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# OPTIMIZATION OF ENZYMATIC HYDROLYSIS OF FIBRE SLUDGE FROM PULP MILL

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**ABSTRACT**

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This thesis is a part of a project which aims at utilizing fibre sludge from pulp mill as a source of biofuel production. The study concentrates on optimizing one of the processing steps, enzymatic hydrolysis, in converting fibre sludge to bioalcohol.

The aim of the thesis was to find optimum process parameters that enable maximum yield of glucose after performing the enzymatic hydrolysis. For this purpose, a series of experiments with changed process parameters was conducted. Also, enzyme mixes from two different manufacturers were used and the outcome was compared.

As a result, the optimum conditions were determined and a more effective enzyme mix is chosen. However, the outcome of glucose may be increased; therefore, further investigation is needed.



**Key words**
Biomass, enzymatic hydrolysis, fibre sludge, glucose, lignocellulose, total reducing sugars

**TABLE OF CONTENTS**

# 1 INTRODUCTION

The concept of sustainable development promotes the increasing interest in renewable energy sources which may partly replace the use of fossil fuels. The possibilities of alternative fuels are diverse, and one of the possibilities is derivation of biofuel from biomass. Although nowadays the level of consumption of renewable energy is quite low comparing to the extent of the use of traditional sources of energy, enormous research in the field of renewable energy is being done and technologies are under development. Biofuel derived from biomass gets great attention as a big amount of feedstock is available and, moreover, the derived fuel is liquid and can be transported easily. Raw materials containing monomeric sugars and starch are ideal feedstocks for biofuel production. Unfortunately their use is quite expensive and brings the concern over food versus fuel issue. Lignocellulosic biomass, agricultural and forest residues, on the other hand, are considered as potential sources of low-cost feedstock for biofuel production.

There are two ways to utilize lignocellulosic biomass for biofuel production: the thermochemical and biochemical method. Each of the ways involves a few steps. In the thermochemical method solid feedstock is first converted into gaseous state and afterwards microbal conversion is applied to receive ethanol. Biochemical method involves chemical pretreatment of biomass and enzymatic hydrolysis followed by microbal fermentation to produce bioalcohol. Pretreatment of biomass is important in order to disrupt the lignin-hemicellulose-cellulose interactions within the lignocellulosic biomass. It increases the accessibility of cellulose for enzymatic attack during the enzymatic hydrolysis which is the next step in processing the biomass. Enzymatic hydrolysis breaks down the cellulose into glucose and other sugars. As an alternative, acidic hydrolysis can be used to obtain glucose. Afterwards, glucose can be converted into biofuel by fermentation.

This thesis concentrates on optimization of one of the processing steps in converting biomass into biofuel which is enzymatic hydrolysis. The biomass used is dried fibre sludge from the pulp mill. Thus, basically, it is lignocellulose. The pretreatment of fibre sludge is done with the ionic liquid [AMIM]Cl. Afterwards, the enzymatic hydrolysis is done. For enzymatic hydrolysis, enzyme mixes from two different manufactures are used – enzyme mix A and enzyme mix B with separate addition of β-glucosidase in different loadings.

After the hydrolysis, concentrations of total reducing sugars as well as concentration of glucose are measured by carrying out assays and measuring the wavelength absorption with the spectrophotometer.

The main goal of the given thesis is to obtain maximum glucose during the enzymatic hydrolysis by defining the optimum enzymes loading and pH of the environment. Also, results of hydrolysis done by enzymes mixtures from two different manufactures are compared. To achieve the goal of the study, which is obtaining maximum amount of glucose, the following questions will be answered:

- What is the pH of the environment that is mostly favourable for the activity of the given enzymes mixes?
- What is the optimum enzyme concentration that allows obtaining maximum glucose within the minimum concentration of enzymes?
- Which enzyme mix is more effective in processing the fibre sludge?

However, the research carried out within the scope of this thesis does not consider the influence of temperature of the environment on hydrolysis which is one more variable that influences the rate of conversion of cellulose to glucose. Moreover, the study is limited as to the time given to conduct the research.

My personal expectation is the professional growth, which will provide a platform for further career as a researcher. Also, I hope to acquire skills of responsible conduct of research to develop an analytical way of thinking.

## 2 THE STRUCTURE OF LIGNOCELLULOSE

Lignocellulose is the major component of biomass. It consists of three polymers: cellulose (35-50%), hemicelluloses (20-35%) and lignin (10-25%) which are arranged in a complex way. Strands of cellulose are encased by hemicelluloses and lignin that act as a barrier for the enzymes to reach cellulose. (Khanal & Surampalli 2010, 174.)

Cellulose is a homopolymer that has the unique ability to form highly ordered, crystalline structures that are stabilized by hydrogen bonding. Cellulose is constructed from different sugars. It is a linear polymer and composed of B-glucose subunits linked by β-1,4 glycosidic bonds forming cellobiose. Linear strands of cellulose known as elemental fibrils aggregate into structures known as protofibres, which then organize to form microfibrils. Hydrogen bonding between cellulose chains makes it more rigid. Also chains of cellulose are linked with intra and intermolecular van der Waals forces. (Pandey, Arroche, Ricke, Dussap & Gnansounou 2011; Khanal & Surampalli 2010, 175.)

Hemicellulose is a chain of five and six carbon sugars with xylose as the sugar backbone. The composition is the following: D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-O-methyl-glucuronic, D- galacturonic, and D-glucuronic acids. The sugars are linked by β-1,3-glycosidic bonds. Hemicellulose forms a protective barrier around the microfibrils of cellulose and held in place by glue-like material lignin. (Pandey et al. 2011; Khanal & Surampalli 2010, 175.)

Lignin is an aromatic polymer that is formed from free radical polymerization from three precursor alcohols: p-hydroxycinnamyl, 4-hydroxy-3-methoxycinnamyl, and 3,5-dimethoxy-4-hydroxycinnamyl. It crosslinks with hemicellulose and prevents chemicals and enzymes to reach cellulose. Lignocellulosic biomass is considered to be a recalcitrant material due to its crystalline structure. (Pandey et al. 2011.)

# 3 PRETREATMENT OF LIGNOCELLULOSIC BIOMASS

The main problem in converting the biomass is its crystalline unreactivity and, consequently, its resistance to hydrolysis. Pretreatment of the lignocellulosic biomass increases the accessibility of cellulose for enzymatic attack by disrupting the structure of lignocellulose. Different pretreatment technologies are used depending on the specific properties of biomass feedstock. (Kumar, Singh & Singh 2008; Khanal & Surampalli 2010, 176.)

## 3.1 Pretreatment methods

Biomass pretreatment encounters different approaches which can be classified into three groups:

1) Physical pretreatment involves reducing the size of substrate increasing the pore volume of the lignocellulosic structure and also increasing the accessibility of enzymes to cellulose. The physical pretreatment methods include (Kumar et al. 2008):

- Comminution which includes ball miling, wet or dry disk refining.
- Steam explosion which includes two forms: heating of biomass with high temperature and pressure and violent reasling it into a collection chamber; and mild bleniding while applying high temperature and pressure until substrate reaches equilibrium with the ambient conditions.
- Hot water washing.

2) Chemical pretreatment involves dissolving hemicelluloses and lignin in acid or base to break down the structure of lignocellulosic biomass. It includes:

- Dilute acid pretreatment which has a high conversion rate of hemicellulose into sugars and low cost. Hydronium ions are believed to break the bonds of hemicellulose chains. As a result pores in structure are generated and therefore making the biomass more vulnerable to enzymatic attack. Among acids used are: perchloric acid, peracetic acid, sulfuric acid, and formic acid.

- Alkaline pretreatment which includes the use of sodium hydroxide, urea, potassium hydroxide, lime, and ammonia. In this method lignin and hemilcellulose are solubilized into liquid phase.

3) Biological pretreatment makes use of natural degradative properties of certain fungal species. With this method it is possible to avoid use of toxic chemicals and save energy required for the process of pretreatment. In biological pretreatment are used:

- Bacteria. There are different species of bacteria that can degradate lignocellulose to cellulases. Bacteria can form multi-enzyme complex which can enhance the hydrolysis of biomass.
- Fungi. Different fungi secrete different amount of enzymes which affects the efficiency of hydrolysis. Some fungi produce more β-glucosidase and less endoglucanase while it's vice versa for other fungi. The most used species is white-rot fungi. They produce extracellular enzymes which degrade the structure of lignocellulose.

Also hybrid pretreatments can be used which implies combination of some of the mentioned above methods. For example, physical pretreatment is combined with chemical or microbial pretreatment. (Kumar et al. 2008; Khanal & Surampalli 2010, 176-181.)

### 3.2 Ionic liquid

As the name implies, ionic liquid consists of ions. They are known as molten salts and do not contain aqueous solution. Usually, it is a eutectic mixture of organic and inorganic salt. Generally, ionic liquids are non-flammable, have a large range of solubilities and miscibilities, negligible vapour pressures, and are liquid at room temperature. They can be designed to have specific properties by giving specific functionalities into anions or cations and have dual functionality. They include organic cation, for example, 1-alkyl-3-methylimidazolium cation in imidazolium ionic liquids, and inorganic anion. The latter can be a halide, nitrate, chloroaluminate, hexafluorophosphate, or alkyl sulfate. Typically, ionic liquids have a shorten name. The name of cation is shortened and can be denoted as the following: $[EMIM]^+$ stands for 1-ethyl-3-methylimidazolium cation. (Freemantle 2010.)
Pretreatment with ionic liquid helps to disrupt the crystalline structure of lignocellulose and provide the greater amount of sites for adsorption of enzymes which leads to better

enzymatic hydrolysis. Ionic liquids are able to dissolve cellulose and, afterwards, can be recovered by adding an anti-solvent such as water or alcohol. However, the rate of solubility depends on the source of lignocellulosic biomass. The mechanism of dissolution has been studied. It is suggested that the hydrogen bond network is being broken by interaction of the chloride ion in the ionic liquid [BMIM]Cl and [AMIM]Cl and the carbohydrate's hydroxyl protons. The ability of ionic liquids to dissolve cellulose depends on a few factors: a nature of cellulose to be dissolved, operating conditions (temperature, reaction time and the amount of cellulose to be dissolved) and the level of impurities in ionic liquid. Water is the main impuritiy that can be found in the ionic liquid, which is why it is necessary to dry it severely; otherwise the solubility of cellulose will be affected. After the pretreatment it is necessary to remove all the ionic liquid traces before the enzymatic hydrolysis as ionic liquid inhibits the activity of enzymes. In addition to a good ability of disrupting the lignocellulosic structure, the other advantages of the use of ionic liquids are the following:

- the operating temperature is quite low comparing to other pretreatment methods which allows to reduce energy costs;
- ionic liquids can be recycled which makes them more environmentally friendly and also helps to reduce costs of pretreatment;
- ionic liquids have low vapour pressure.

However, the remaining challenge in an application of ionic liquid is to find its effective recovery for large-scale industrial use. (Liu, Wang, Stiles & Guo 2011; Holm & Lassi 2011.)

# 4 ENZYMES

Enzymes are special molecules that catalyze biological transformations that cater for almost all the needs of living systems. They are mostly proteins in nature with a few exceptions of the self-slicing RNA molecules. The word “enzyme” means “in yeast”, a term proposed by Kuhn in the beginning to indicate their association with yeast cells. (Kulkarni & Deshpande 2007, 2.)

Enzymes have a number of advantages over conventional chemical catalysts. They include: selectivity and specificity for particular reactions, discrimination between similar parts of molecules or optical isomers, catalysis of reactions of narrow ranges of substrates, absence of contamination of the product of reaction. (Shanmugam & Sathishkumar 2009.)

## 4.1 Properties of enzymes

Enzymes possess a number of characteristic properties that can be described as follows:

1) Enzymes can be recovered after being used in reaction.
2) Enzymes catalyze the reaction reducing the time of reaction without interfereing with chemical equilibrium.
3) Enzymes are environmentally friendly. They are completely biodegradable which makes them a better choice comparing to heavy metals catalysts.
4) Enzymes have a high molecular weight, colloidal state and show slow rate of diffusion.
5) Enzymes have exceptionally high catalytic efficiency. Comparing the rate of noncatalytic reaction and the one catalyzed by enzymes, the rate of a catalyzed reaction is $10^{8}$-$10^{10}$ times faster. The rate is much higher than the one that can be achieved by using a chemical catalyst.
6) To perform catalysis just a minute concentration of enzymes is needed. Taking into account the rate of reaction enzymes can catalyze, a mole percentage of 0.1-1% of catalyst is employed. In some cases a mole percentage of $10^{-3}$-$10^{-4}$ % of catalyst is sufficient to have a reasonable rate of reaction.
7) Slight change in environmental parameters can inhibit the activity of enzymes. However, if certain precautions are met, enzymes can be stable. There are enzymes that can tolerate

temperatures greater than 100°C and pressures over 10 MPa. (Kulkarni & Deshpande 2007, 2; Faber 2011, 2-4.)

