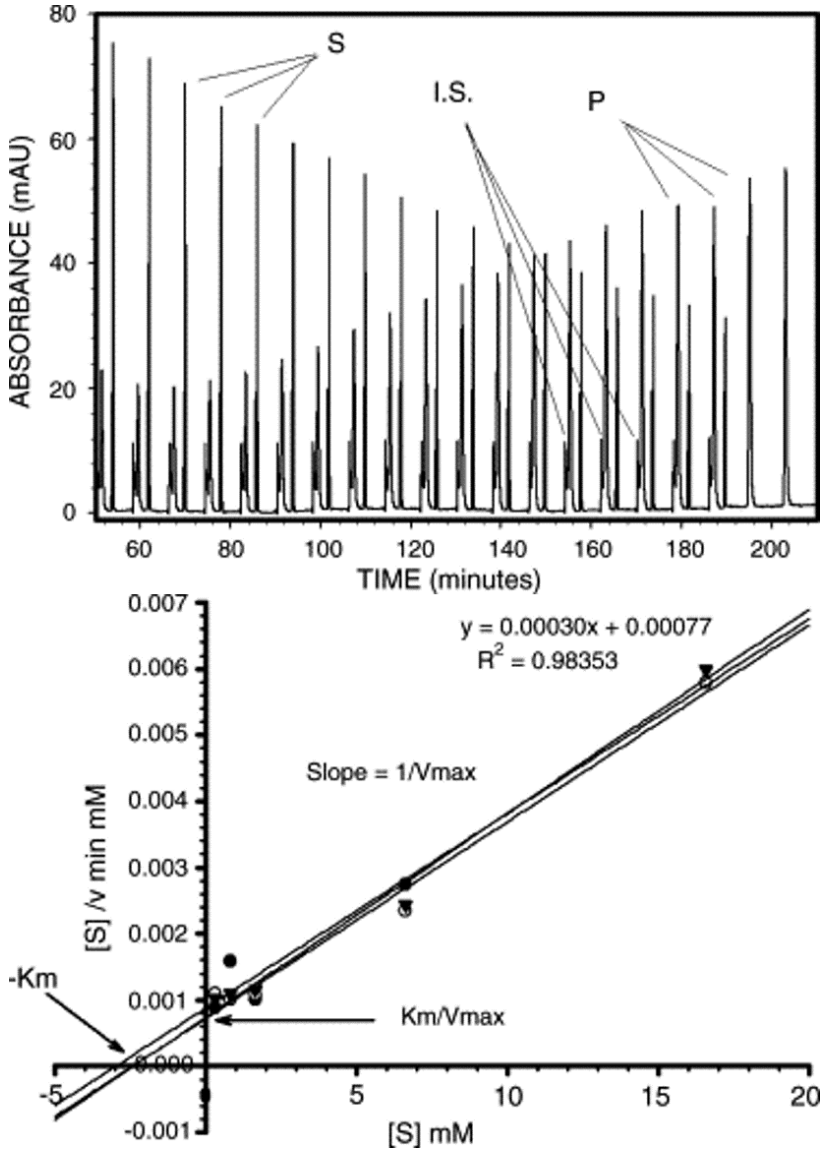


Figure 1.9 Determination of Michaelis-Menten kinetic parameters using microdialysis coupled to HPLC-UV. (Top) Chromatogram of enzymatic solution after addition of enzyme with internal standard 1-(4-nitrophenyl)glycerol (IS), decrease in substrate (S), and growth of product 4-nitrophenol (P) as the reaction progresses. (Bottom) Hanes plot for the determination of K_m value for the substrate 4-nitrophenyl-β-D-glucoside by MD-HPLC-UV.[41]

1.5 Dissertation Overview

1.5.1 Significance

Fermentation broths and enzymatic bioprocesses are used in the manufacturing of biotherapeutics and many other biological materials produced using recombinant genetic technology, to produce methanol and ethanol as alternative energy sources to fossil fuels, and for the manufacturing of alcoholic beverages.[1, 2] Nature has developed very efficient, highly precise methods of controlling bioprocesses. The challenge is to transfer these principles into technically applicable and precise analytical systems that can be used for many industrial applications.[3] Bioprocess monitoring is generally performed by measuring chemical parameters such as pH and dissolved oxygen[4], but because carbon sources and metabolic by-products can impact the yield or quality of the desired products[1] it is important to characterize the concentrations of these analytes in order to optimize manufacturing productivity. Enzymatic bioprocesses and fermentation broths are complex mixtures of nutrients, waste products, cells, cell debris, and desired products[1] and, therefore, a sampling technique that allows for cleanup without additional sample handling and preparation prior to its analysis would prove beneficial.

Carbohydrates are polymers that can be used in the production of renewable sources. To use these polymers, it is necessary to characterize and profile them. This requires fast and efficient hydrolysis, sampling and sample clean-up, separation and detection steps. Enzymatic hydrolysis, as opposed to chemical methods, of carbohydrate polymers is preferred because it is environmentally friendly and selective.[1] Most hydrolysis steps are performed off-line and this sampling method of

bioprocesses typically involves manual removal of an aliquot from the bioreactor, quenching of the reaction, with subsequent filtration prior to separation and detection.[3, 4] These steps can result in contamination, are time-consuming and can alter the kinetics of the reaction which could disturb manufacturing performance.[8, 24, 25] Availability and concentration of carbohydrates in enzymatic bioprocesses can greatly affect their yield. Thus, it is important, to monitor these analytes during bioprocesses in order to minimize manufacturing cost and maximize manufacturing performance. On-line monitoring systems for bioprocesses are desirable, offering the advantages of quick feedback and reduced time analysis. *In vitro* microdialysis sampling coupled to high performance anion exchange chromatography and pulsed amperometric detection (MD-HPAEC-PAD) is a powerful tool for carbohydrate analysis in complex matrices.

Microdialysis sampling reduces analysis time and overcomes the aforementioned problems because it offers the benefits of sampling and sample cleanup, eliminating the enzymatic reaction quenching and filtration steps. Additionally, sample loss is not an issue when microdialysis is employed since no sample is removed from the bioreactor. This sampling technique can sample from complex matrices without depleting the enzymes and without perturbing the reaction.[5] Microdialysis sampling does not disturb the kinetics of the reaction under study[6], allows for continuous dynamic time profile changes in a biological matrix without affecting the environment of the bioreactor[7], and can be coupled to an analytical system in an on-line setup[26]. Importantly, this technique minimizes sample handling and contamination issues associated with bioprocess sampling.[27]

Microdialysis is a sampling technique that employs a semi-permeable membrane that achieves online dilution, online cleanup, and *in situ* sampling in both *in vivo* and *in vitro* applications. Microdialysis sampling takes advantage of selective diffusion of small molecules out of the bioreactor by passing a perfusate fluid through the inside of a low-volume, tubular membrane; analytes are transported from areas of high concentration (i.e., bioreactor) to areas of low concentration through a semi-permeable membrane. The selectivity of this sampling technique in a complex matrix is due to the MWCO of the probe, where freely diffusing species in the sample solution may be transported along a concentration gradient over the membrane into a continuous perfusion flow. This principle allows for the continuous removal of small and representative samples, which do not affect the volume of the bioreactor, and excludes large molecules, such as proteins. The fluid continuously pumped into the probe is termed perfusate, and the fluid collected after sampling containing the analyte(s) is known as the dialysate. The dialysate can be assayed to develop a dynamic concentration versus time profile of the system under study.

The goal of this research was to develop and improve on-line *in vitro* microdialysis sampling as an analytical tool for the quantitative monitoring of enzymes in bioprocesses.

1.5.2 Specific Aims

1. ***Improve online in vitro microdialysis sampling by reducing system noise, improving recovery, and minimizing dead volume.*** (Chapter 3)
2. ***Validate the improvements and apply microdialysis sampling to develop an assay for monitoring β-mannanase.*** (Chapter 4)
3. ***Apply the technical improvements of microdialysis sampling by monitoring the enzymatic breakdown of mannan using the model assay.*** (Chapter 4)

2 Experimental: Materials and Methods

2.1 Standard Solutions and Reagents

2.1.1 Technical Improvement Studies

High performance liquid chromatography grade sodium hydroxide (50/50, w/w) was used for eluent preparation (J.T. Baker, Phillipsburg, NJ). Acetone was purchased from Fisher Scientific (Springfield, NJ). All standard solutions were prepared in purified, deionized water acquired from a Milli-Q water purification system (Millipore, Billerica, MA). The compounds lactose ($C_{12}H_{22}O_{11}$, 342.38 g/mol), glucose ($C_6H_{12}O_6$, 180.16 g/mol), galactose ($C_6H_{12}O_6$, 180.16 g/mol), and 2-deoxy-ᴅ-glucose ($C_6H_{12}O_5$, 164.16 g/mol) were purchased from Sigma (St. Louis, MO).

2.1.2 Mannanase Assay Studies

High performance liquid chromatography grade sodium hydroxide (50/50, w/w) was used for eluent preparation (J.T. Baker, Phillipsburg, NJ). Mannose (M1, $C_6H_{12}O_6$, 180.2 g/mol) was purchased from Sigma (St. Louis, MO). All standard solutions were prepared in ultrapure NERL™ reagent grade water from Thermo Fisher Scientific (Pittsburg, PA). The 1,4-β-mannooligosaccharide standards mannobiose (M2, $C_{12}H_{22}O_{11}$, 342.3 g/mol), mannotriose (M3, $C_{18}H_{32}O_{16}$, 504.4 g/mol), mannotetraose (M4, $C_{24}H_{24}O_{21}$, 666.6 g/mol), mannopentaose (M5, $C_{30}H_{52}O2_6$, 828.7 g/mol), and mannohexaose (M6, $C_{36}H_{62}O_{31}$, 990.9 g/mol) were all purchased from Megazyme Inc. (Bray, Republic of Ireland) (Figure 2.1).

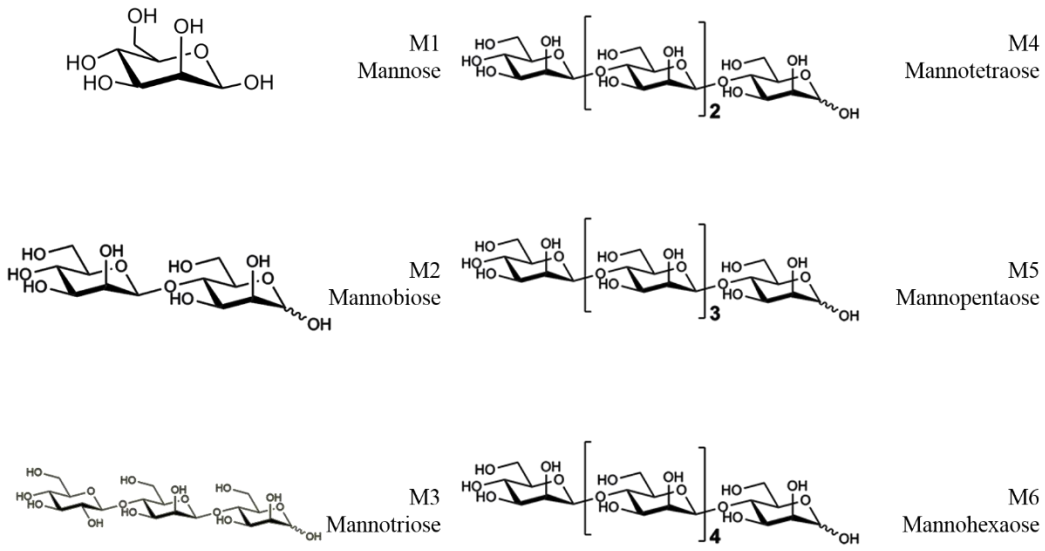


Figure 2.1 Chemical structure of mannose and 1,4-β-D-mannooligosaccharides.

Three β-mannanase enzymes (EC 3.2.1.78) were purchased from Megazyme Inc. Their sources, specific activities, optimum pH and optimum temperature are reported in Table 2-1. The specific activities are listed as Units, where one unit is defined as the amount of enzyme per milligram or milliliter of protein that produces one micromole of mannose-reducing-sugar equivalents per minute. High purity 1,4-β-mannan substrate was purchased from Megazyme. This substrate was reportedly prepared from controlled hydrolysis of galactomannan from carob seed with DP ~15.

Table 2-1 The reported physiochemical properties of three β-mannanase enzymes.[42-44]

Enzyme Source	Specific Activity (U/mg protein)	Specific Activity (U/mL)	pH Optima (Stability Range)	Temperature Optima (Stability Range)
Bacillus sp.	55[a]	400[a]	8.8 (6.0 – 10.0)	65 °C (< 60 °C)
Cellvibrio japonicus	400[b]	5000[b]	7.0 – 10.0 (7.0 – 9.0)	60 °C (up to 50 °C)
Bacillus circulans	49[c]	390[c]	7.0 (5.0 – 9.0)	50 °C (up to 50 °C)

[a]One Unit of β-mannannase activity is the amount of enzyme which releases one micromole of mannose-reducing-sugar equivalents per minute from low viscosity carob galactomannan (10 mg/mL) in 0.1 M glycine buffer (pH 8.8) at 40 °C.
[b]One Unit of β-mannannase activity is defined as the amount of enzyme required to release one µmole of mannose-reducing-sugar equivalents per minute from carob galactomannan (5 mg/mL) in sodium phosphate buffer (100 mM) pH 7.0 at 40 °C.
[c]One Unit of β-mannannase activity is defined as the amount of enzyme which releases one µmole of mannose-reducing-sugar equivalents per minute from low viscosity carob galactomannan (5 mg/mL) in sodium phosphate buffer (100 mM), pH 7.5 at 40 °C.

2.1.3 Reducing Sugar Assay

Sodium carbonate anhydrous (Na_2CO_3, 105.99 g/mol) and concentrated sulfuric acid (H_2SO_4, 98.08 g/mol) were purchased from J.T. Baker. Sodium sulfate (Na_2SO_4, 142.04 g/mol) was purchased from Acros Organics. Copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$, 249.68 g/mol), sodium phosphate monobasic (NaH_2PO_4, 119.98 g/mol) and sodium phosphate dibasic (Na_2HPO_4, 141.96 g/mol) were purchased from Fisher Scientific. Glycine ($C_2H_5NO_2$, 75.07 g/mol) was purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, MD). The compounds potassium sodium tartrate tetrahydrate ("Rochelle Salt", $KNaC_4H_4O_6 \cdot 4H_2O$, 282.22 g/mol), ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1235.86 g/mol), sodium arsenate heptahydrate ($Na_2HAsO_4 \cdot 7H_2O$, 312.01 g/mol), benzoic acid ($C_7H_6O_2$, 122.12 g/mol), ammonium bicarbonate (NH_4HCO_3, 79.06 g/mol), and Albumin from Bovine serum

(BSA, 66kDa) were all purchased from Sigma-Aldrich. All solutions were prepared in ultrapure NERL™ reagent grade water from Thermo Fisher Scientific. A summary and brief description of reagents are summarized in Table 2-2. All solutions were prepared quantitatively, unless otherwise noted, and in a fume hood. It is important to note that the preparation of the copper and arsenomolybdate reagents require special safety consideration, including wearing proper attire of a lab coat, gloves, and protective eyewear.

Table 2-2 Summary of five solutions for Nelson-Somogyi reducing sugar assay.

Solution	Preparation
A	25 g Na_2CO_3 + 25 g Rochelle Salt + 200 g Na_2SO_4 were dissolved in 800 mL of water with stirring, diluted to 1.00 L and filtered through Whatman 125 mm cellulose filter paper.
B	30 g $CuSO_4 \cdot 5H_20$ were dissolved in 200 mL of water with 4 drops of conc. H_2SO_4.
C	25 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ were dissolved in 400 mL of water, then 21 mL of conc. H_2SO_4 were added. 3 g $Na_2HAsO_4 \cdot 7H_2O$ were dissolved in 25 mL of water and then this solution was added to the molybdate solution and diluted to 0.500 L.
Copper Reagent D	1 mL of Solution B + 25 mL of Solution A
Arsenomolybdate Color Reagent E	1:5 dilution with water immediately prior to use.

2.2 Instrumentation

2.2.1 Detection

For separation and detection, a DX-500 liquid chromatography system (Dionex, Sunnyvale, CA) with a Dionex Model ED40 electrochemical detector was used. The electrochemical cell was equipped with a 1.0 mm Au electrode, combination pH and Ag/AgCl reference electrode, and titanium auxiliary electrode. Carbohydrate solutions were analyzed using a standard quadruple-pulsed potential-time waveform controlled with PeakNet software (Dionex, Version 5.21). The 500 ms waveform potentials are as follows: E_1 = +0.10V, E_2 = -2.00V, E_3 = +0.60V, and E_4 = -0.10V. The potential E_1 is maintained for 400 ms, including a 200 ms delay time followed by 200 ms integration time. There is a 10 ms cleaning step at E_2 then a pulse to E_3 for a total of 40 ms, which allows for reductive cleaning of the working electrode surface followed by gold oxide formation. The final potential E_4 is held for 60 ms to reduce the gold oxide formed in the previous step.

2.2.2 Separation

Solutions were monitored by on-line flow onto an injection valve (Model 9126, Rheodyne, Cotati, CA) fitted with an 8 µL injection loop. Data collection and system control was accomplished using PeakNet software. Separations of carbohydrates were achieved using a CarboPac PA100 microbore column (2x250 mm) fitted with a CarboPac microbore PA100 guard column (2x50 mm) (Dionex). The columns were temperature controlled at 30 °C with an LC-25 chromatography oven (Dionex). The

eluent was 100 mM NaOH and delivered at a flow rate of 0.25 mL/min. Mobile phase solvent was kept under pressure ($N_{2(g)}$, ca. 9 psi).

2.2.3 Other Instrumentation: UV-VIS

Nelson-Somogyi reducing sugar assays were monitored by UV-VIS using a JASCO Model V-560 UV/VIS Spectrophotometer (JASCO, Easton, MD) with 1.6 mL transparent polystyrene Sarstedt cuvettes (Sarstedt, Nümbrecht, Germany). Data collection and system control was accomplished using Spectra Manager software (JASCO, Version 1.33.01). Spectrum measurements of standard mannose sugar solutions were scanned across 400 – 800 nm at 0.5 nm increments at a rate of 400 nm/min. For fixed wavelength measurements, the absorbance was averaged from six absorbance readings collected at a rate of 2 seconds/reading at 520 nm.

2.3 Microdialysis Improvement Studies

2.3.1 Pumps for Fluid Delivery

Fluid delivery by three pumps was explored for microdialysis studies. The two syringe pumps used for pump comparison studies were Bee Stinger® syringe pump and Busy Bee® controller with a 2.5 mL glass syringe (BASi, West LaFayette, IN), and model KDS-100 syringe pump (KD Scientific, Holliston, MA) with a 5 mL gas-tight syringe (Fisher Scientific). The milliGAT® pump (Global FIA, Fox Island, WA) is computer programmable and controlled by serial communication between a MicroLynx-4 micro-electric controller (Global FIA) and Intelligent Motion Systems, Inc (IMS) Terminal software. To compare the pumps for fluid delivery, a 0.50% (v/v) acetone in water solution was continuously delivered at 1 µL/min and the peak-to-peak

noise from the ultraviolet absorbance (254 nm) was measured using a Linear UV-106 detector (Rainin, Columbus, OH).

2.3.2 Microdialysis Characterization

For microdialysis characterization, perfusate was delivered at various flow rates by the milliGAT® pump and the dialysate was analyzed to measure the relative recovery of a standard glucose solution across the dialysis membrane interfaced to the chromatography system. Polyacyrlonitrile loop microdialysis probes (3 cm membrane, 30 kDa molecular weight cut-off) (BASi) were received dry with a protective layer of glycerin, and were placed in and perfused with water at 5 µL/min for >2 hours prior to experimental use. Fluorinated ethylene propylene connective tubing (BASi) was used to connect the pump to the inlet of the loop probe, and from the outlet to a conical adapter (Upchurch Scientific, Oak Harbor, WA) that was used to interface the microdialysis tubing to the mixing tee (VICI®, Baton Rouge, LA) leading to the chromatography injection valve. This on-line setup is visualized in Figure 2.2, and the volume injection loop was continuously filled and injections were made at fixed time intervals.

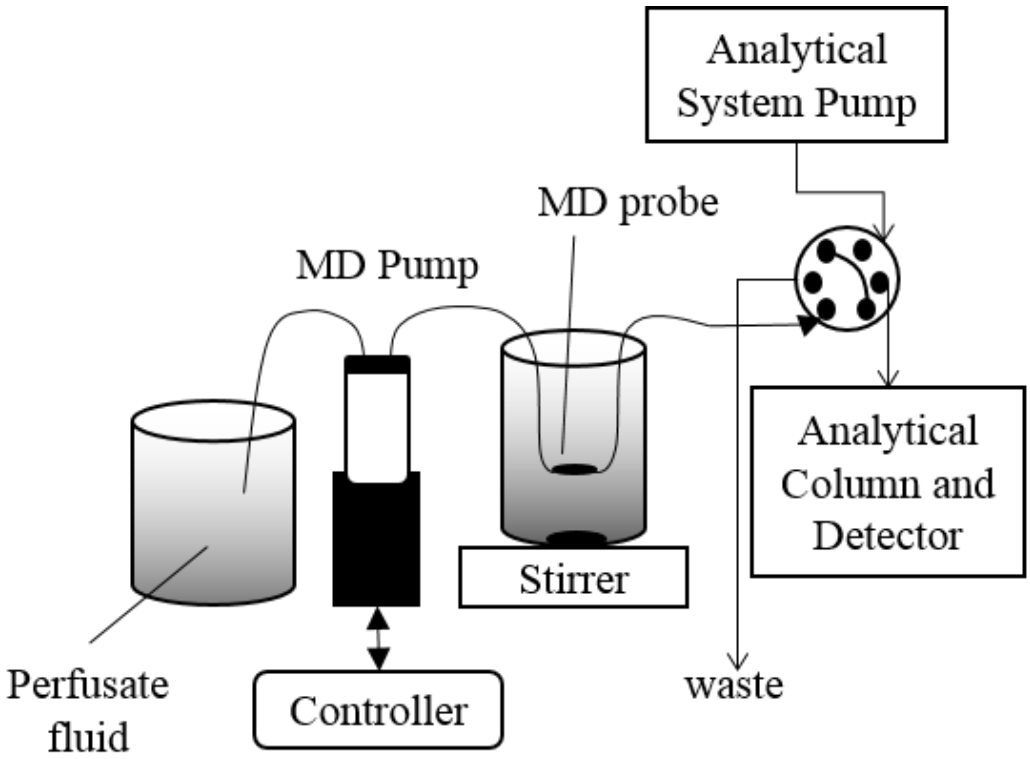


Figure 2.2 A schematic representation of online microdialysis coupled to a chromatography system.[45]

2.4 MD-HPAEC-PAD Assay

2.4.1 MD-HPAEC-PAD Characterization

For microdialysis characterization, water perfusate was delivered at 1 µL/min by the milliGAT® pump and the dialysate was analyzed to measure the relative recovery of a standard mixture consisting of each mannooligosaccharide (M1 – M6) and 2-deoxy-D-glucose across the dialysis membrane interfaced to the chromatography system. Figure 2.3 is a schematic depiction of the on-line configuration of microdialysis sampling coupled to HPAEC-PAD. This same setup was used for enzyme assays.

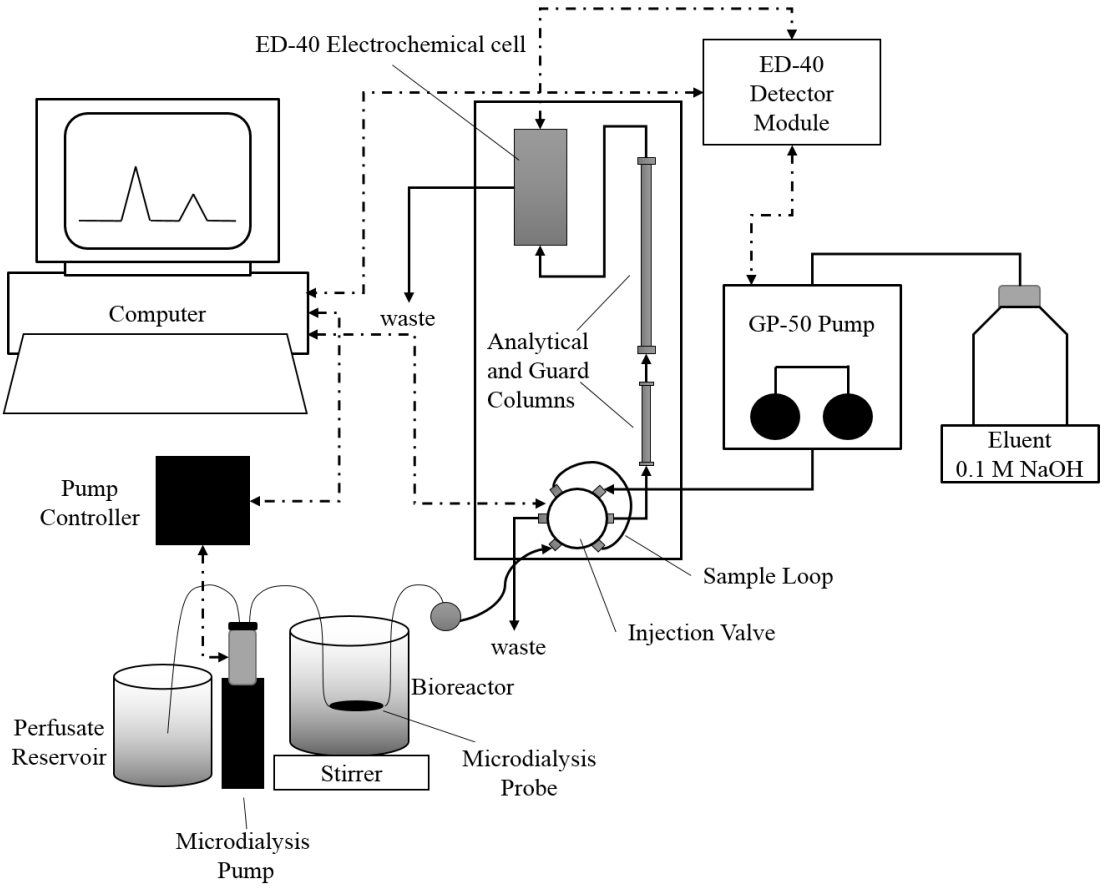


Figure 2.3 Schematic representation of the MD-HPAEC-PAD setup.

2.4.2 Substrate and Enzyme Reactions

For initial enzyme studies, standard mannooligosaccharide solutions of mannotetraose, mannopentaose or mannohexaose were used as substrate with a concentration that was toward the limit of linearity. For β-1,4-mannan assays, perfusate solutions consisted of water. After steady-state recovery of the internal standard mixed in a β-1,4-mannan substrate solution was achieved, a 0.500 mL aliquot of enzyme solution was added to the bioreactor for each assay. The details for preparing mannan substrate is outlined in 2.5.1. Enzyme solutions for MD-HPAEC-PAD assays were prepared by diluting 100.0 µL of standard enzyme in 10.00 mL of water for a 1:100

dilution. The injection valve was continuously filled by dialysate and injections were made at specific time intervals.

2.5 Nelson-Somogyi Reducing Sugar Assay

The Nelson-Somogyi Reducing Sugar assay was used for the determination of mannose-reducing sugars released upon hydrolysis of mannan.[46, 47] Figure 2.4 is a schematic of the general protocol that involves: hydrolyzing substrate via enzymatic reaction, quenching at timed intervals, heating the solutions to oxidize sugars and reduce copper ($Cu^{2+} + e^- \rightarrow Cu^{1+}$), then cooling and adding an arsenomolybdate reagent that oxidizes the copper back to Cu^{2+}. The developed color is directly proportional to the amount of reducing sugars in the solution. The amount of reducing sugars increases as the mannanase enzymatic reaction takes place, breaking down β-1,4-mannan substrate to produce β-1,4-manno-oligosaccharides.

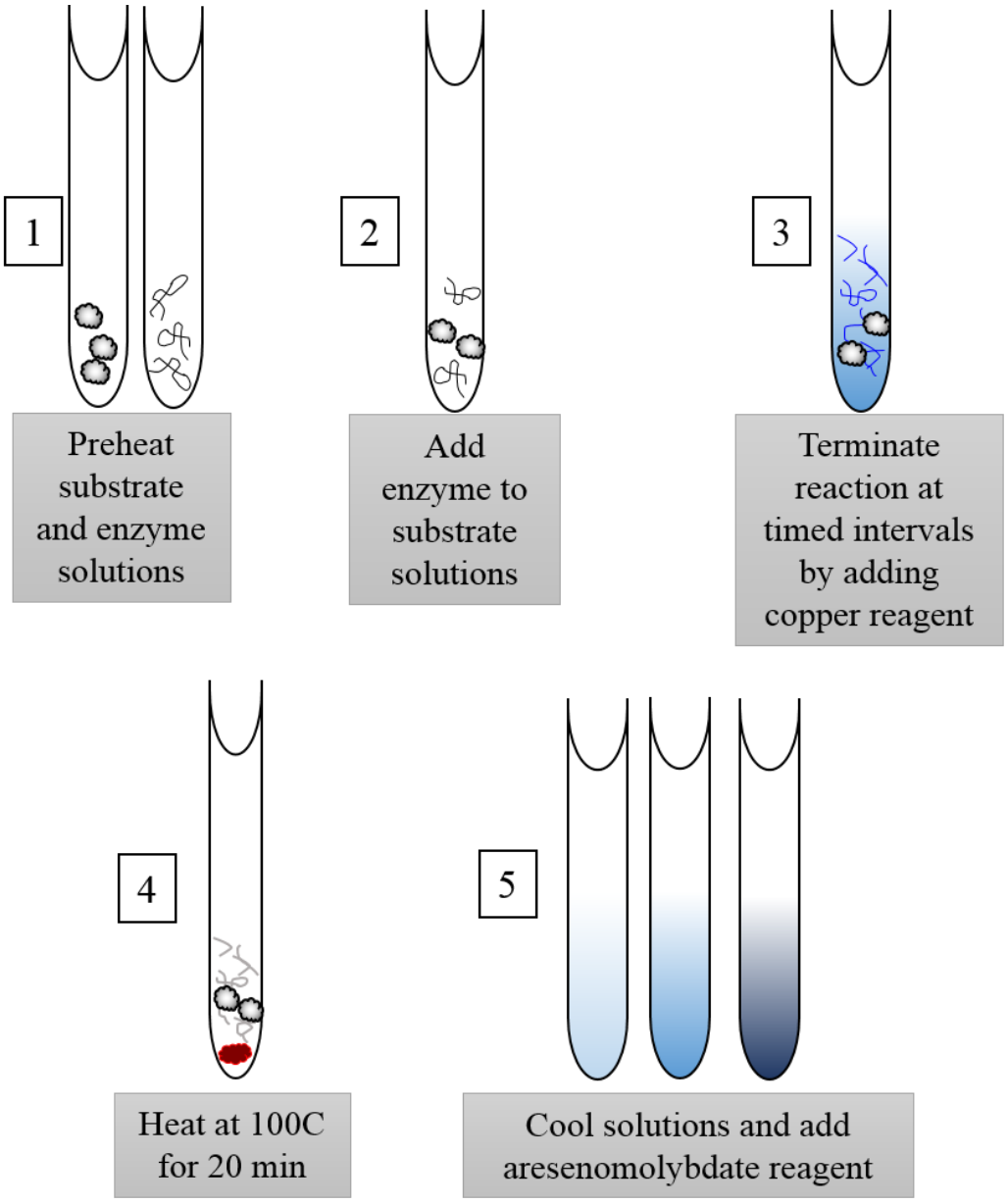


Figure 2.4 General schematic representation the Nelson-Somogyi reducing sugar assay protocol. The color development is directly proportional to the amount of reducing sugars produced through enzymatic action of mannanase on mannan substrate. (1) Enzyme and substrate solutions are preheated to 40 °C. (2) Aliquots of enzyme solution are added to substrate at carefully timed 15 second intervals, mixed and incubated at 40 °C. (3) At time (T) = 3, 6, 9, and 12 minutes, reactions are quenched by adding copper reagent and mixing. (4) All solutions (mixtures, sugar standards, and reagent and reaction blanks) are heated in a boiling water bath at 100 °C for 20 minutes. (5) Solutions are cooled to room temperature, an aliquot of arsenomolybdate solution is added, and then mixed. After centrifuging, the absorbance of each solution is measured at 520 nm to determine the enzyme activity.

2.5.1 Substrate and Enzyme Reactions

Substrate was prepared by dissolving mannan DP ~15 in 5% (w/v) NaOH to a concentration of 20 mg/mL and then neutralized with 50% acetic acid. Upon neutralization, the mannan substrate precipitated to form a colloid. This solution was diluted to 5 mg/mL in the enzyme's buffer for reducing sugar assays and 0.1 mg/mL microdialysis assays.

Enzyme buffers were prepared to match the buffer composition in the manufacturer's reported assay conditions, and their compositions are detailed in Table 2-3. 100 mM phosphate buffer (pH = 7) was prepared by mixing 39.0 mL of 200 mM monobasic sodium phosphate with 61.0 mL of 200 mM dibasic sodium phosphate, adjusting to pH 7.0, and then diluting to 200 mL with water. 100 mM phosphate buffer (pH = 7.5) was prepared by mixing 16.0 mL of 200 mM monobasic sodium phosphate with 84.0 mL of 200 mM dibasic sodium phosphate, adjusting to pH 7.5, and then diluting to 200 mL with water. Both phosphate buffers were also prepared with concentrations of 0.5 mg/mL and 1 mg/mL BSA for pH 7.0 and 7.5, respectively. Enzyme solutions were prepared in buffer containing BSA, if needed, whereas substrate solutions were diluted in buffer without BSA. Original enzyme solution was prepared for each enzyme by diluting 0.250 mL of enzyme to 10.00 or 25.00 mL in appropriate buffer, 1:40 and 1:100 dilution, respectively.

Table 2-3 Summary of the enzyme buffers' compositions for confirming manufacturer's reported enzyme specific activies.

1,4-β-mannanase enzyme source	Assay Buffer Composition
Cellvibrio japonicus[b]	100 mM sodium phosphate with 1 mg/mL BSA, pH = 7.5
Bacillus circulans[c]	100 mM sodium phosphate with 0.5 mg/mL BSA, pH = 7.0

Enzyme solutions for assays was prepared by diluting the original solution in buffer. Test tubes containing 0.500 mL of substrate and a small vial of diluted enzyme were pre-heated for five minutes at 40 °C. After pre-heating, 0.200 mL of enzyme was added to each reaction tube and mixed at carefully timed intervals 15 seconds apart. At time intervals of 3, 6, 9, and 12 min., 0.500 mL of Copper Reagent D was added to each test tube and mixed thoroughly to quench the reaction.