### 4.2 Inhibitors of enzyme activity

Inhibitors are substances that negatively affect the enzymatic activity by affecting the rate of catalysis by reducing it or stopping the reaction (Kulkarni & Deshpande 2007, 15). Molecules of inhibitor bind to enzymes and block its activity. Not all the molecules that bind to enzymes are inhibitors; there are also enzyme activators that bind to enzyme and increase enzymatic activity. There are two types of inhibitons: irreversible and reversible. (Shanmugam & Sathishkumar 2009, 80.)

Irreversable inhibitors are also known as enzyme poisons. They bind to the active site residues forming stable covalent bonds which irreversibly decrease the reaction rate. Irreversible inhibition results in a time-dependant loss of enzyme concentration, or in case of incomplete inactivation, time-dependant changes in maximal velocity and Michaelis constant. (Shanmugam & Sathishkumar 2009, 81; Kulkarni & Deshpande 2007, 15.)

Reversable inhibitors are substances that reversibly bind to free enzymes and temporary reduce the rate of the catalyzed reaction. Reversible inhibitors are divided into three groups: competitive noncompetitive and uncompetitive inhibitors. Reversible inhibitors bind to enzymes with non-covalent interactions such as hydrogen bonds, hydrophobic interactions and ionic bonds. Reversible inhibitors do not undergo chemical reaction when bounding to enzymes and therefore can be easily removed by dilution or dialysis. (Shanmugam & Sathishkumar 2009, 81; Kulkarni & Deshpande 2007, 15.)

In competitive inhibition, a substance that is a substrate analogue competes with the substarate for the active site of the enzyme. As a result, an unproductive complex is formed. It affects Michaelis constant. It represents half-maximal velocity and characterizes the given enzyme-substrate pair and indicates affinity between them; the more the value, the less the affinity. The effect can be overcome by increasing the substrate concentration. (Kulkarni & Deshpande 2007, 15-16.)

In non-competitive inhibition, a substance does not compete with the substrate but it binds at some other site with free enzymes, or complexes are formed. It affects the maximal velocity of the reaction. Maximal velocity of reaction stands for the maximal velocity of reaction at complete saturation of the enzyme active sites. It is a constant for a specific enzyme substrate. Increasing the substrate concentration does not eliminate the effect of the inhibitor. In uncompetitive inhibition a substance binds to the ES complex only forming ESI complex. It affects maximal velocity and Michaelis constant. (Kulkarni & Deshpande 2007, 15-16.)

### 4.3 Activity of the enzyme

Catalytic activity of enzymes is measured in different systems. According to SI system of units, activity of an enzyme is measured by katal which is equal to one mol of substrate transformed per second. However, the unit is not widely accepted as the magnitude of the unit is too big and suitable for industrial-scale reaction only. Consequently, another unit was accepted which is International Unit (IU). It is equal to one micromol of substrate transformed per minute under defined conditions. (Faber 2011, 24.)

However, in practice it is difficult to define the stoichiometric amount of product formed for some enzymes. Therefore, the enzymatic activity cannot always be expressed in standardized units and units, for different enzymes can differ. For example, the unit used for restriction endonuclease is based on yield of a cleaveage pattern with an amount of particular DNA under defined conditions. (Kulkarni & Deshpande 2007, 16; Aehle 2007, 21.)

Thus, different units to denote an activity of a specific enzyme are used. Among units that measure the activity of enzymes for lignocellulose conversion are the following: AmyloGlucosidase Unit (AGU), CelloBiase Unit (CBU), Biomass Hydrolysis Unit (BHU2), Fungal Beta-Glucanase Unit (FBG), Filter Paper Unit (FPU), Fungal Xylanase Unit (FXU), Polygalacturonase Unit (PGU).

### 4.4 Enzyme specificity

The ability of the enzyme to react with one of substrates determines its specificity. Three types of enzyme specificity are defined: absolute, group and broad specificity. Absolute specificity means that an enzyme reacts only with a single substrate. For example, glucokinase can only react with glucose as its substrate. It does not react with other hexoses. In group specificity, an enzyme can react with a range of related compounds. In broad specificity, an enzyme reacts with varied substrates. For example, trypsin can react with proteins, shorter peptides and esters. (Kulkarni & Deshpande 2007, 17.)

Another type of specificity defined is steriochemical specificity. In this case an arrangement of specific groups of atoms in the substrate plays a main role. An enzyme will act on one isomer but will not react on another one. (Shanmugam & Sathishkumar 2009, 20.)

### 4.5 Enzyme nomenclature principle

Currently, about 3,700 enzymes are recognized by the International Union of Biochemistry and Molecular Biology. There are general rules used for naming and coding of enzymes for their classification. They are the following:

1) Enzymes names ending in –ase should be used only for single enzymes and not applied to systems containing more than one enzyme. Those systems should contain the word “complex”.
2) All enzymes are principally classified and termed according to the reaction they catalyze, for example, proteases, lipases, and glycosidases.
3) The enzymes are divided into groups on the basis of the type of reaction catalyzed. This is the basis for classification and code numbers.
4) Enzymes are assigned code numbers which contain four elements separated by points and show the class and subclass of the enzyme. For example, [EC A.B.C.D.], where EC stands for Enzyme Comission, A. indicates the main type or reaction, B. stands for subtype, C. denotes the nature of co-substrate and D. is the number of enzyme. (Faber 2011; Kulkarni & Deshpande 2007.)

### 4.7 Enzyme denaturation

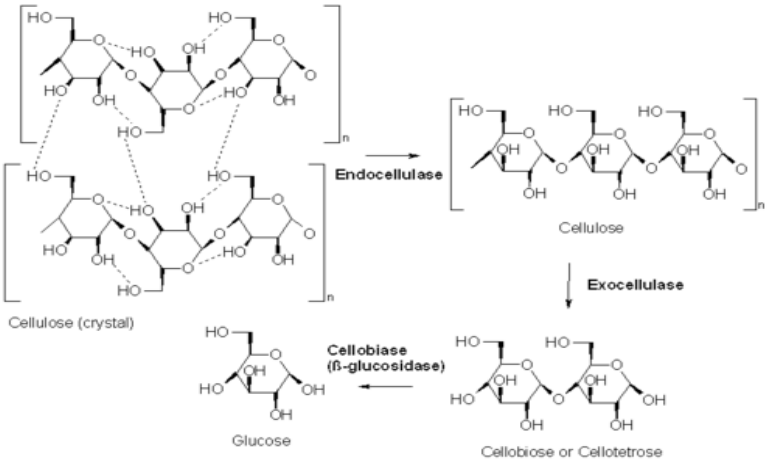
Some physical and chemical agents can cause enzyme to lose its three dimensional form which leads to the loss of enzyme activity. It is known as enzyme denaturation. Among the denaturating agents are changes in pH and temperature. Also, enzymes can be inhibited and deactivated by compounds such as oxidising and reducing agents, metal ions, salts, solvents, and surfactants. For example, at around 40-50°C rearrangement of peptide chains in enzymes starts. In some cases enzymes can be inhibited by their end-products and can be denaturated within a certain period of time. Some enzymes, however, can gain their active from back after removing the denaturating conditions. It is known as renaturation. (Tsao 1999; Kulkarni & Deshpande 2007, 18.)

# 5 ENZYMATIC HYDROLYSIS

After pretreating the lignocellulosic biomass with ionic liquid and, therefore, breaking its structure, it is porous and cellulose is available for enzymatic attack. For effective conversion of lignocellulosic biomass into fermentable sugars, a mixture of different enzymes is needed. This enzyme cocktail is referred to as cellulases which are capable of degrading cellulose. Cellulase enzymes are produced using such fungi as Trichoderma reesei, Penicillium funiculosum, and Aspergillus niger. (Khanal & Surampalli 2010, 203.)

## 5.1 Cellulases

Cellulases include three different systems of enzymes: endo-β-1,4-glucanases, exo-β-1,4-glucanases (cellobiohydrolase), and β-1,4-glucosidases. The difference of the enzymatic attack of the mentioned cellulases is that they attack cellulose chains at different sites in order to break cellulose to glucose units. Endoglucanases act in the middle of cellulose chain and cut it randomly. As a result, shorter and shorter cellodextrines which are mixed 1,3-1,4-β-dextrines. Cellodextrines can be further degraded with exoglucanases. Exoglucanases (cellobiohydrolase) act in the end of the cellulose chain. As a result, cellobiohydrolase give celobiose as a reaction product. Cellobiase, also known as β-glucosidase, hydrolyzes cellobiose and gives glucose as a reaction product. The mechanism of cellulase degradation of cellulose is presented in Graph 1 below.




GRAPH 1. The mechanism of enzymatic hydrolysis by cellulase (adapted from Answers 2012)

B-glucosidase is considered to be a rate-limiting step in the enzyme hydrolysis as it breaks cellobiose and in case of cellobiose accumulation endoglucanase and cellobiohydrolase activity is inhibited. (Kumar, Singh & Singh 2008; Khanal & Surampalli 2010, 204-205.)

### 5.2 Multi enzyme complexes

Some catalyses are performed by a group of enzymes that are bonded to each other and form a complex (Kulkarni & Deshpande 2007, 17). Mixes of enzymes can form complex or non-complex systems. Non-complex system can be described as a system which possesses components that act rather discretely than as a stable complex. For example, fungal cellulases are non-complexed systems, while anaerobic microorganisms possess complexed cellulase systems. It means when fungal cellulase complex acts on cellulose three processes occur simultaneously as cellulase complex contains three enzyme systems. Complex and non-complex systems should not be confused with synergism. Synergism occurs when the activity of different components in a system is greater than the activity of each component acting separately. There are a few types of synergism acting in a cellulase complex. They are between endoglucanases, exoglucanases, endo- and exoglucanases, endoglucanases, exoglucanases and β-glucosidases. (Zhang & Lynd 2004.)

### 5.3 Mechanism of enzymatic hydrolysis

A widely accepted mechanism of enzymatic hydrolysis is a parallel one. Three processes occur simultaneously. The first one is chemical and physical changes in not yet solubilised cellulose which is in a solid state. The other two are primary and secondary hydrolysis. The primary hydrolysis involves the release of soluble intermediates and the secondary one involves hydrolysis of released intermediates into intermediates of lower molecular weight. Chemical changes in cellulose are performed by endoglucanases. They attack amorphous regions of interior part of cellulose producing cello-oligomers and increasing the concentration of chain ends. Exoglucanases shorten the chain ends which are attacked by β-glucosidases and are broken down to cellobiose and glucose. At the same time, the

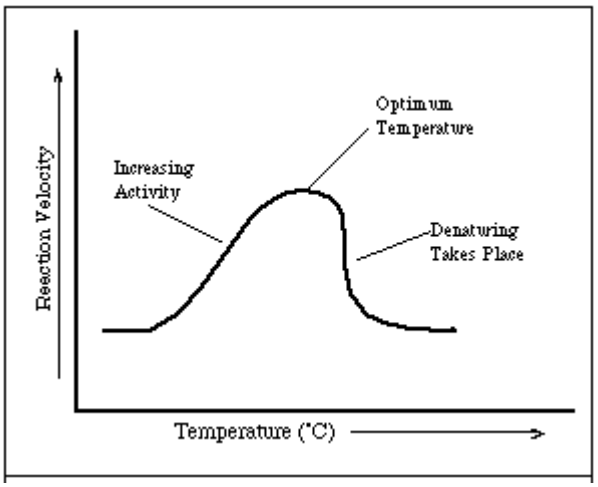
crystalline regions of cellulose are attacked by cellobiohydrolase and endoglucanase resulting in cellobiose. Cellobiase convert cellobiose into glucose units. The main requirement to the successful hydrolysis is the presence of both exo- and endoglucanase along with β-glucosidase enzymes. (Zhand & Lynd 2004; Khanal & Surampalli 2010, 204-205.)

The kinetics of reaction depends on several factors which are the following: enzyme adsorption to the substrate, formation of intermediate complex between enzymes and substrate, degradation rates, product inhibitions, and crystallinity of cellulose samples. Taking into account all the factors it is difficult to predict the extent of synergism between cellulase and substrate. (Khanal & Surampalli 2010, 204-205.)

# 6 PROCESS PARAMETERS

## 6.1 Temperature

Temperature has a significant effect on the conversion of lignocellulosic biomass. Generally, like in most chemical reactions, the rate of enzyme-catalyzed reaction increases as the temperature rises. Variations in temperature in 1 or 2 degrees may introduce changes of about 10-20% in the reaction. However, every enzyme has a range of temperature for optimal activity. Outside the range the activity of enzyme is inhibited and may be fully stopped. As presented in Graph 2 below, enzyme activity increases with the rise of temperature and reaches the maximum level. With the further increase of temperature the activity of enzymes declines abruptly.




GRAPH 2. The correlation between temperature change and enzyme activity (adapted from Worthington Biochemical Corporation 2012)

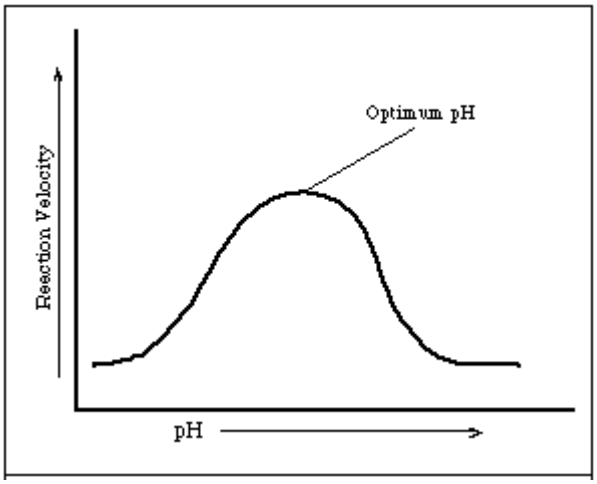
The activity of enzyme declines due to the fact that temperature rise supplies enough thermal energy to break some of the intermolecular attractions between polar molecules like hydrogen bonding, dipole-dipole attractions, ionic interactions and hydrophobic forces between non-polar groups within the enzyme structure. As a result, enzymes become denatured. Thus, enzymes lose their activity when frozen. The optimum temperatures for cellulase activity are within the range of 50-65°C. Moreover, the temperature has an influence on cellulase absorption which is positive at temperatures below 60°C. However, it was analyzed that the maximum absorption of cellulases occurred at temperature 50°C.

(Kumar 2008; Shanmugam & Sathishkuma 2009, 73-75; Worthington Biochemical Corporation 2012.)

### 6.2 pH of the environment

By nature enzymes are amphoteric containing a large amount of acidic and basic groups that are situated on their surface as well as interior in the molecule. The charges on these groups will vary, according to their dissociation constants with the pH of the environment. In particular pH the net charge of the enzyme molecule is zero. This pH is called isotonic point. Naturally enzyme molecule will be cationic when the pH of the environment is lower than its isoelectric pH and anionic when the pH is higher. The change in pH affects the polar and non-polar intramolecular attractive and repulsive interactions, therefore, the shape and active site of the enzyme will be changed and the substrate molecule cannot fit. Every enzyme has an optimum pH range beyond the limit of which the enzymatic activity will be inhibited. (Shanmugam & Sathishkumar 2009, 77.)

Graph 3 below illustrates the variations in enzyme activity rate within the change of pH of environment.




GRAPH 3. The correlation between pH change and enzyme activity (adapted from Worthington Biochemical Corporation 2012)

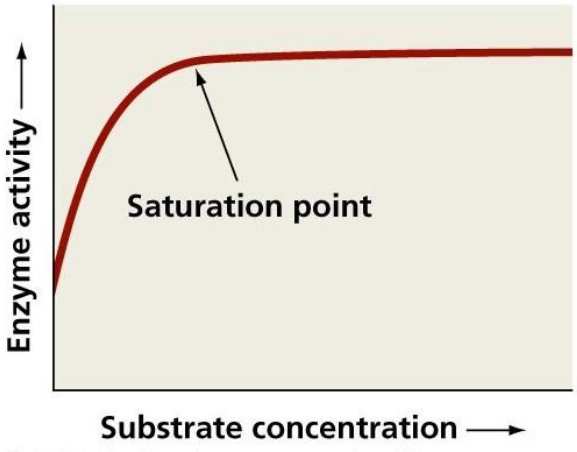
As it can be seen, extremely low or high pH value results in the loss of enzyme activity. Also there is an optimum pH level for every enzyme which is mostly favourable for the given enzyme activity. Optimal level of pH can differ greatly for different enzymes.

Optimum pH for cellulase activity is within the range 4.2-5.2 as cellulase is a complex of enzymes and each of those has slightly different optimum pH range. For this reason it is necessary to optimize the pH of environment for enzyme complex so that the enzymatic hydrolysis would be accurate and reproducible. (Worthington Biochemical Corporation 2012.)

### 6.3 Substrate level

For any enzyme mediated catalysis, the rate of the reaction is directly proportional to the concentration of the substrate. At low substrate concentration the initial velocity of the reaction is directly proportional and at high substrate concentration the velocity of the reaction remains constant following a zero order reaction rate. (Shanmugam & Sathishkumar 2009, 78-79.)

The correlation between substrate level and enzyme activity is illustrated in Graph 4 below.



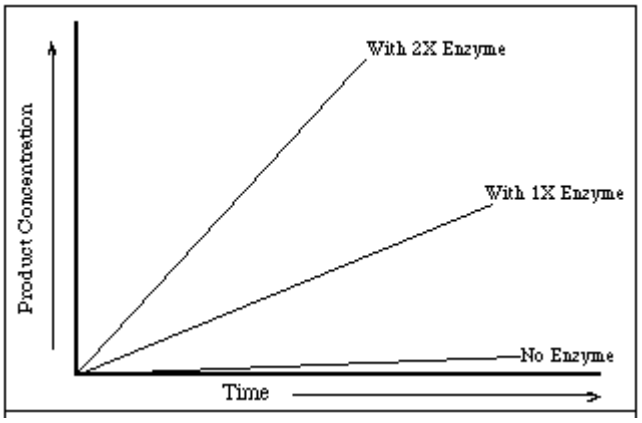

GRAPH 4. The correlation between substrate level and enzyme activity (adapted from Worthington Biochemical Corporation 2012)

It can be seen that in case of increase of substrate level, the reaction velocity will increase, too. However, the reaction velocity reaches its maximum at some point and the further increase of substrate level will not influence the speed of reaction. It should be mentioned that the amount of enzyme is kept constant. (Worthington Biochemical Corporation 2012.)

### 6.4 Enzyme loading

Enzyme loading is also referred to as enzyme concentration. The changes in product formed will be dependant solely on enzyme concentration only if the substrate is in excess amount. In this case, the formation of product proceeds at a rate linear with time. Allowing reaction to proceed double time will results in double amount of product. The activity catalyzed by enzyme is proportional to the amount of enzyme present only if other factors like temperature or pH are favourable and do not affect the relationship between activity of catalysis and enzyme concentration. (Shanmugam & Sathishkumar 2009, 79.)

In Graph 5 below it can be seen that "zero order" reaction rate is independent of substrate concentration.




GRAPH 5. Zero order rate reaction independent on substrate concentration (adapted from Worthington Biochemical Corporation 2012)

Also, according to the graph, the product will be formed at a rate linear with time. It is absolutely necessary that other parameters that affect the rate of reaction are in their optimum range. In this case, the enzyme concentration is the only limiting factor and the reaction is of zero order. As a rule, the best enzyme activity is observed when substrate concentration is unlimiting. (Worthington Biochemical Corporation 2012.)

# 7 EXPERIMENTAL

## 7.1 IL preptreatment

The oil bath was preheated to 100°C. The dry fibre sludge was weighed. The mass used was 0.1 g. Dry fibre sludge is illustrated in Graph 6 below.

GRAPH 6. Dry fibre sludge (adapted from Holm & Lassi 2011)

Afterwards, 0.5 g of ionic liquid was weighed in Erlenmeyer bulb. Then fibre sludge was added along with the magnetic stirrer. Erlenmeyer bulbs were connected to the condenser and placed into preheated oil. The pretreatment was done within 30 minutes. Afterwards, 20 ml of deionized water was added to the flask and it was shaked for 2 minutes to mix ionic liquid with anti-solvent. The flasks were left for 15 minutes to recover cellulose. Afterwards, the filtration was done and cellulose was left to dry overnight.

Some of pretreatment procedureds are done with the reduced amount of dry fibre sludge and ionic liquid. The amount of ionic liquid and fibre sludge taken was twice less comparing to the initial amount. Consecutive enzymatic hydrolysis also implied the use of reduced amount of buffer and enzyme concentration proportionally.

## 7.2 Enzymatic hydrolysis

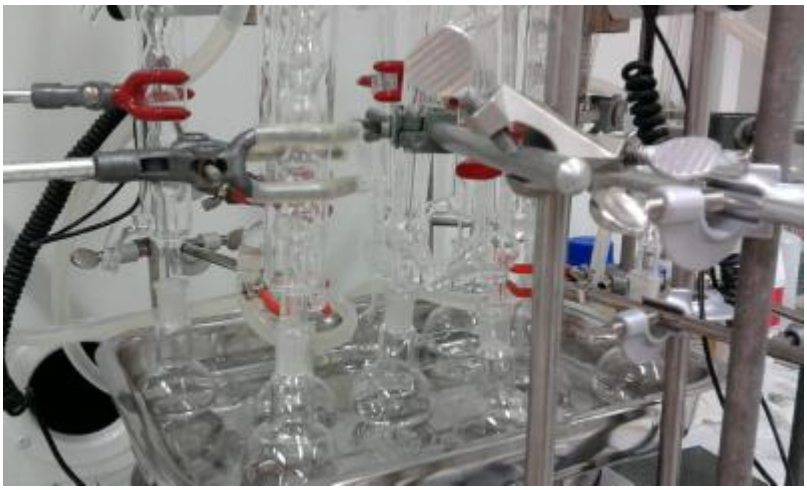
To perform enzymatic hydrolysis the following was needed: citric buffer, enzyme mix and fibre sludge.

### 7.2.1 Citric buffer preparation

Citric buffer 0.1 M with pH 5.00 containing citric acid 0.1 M and sodium citrate 0.1 M was prepared in the following way: 40 ml of 0.1 M citric acid mixed with 80 ml of 0.1 M sodium citrate resulted in buffer with pH 5.05.
Citric acid 0.1 M preparation: 250 ml of 0.1 M citric acid contains 5.2555 g of citric acid.
Sodium citrate 0.1 M preparation: 250 ml of 0,1 M sodium citrate contains 7.3532 g of sodium citrate.

### 7.2.2 Procedure of enzymatic hydrolysis

Dried fibre sludge sample was weighed and placed into Erlenmeyer bulb. Citric buffer was added to the Erlenmeyer bulb as well as magnetic stirrer. Enzymes were added just before placing the Erlenmeyer bulb into the oil bath and connecting it to the condenser. Oil bath was preheated to maintain 50° C, and allowed placing of 5-6 Erlenmeyer bulbs for simultaneous hydrolysis. Enzymatic hydrolysis was being done with gentle stirring at 150 rpm. The system for hydrolysis can be seen from Graph 7 below.



GRAPH 7. The system designed for performing the enzymatic hydrolysis

Samples for DNS and glucose assay were taken within a certain time interval. Depending on the experiment, one or two samples were taken from one Erlenmeyer bulb. If two samples were taken out from the same bulb, then after taking the first sample the same amount of buffer was added. Each sample was pipetted into Eppendorf tube and immediately placed into boiling water for about 5 minutes to stop enzymatic activity. Samples were further analyzed for total reducing sugars and glucose content with DNS and glucose assays.

### 7.3 DNS assay

DNS assay allows measuring the concentration of total reducing sugars obtained as a result of enzymatic hydrolysis of lignocellulosic biomass. It is the most common assay for determination of accumulation of reducing sugars.

#### 7.3.1 DNS reagent preparation

125 ml of water was heated to approximately 50°C in an Erlenmeyer flask. Afterwards, 45.5 g of potassium sodium tartrate, 1.575 g of 3.5-dinitrosalysilic acid and 65.5 ml of 2 M sodium hydroxide are added. When all the reagents are dissolved, 1.25 g of phenol and 1.25 g of sodium sulphate are added and the mixture was being stirred until the solution

was homogeneous. The solution was cooled down to room temperature and diluted with deionized water to make 250 ml of DNS reagent. The reagent was stored in an amber bottle in the refrigerator. (Miller 1959.)

### 7.3.2 DNS assay procedure

Depending on the experiment, different dilution ratios were used for the assay. An amount of 0.1 ml or 0.01ml of sample was pipetted into a tube. Afterwards, distilled water was added to have the total volume of the sample 0.3 ml. Correspondingly, 0.2 ml of distilled water in the first case and 0.99 ml in the second one. Then 0.3 ml of DNS reagent was added into each tube with separate sample and mixed. Also, for blank sample, 0.3 ml of water was taken and 0.3 ml of DNS reagent was added. All the tubes were placed into boiling water for exactly 5 minutes. The initial bright yellow colour of the mixture of DNS reagent and sample turned into brown colour. The intensity of brown colour indicated the level of total reducing sugars in the sample. Afterwards, the colour intensity was measured with spectrophotometer at wavelength 540 nm. (Miller 1959.)