The Nelson-Somogyi Reducing Sugar assay was repeated for each enzyme, as outlined here, but with reaction conditions of pH = 7 and T = 23 °C. Each enzyme and substrate solution was prepared in water and no BSA was added.

2.5.2 Reagent and Reaction Blanks

A reaction blank was prepared by adding 0.500 mL Copper Reagent D to 0.500 mL of substrate, and then adding 0.200 mL 0.1 M enzyme buffer. A reagent blank was prepared by adding 0.500 mL of Copper Reagent D to 0.500 mL of substrate, and then adding 0.200 mL of enzyme solution. Mannose sugar standards were prepared by mixing 0.200 mL of 250 µg/mL or 500 µg/mL mannose with 0.500 mL of substrate, and then adding 0.500 mL of Copper Reagent D.

2.5.3 Copper Reduction and Color Development

Once the Copper Reagent D was added to each test tube, the resulting solutions were light blue (from Cu^{2+}). All the test tubes were heated in a boiling water bath (100 °C) for 20 min. After heating, the solutions were clear with reddish-orange precipitate at the bottom, from the reduction of Cu^{2+} to Cu^{1+} and formation of copper oxide (Cu_2O). All solutions (reactions, blanks, and mannose standards) were cooled to room temperature. After cooling, 3 mL of Arsenomolybdate Reagent E were added to each test tube, mixed thoroughly, allowed to sit for ten minutes, and then mixed again. The resulting blue color that develops is from the oxidation of copper to reform Cu^{2+}. All solutions were centrifuged for 10 min. at 3,000 RPM, and 0.500 mL of supernatant was diluted to 1.00 mL for each absorbance measurement.

3 Technical Improvements of Quantitative *In Vitro* Microdialysis Sampling of Carbohydrates

This Chapter is reproduced in part from: ***Bass, Sarah M.****, and LaCourse, William R. Instrumentation Science & Technology;* ***2015****, 43, 601-610.*

3.1 Introduction

Enzymes, the reaction catalysts of biological systems, often exhibit a high degree of substrate specificity and can accelerate specific chemical reactions. These attributes are harnessed for enzymatic bioprocessing in numerous research and industrial applications including the production of biotherapeutics and biofuels, fermentation of foods, and the modification of commercial additives and goods.[1, 2] In many bioprocesses, the substrate and product(s) of interest are contained within a complex matrix, which often complicates the monitoring of these reactions.

Microdialysis facilitates the continuous sampling of dynamic processes. Small molecules, able to diffuse through a semi-permeable membrane of a specific molecular weight cut-off, are collected by a perfusate fluid delivered by a pump.[40] The resultant dialysate can be assayed by various analytical techniques to develop a concentration versus time profile of the system under study.[40] Figure 3.1 is a schematic view of the microdialysis sampling process.

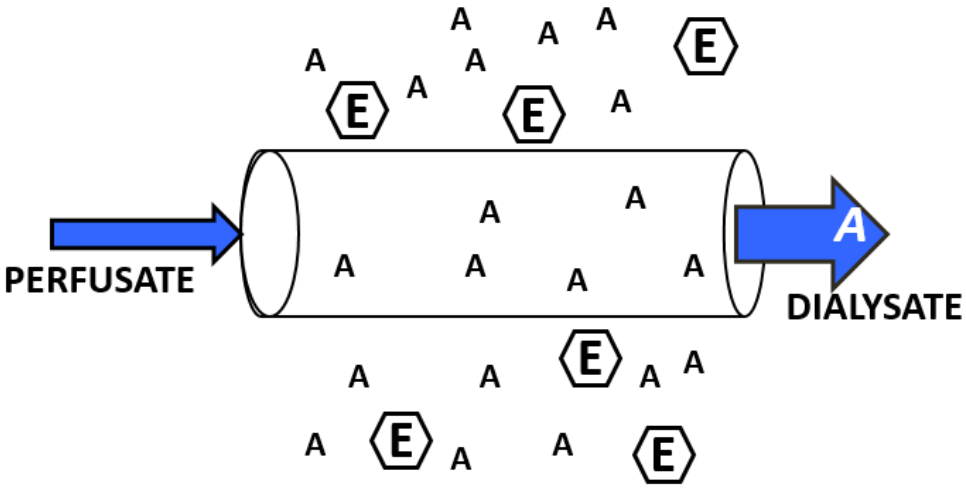


Figure 3.1 A schematic representation of the microdialysis sampling process. A perfusate fluid is delivered through the microdialysis probe. The fluid that is collected on the other side of the probe is called the dialysate, which contains any molecules that diffused into the probe.

For bioprocesses, sampling by microdialysis offers on-line sampling and a limited degree of sample clean-up, avoids contamination and volume change introduced by repetitive manual sampling, and quenches substrate conversion immediately upon collection by excluding enzymes. Microdialysis is easily coupled to chromatographic separation techniques, which provides additional clean-up of complex matrices and allows for the separation of substrate from product, resulting in continuous monitoring of a bioprocess' complex reaction.[24, 48] Microdialysis sampling is most commonly used for *in vivo* studies in the area of pharmacokinetics and biological responses to various stimuli.[49, 50] Figure 3.2 is an illustration of the typical *in vivo* and *in vitro* microdialysis setup.

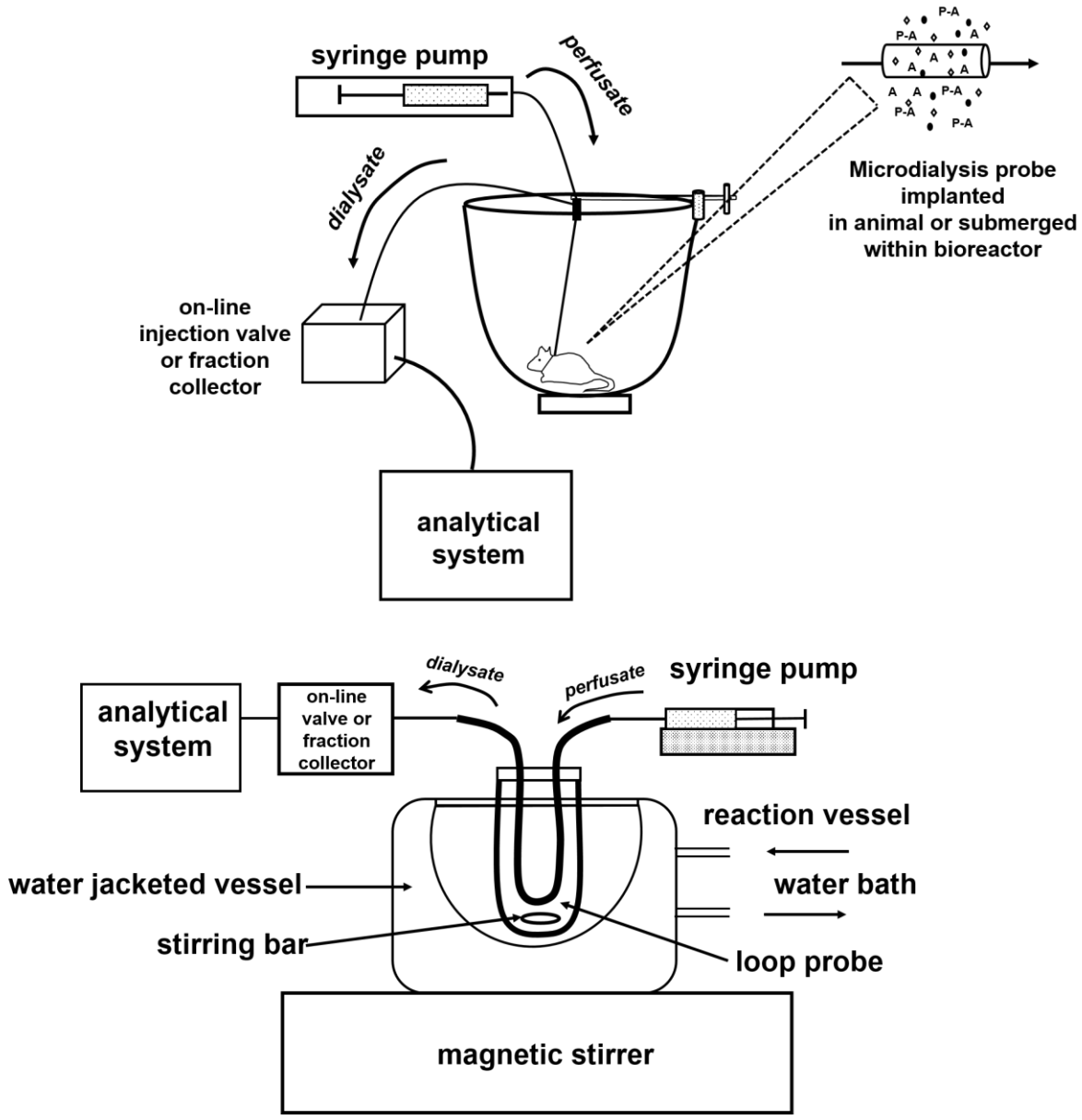


Figure 3.2 A schematic representation of the typical *in vivo* (top) and *in vitro* (bottom) microdialysis sampling setup.[22]

The quantitative evaluation of microdialysis in bioprocesses can be described by the extraction fraction (EF), defined in Equation 5.[22] C_d, C_{in}, and C_b are the analyte concentrations in the dialysate, perfusion fluid, and bioreactor, respectively, and Q_d is the perfusion rate. Microdialysis recovery is governed by the resistance of the analyte transport through the medium exterior of the probe, the membrane and the dialysate, and summarized in the permeability and surface area constant, PS.[22]

$$EF=\frac{C_d-C_{in}}{C_b-C_{in}}=1-e^{(-\frac{PS}{Q_d})}$$

Equation 5

When the analyte is absent from the perfusate the equation simplifies and is typically expressed as the ratio between the concentration of analyte in the dialysate (C_d) and the bulk concentration (C_b) in the sample, and the percent recovery, or relative recovery (RR), is expressed as Equation 6.[22] Variance of the RR value has been attributed to analytes, matrix, temperature, mass-transport, perfusion flow rate, and membrane characteristics.[51, 52] As such, the recovery values for each analyte are difficult to obtain, especially in complex matrices, and as a consequence, the majority of in vitro studies have been qualitative or semi-quantitative.[53]

$$RR=\frac{C_d}{C_b}*100$$

Equation 6

Fundamental studies of improving the quantitative aspects of *in vitro* microdialysis for on-line monitoring have been reported previously by our group for several carbohydrate-based enzymatic reactions (i.e., glucose oxidase, β-galactosidase, and isoamylase).[40, 41, 54] In 2006, on-line *in vitro* microdialysis sampling followed by high performance liquid chromatography separation and ultraviolet absorbance detection was used to monitor the enzymatic hydrolysis of carbohydrate substrates by β-glucosidase to obtain Michaelis-Menten constants. Accurate quantitation was achieved via internal standard methodology and compared to spectrophotometric data and literature Michaelis-Menten constant values. The Michaelis-Menten constant for a system is the substrate concentration where the rate of the reaction is half of the maximum rate achieved. A previously unpublished Michaelis-Menten constant value for the substrate salicin was determined by this method.[41]

Perfusate fluid is typically delivered to microdialysis probes via syringe pump, which is prone to reproducibility and precision problems. The syringe volume limits the volume of perfusate fluid that can be delivered, which is an issue if the syringe needs to be refilled or replaced while monitoring a process over an extended period. Additionally, the use of larger syringe volumes leads to flow rate limitations, an issue when low perfusate flow rate is desired. This work introduces a further improvement in the quantitative aspects of on-line microdialysis: a computer-programmable pump designed for precision fluid delivery, with flow rates in the nano- and microliter per minute range. In contrast to the more commonly used syringe pump, this bi-directional positive displacement pump does not require perfusate reloading, exhibits improved reproducibility, and enables high analyte recoveries using accurate low flow rates.

This pump technology was compared against two other commercially available syringe pumps and characterized in regard to microdialysis sampling. Fluctuations in fluid delivery, and thus microdialysis recovery, can be traced back to the worm-gear of the syringe pump and can be visualized by the sinusoidal variation in analyte recovery over time. Slow perfusate flow rate allows for high recovery of analyte(s) from a complex mixture, but can lead to lengthy sample collection times for subsequent analysis. A second pump was added in-line to the setup to achieve on-line dilution, where a faster flow rate was delivered to dilute an incoming microdialysis flow rate of 1 µL/min. This setup compensates for the long acquisition time of samples associated with slow perfusate flow rates and the dead volume prior to the injection valve (Figure 3.3), which allows for real-time analysis, overfilling of the injection loop to avoid carryover from previous injections, and diluting dialysate samples for quantitative analysis of enzymatic processes.

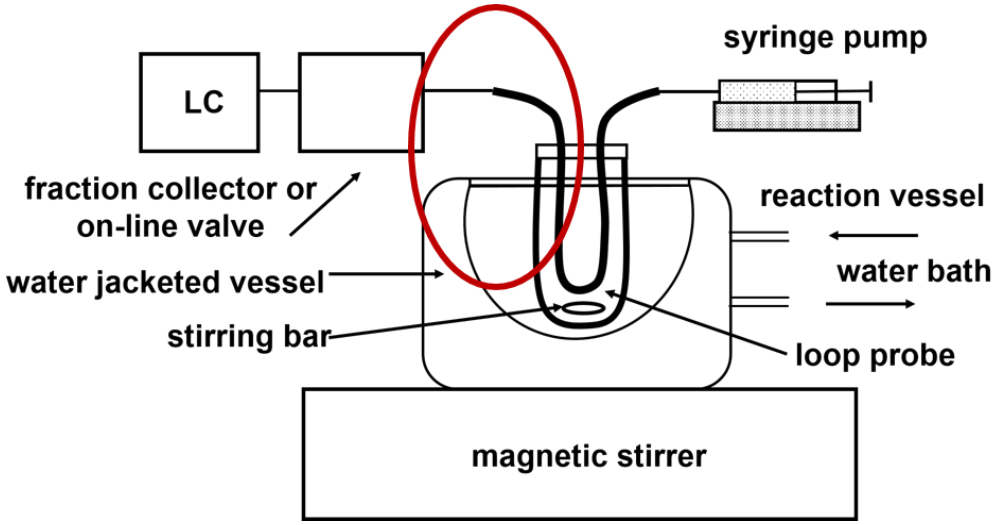


Figure 3.3 The red line highlights the dead volume between the microdialysis probe and the injection valve. This dead volume leads to long acquisition times, especially when slow perfusion rates are used.

3.2 Microdialysis Pump Comparison

The milliGAT® pump was investigated as a replacement for perfusate delivery to determine whether more precise fluid delivery is attainable for microdialysis experiments. Fluid delivery by the milliGAT® pump was compared to two other commonly used pumps, the BASi syringe and KD Scientific syringe pumps. This milliGAT® pump is advertised to be capable of seamless bi-directional flow and precise fluid delivery, dispensing volumes at flow rates ranging from 6 nL/min to 6 mL/min, and eliminates volume limitations associated with syringe pumps. The ultraviolet absorbance peak-to-peak noise of a 0.50% (v/v) acetone in water solution, flowing at 1 µL/min, was averaged over one minute for three runs for each pump, and the results are reported in the Table 3-1. Figure 3.4 illustrates that the sinusoidal signal fluctuation from the two syringe pumps (BASi and KD Scientific) is reduced by using the milliGAT® pump. The signals were offset so that the minimum point was at zero on the y-axis in Fig. 3.4. The peak-to-peak noise of the ultraviolet signal of a flowing acetone solution was reduced by up to 60 times with the new pump and so it was further investigated for microdialysis experiments.

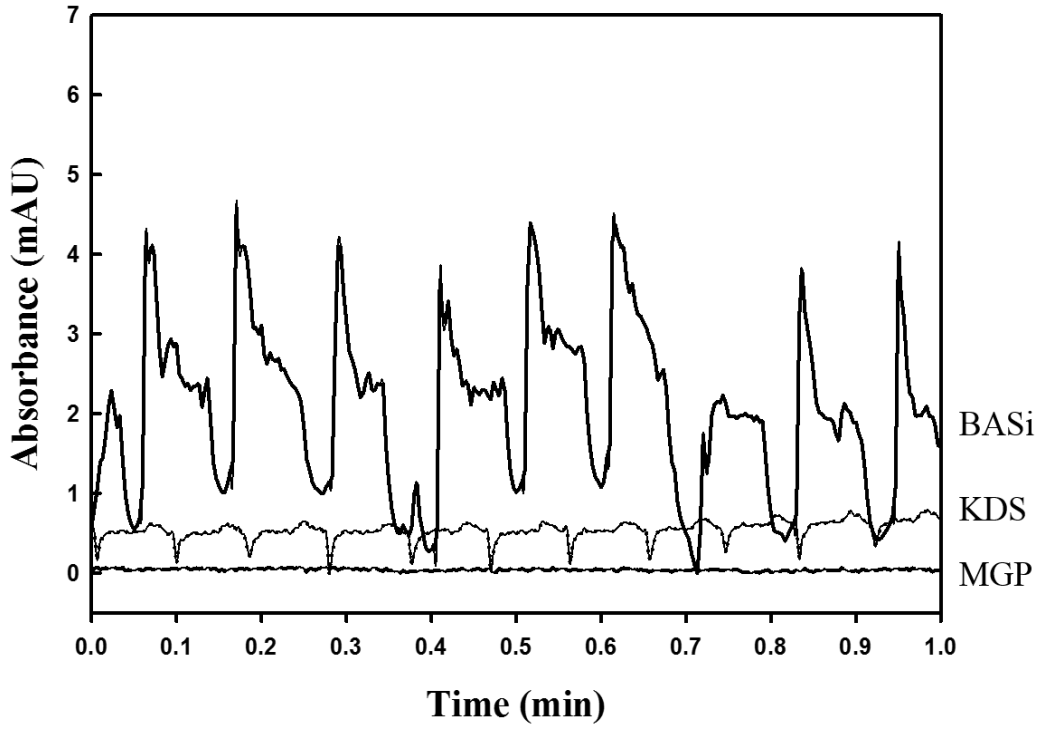


Figure 3.4 A plot of the fluctuation in the mean UV absorbance signal of acetone flowing at 1 µL/min by three pumps (BASi syringe pump (BASi), KD Scientific syringe pump (KDS), and milliGAT® pump (MGP)).