## 7.4 Glucose assay

Glucose assay allows measuring the concentration of glucose apart from total reducing sugars in the given sample. As obtaining the maximum concentration of glucose is one of the aims of the conducted experiments, glucose assay reveals the final outcomes of the research.

### 7.4.1 Glucose assay reagent preparation

For glucose assay the Glucose assay kit (product code GAGO-20, Sigma) was used. It is designed for quantitive, enzymatic determination of glucose in food and other materials. Assay reagent contained mixed beforehand 0,8 ml of o-Dianisidine reagent and 39,2 ml of

Glucose oxidize/peroxidise reagent. The quantity of assay reagent was sufficient for 20 glucose assays.

The mechanism of glucose assay kit is the following:

$$D\text{-}Glucose + H_2O + O_2 \xrightarrow{glucose\ oxidase} D\text{-}Gluconic\ Acid + H_2O$$

$$H_2O + reduced\ o\text{-}Dianisidine\ (colourless) \xrightarrow{peroxidase} oxidized\ o\text{-}Dianisidine\ (brown)$$

$$Oxidized\ o\text{-}Dianisidine \xrightarrow{H_2SO_4} oxidized\ o\text{-}dianisidine\ (pink)$$

Glucose is oxidized to gluconic acid and hydrogen peroxide by glucose oxidase. Hydrogen peroxide reacts with o-dianisidine in the presence of peroxide to form a colored product. Oxidised o-dianisidine reacts with sulfuric acid to form a more stable colored product. The intensity of the pink colour is measured at 540 nm in spectrophotometer is proportional to the original concentration of glucose in the product. (Sigma 2012.)

### 7.4.2 Procedure of glucose assay

Standards were prepared in order to obtain standard curve on spectrophotometer for further analysis. The Table 1 shows the proportion of glucose standard solution (D-glucose, 1.0 mg/ml in 0.1% benzoic acid, product code G 3285, Sigma) and distilled water taken for each standard.

TABLE 1. Standards solutions preparation

| Tube | Water (ml) | Glucose standard (ml) |
|---|---|---|
| **Standard #1** | 0.98 | 0.02 |
| **Standard #2** | 0.96 | 0.04 |
| **Standard #3** | 0.94 | 0.06 |
| **Standard #4** | 0.92 | 0.08 |
| **Reagent blank** | 1.00 | --- |

Test samples were prepared by diluting the samples taken during the enzymatic hydrolysis with distilled water in the following way:

At zero time, 2.0 ml of assay reagent was added to the first tube, mixed and placed into water bath at 37C. With 1 minute interval, assay reagent was added into each tube and tubes were placed into water bath for exactly 30 minutes. While in the water bath, the samples were changing colour to reddish yellow. In 30 minutes, starting from the first tube, the reaction was stopped by adding 2 ml of 6M $H_2SO_4$ into each tube and then mixed. On adding the sulfuric acid, the samples changed their colour to pink. Afterwards, the absorbance of each sample was measured in spectrophotometer (See APENDIX 1) against the reagent blank. (Sigma 2012.)

### 7.5 Enzymatic hydrolysis with enzyme mix A

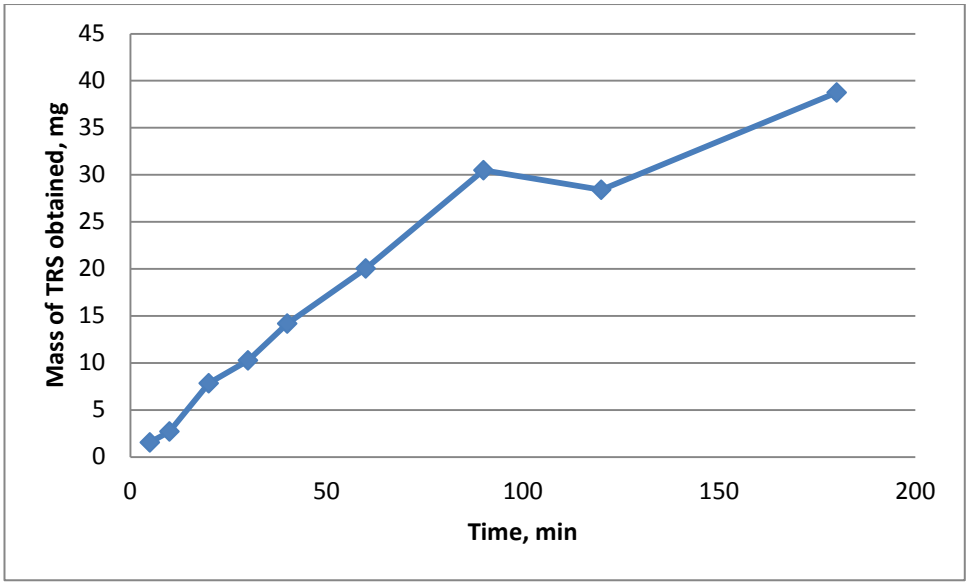
#### 7.5.1 Experiment 1

Samples of 0.1 g of fibre sludge were pretreated with 5 g of ionic liquid [AMIM]Cl. The yield of pretreated fibre sludge was in the range 81-90 % of initial mass of fibre sludge. The volume of citric buffer with pH 5.0 taken was 9.8 ml. Volume of enzyme mix A taken was 0.2 ml. Samples were taken with the automatic pipette within a certain time interval. DNS assay was performed and results are presented in Table 2 below.

TABLE 2. Outcome of TRS in experiment 1

| Sample | Time, min | Concentration, mg/ml | Mass of TRS obtained, mg | Percentage of intitial FS mass, % |
|---|---|---|---|---|
| 1 | 5 | 0.171 | 1.539 | 1.5 |
| 2 | 10 | 0.3 | 2.7 | 2.7 |
| 3 | 20 | 0.87 | 7.83 | 7.8 |
| 4 | 30 | 1.14 | 10.26 | 10.3 |
| 5 | 40 | 1.575 | 14.175 | 14.2 |
| 6 | 60 | 2.226 | 20.034 | 20.0 |
| 7 | 90 | 3.387 | 30.483 | 30.5 |
| 8 | 120 | 3.156 | 28.404 | 28.4 |
| 9 | 180 | 4.305 | 38.745 | 38.7 |

Mass of total reducing sugars obtained is plotted against processing time of hydrolysis in Graph 9 below.



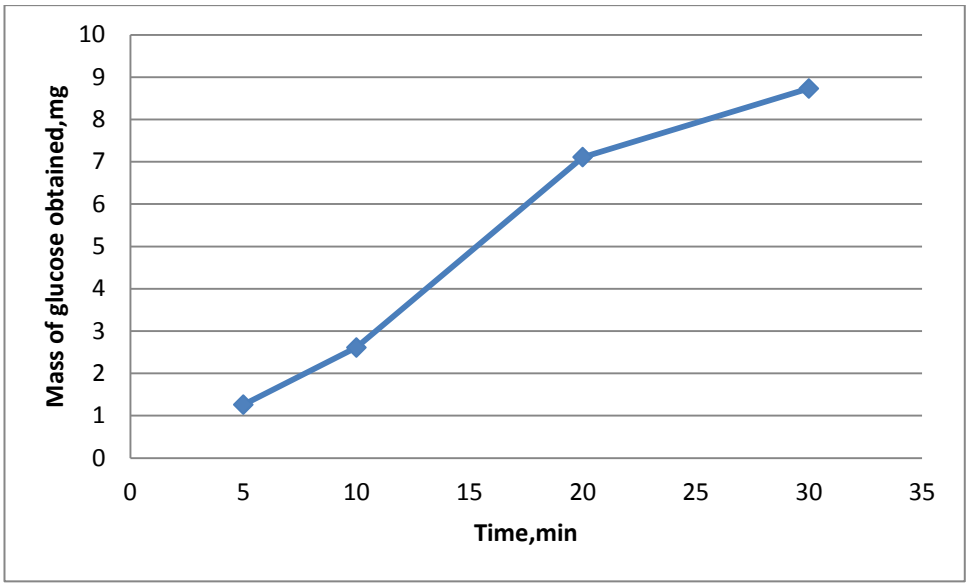

GRAPH 9. DNS assay outcome for experiment 1 with enzyme mix A

Also, glucose assay of the samples was performed. In glucose assay the results starting from 40 minutes time were not available as the concentration of glucose was high and they were beyond the concentrations of the standard curve. The samples should have been diluted more for their glucose concentration to be detectable. The glucose concentration of 4 samples is presented in Table 3 below.

TABLE 3. Outcome of glucose assay in experiment 1 with enzyme mix A

| Sample | Time, min | Concentration, mg/ml | Mass of glucose, mg | Percentage of TRS mass, % |
|---|---|---|---|---|
| 1 | 5 | 0.14 | 1.26 | 1.3 |
| 2 | 10 | 0.29 | 2.61 | 2.6 |
| 3 | 20 | 0.79 | 7.11 | 7.1 |
| 4 | 30 | 0.97 | 8.73 | 8.7 |

Concentration of glucose in samples was plotted against processing time of hydrolysis and presented in the Graph 10 below.




GRAPH 10. Glucose assay outcome for experiment 1

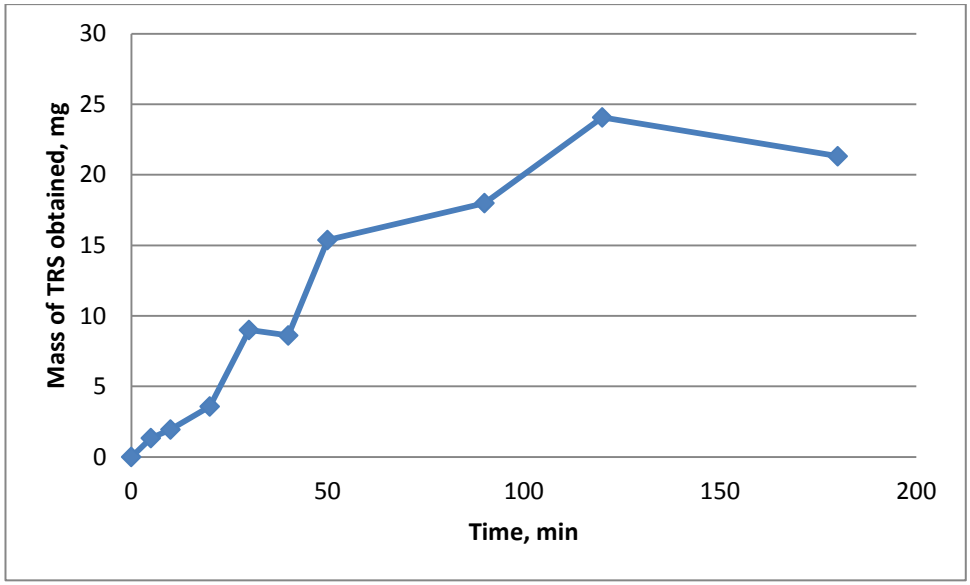
### 7.5.2 Experiment 2 with reduced reagents by half

Samples of 0.05 g of fibre sludge were pretreated with 2.5 g of ionic liquid [AMIM]Cl. The yield of pretreated fibre sludge was in the range 40-55 % of initial mass of fibre sludge. The volume of citric buffer with pH 5.0 taken was 4.9 ml. Enzyme mix used was Enzyme mix A, volume taken was 0.1 ml. Samples were taken within a certain time interval and results of TRS assay are presented below in Table 4.

TABLE 4. Outcome of TRS in experiment 2

| Sample | Time, min | Concentration, mg/ml | Mass of TRS obtained, mg | Percentage of intitial FS mass, % |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 1 | 5 | 0.297 | 1.337 | 2.7 |
| 2 | 10 | 0.432 | 1.944 | 3.9 |
| 3 | 20 | 0.795 | 3.578 | 7.2 |
| 4 | 30 | 2.000 | 9.000 | 18.0 |
| 5 | 40 | 1.914 | 8.613 | 17.2 |
| 6 | 50 | 3.417 | 15.377 | 30.8 |
| 7 | 60 | 2.400 | 10.800 | 21.6 |
| 8 | 90 | 3.999 | 17.996 | 36.0 |
| 9 | 120 | 5.349 | 24.071 | 48.1 |
| 10 | 180 | 4.737 | 21.317 | 42.6 |

Mass of total reducing sugars obtained was plotted against processing time of hydrolysis in Graph 11 below.