Table 3-1 The average peak to peak noise for each perfusate delivery pump.

Pump	Average Noise (peak-peak (mAU), n=3)
BASi syringe	4.5 ± 0.2
KD Scientific syringe	0.71 ± 0.02
milliGAT®	0.073 ± 0.007

3.3 Microdialysis Characterization Studies

Several factors, like perfusate flow rate, affect analyte recovery across the microdialysis membrane, Figure 3.5 is an overlay of chromatograms from a solution of 4.86 ppm of glucose recovered across the microdialysis membrane when coupled on-line to high performance anion exchange with pulsed amperometric detection (HPAEC-PAD). Calibration of the microdialysis probe with respect to analyte recovery is necessary for each set of experiments to ensure accurate sampling and sample cleanup prior to chromatographic analysis. The recovery of a glucose solution across the dialysis membrane was analyzed by microdialysis coupled on-line to HPAEC-PAD with sequential injections for 90 minutes. The results are summarized in Table 3-1. As the perfusate flow rate decreases, the relative analyte recovery across the membrane approaches 100% and the sampling precision increases, consistent with the theoretical plot of recovery based on flow rate (Figure 3.6).

The theoretical plot of recovery of a glucose solution across the microdialysis probe was calculated based upon Equation 5. [8] The permeability constant (PS) used to generate the theoretical line was calculated from the slope of the best fit line of the plot of –ln(1-EF) versus inverse flow rate, where extraction fraction (EF) was calculated from the relative recovery (RR) at each flow rate.[8] The results in Fig. 3.6 illustrate that the milliGAT® pump is a suitable choice for quantitative *in vitro* microdialysis experiments because of precise and high recovery of analyte at low flow rates. In order to further obtain accurate quantitation in these types of studies, the addition of an internal standard can be used to mathematically correct for any fluctuations in recovery.

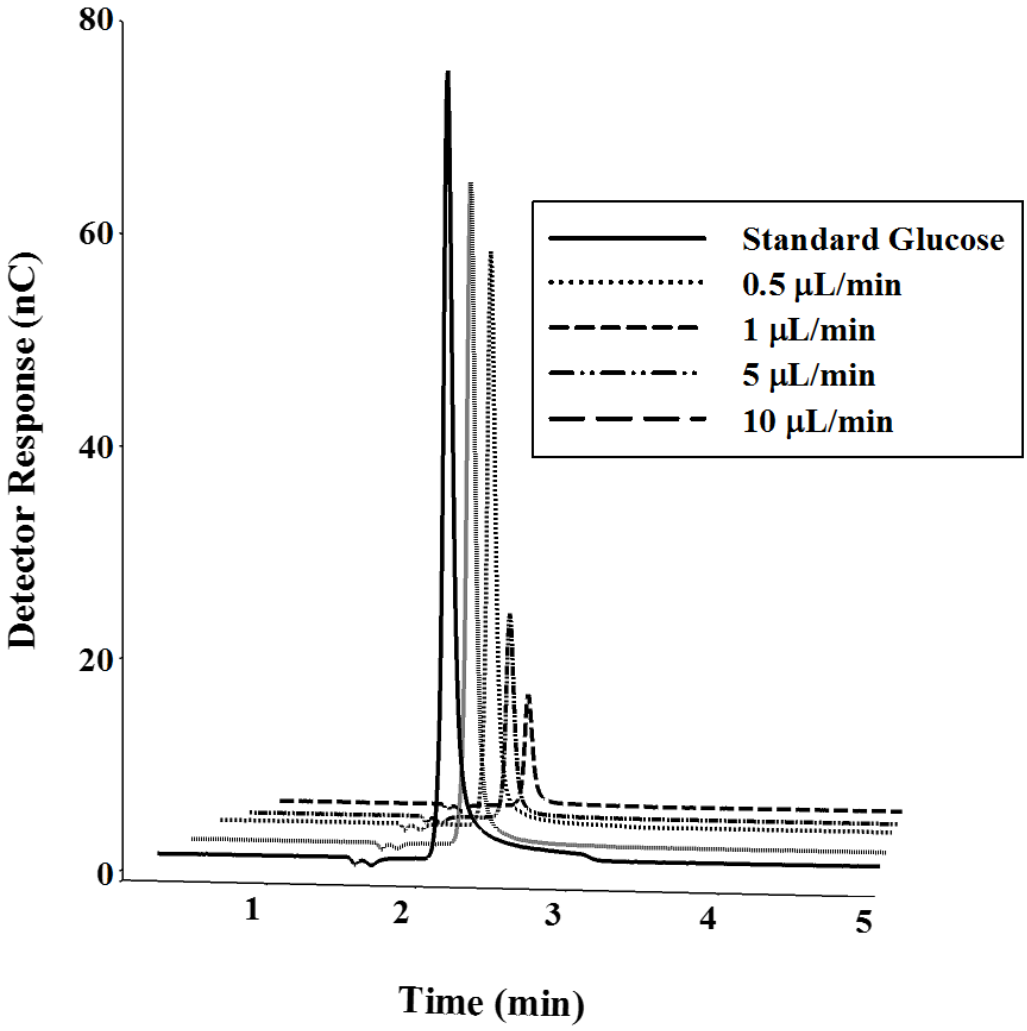


Figure 3.5 Chromatogram overlay of glucose recovery across the microdialysis membrane coupled to high performance anion exchange chromatography with pulsed amperometric detection at various perfusate flow rates.

Table 3-2 The relative recovery of a standard glucose solution across the microdialysis membrane at various flow rates.

Flow Rate (µL/min)	Relative Recovery (%)	%RSD
0.5	91 ± 0.3	0.3
1	80 ± 0.7	0.9
5	30 ± 1	4
10	17 ± 1	6

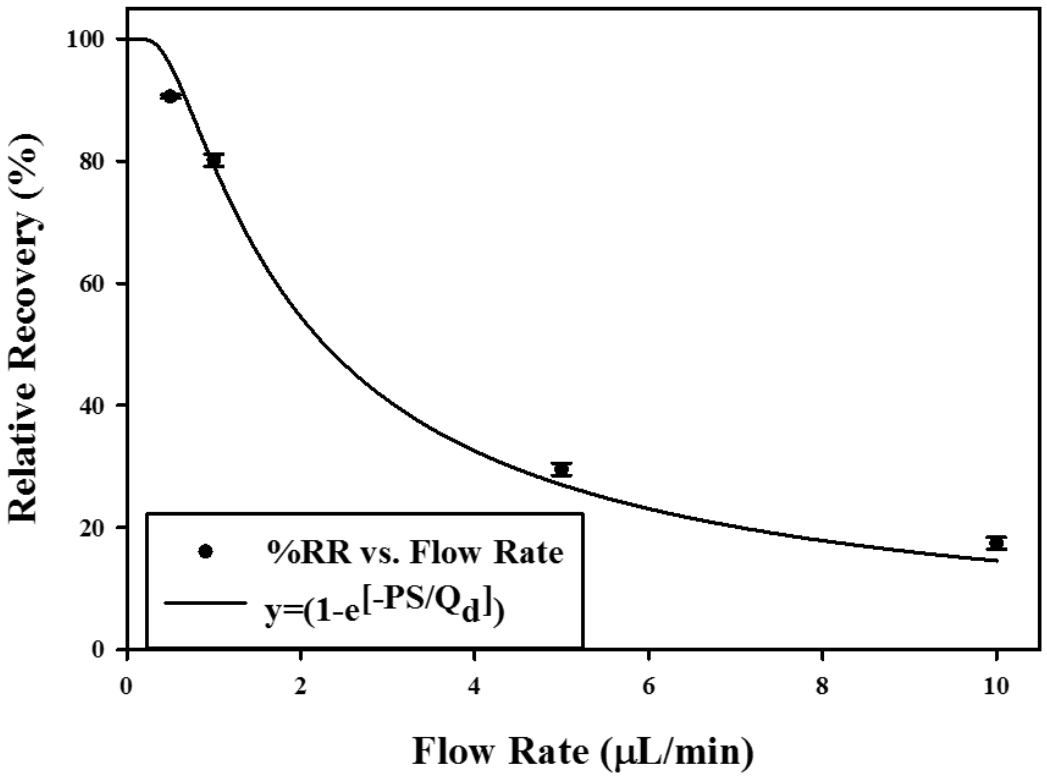


Figure 3.6 Relative recovery of 4.86 ppm glucose solution across the microdialysis membrane at various flow rates (·) with the calculated theoretical plot (-) of extraction fraction (EF), where PS is the permeability constant and Q_d is the flow rate.

3.4 On-line Dilution

The determination of enzymatic kinetic parameters relies on the ability to observe a reaction's progress. While slow perfusate flow rates are ideal for microdialysis sampling, it is a drawback when real time analysis is desired. The dead volume between the microdialysis probe and injection loop, along with slow flow rate, contribute to a delay in delivery of dialysate for analysis. Additionally, temporal resolution in enzymatic reaction monitoring, and short and consistent sampling intervals are important to clearly observe the progress of a reaction for determining

enzymatic kinetic parameters. To overcome these issues, a second milliGAT® pump was incorporated in-line to deliver a dilutant at a faster flow rate and represented by the schematic in Figure 3.7. The fluid delivery from the newly incorporated pump is joined to the fluid from the other pump via a mixing tee, and the resulting mixture is then directed to the injection valve of the chromatography system.

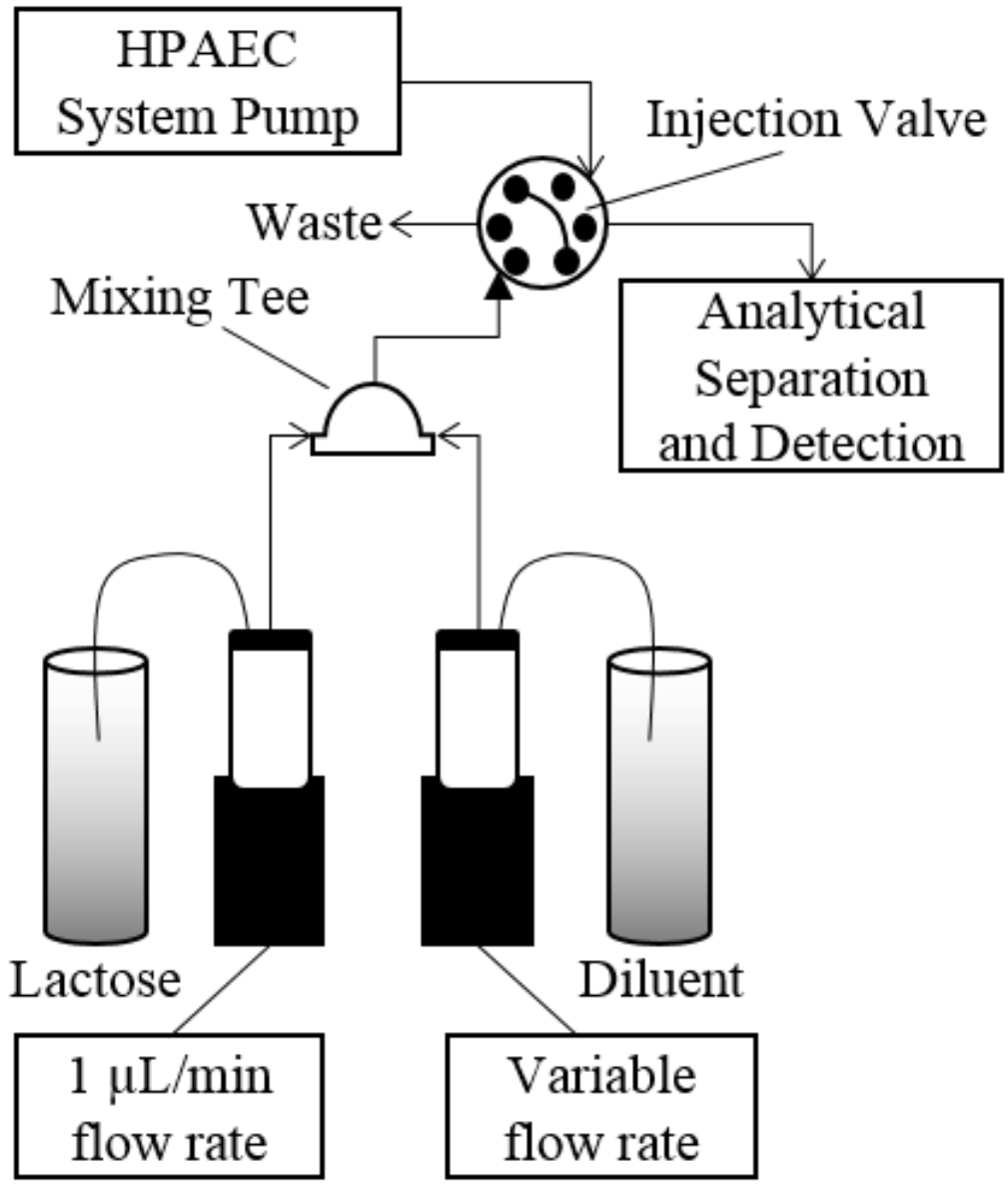


Figure 3.7 A schematic representation of the two milliGAT® pumps setup. The flows from the two pumps are joined by a mixing tee that leads the resulting mixture to the injection valve of an HPAEC-PAD system.

The use of a known faster flow rate provides the dilution factor for quantitation of dialysate concentrations, as well as delivering dialysate without a loss in time resolution for sampling analysis. The goal was to dilute higher concentrations of lactose solutions to 1 ppm by adding an in-line diluting agent at a faster flow rate. Each lactose solution was delivered at a flow rate of 1 µL/min, an ideal flow rate for microdialysis sampling, whereas the diluting agent was delivered at faster flow rates of 1, 9, and 99 µL/min to achieve dilutions of 50, 10 and 1%, respectively. The flow rates used by the pumps are summarized in Table 3-3. It is important to note that the computer software language requires flow rates be inputted in units of µL/s, and after confirmation from the manufacturer, the fraction equivalents of µL/s flow rates were used. The resulting diluted peak area responses were compared to that of a standard 1.00 ppm lactose solution (Figure 3.8).

Table 3-3 Description of the faster diluent flow rates used for each lactose concentration and its resulting dilution as a percentage.

[Lactose][a] (ppm)	Diluent Flow Rate (µL/min)[b]	Resultant (%)
1.00	0	100
2.01	1	50
10.05	9	10
100.5	99	1

[a]Delivered at 1 µL/min

[b]Computer software language requires flow rates to be inputted in units of µL/sec. The fractions 1/60, 1/30, 3/20, and 33/20 were used for flow rates 1, 2, 9, and 99 µL/min, respectively.

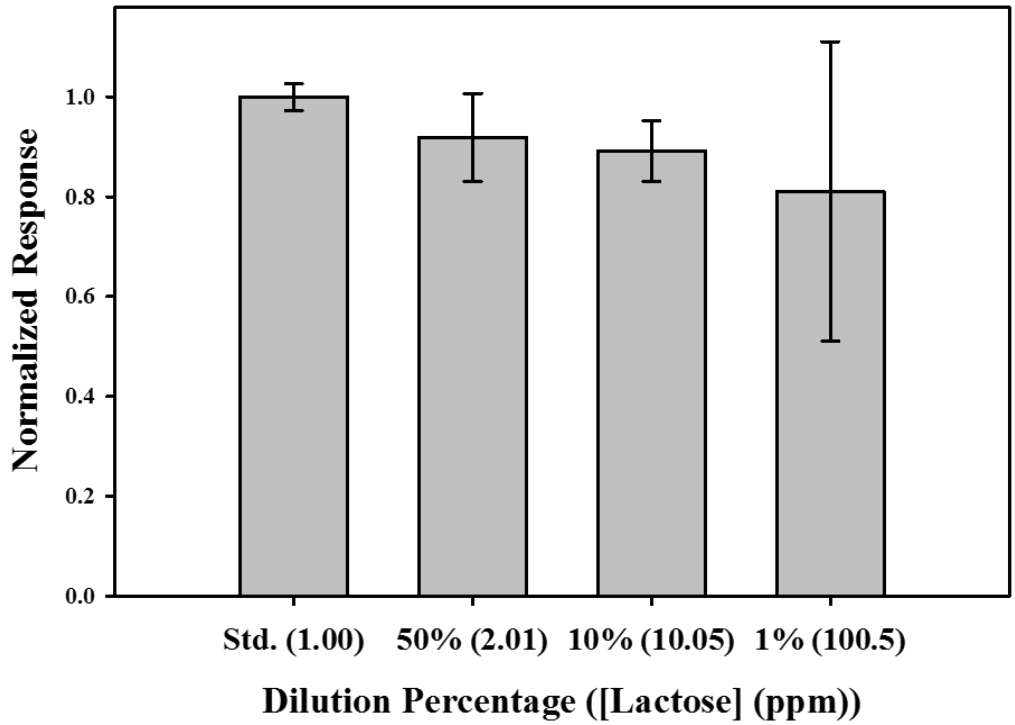


Figure 3.8 The normalized peak area of lactose solutions, delivered at 1 µL/min, and diluted by faster flow rates of 1, 9, and 99 µL/min to achieve dilutions of 50, 10, and 1%, respectively.

The results of all the dilution setups were analyzed using one-way ANOVA, and with 95% confidence the null hypothesis is retained; there is no significant difference between the standard 1.00 ppm lactose solution and the lactose solutions that were diluted at faster flow rates in this setup. Thus, it was concluded that the setup accurately and precisely dilutes 1 µL/min sample flow rates and is amenable to quantitative analysis of microdialysis sampling experiments. It was noted that the precision of sequential injections decreased with increasing dilution flow rate, which may stem from limitations with the current in-line mixer and could be overcome with a better mixing apparatus.

3.5 Conclusion

The use of quantitative *in vitro* microdialysis has far-reaching applications to monitoring a wide variety of bioprocesses without complex sample preparation procedures over an extended period of time. On-line *in vitro* microdialysis sampling followed by high performance anion exchange chromatography and pulsed amperometric detection has been applied to the monitoring of carbohydrate enzyme kinetics. In this work, a key improvement to the microdialysis perfusate delivery system is made to enhance reproducibility, accuracy, and recovery. A computer-programmable pump designed for precision fluid delivery, with flow rates in the nano- and microliter per minute range that does not require perfusate reloading, is used in place of the commonly used syringe pump. Peak-to-peak noise levels have been reduced by three orders of magnitude and recoveries at low flow rates (less than 1 μL/min) are greater than 90% for glucose. In addition, accurate and reproducible delivery allows for on-line dilution of over two orders of magnitude of flow rate. The technical improvements introduced further the utility of microdialysis sampling as an on-line sampling and sample clean-up method for monitoring bioprocesses.

4 Quantitative Assessment of Mannan-Degrading Enzymes Using *In Vitro* Microdialysis Sampling Coupled to HPAEC-PAD

4.1 Introduction

Carbohydrates are the most abundant biomolecules on Earth, and fall into one of three major size classes: monosaccharides, oligosaccharides, and polysaccharides. Oligo- and polysaccharides consist of monosaccharide residues joined together by characteristic linkages known as glycosidic bonds. Carbohydrate polymers serve many functions in biology, such as plant cell wall structure, as animal connective tissue, and in cell signaling. Enzymes that hydrolyze polysaccharides are carbohydrases (EC 3.2.1.-). Carbohydrases, such as mannanases, cellulases, pectinases, etc., represent a large class of enzymes and are classified per their specificity toward natural glycoside substrates. These enzymes are found extensively in biotechnology applications.

Most methods for determining carbohydrase activity are based on reducing sugar analysis. Under alkaline conditions, a reducing sugar has a free ketone or aldehyde functional group, which allows it to act as a reducing agent (Figure 4.1). Reducing sugar assays rely on the oxidation of the aldehyde group of a sugar to reduce a reagent. Reducing sugars that are produced by the enzymatic action on glycosidic bonds produce a colorimetric response that is directly proportional to their amount. The two most popular methods for reducing sugar analysis are the Nelson-Somogyi (NS) assay and the 3,5-dinitrosalicylic acid (DNS) assay.[46, 47, 55]

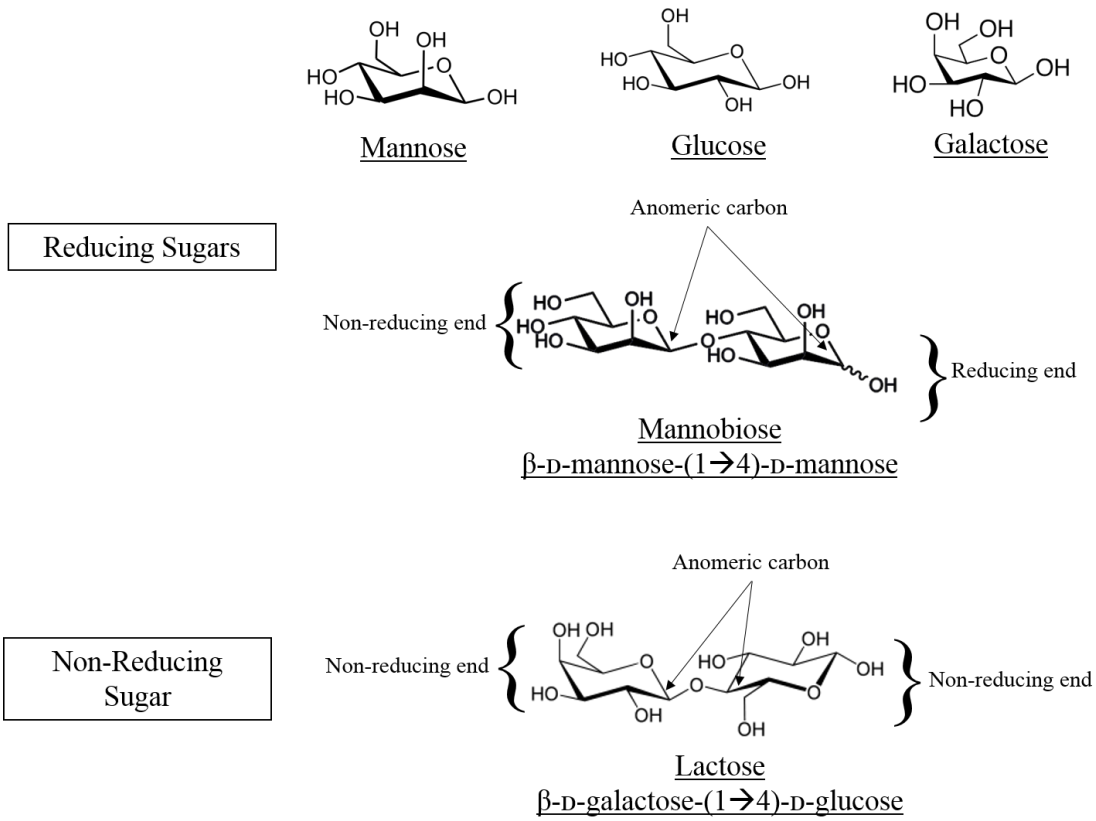


Figure 4.1 Examples of reducing and non-reducing sugars. A reducing sugar tautomerizes with its open-chain form where the carbonyl group on the anomeric carbon acts as a reducing agent and can be oxidized to a carboxyl group. All monosaccharides are reducing sugars, but not all disaccharides are reducing sugars. Non-reducing sugars have a glycosidic bond between anomeric carbons, preventing the reducing ability of the sugar. The disaccharide mannobiose is a reducing sugar; the disaccharide lactose is a non-reducing sugar.

In the DNS assay, 3,5-dinitrosalicylic acid is reduced to 3-amino-5-nitrosalicylic acid while the aldehyde groups of reducing sugars are oxidized to carboxyl groups (Figure 4.2). The IUPAC commission recommends the DNS assay for determining standard cellulose activities, and has been used for measuring the enzyme activities of β-mannanases, for which there is no current standard method. However, the DNS assay is approximately ten times less sensitive than the NS assay, overestimating the amount of reducing sugars produced during assay. For example, the DNS assay has been found to overestimate β-mannanase activity by a ratio of 7.3:1

when compared to the NS assay.[56] Additionally, other researchers found enzyme activity measurements by the NS assay to be significantly different from the DNS assay for cellulase, but that the NS assay and HPLC-RI produce similar results.[57] Researchers have modified the DNS assay and improved the correlation between product yields, but differences between activity measurements and procedures (through modifications), make it difficult to compare experimental results between researchers.

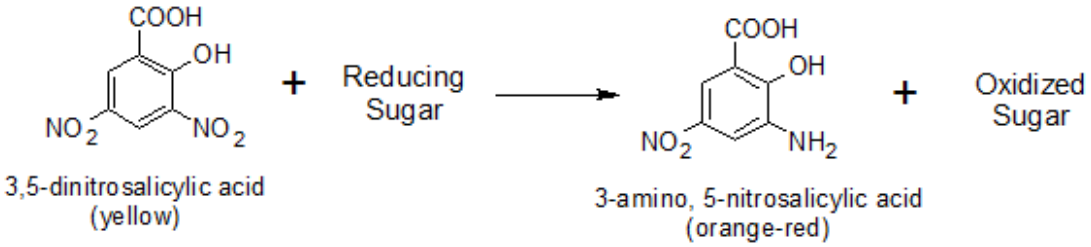


Figure 4.2 The development of color, and increase of UV-VIS absorbance at a wavelength of 575 nm, is a result of the reduction of 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid by a reducing sugar, where the sugar's aldehyde group is oxidized to a carboxyl group.

In the NS assay, Cu^{2+} is reduced to Cu^{+} by reducing sugars (Figure 4.3). Then, Cu^{+} is oxidized back to Cu^{2+} by an aresenomolybdate reagent, where molybdic acid is reduced to molybdenum blue. The blue color that develops is directly proportional to the amount of reducing sugars. The NS assay has improved accuracy of activity measurements and offers improved sensitivity and lower detection limits compared to the DNS assay. However, the NS assay is limited because it cannot be automated, is not appropriate for large batch samples, is labor intensive, and uses harsh reagents.

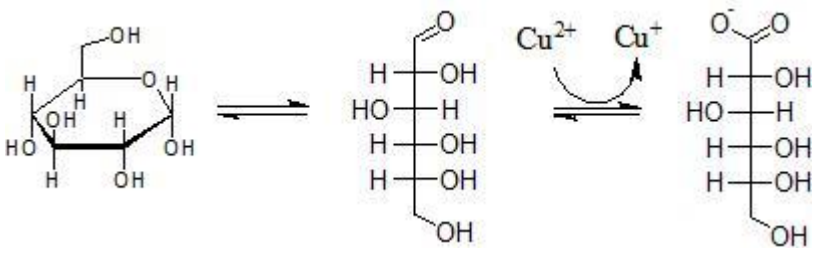


Figure 4.3 In the Nelson-Somogyi assay, the open-chain form of D-glucose is oxidized to gluconate and Cu^{2+} is reduced to Cu^{+}. By adding arsenomolybdate, Cu^{+} is oxidized back to Cu^{2+}. The subsequent development of color is directly proportional to the amount of Cu^{+} that was in solution.

Despite the documented shortcomings of the DNS method, it is still used internationally for characterizing enzymes such as β-mannanases, pectinases, and xylanases, and is the IUPAC recommended assay for determining cellulase activities.[56] Both the DNS and NS assays equate total reducing sugar production to enzymatic activity, but cannot account for the influence of the hydrolyzates with varying DP on enzyme activity, thereby misrepresenting the determined activity values. Using an assay that can quantify and characterize the hydrolysis products during enzymatic action can overcome the issues associated with traditional colorimetric methods for determining enzyme activity.

Microdialysis coupled to HPAEC-PAD was developed as a tool to characterize β-mannanase enzymes. This on-line sampling and monitoring tool automates enzyme activity measurement procedures, reduces sample handling, and offers selective enzyme and substrate characterization through the direct detection of the sugars in a dynamic process. Furthermore, the development of this tool offers a procedure that allows researchers to screen mannanase enzymes for their desired applications, which satisfies the goals of PAC to improve bioprocess understanding to increase efficiencies, decrease costs, prevent waste, and facilitate continuous monitoring of a dynamic process. This method was compared to the accepted literature Nelson-Somogyi reducing sugar method for measuring specific enzyme activity of β-mannanase enzymes from various sources.

4.2 MD-HPAEC-PAD Assay Characterization

Analytical figures of merit were assessed for 2-deoxy-D-glucose, mannose and mannooligosaccharides using HPAEC-PAD, and are summarized in Table 4-1. The LODs and LOQs range from 10 – 60 µg/L and 20 – 200 µg/L, respectively. The calibration sensitivity and system bias were determined from the slope and y-intercept of the linear regression lines for each sugar (Figure 4.4). The correlation coefficient values R^2 were all greater than 0.999, and the linear ranges were defined over three orders of magnitude.

Table 4-1 Analytical figures of merit for the calibration of HPAEC-PAD analysis of mannose and mannooligosaccharides.

$$Response\ (nC) = a\left(\frac{nC * mL}{\mu g}\right) * [Sugar]\left(\frac{\mu g}{mL}\right) + b(nC)$$

Sugar	a (µg/mL)	b (µg/mL)	R^2	LOD (µg/mL)	LOQ (µg/mL)
2-deoxy-D-glucose	12.26 (±0.138)	0.83 (±1.6)	0.999981	7×10^{-3}	2×10^{-2}
Mannose	15.87 (±0.33)	0.25 (±3.7)	0.999936	5×10^{-3}	2×10^{-2}
Mannobiose	6.234 (±0.211)	1.7 (±2.4)	0.999863	2×10^{-2}	4×10^{-2}
Mannotriose	3.699 (±0.182)	1.1 (±2.1)	0.999594	3×10^{-2}	8×10^{-2}
Mannotetraose	2.046 (±0.032)	0.17 (±0.36)	0.999959	4×10^{-2}	1.5×10^{-1}
Mannopentaose	1.282 (±0.027)	.31 (±0.31)	0.999932	5×10^{-2}	1.6×10^{-1}
Mannohexaose	1.013 (±0.029)	0.0263 (±0.15)	0.999757	6×10^{-2}	1.7×10^{-1}

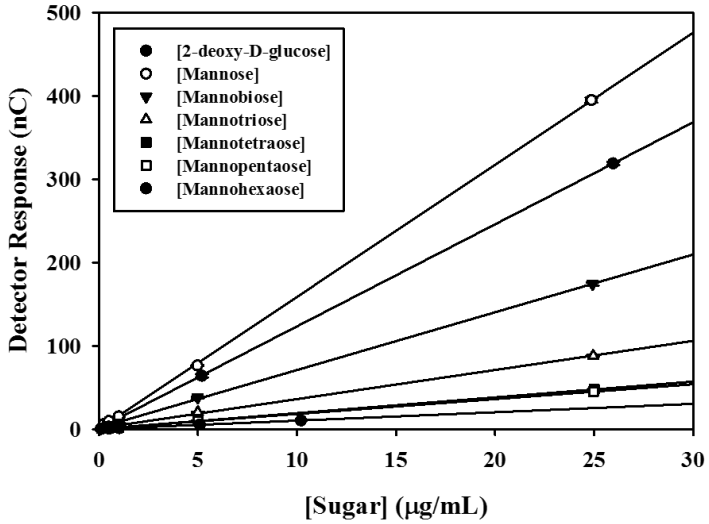


Figure 4.4 Calibration plots for mannose and mannooligsaccharides using HPAEC-PAD with isocratic elution using 0.1 M NaOH on a Dionex PA-100 microbore guard and analytical column.

The slope a was calculated from the slope of the least squares line for each analyte. The confidence limit of a is given as the slope plus or minus the standard deviation times the t-value at 95% for n-2 degrees of freedom, $a \pm t_{(n-2)} * s_a$. The y-intercept b was calculated from the intercept of the least squares line. The confidence limit of b is given as the y-intercept plus or minus the standard deviation times the t-value at 95% for (n-2) degrees of freedom, $b \pm t_{(n-2)} * s_b$. The standard deviation of b was calculated from the estimated errors in the y-direction of the regression line. The LOD and LOQ were calculated from three and ten times, respectively, the root mean square (RMS) noise of the baseline.

After characterizing the analytical figures of merit for the sugars of interest, their relative recoveries were assessed using a 5.00 ppm standard mixture. The dialysate concentration was measured by sampling from the standard solution with continual stirring and a perfusate flow rate of 1 µL/min. The relative recovery was calculated by comparing the ratio of the measured dialysate concentration to the concentration of the standard mixture from direct injection. The relative recovery was also determined by calculating the ratio of the dialysate peak height response to the standard peak height response, as displayed in Figure 4.5. A student's t-test was performed to assess whether the relative recovery determination differs between both methods. The t_{calc} (2.23) did not exceed t_{crit} (2.57, n = (6-1)). The null hypothesis was not rejected and there is no significant difference in methods for determining relative recoveries. This allowed for quicker calibration of microdialysis by comparing analyte recoveries to a standard solution.