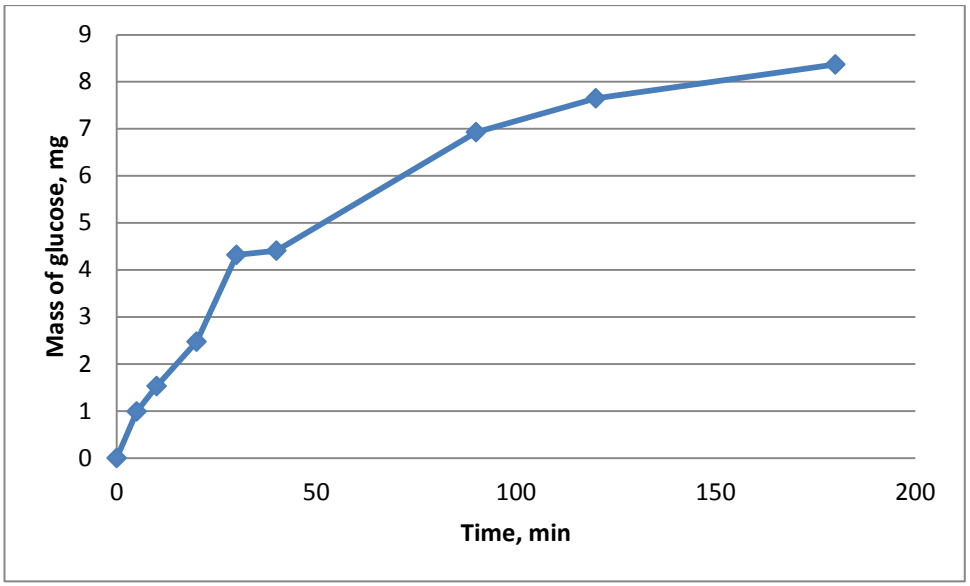

GRAPH 11. DNS assay outcome for experiment 2

Glucose assay of the samples was performed as well. Concentrations of glucose obtained are presented in Table 5 as well as processing time of hydrolysis.

TABLE 5. Outcome of glucose in experiment 2

| Sample | Time, min | Concentration, mg/ml | Mass of glucose, mg | Percentage of TRS mass, % |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 1 | 5 | 0.22 | 0.99 | 2.0 |
| 2 | 10 | 0.34 | 1.53 | 3.1 |
| 3 | 20 | 0.55 | 2.475 | 5.0 |
| 4 | 30 | 0.96 | 4.32 | 8.6 |
| 5 | 40 | 0.98 | 4.41 | 8.8 |
| 8 | 90 | 1.54 | 6.93 | 13.9 |
| 9 | 120 | 1.7 | 7.65 | 15.3 |
| 10 | 180 | 1.86 | 8.37 | 16.7 |

Concentration of glucose in samples was plotted against processing time of hydrolysis and presented in Graph 12 below.




GRAPH 12. Glucose assay outcome for experiment 2

### 7.6 Enzymatic hydrolysis with enzyme mix B

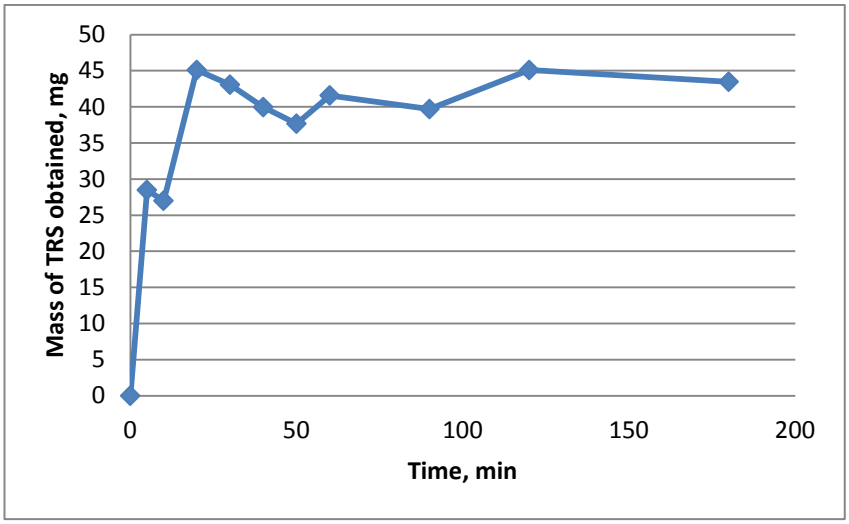
#### 7.6.1 Experiment 1

Samples of 0.05 g of fibre sludge were pretreated with 2.5 g of ionic liquid [AMIM]Cl. The yield of pretreated fibre sludge was around 40 % of initial mass of fibre sludge. Citric buffer with pH 5.0, volume used was 4.9 ml. Volume of enzyme mix B taken was 0.1 ml and volume of β-glucosidase taken was 0.004 ml. Samples of volume 0.3 ml were taken during the hydrolysis within a certain time interval and results of DNS assay are presented below in Table 6.

TABLE 6. Outcome of TRS in experiment 1

| Sample | Time, min | Concentration, mg/ml | Mass of TRS obtained, mg | Percentage of intitial FS mass, % |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 1 | 5 | 6.33 | 28.485 | 57.0 |
| 2 | 10 | 6 | 27 | 54.0 |
| 3 | 20 | 10.02 | 45.09 | 90.2 |
| 4 | 30 | 9.57 | 43.065 | 86.1 |
| 5 | 40 | 8.88 | 39.96 | 79.9 |
| 6 | 50 | 8.37 | 37.665 | 75.3 |
| 7 | 60 | 9.24 | 41.58 | 83.2 |
| 8 | 90 | 8.82 | 39.69 | 79.4 |
| 9 | 120 | 10.02 | 45.09 | 90.2 |
| 10 | 180 | 9.66 | 43.47 | 86.9 |

Mass of total reducing sugars obtained was plotted against processing time of hydrolysis in Graph 13 below.



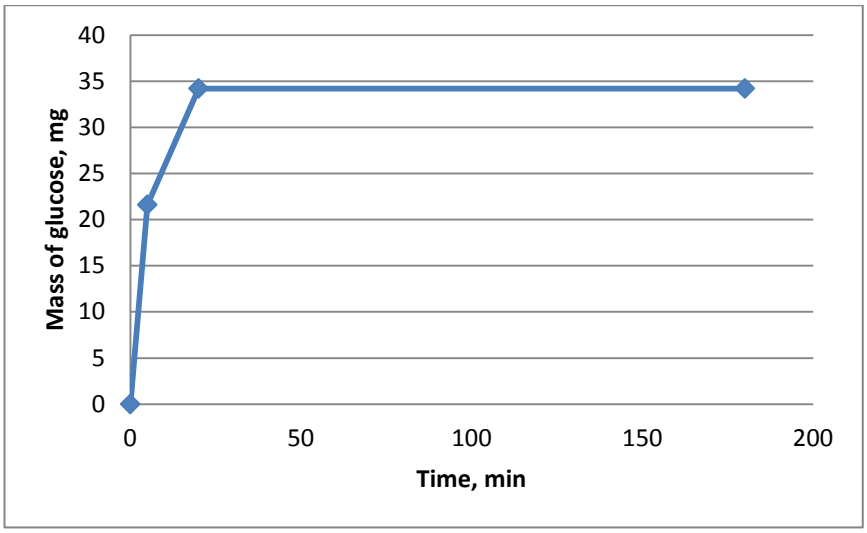

GRAPH 13. DNS assay outcome for experiment 1

Glucose assay of the samples was performed as well. Concentrations of glucose obtained are presented in Table 7 below as well as processing time of hydrolysis.

TABLE 7. Outcome of glucose in experiment 1

| Sample | Time, min | Concentration, mg/ml | Mass of glucose, mg | Percentage of TRS mass, % |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 1 | 5 | 4.8 | 21.6 | 43.2 |
| 3 | 20 | 7.6 | 34.2 | 68.4 |
| 10 | 180 | 7.6 | 34.2 | 68.4 |

Concentration of glucose in samples was plotted against processing time of hydrolysis and presented in Graph 14 below.




GRAPH 14. Glucose assay outcome for experiment 1

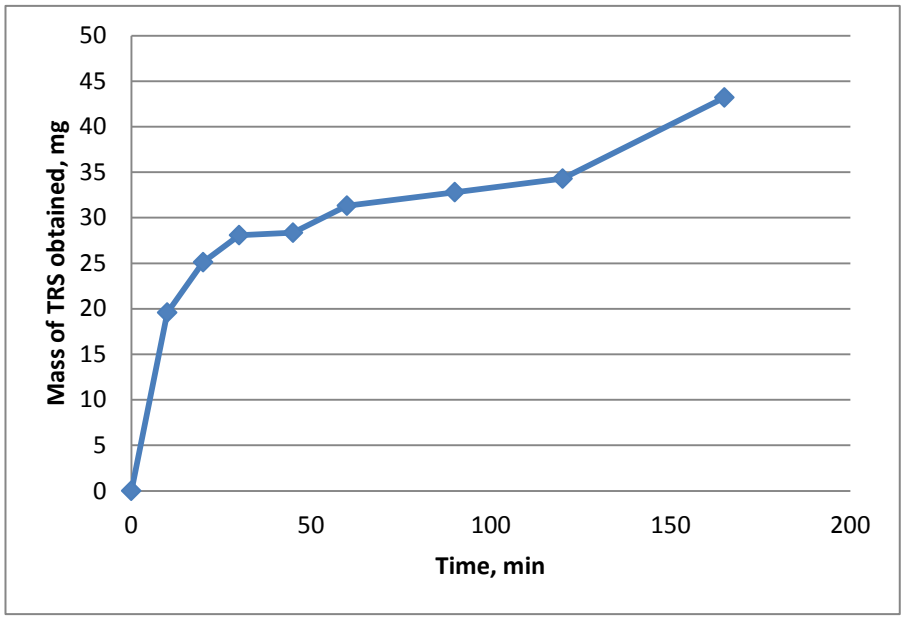
### 7.6.2 Experiment 2 with reduced amount of enzyme mix B by half

Samples of 0.05 g of fibre sludge were pretreated with 2.5 g of ionic liquid [AMIM]Cl. The yield of pretreated fibre sludge was around 80-85% of initial mass of fibre sludge. Citric buffer used of pH 5.04, volume used was 4.95 ml. Volume of enzyme mix B taken was 0.05 ml and volume of β-glucosidase taken is 0.004 ml. Samples of volume 0.1 ml were taken during the hydrolysis within a certain time interval. Sampling was done twice from the same Erlenmeyer bulb. After the first sampling, citric buffer of the same volume 0.1 ml was added. Results of DNS assay are presented in Table 9.

TABLE 9. Outcome of TRS in experiment 2

| Sample | Time, min | Concentration, mg/ml | Mass of TRS obtained, mg | Percentage of intitial FS mass, % |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 1 | 10 | 4.35 | 19.575 | 39.2 |
| 2 | 20 | 5.58 | 25.11 | 50.2 |
| 3 | 30 | 6.24 | 28.08 | 56.2 |
| 4 | 45 | 6.3 | 28.35 | 56.7 |
| 5 | 60 | 6.96 | 31.32 | 62.6 |
| 6 | 90 | 7.29 | 32.805 | 65.6 |
| 7 | 120 | 7.62 | 34.29 | 68.6 |
| 8 | 165 | 9.6 | 43.2 | 86.4 |

Mass of total reducing sugars obtained was plotted against processing time of hydrolysis in Graph 15 below.



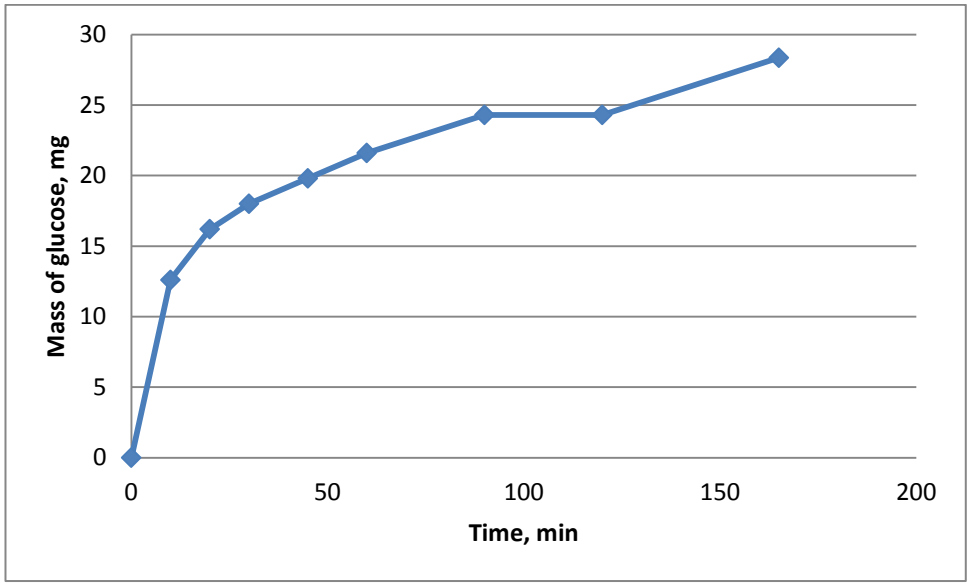

GRAPH 15. DNS assay outcome for experiment 2

Glucose assay of the samples was performed as well. Concentrations of glucose obtained are presented in Table 10 below as well as processing time of hydrolysis.