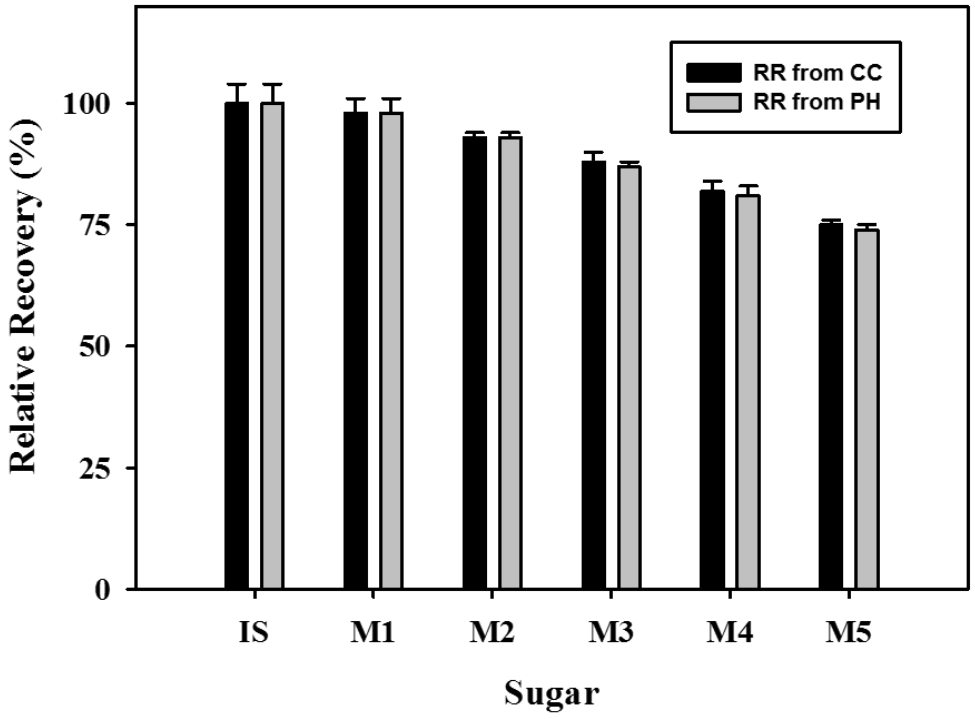


Figure 4.5 A comparison of relative recovery values (RR) of a 5.00 ppm mixture of sugars 2-deoxy-d-glucose (IS), mannose (M1), mannobiose (M2), mannotriose (M3), mannotetraose (M4), and mannopentaose (M5). RR was calculated by comparing the ratio of the measured dialysate concentration to the concentration of the standard mixture from direct injection (RR from CC), or by calculating the ratio of the dialysate peak height response to the standard peak height response (RR from PH).

The microdialysis setup was applied to monitor the action of 1,4-β-mannanase from *Bacillus sp.* on mannotetraose, mannopentaose, and mannohexaose to explore the smallest mannooligosaccharide that could be used as a substrate. Dialysate was sequentially injected at twenty minute intervals for two hours. An example chromatogram of a digestion is shown in Figure 4.6. In the chromatogram, the substrate mannohexaose is fully broken down to produce the mannooligosaccharides mannobiose, mannotriose, and mannotetraose.

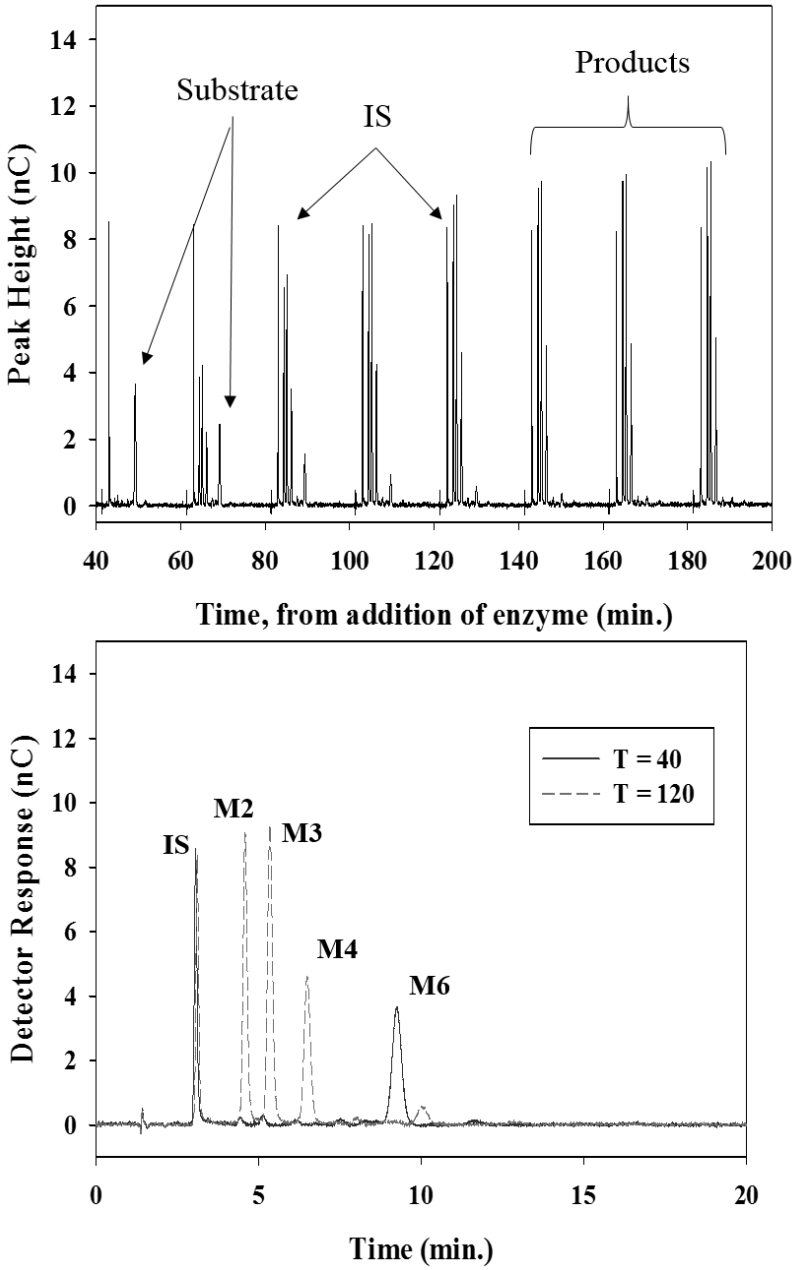


Figure 4.6 (Top) An example chromatogram of the digestion of a small mannan substrate. 16.6 ppm mannohexaose (substrate) was digested by 1,4-β-mannanase from *Bacillus sp.* to form multiple mannooligosaccharides (products) at T = 23 °C and pH = 7.0. The response from the deoxy-d-glucose (IS) remains constant throughout the process. (Bottom) A zoomed in view of the chromatogram showing the digestion of substrate (M6) to form products mannobiose (M2), mannotriose (M3) and mannotetraose (M4) after adding enzyme while the internal standard (IS) remains constant through the process.

An internal standard was added to correct for any variability of sampling recovery over the duration of the process. The ratio of the mannotetraose response relative to the 2-deoxy-d-glucose response remains constant over the course of the reaction, showing that the addition of an internal standard can be used to correct for fluctuations. The results also showed that there was no action of the enzyme on mannotetraose (Figure 4.7), but the breakdown of mannopentaose and mannohexaose by 1,4-β-mannanase from *Bacillus sp.* could be observed. (Figure 4.8)

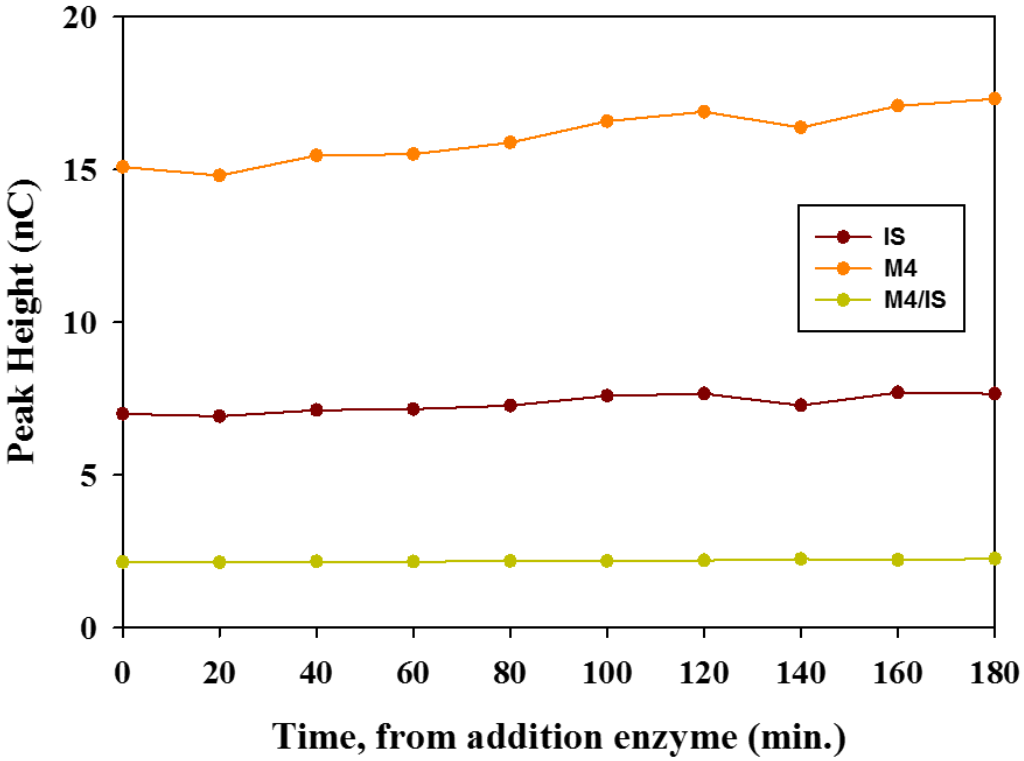


Figure 4.7 The detector response of deoxy-d-glucose (IS) and mannotetraose (M4) after the addition of 1,4-β-mannanase from *Bacillus sp.* at T = 23 °C and pH = 7.0. An internal standard can be used to correct for any variations in microdialysis sampling recovery over the course of a reaction, as shown by the consistent ratio of mannotetraose to 2-deoxy-d-glucose responses.

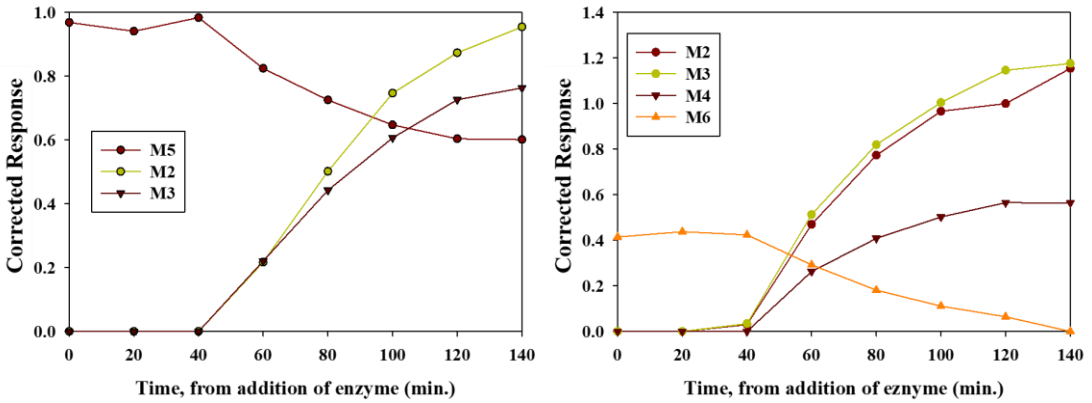


Figure 4.8 (Left) Mannopentaose (M5) substrate was consumed by 1,4-β-mannanase from *Bacillus sp.* to produce the mannooligosaccharides mannobiose (M2) and mannotriose (M3) at T = 23 °C and pH = 7.0. (Right) Mannohexaose (M6) substrate was consumed by 1,4-β-mannanase from *Bacillus sp.* to produce the mannooligosaccharides mannobiose (M2), mannotriose (M3), and mannotetraose (M4) at T = 23 °C and pH = 7.0.

The MD-HPAEC-PAD setup was applied for monitoring the action of various β-mannanase enzymes on the mannooligosaccharide mannopentaose. For each experiment, a 50.0 mL solution of 11.4 ppm mannopentaose and 1.00 ppm 2-deoxy-d-glucose was monitored until steady-state recovery was achieved, and then an aliquot of β-mannanase was added. A 25 µL aliquot of β-mannanase enzyme from *Bacillus sp.* and *Bacillus circulans* was added, and an 8 µL aliquot of enzyme from *Cellvibrio japonicus* was added. The results are shown in Figure 4.9. The microdialysis setup could directly monitor the breakdown of a mannooligosaccharide and the breakdown products could be observed over time. A closer look showed interesting results with respect to β-mannanase from *Cellvibrio japonicus*. Not only was mannose one of the unexpected products, but after the quick depletion of mannopentaose as substrate, mannotriose was then digested after its production.

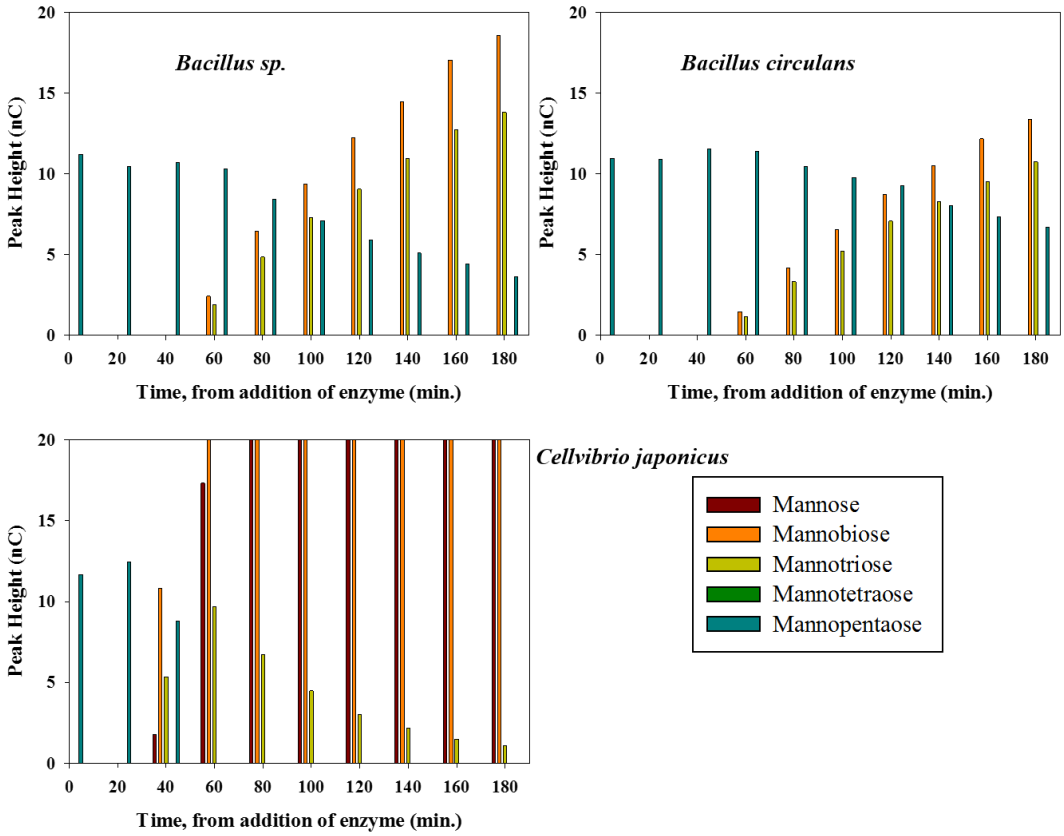


Figure 4.9 The breakdown of a 50.0 mL 11.4 µg/mL standard solution of mannopentaose by β-mannanase enzymes from various sources at T = 23 °C and pH = 7.0.

4.3 Reducing Sugar Assay

Absorption spectra for mannose standards at various concentrations were collected over a wavelength range of 400 – 800 nm (Figure 4.10). Each standard was subjected to the Nelson-Somogyi method, outlined previously in Chapter 2.4. The blue color that develops is directly proportional to the amount of reducing sugar. Though absorption maximum occurs at a wavelength of 660 nm[46], there are difficulties associated with using longer wavelengths due to the formation of the colored complex at higher sugar concentrations.[58] A wavelength of 520 nm was chosen for absorption measurements using the Nelson-Somogyi reducing sugar assay. Figure 4.11 shows the

linear relationship between the absorption at 520 nm and the concentration of mannose. This wavelength was used because it offers sensitivity, while minimizing the effects of variation due to assay factors.[46] Analytical figures of merit were not assessed for standard mannose solutions in the Nelson-Somogyi assay since a calibration curve was unnecessary. An exo-acting β-mannanase enzyme releases mannobiose from the non-reducing end of an undecorated 1,4-β-mannan. An endo-acting enzyme acts by breaking glycosidic bonds in the middle of a polysaccharide, rather than on an end. The 1,4-β-mannanase enzymes used in this research are all endo-acting and they release mannooligosaccharides.[59] This assay assumes that all reducing sugars produced via hydrolysis react similarly to the monosaccharide mannose.