TABLE 10. Outcome of glucose in experiment 2

| Sample | Time, min | Concentration, mg/ml | Mass of glucose, mg | Percentage of TRS mass, % |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 1 | 10 | 2.8 | 12.6 | 25.2 |
| 2 | 20 | 3.6 | 16.2 | 32.4 |
| 3 | 30 | 4 | 18 | 36.0 |
| 4 | 45 | 4.4 | 19.8 | 39.6 |
| 5 | 60 | 4.8 | 21.6 | 43.2 |
| 6 | 90 | 5.4 | 24.3 | 48.6 |
| 7 | 120 | 5.4 | 24.3 | 48.6 |
| 8 | 165 | 6.3 | 28.35 | 56.7 |

Concentration of glucose in samples was plotted against processing time of hydrolysis and presented in Graph 16 below.




GRAPH 16. Glucose assay outcome for experiment 2

### 7.6.3 Experiment 3 with reduced amount of enzyme mix B by half

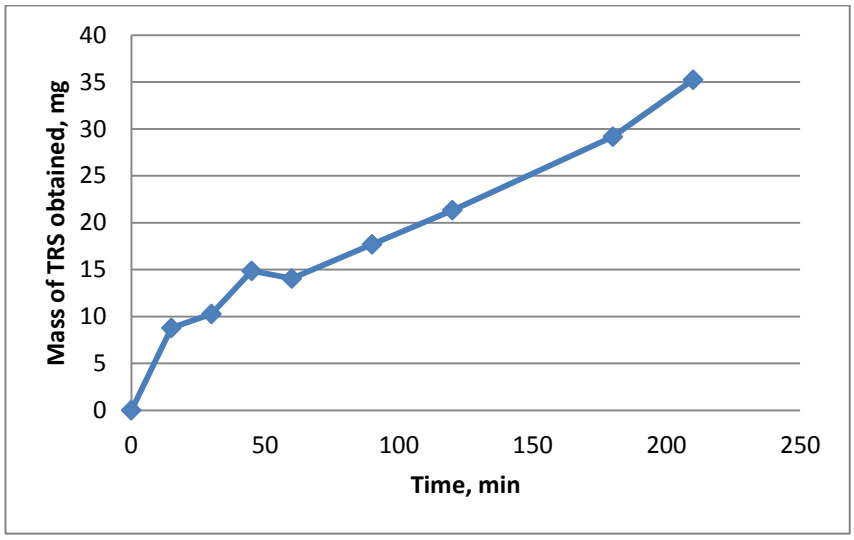
Samples of 0.05 g of fibre sludge were pretreated with 2.5 g of ionic liquid [AMIM]Cl. The yield of pretreated fibre sludge was around 85% of initial mass of fibre sludge. Citric buffer with pH 5.00, volume used was 4.97 ml. Volume of enzyme mix B taken was 0.025 ml and volume of β-glucosidase taken was 0.006 ml. Samples of volume 0.1 ml were taken

during the hydrolysis within a certain time interval. Sampling was done twice from the same Erlenmeyer bulb. After the first sampling, citric buffer of the same volume 0.1 ml was added. Results of DNS assay are presented in Table 11.

TABLE 11. Outcome of TRS in experiment 3

| Sample | Time, min | Concentration, mg/ml | Mass of TRS obtained, mg | Percentage of intitial FS mass, % |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 1 | 15 | 1.95 | 8.775 | 17.6 |
| 2 | 30 | 2.28 | 10.26 | 20.5 |
| 3 | 45 | 3.3 | 14.85 | 29.7 |
| 4 | 60 | 3.12 | 14.04 | 28.1 |
| 5 | 90 | 3.93 | 17.685 | 35.4 |
| 6 | 120 | 4.74 | 21.33 | 42.7 |
| 7 | 180 | 6.48 | 29.16 | 58.3 |
| 8 | 210 | 7.83 | 35.235 | 70.5 |

Mass of total reducing sugars obtained was plotted against processing time of hydrolysis in the Graph 17 below.



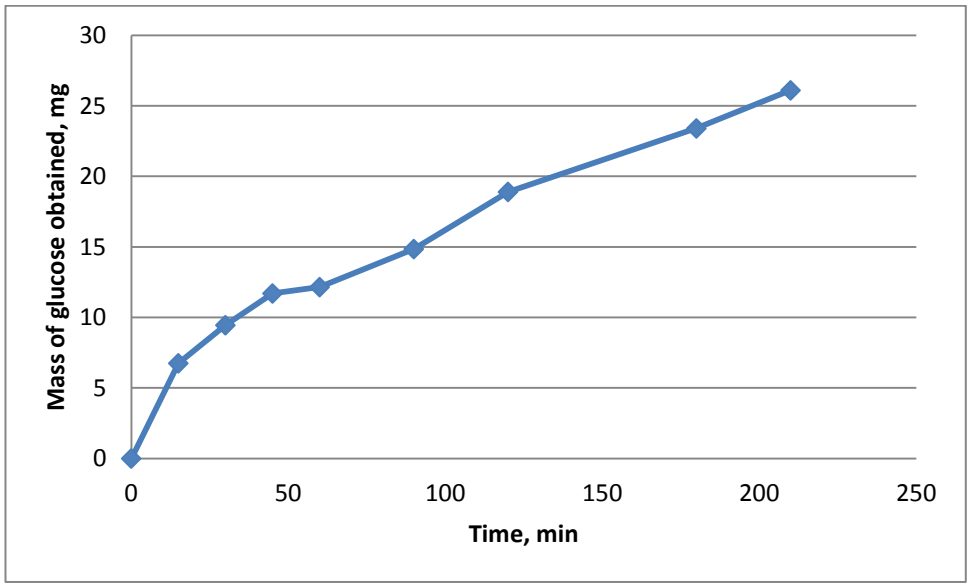

GRAPH 17. DNS assay outcome for experiment 3

Glucose assay of the samples was performed as well. Concentrations of glucose obtained are presented in Table 12 as well as processing time of hydrolysis.

TABLE 12. Outcome of glucose in experiment 3

| Sample | Time, min | Concentration, mg/ml | Mass of glucose, mg | Percentage of FS mass, % |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 1 | 15 | 1.5 | 6.75 | 13.5 |
| 2 | 30 | 2.1 | 9.45 | 18.9 |
| 3 | 45 | 2.6 | 11.7 | 23.4 |
| 4 | 60 | 2.7 | 12.15 | 24.3 |
| 5 | 90 | 3.3 | 14.85 | 29.7 |
| 6 | 120 | 4.2 | 18.9 | 37.8 |
| 7 | 180 | 5.2 | 23.4 | 46.8 |
| 8 | 210 | 5.8 | 26.1 | 52.2 |

Concentration of glucose in samples was plotted against processing time of hydrolysis and presented in Graph 18 below.




GRAPH 18. Glucose assay outcome for experiment 3

### 7.6.4 Experiment 4 with pH changed to 5.2

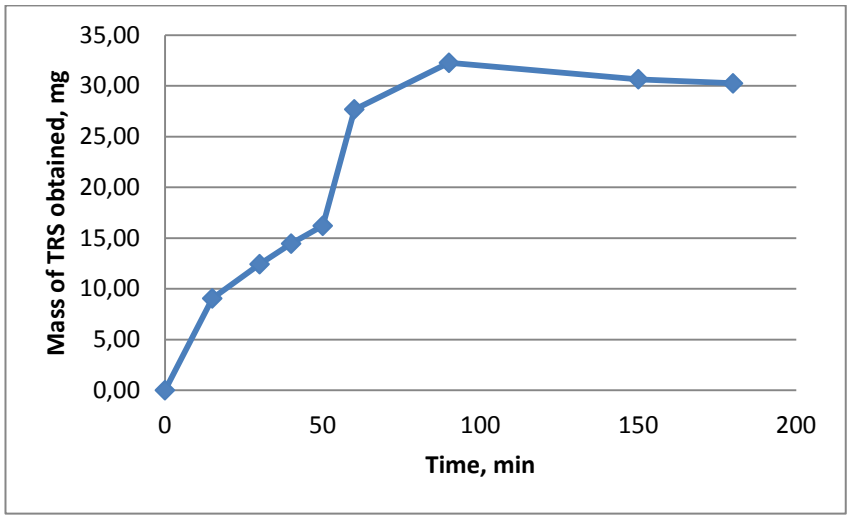
Samples of 0.05 g of fibre sludge were pretreated with 2.5 g of ionic liquid [AMIM]Cl. The yield of pretreated fibre sludge was around 85% of initial mass of fibre sludge. Citric buffer with pH 5.19, volume used is 4.97 ml. Volume of enzyme mix B taken was 0.025 ml and volume of β-glucosidase taken was 0.006 ml. Samples of volume 0.1 ml were taken

during the hydrolysis within a certain time interval. Sampling was done twice from the same Erlenmeyer bulb. After the first sampling, citric buffer of the same volume 0.1 ml was added. Results of DNS assay are presented in Table 13.

TABLE 13. Outcome of glucose in experiment 3

| Sample | Time, min | Concentration, mg/ml | Mass of TRS obtained, mg | Percentage of intitial FS mass, % |
|---|---|---|---|---|
| 0 | 0 | 0 | 0,00 | 0 |
| 1 | 15 | 2.01 | 9.05 | 18.1 |
| 2 | 30 | 2.76 | 12.42 | 24.8 |
| 3 | 40 | 3.21 | 14.45 | 28.9 |
| 4 | 50 | 3.6 | 16.20 | 32.4 |
| 5 | 60 | 6.15 | 27.68 | 55.4 |
| 6 | 90 | 7.17 | 32.27 | 64.5 |
| 7 | 150 | 6.81 | 30.65 | 61.3 |
| 8 | 180 | 6.72 | 30.24 | 60.5 |

Mass of total reducing sugars obtained is plotted against processing time of hydrolysis in the Graph 19 below.



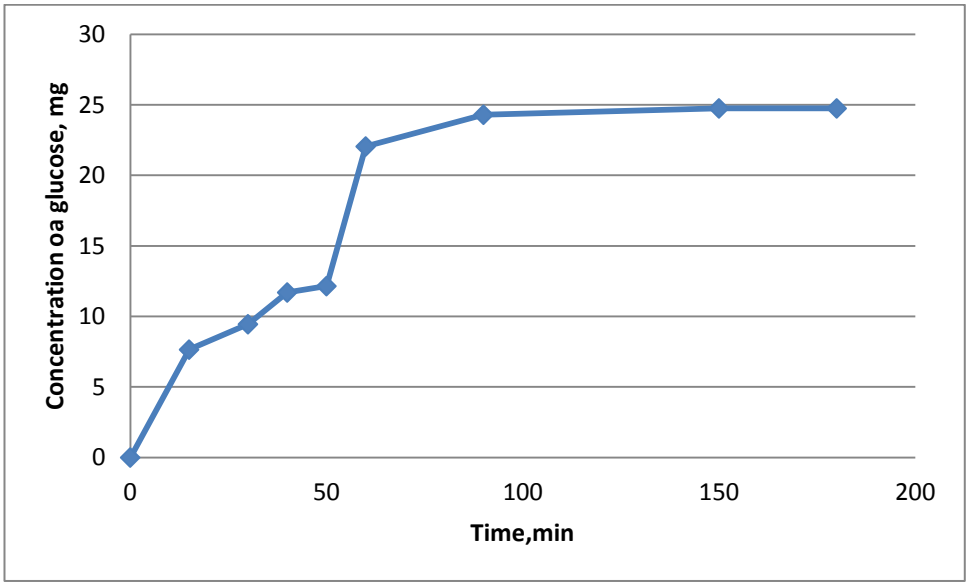

GRAPH 19. DNS assay outcome for experiment 4

Glucose assay of the samples was performed as well. Concentrations of glucose obtained are presented in the Table 14 as well as processing time of hydrolysis.

TABLE 14. Outcome of glucose in experiment 4

| Sample | Time, min | Concentration, mg/ml | Mass of glucose, mg | Percentage of FS mass, % |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 1 | 15 | 1.7 | 7.65 | 15.3 |
| 2 | 30 | 2.1 | 9.45 | 18.9 |
| 3 | 40 | 2.6 | 11.7 | 23.4 |
| 4 | 50 | 2.7 | 12.15 | 24.3 |
| 5 | 60 | 4.9 | 22.05 | 44.1 |
| 6 | 90 | 5.4 | 24.3 | 48.6 |
| 7 | 150 | 5.5 | 24.75 | 49.5 |
| 8 | 180 | 5.5 | 24.75 | 49.5 |

Concentration of glucose in samples was plotted against processing time of hydrolysis and presented in Graph 20 below.