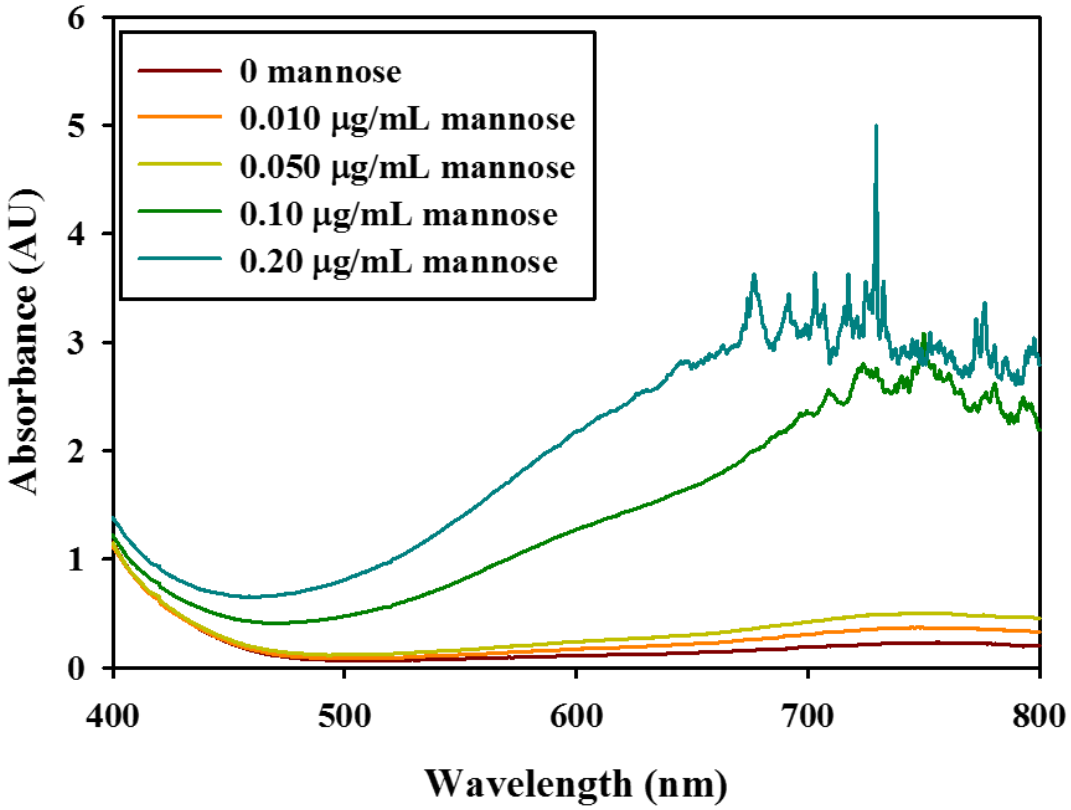


Figure 4.10 The absorption spectra of the developed color from the Nelson-Somogyi assay for various mannose sugar concentrations.

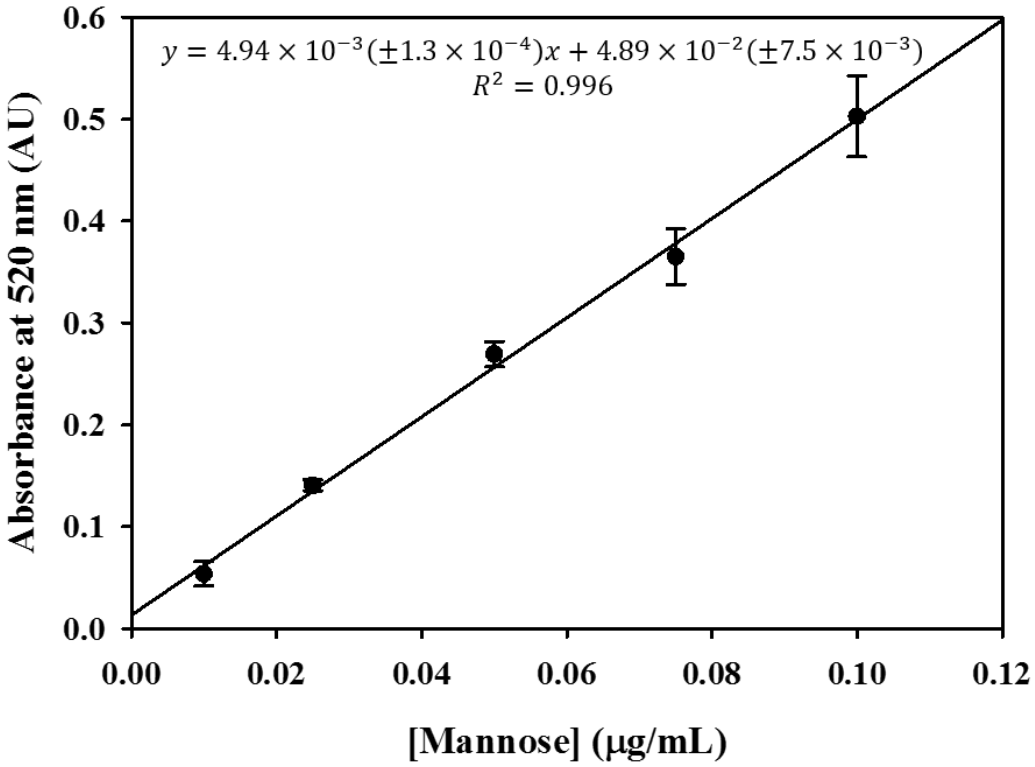


Figure 4.11 The correlation between the absorbance at 520 nm for various mannose concentrations used in the Nelson-Somogyi assay.

For each enzymatic reaction, it is important to capture the initial velocity of the reaction for determining the enzyme kinetic parameters. A general plot of product concentration relative to the amount of time a reaction progresses will show the concentration increasing steadily, and then leveling as the product concentration reaches its maximum. During the initial part of a progress-curve, the slope of the tangent line is linear. It was the goal to find an enzyme concentration for assay that extends the initial velocity for the time length of the assay, 12 minutes. The progress curves for 1,4-β-mannanase enzymes from *Cellvibrio japonicus* and *Bacillus circulans* were examined using initial dilutions of 1:100 and 1:40, respectively. The results are shown in Figure 4.12. Both progress curves show the characteristic hyperbolic shape,

indicating that the initial enzyme concentrations used for assay are too high and required further dilution.

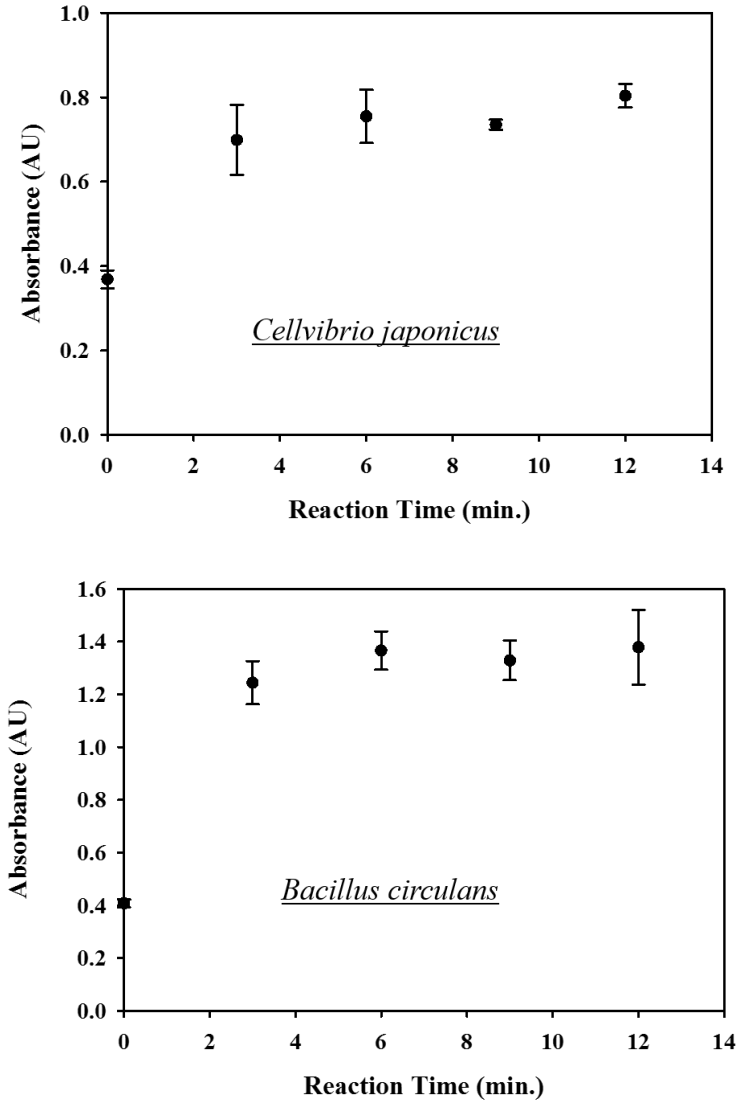


Figure 4.12 Progress curves for 1,4-β-mannanase enzymes from *Cellvibrio japonicus* and *Bacillus circulans* with initial dilutions of 1:40 and 1:100, respectively.

To capture the initial velocity of the enzymatic reactions from the 1,4-β-mannanase enzymes from *Cellvibrio japonicus* and *Bacillus circulans*, the original enzyme solutions were further diluted by 1:15 and 1:400, respectively. The assay dilutions were ultimately 1:1500 for *Cellvibrio japonicus* and 1:16000 for *Bacillus circulans*. Each assay was repeated four times, and the change in absorbance at 520 nm relative to the reaction time are shown in Figure 4.13. Linear regression statistics using Sigma Plot software (Windows version 11.0) were run for each assay to calculate the slope of the line and are reported in Table 4-2. The slope of the regression line is the change in absorbance relative to the amount of reaction time. Using the slope, the specific activity for each assay run (n = 4) was calculated and the values for each run are summarized in Table 4-2. Though two of the assays for β-mannanase from *Cellvibrio japonicus* had poor correlation values (R^2 = <0.99), neither run was excluded in calculation of average Specific Activity.

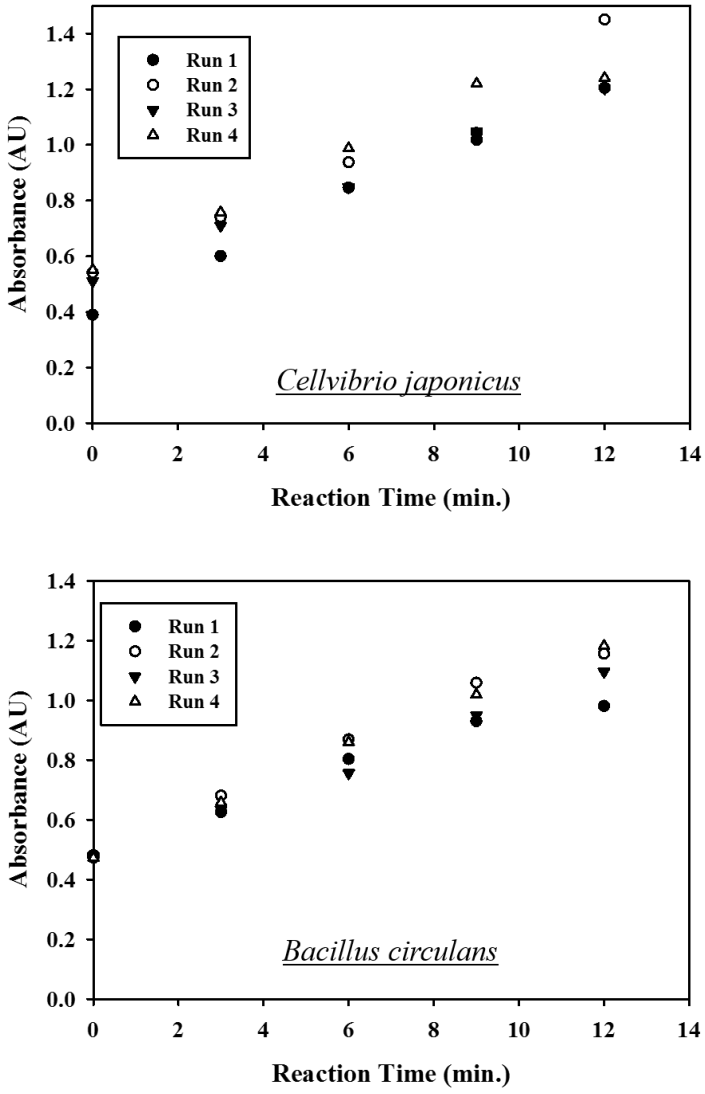


Figure 4.13 Progress curves for 1,4-β-mannanase enzymes from *Cellvibrio japonicus* and *Bacillus circulans* after dilution of Original solutions to final ratios of 1:1500 and 1:16000.

Table 4-2 A summary of the regression line slope statistics for each assay for each β-mannanase enzyme. The slope of each regression line was used to calculate the measured Specific Activity for each enzyme.

β-mannanase enzyme source	Slope of regression line (ΔAU/Δt)	Measured Specific Activity (U/mL)
Cellvibrio japonicus	0.0682 (±0.0025) $R^2 = 0.996$	5240[a]
	0.0707 (±0.0086) $R^2 = 0.957$	4710[a]
	0.0574 (±0.0017) $R^2 = 0.997$	6610[a]
	0.0613 (±0.0077) $R^2 = 0.955$	5150[a]
Bacillus circulans	0.0434 (±0.00442) $R^2 = 0.970$	410[b]
	0.0707 (±0.0039) $R^2 = 0.986$	460[b]
	0.0574 (±0.0018) $R^2 = 0.996$	470[b]
	0.0613 (±0.0016) $R^2 = 0.998$	550[b]

[a]One Unit of β-mannannase activity is defined as the amount of enzyme required to release one µmole of mannose-reducing-sugar equivalents per minute from 1,4-β-mannan (DP15) (5 mg/mL) in sodium phosphate buffer (100 mM) pH 7.0 at 40 °C.
[b]One Unit of β-mannannase activity is defined as the amount of enzyme which releases one µmole of mannose-reducing-sugar equivalents per minute from 1,4-β-mannan (DP15) (5 mg/mL) in sodium phosphate buffer (100 mM), pH 7.5 at 40 °C.

1,4-β-mannan substrate was chosen for these assays instead of the low viscosity carob galactomannan, which was the substrate used by the manufacturer for determining the specific activity for each enzyme. The low viscosity galactomannan has a galactose:mannose ratio of 22:78.[60] During enzymatic action the mannan backbone is broken apart to produce mannooligosaccharides with galactose residues. For the Nelson-Somogyi reducing sugar assay, substitution patterns do not affect the quantification of the assay since it is a bulk measurement of the total amount of reducing sugars produced during enzymatic action. However, to be able to compare the reducing sugar assay to the microdialysis assay, 1,4-β-mannan substrate was chosen because it has no galactose residues. This substrate was chosen for assay experiments for a direct comparison to the microdialysis assay. Since there are mannooligosaccharides standards available up to mannohexaose (M6), the microdialysis assay with HPAEC-PAD allows for direct determination of product formation and, thus, the rate of reaction.

The average specific activity for each enzyme was compared to the manufacturer's reported value using a student's t-test. The certificate of analysis for each enzyme from the manufacturer's website accepts specific activity values up to 10% higher than the reported value. The assay conditions used for each enzyme are summarized in Table 4-3. All the conditions of pH, temperature, and substrate concentration were controlled. For β-mannanase from *C. japonicus*, t_{calc} (1.04) did not exceed t_{crit} (3.19, n = (4-1)). Therefore, the null hypothesis is retained and there is no significant difference between the manufacturer's and the experimentally determined specific activity. For β-mannanase from *B. circulans*, t_{calc} (2.78) did not exceed t_{crit}

(3.19, n = (4-1)). Therefore, the null hypothesis is retained and there is no significant difference between the manufacturer's reported specific activity and the experimentally determined specific activity. The experimentally determined specific activities using the literature method for each enzyme were aligned with the manufacturer's reported activities.

Table 4-3 Summary of the conditions, reported specific activity, and experimentally determined specific activity for each β-mannanase enzyme using the Nelson-Somogyi Reducing Sugar assay.

β-mannanase enzyme source	Assay Conditions		Reported Specific Activity[a] (U/mL)	Measured Specific Activity[a] (U/mL)
Bacillus circulans	[Substrate]	5 mg/mL	390	470 (±60)
	[BSA]	0.5 mg/mL		
	pH	7.0		
	Temp.	40 °C		
Cellvibrio japonicus	[Substrate]	5 mg/mL	5000	5430 (±820)
	[BSA]	1 mg/mL		
	pH	7.5		
	Temp.	40 °C		

[a]One Unit (U) of β-mannannase activity is defined as the amount of enzyme required to release one µmole of mannose-reducing-sugar equivalents per minute under the specified assay conditions.

4.4 MD-HPAEC-PAD and Nelson-Somogyi Reducing Sugar Assay Comparison

The MD-HPAEC-PAD assay was applied for the determination of specific activity for 1,4-β-mannanase enzymes from *B. circulans* and *C. japonicus*. Each 50.00 mL bioreactor consisted of 1.00 ppm 2-deoxy-d-glucose and 0.1 mg/mL 1,4-β-mannan substrate. After steady-state recovery of the internal standard was achieved, a 0.500 mL aliquot of enzyme solution was added and injections were made every 20 minutes for three hours. Example chromatograms are shown below in Figure 4.14. To determine the μmole of sugar(s) produced, the response was corrected from the relative recovery of the internal standard and the ratio of the relative recovery of the internal standard/sugar for each injection. The concentration of each sugar was determined by its response factor, and the amount produced was determined for each time point.

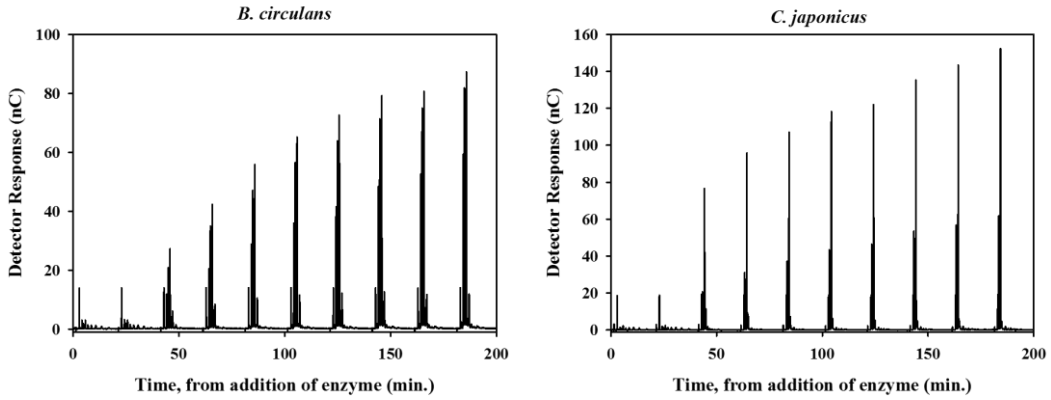


Figure 4.14 Example chromatograms from the digestion of 1,4-β-mannan substrate by 1,4-β-mannanase enzymes from *Bacillus circulans* (left) and *Cellvibrio japonicus* (right).