GRAPH 20. Glucose assay outcome for experiment 4

### 7.6.5 Experiment 5 with pH changed to 5.5

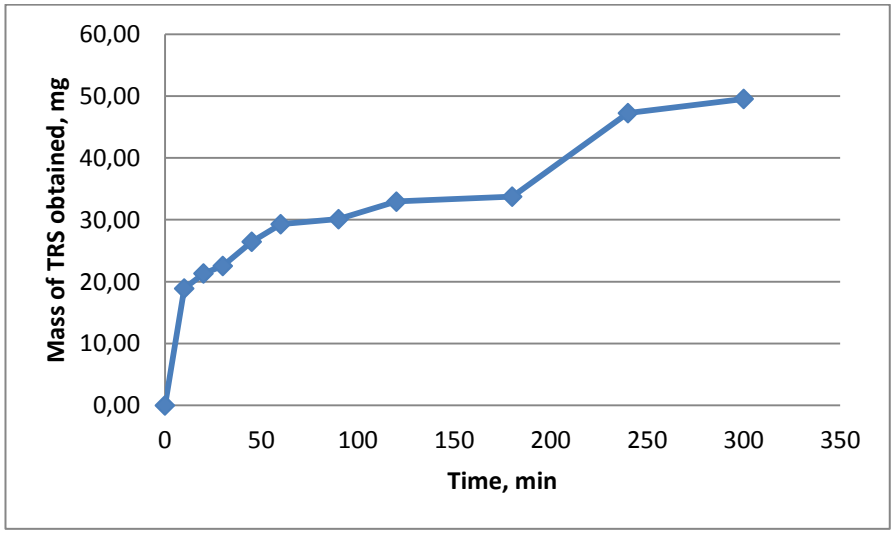
Samples of 0.05 g of fibre sludge were pretreated with 2.5 g of ionic liquid [AMIM]Cl. The yield of pretreated fibre sludge was around 85% of initial mass of fibre sludge. Citric buffer with pH 5.49, volume used was 4.95 ml. Volume of enzyme mix B taken was 0.05 ml and volume of β-glucosidase taken was 0.006 ml. Samples of volume 0.1 ml were taken

during the hydrolysis within a certain time interval. Sampling was done twice from the same Erlenmeyer bulb. After the first sampling, citric buffer of the same volume 0.1 ml was added. Results of DNS assay are presented in Table 15.

TABLE 15. Outcome of TRS in experiment 5

| Sample | Time, min | Concentration, mg/ml | Mass of TRS obtained, mg | Percentage of intitial FS mass, % |
|---|---|---|---|---|
| 0 | 0 | 0 | 0,00 | 0 |
| 1 | 10 | 4.2 | 18.90 | 37.8 |
| 2 | 20 | 4.74 | 21.33 | 42.7 |
| 3 | 30 | 5.01 | 22.55 | 45.1 |
| 4 | 45 | 5.88 | 26.46 | 52.9 |
| 5 | 60 | 6.51 | 29.30 | 58.6 |
| 6 | 90 | 6.69 | 30.11 | 60.2 |
| 7 | 120 | 7.32 | 32.94 | 65.9 |
| 8 | 180 | 7.5 | 33.75 | 67.5 |
| 9 | 240 | 10.5 | 47.25 | 94.5 |
| 10 | 300 | 11.01 | 49.55 | 99.1 |

Mass of total reducing sugars obtained was plotted against processing time of hydrolysis in Graph 21 below.



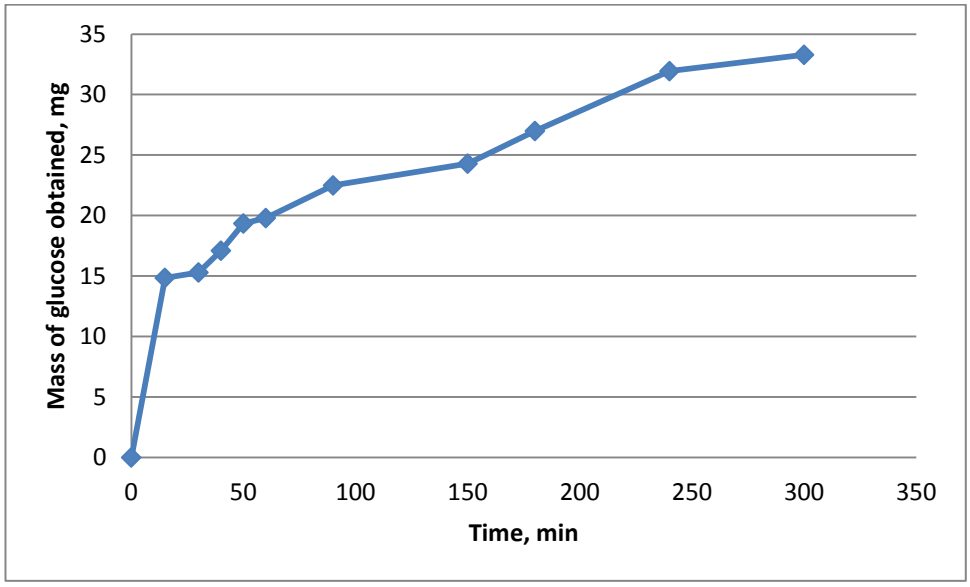

GRAPH 21. DNS assay outcome for experiment 5

Glucose assay of the samples was performed as well. Concentrations of glucose obtained are presented in Table 16 below as well as processing time of hydrolysis.

TABLE 16. Outcome of glucose in experiment 5

| Sample | Time, min | Concentration, mg/ml | Mass of glucose, mg | Percentage of FS mass, % |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 1 | 15 | 3.3 | 14.85 | 29.7 |
| 2 | 30 | 3.4 | 15.3 | 30.6 |
| 3 | 40 | 3.8 | 17.1 | 34.2 |
| 4 | 50 | 4.3 | 19.35 | 38.7 |
| 5 | 60 | 4.4 | 19.8 | 39.6 |
| 6 | 90 | 5 | 22.5 | 45.0 |
| 7 | 150 | 5.4 | 24.3 | 48.6 |
| 8 | 180 | 6 | 27 | 54.0 |
| 9 | 240 | 7.1 | 31.95 | 63.9 |
| 10 | 300 | 7.4 | 33.3 | 66.6 |

Concentration of glucose in samples was plotted against processing time of hydrolysis and presented in Graph 22 below.




GRAPH 22. Glucose assay outcome for experiment 5

### 7.6.6 Experiment 6 with increased amount of enzyme mix B and β-glucosidase

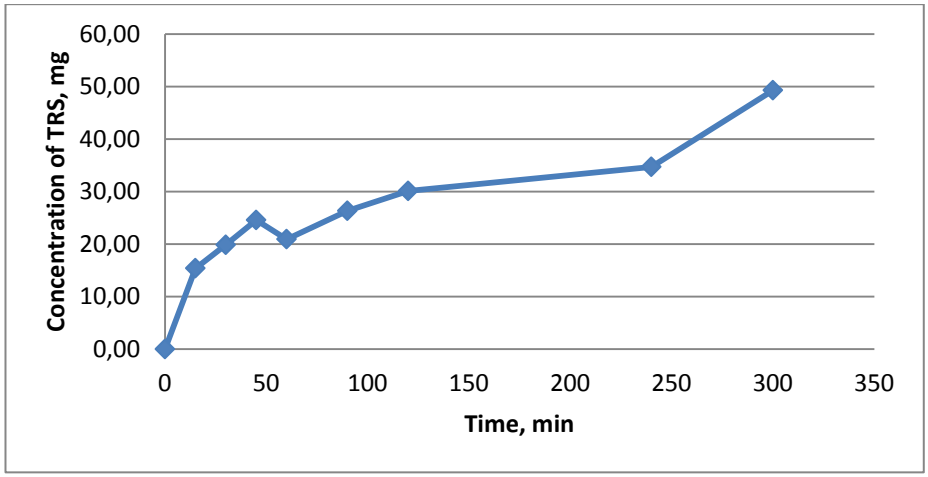
Samples of 0.05 g of fibre sludge were pretreated with 2.5 g of ionic liquid [AMIM]Cl. The yield of pretreated fibre sludge was around 85% of initial mass of fibre sludge. Citric

buffer with pH 5.00, volume used was 4.96 ml. Volume of enzyme mix B taken was 0.03 ml and volume of β-glucosidase taken was 0.01 ml. Samples of volume 0.1 ml were taken during the hydrolysis within a certain time interval. Sampling was done twice from the same Erlenmeyer bulb. After the first sampling, citric buffer of the same volume 0.05 ml was added. Results of DNS assay and glucose assay are presented in Table 17.

TABLE 17. Outcome of TRS in experiment 6

| Sample | Time, min | Concentration, mg/ml | Mass of TRS obtained, mg | Percentage of intitial FS mass, % |
|---|---|---|---|---|
| 0 | 0 | 0 | 0,00 | 0 |
| 1 | 15 | 3.42 | 15.39 | 30.8 |
| 2 | 30 | 4.41 | 19.85 | 39.7 |
| 3 | 45 | 5.46 | 24.57 | 49.1 |
| 4 | 60 | 4.65 | 20.93 | 41.9 |
| 5 | 90 | 5.85 | 26.33 | 52.7 |
| 6 | 120 | 6.69 | 30.11 | 60.2 |
| 8 | 240 | 7.71 | 34.70 | 69.4 |
| 9 | 300 | 10.95 | 49.28 | 98.6 |

Mass of total reducing sugars obtained was plotted against processing time of hydrolysis in Graph 23 below.



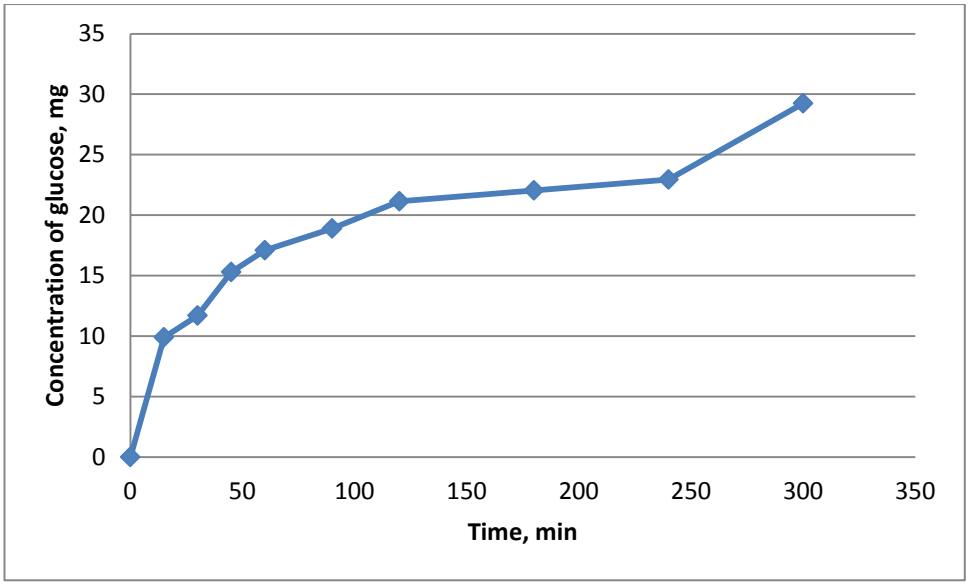

GRAPH 23. DNS assay outcome for experiment 6

Glucose assay of the samples was performed as well. Concentrations of glucose obtained are presented in Table 18 below as well as processing time of hydrolysis.

TABLE 18. Outcome of glucose in experiment 6

| Sample | Time, min | Concentration, mg/ml | Mass of glucose, mg | Percentage of FS mass, % |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 1 | 15 | 2.2 | 9.9 | 19.8 |
| 2 | 30 | 2.6 | 11.7 | 23.4 |
| 3 | 45 | 3.4 | 15.3 | 30.6 |
| 4 | 60 | 3.8 | 17.1 | 34.2 |
| 5 | 90 | 4.2 | 18.9 | 37.8 |
| 6 | 120 | 4.7 | 21.15 | 42.3 |
| 7 | 180 | 4.9 | 22.05 | 44.1 |
| 8 | 240 | 5.1 | 22.95 | 45.9 |
| 9 | 300 | 6.5 | 29.25 | 58.5 |

Concentration of glucose in samples was plotted against processing time of hydrolysis and presented in Graph 24 below.