The total amount of mannooligosaccharides, as well as the amount of each mannooligosaccharide, produced by enzymatic action from 1,4-β-mannanase from *B. circulans* are shown in Figure 4.14. It was observed that there is no mannose produced during this enzymatic reaction, and mannotetraose was produced in the largest amount. Small amounts of mannopentaose were produced, and consumed over the course of the reaction. The total amount of mannooligosaccharides, as well as the amount of each mannooligosaccharide, produced by enzymatic action from 1,4-β-mannanase from *C. japonicus* is shown in Figure 4.15. It was observed that there is no mannotetraose produced during this enzymatic reaction, and mannobiose was produced in the largest amount. Small amounts of mannotriose and mannopentaose were produced and subsequently consumed over the course of the reaction. These results are shown in Figure 4.15.

To determine the specific activity for each reaction, the slope of the line of regression for the total amount of sugars produced over time was used to determine the Units of the enzyme. The first time point was not included since the injection loop may not have been filled from dialysate, which occurred at 40 minutes for each run. The slope of the regression line was determined from the second time point and the subsequent five points, which began at 60 minutes after the addition of enzyme. Table 4-4 summarizes the slope of the regression line statistics and the experimentally determined specific activities for each run.

Table 4-4 A summary of the regression line slope statistics for each β-mannanase enzyme using MD-HPAEC-PAD. The slope of each regression line was used to determine the Specific Activity for each run.

β-mannanase enzyme source	Slope of regression line ($\Delta nC/\Delta t$)	Experimentally determined Specific Activity[a] (U/mL)
Bacillus circulans	0.0299 (±0.0064) $R^2 = 0.845$	6.0
	0.0365 (±0.0036) $R^2 = 0.963$	7.3
	0.0340 (±0.0036) $R^2 = 0.958$	6.8
Cellvibrio japonicus	0.0277 (±0.0017) $R^2 = 0.985$	5.5
	0.0203 (±0.0040) $R^2 = 0.867$	4.1
	0.0147 (±0.0020) $R^2 = 0.931$	3.0

[a]One Unit (U) of β-mannannase activity is defined as the amount of enzyme required to release one µmole of mannose-reducing-sugar equivalents per minute from 1,4-β-mannan (DP15) (0.1 mg/mL) in water at pH 7.0 and 23 °C.

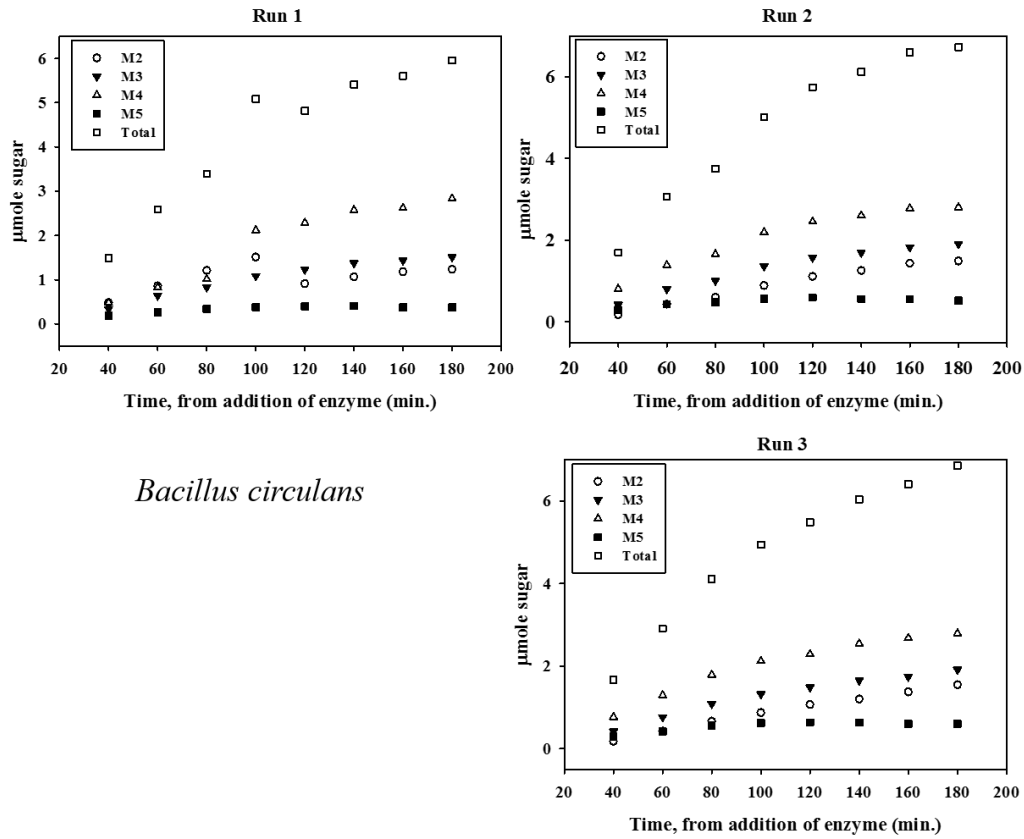
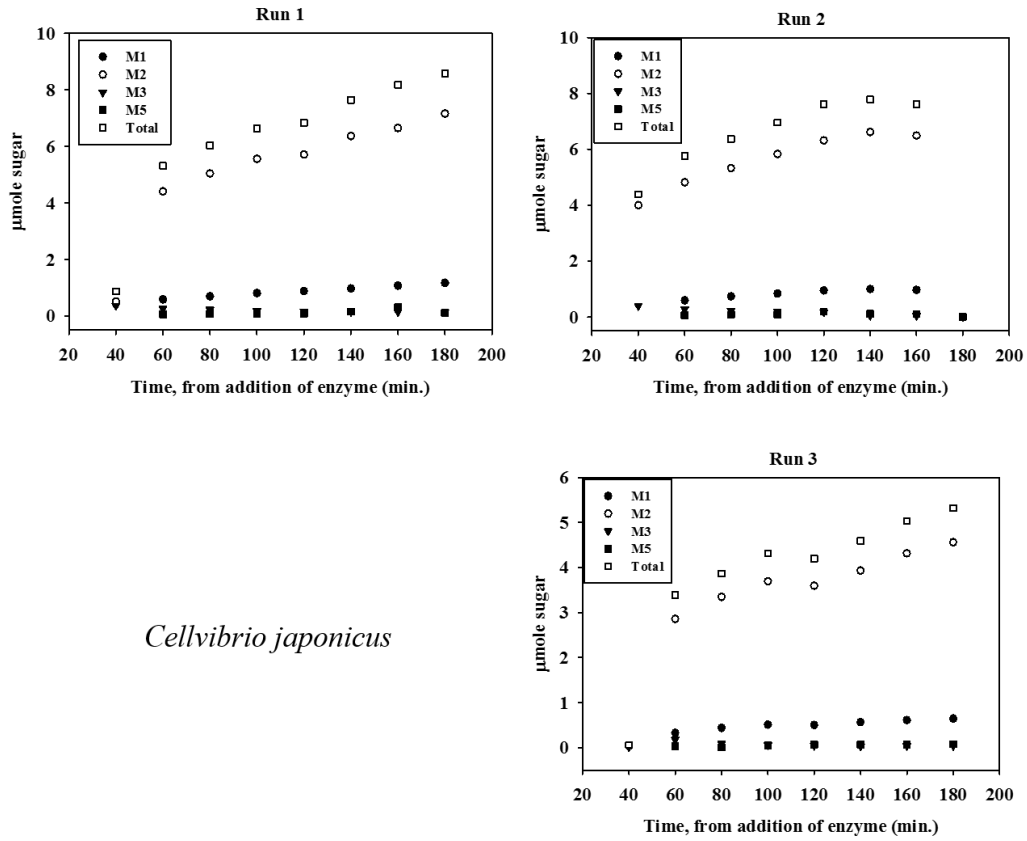


Figure 4.15 Total amount of mannooligosaccharides produced in µmoles by enzymatic action of 1,4-β-mannanase from *Bacillus circulans* on 1,4-β-mannan substrate.



Cellvibrio japonicus

Figure 4.16 Total amount of mannooligosaccharides produced in µmoles by enzymatic action of 1,4-β-mannanase from *Cellvibrio japonicus* on 1,4-β-mannan substrate.

The Nelson-Somogyi reducing sugar assay was applied for both enzymes under the same reaction conditions as the MD-HPAEC-PAD assay, as outlined previously in 4.3. The experimentally determined specific and relative activities for each run are summarized in Table 4-5, and with the conditions in Table 4-6.

Table 4-5 A summary of the regression line slope statistics for each assay for each β-mannanase enzyme. The slope of each regression line was used to calculate the measured Specific Activity for each enzyme.

β-mannanase enzyme source	Slope of regression line (ΔAU/Δt)	Determined Specific Activity[a] (U/mL)	Relative Specific Activity[b]
Bacillus circulans	0.0230 (±0.0018) R^2 = 0.982	120	31%
	0.0127 (±0.0014) R^2= 0.976	90	23%
	0.0148 (±0.0070) R^2 = 0.696	110	28%
	0.0122 (±0.0037) R^2 = 0.844	64	16%
Cellvibrio japonicus	0.0158 (±0.0018) R^2 = 0.963	530	11%
	0.0141 (±0.0016) R^2= 0.965	470	9.4%
	0.0160 (±0.0009) R^2 = 0.991	580	12%

[a]One Unit (U) of β-mannannase activity is defined as the amount of enzyme required to release one µmole of mannose-reducing-sugar equivalents per minute from 1,4-β-mannan (DP15) (5 mg/mL) in water at pH 7.0 and 23 °C.
[b]Relative specific activity is determined by comparing the measured specific activity under the specified assay conditions to the manufacturer's reported specific activity under the specific assay conditions reported in Table 4-3.

Table 4-6 Summary of the conditions, experimentally determined specific activity, and relative specific activity for each β-mannanase enzyme using the Nelson-Somogyi Reducing Sugar assay.

β-mannanase enzyme source	Assay Conditions		Measured Specific Activity[a] (U/mL)	Relative Specific Activity[b]
Bacillus circulans	[Substrate]	5 mg/mL	100 (±25)	25%
	pH	7.0		
	Temp.	23 °C		
Cellvibrio japonicus	[Substrate]	5 mg/mL	530 (±55)	11%
	pH	7.0		
	Temp.	23 °C		

[a]One Unit (U) of β-mannannase activity is defined as the amount of enzyme required to release one µmole of mannose-reducing-sugar equivalents per minute from 1,4-β-mannan (DP15) (5 mg/mL) in water at pH 7.0 and 23 °C.
[b]Relative specific activity is determined by comparing the measured specific activity under the specified assay conditions to the manufacturer's reported specific activity from conditions reported in Table 4-3.

The comparison of the Nelson-Somogyi assay to the MD-HPAEC-PAD assay is summarized in Table 4-7. The experimentally determined specific activities from the MD-HPAEC-PAD assay were consistently lower than the values for both enzymes determined from the Nelson-Somogyi reducing sugar assay. The determination of specific activity cannot account for the influence of higher DP hydrolyzates on enzyme activity. Yet, in the calculation for determining the specific activity via the Nelson-Somogyi reducing sugar assay, the underlying assumption is that all the reducing sugar products behave similarly to the sugar monomer mannose. However, the MD-HPAEC-PAD assay showed that β-mannanase from *Bacillus circulans* does not produce any mannose, and that β-mannanase from *Cellvibrio japonicus* primarily produces mannotetraose.

Table 4-7 Summary of mannooligosaccharides produced from 1,4-β-mannan from each β-mannanase enzyme using the MD-HPAEC-PAD assay.

β-mannanase enzyme source	Main products	Intermediate product(s)
Bacillus circulans	Mannobiose Mannotriose Mannotetraose	Mannopentaose
Cellvibrio japonicus	Mannotetraose Mannobiose Mannose	Mannopentaose Mannotriose

MD-HPAEC-PAD facilitated the continuous monitoring of the enzymatic reactions by 1,4-β-mannanase enzymes from various sources. Additionally, the enzymes were characterized by direct determination of product formation over time. The MD-HPAEC-PAD assay can account for the influence of hydrolyzates of various DP on enzyme activity, when compared to the traditional Nelson Somogyi reducing sugar assay. Additionally, the MD-HPAEC-PAD assay overcomes other issues associated with the traditional colorimetric technique by avoiding the use of harsh reagents and allowing for automation. MD-HPAEC-PAD can be implemented to improve understanding of processes with continuous real-time monitoring which, in turn, can increase efficiencies, decrease costs, and prevent waste by improving energy and material consumption in bioprocess monitoring.

5 Summary and Conclusions

5.1 Dissertation Summary: Major Findings

The overall goal of this research project was to illustrate the ability of microdialysis sampling coupled to high performance anion exchange chromatography with pulsed amperometric detection (MD-HPAEC-PAD) to characterize β-mannanase enzymes with respect to their specific activity. The objectives of this research were to first improve the technical aspects related to microdialysis sampling by improving perfusate delivery, and then to develop an assay for determining β-mannanase enzyme specific activity. The first objective was accomplished by incorporating a computer programmable pump for perfusate delivery and characterizing microdialysis sampling of a standard glucose solution. A second pump was added in-line and the on-line setup was characterized. The power of a flowing fluid, delivered at various flow rates, to dilute a slow flowing fluid was investigated to illustrate further improvements and setup flexibilities. The second objective was accomplished by developing an on-line microdialysis monitoring system for β-mannanase enzymes. The on-line MD-HPAEC-PAD setup was characterized and applied to monitor β-mannanase enzyme digestions of simple mannooligosaccharides. The third objective was to determine β-mannanase enzyme activities using the MD-HPAEC-PAD assay. The third objective was accomplished by quantitatively monitoring the breakdown of a mannan substrate by various β-mannanase enzymes and comparing the results against the Nelson-Somogyi reducing sugar assay.

5.2 Significance

Currently, there is no standard method for the assessment of β-mannanase enzymes. As the use of mannan and mannanase enzymes increases in biotechnology applications, it is important to enable the comparison of enzyme efficiencies between laboratories and researchers. The application of quantitative on-line microdialysis sampling for characterizing enzymes eliminates issues associated with current methodologies for assessing enzyme activities. Reducing sugar assays, the common methods for characterizing β-mannanase enzymes, rely on harsh chemicals, time-consuming procedures and indirect detection that cannot be automated. MD-HPAEC-PAD can characterize β-mannanase enzymes by directly monitoring the carbohydrate products of the reaction. Employing microdialysis sampling of a bioprocess eliminates the need to collect samples from the bioreactor, which can introduce volume loss and contamination. Additionally, the microdialysis probe offers inherent selectivity with the molecular weight cut-off of the probe, eliminating the need to clean up the sample prior to analysis. Finally, by coupling microdialysis sampling to a separation and/or detection system, analytes of interest can be directly monitored.

In this work, MD-HPAEC-PAD was successfully applied and validated for the characterization of β-mannanase enzymes, highlighting the utility of MD-HPAEC-PAD as a powerful tool for characterizing carbohydrate-based enzymes. Moreover, by employing microdialysis sampling in addition to monitoring other bio- and physiochemical properties of a bioreactor, a comprehensive picture of the dynamic process can be developed for multi-variate data analysis. This big picture will satisfy

the goals of process analytical chemistry, especially toward increasing process efficiencies, decreasing costs, and preventing waste.

5.3 Future Outlook

MD-HPAEC-PAD is a tool that can be extended to other carbohydrate-based bioprocesses, especially for substrates that are not amenable to reducing sugar assays. For example, polysaccharide degradation can be accelerated by the conditions of the reducing sugar assays (e.g., high temperature and pH). By employing MD-HPAEC-PAD for characterizing these systems, the types of substrates that can be used are expanded. This setup was validated using commercially available standards that are the products of enzymatic breakdown of an unbranched polysaccharide. However, for profiling and characterizing branched polysaccharides for which there are no standards available, mass spectrometry coupled to microdialysis sampling should be considered. Anion exchange chromatography can separate the breakdown products of carbohydrate substrates, but mass spectrometry can elucidate the oligosaccharides for which there are no current standards. The combination of these techniques for enzyme and/or substrate characterization will provide complimentary information.

The technological improvements documented in this work highlight the capabilities of a new perfusate delivery pump. The pump was operated in a continuous flow mode, but the computer program abilities should be further explored. For example, stopped-flow microdialysis sampling should be explored with programming of the two-pump on-line dilution setup. Stopped-flow microdialysis sampling allows for equilibrium to be established across the membrane and programming of the perfusate fluid delivery should be explored to enable this type of microdialysis sampling. Then,

at set time intervals, the dialysate contained within the probe can be collected by flowing of the perfusate fluid and delivered to instrumentation via a second pump for sample analysis. This type of system could reduce the effects of band broadening that occur within the tubing between the membrane and injection valve.

Finally, this work offers a unique educational opportunity in various fields touching on some key STEM topics including, but not limited to, engineering, equilibrium, kinetics, and calibration. For young researchers, studying microdialysis sampling applied to various processes offers a hands-on experience to the various topic components, as well as a unique opportunity to bridge various STEM fields together.

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