GRAPH 24. Glucose assay outcome for experiment 6

### 7.6.7 Experiment 7 with increased amount of β-glucosidase

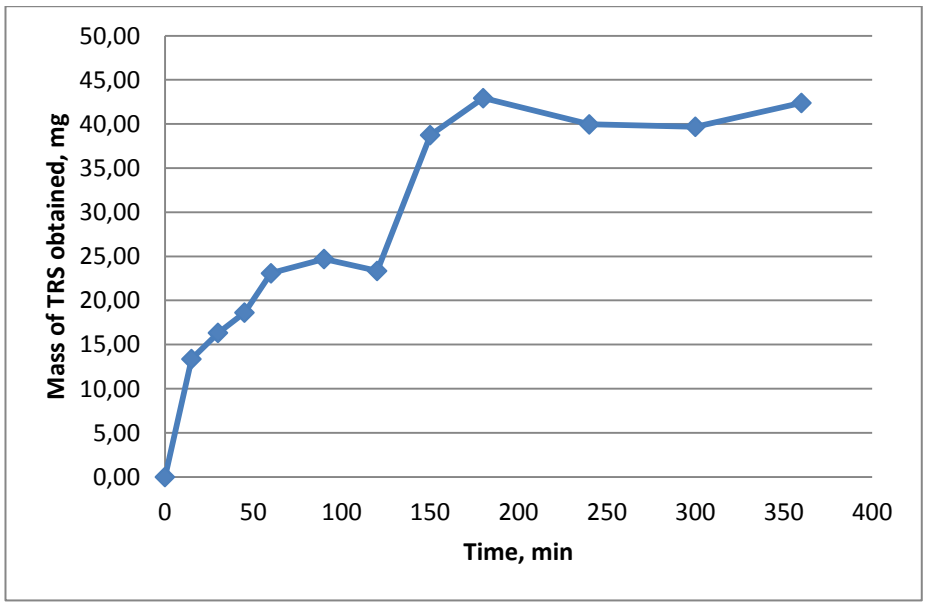
Samples of 0.05 g of fibre sludge are pretreated with 2.5 g of ionic liquid [AMIM]Cl. The yield of pretreated fibre sludge was around 85% of initial mass of fibre sludge. Citric buffer with pH 5.00, volume used was 4.955 ml. Volume of enzyme mix B taken was 0.03

ml and volume of β-glucosidase taken was 0.015 ml. Samples of volume 0.1 ml were taken during the hydrolysis within a certain time interval. Sampling was done twice from the same Erlenmeyer bulb. After the first sampling, citric buffer of the same volume 0.05 ml is added. Results of DNS assay are presented in Table 19.

TABLE 19. Outcome of TRS in experiment 7

| Sample | Time, min | Concentration, mg/ml | Mass of TRS obtained, mg | Percentage of intitial FS mass, % |
|---|---|---|---|---|
| 0 | 0 | 0 | 0,00 | 0 |
| 1 | 15 | 2.97 | 13.37 | 26.7 |
| 2 | 30 | 3.63 | 16.34 | 32.7 |
| 3 | 45 | 4.14 | 18.63 | 37.3 |
| 4 | 60 | 5.13 | 23.09 | 46.2 |
| 5 | 90 | 5.49 | 24.71 | 49.4 |
| 6 | 120 | 5.19 | 23.36 | 46.7 |
| 7 | 150 | 8.61 | 38.75 | 77.5 |
| 8 | 180 | 9.54 | 42.93 | 85.9 |
| 9 | 240 | 8.88 | 39.96 | 79.9 |
| 10 | 300 | 8.82 | 39.69 | 79.4 |
| 11 | 360 | 9.42 | 42.39 | 84.8 |

Mass of total reducing sugars obtained was plotted against processing time of hydrolysis in Graph 25 below.



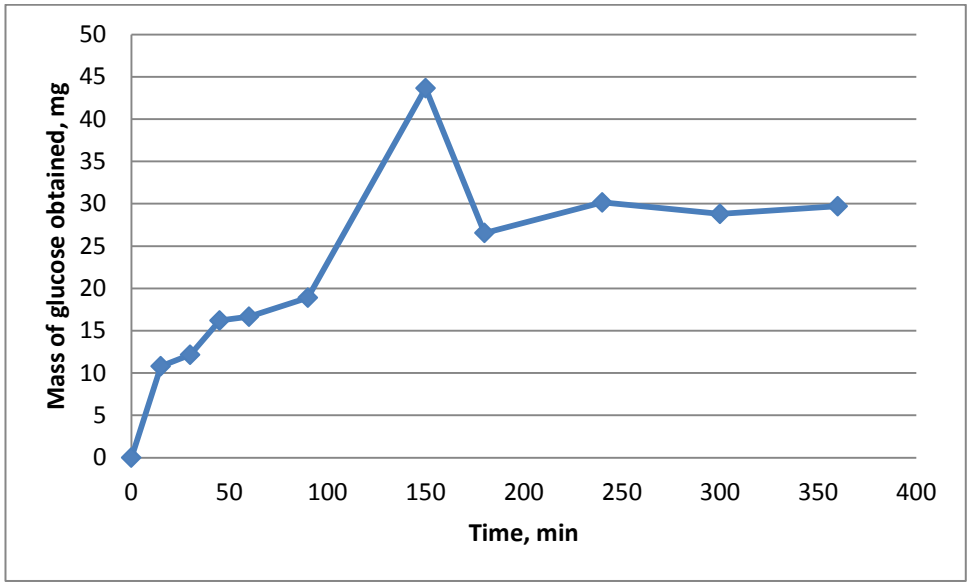

GRAPH 25. DNS assay outcome for experiment 7

Glucose assay of the samples was performed as well. Concentrations of glucose obtained are presented in Table 20 below as well as processing time of hydrolysis.

TABLE 20. Outcome of glucose in experiment 7

| Sample | Time, min | Concentration, mg/ml | Mass of glucose, mg | Percentage of FS mass, % |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 1 | 15 | 2.4 | 10.8 | 21.6 |
| 2 | 30 | 2.7 | 12.15 | 24.3 |
| 3 | 45 | 3.6 | 16.2 | 32.4 |
| 4 | 60 | 3.7 | 16.65 | 33.3 |
| 5 | 90 | 4.2 | 18.9 | 37.8 |
| 7 | 150 | 9.7 | 43.65 | 87.3 |
| 8 | 180 | 5.9 | 26.55 | 53.1 |
| 9 | 240 | 6.7 | 30.15 | 60.3 |
| 10 | 300 | 6.4 | 28.8 | 57.6 |
| 11 | 360 | 6.6 | 29.7 | 59.4 |

Concentration of glucose in samples was plotted against processing time of hydrolysis and presented in Graph 26 below.




GRAPH 26. Glucose assay outcome for experiment 7

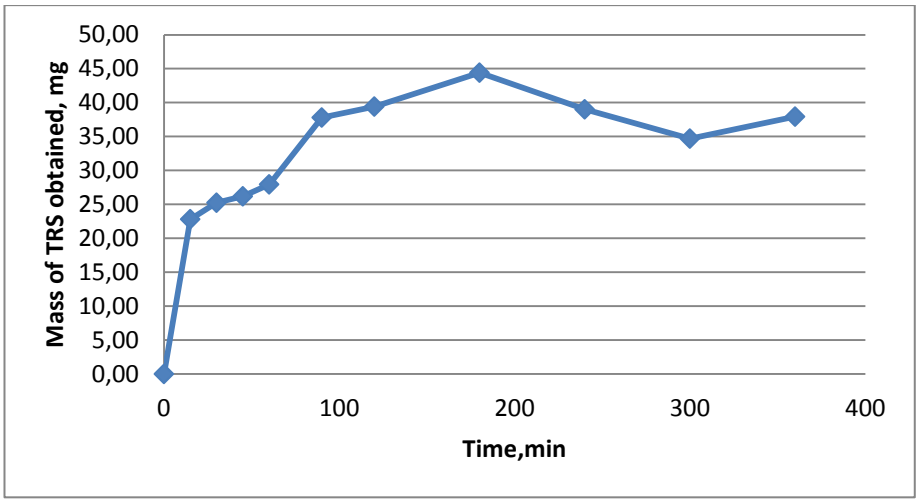
### 7.6.8 Experiment 8 with increased amount of enzyme mix B

Samples of 0.05 g of fibre sludge were pretreated with 2.5 g of ionic liquid [AMIM]Cl. The yield of pretreated fibre sludge was around 85% of initial mass of fibre sludge. Citric buffer of pH 5.00, volume taken was 4.935 ml. Volume of enzyme mix B taken was 0.05 ml and volume of β-glucosidase taken was 0.015 ml. Samples of volume 0.1 ml were taken during the hydrolysis within a certain time interval. Sampling was done twice from the same Erlenmeyer bulb. After the first sampling, citric buffer of the same volume 0.05 ml was added. Results of DNS assay are presented in Table 21.

TABLE 21. Outcome of TRS in experiment 8

| Sample | Time, min | Concentration, mg/ml | Mass of TRS obtained, mg | Percentage of intitial FS mass, % |
|---|---|---|---|---|
| 0 | 0 | 0 | 0,00 | 0 |
| 1 | 15 | 5.07 | 22.82 | 45.6 |
| 2 | 30 | 5.61 | 25.25 | 50.5 |
| 3 | 45 | 5.82 | 26.19 | 52.4 |
| 4 | 60 | 6.21 | 27.95 | 55.9 |
| 5 | 90 | 8.4 | 37.80 | 75.6 |
| 6 | 120 | 8.76 | 39.42 | 78.8 |
| 7 | 180 | 9.87 | 44.42 | 88.8 |
| 8 | 240 | 8.67 | 39.02 | 78.0 |
| 9 | 300 | 7.71 | 34.70 | 69.4 |
| 10 | 360 | 8.43 | 37.94 | 75.9 |

Mass of total reducing sugars obtained was plotted against processing time of hydrolysis in Graph 27 below.



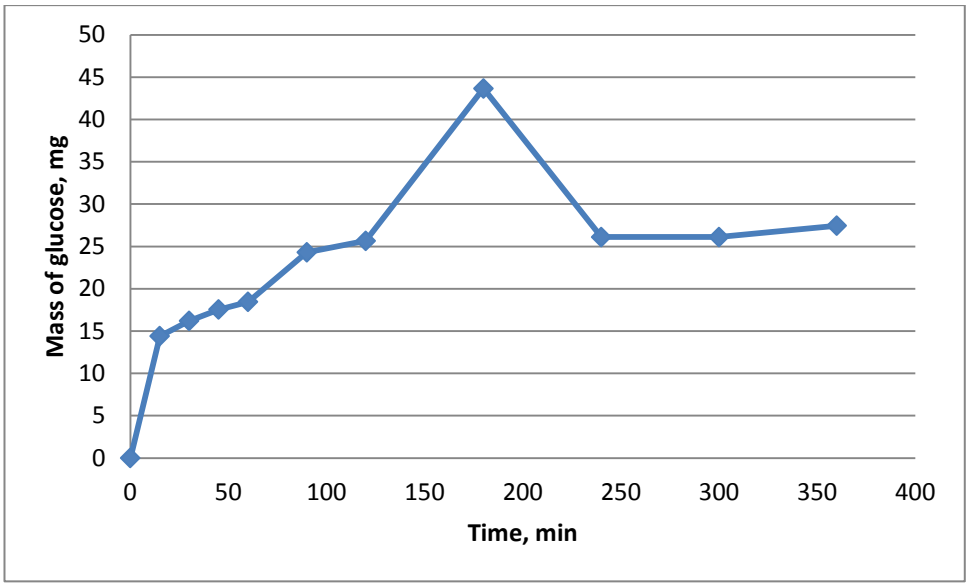

GRAPH 27. DNS assay outcome for experiment 8

Glucose assay of the samples was performed as well. Concentrations of glucose obtained as well as processing time of hydrolysis are presented in Table 22 below.

TABLE 22. Outcome of glucose in experiment 8

| Sample | Time, min | Concentration, mg/ml | Mass of glucose, mg | Percentage of FS mass, % |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 1 | 15 | 3.2 | 14.4 | 28.8 |
| 2 | 30 | 3.6 | 16.2 | 32.4 |
| 3 | 45 | 3.9 | 17.55 | 35.1 |
| 4 | 60 | 4.1 | 18.45 | 36.9 |
| 5 | 90 | 5.4 | 24.3 | 48.6 |
| 6 | 120 | 5.7 | 25.65 | 51.3 |
| 7 | 180 | 9.7 | 43.65 | 87.3 |
| 8 | 240 | 5.8 | 26.1 | 52.2 |
| 9 | 300 | 5.8 | 26.1 | 52.2 |
| 10 | 360 | 6.1 | 27.45 | 54.9 |

Concentration of glucose in samples was plotted against processing time of hydrolysis and presented in Graph 28 below.




GRAPH 28. Glucose assay outcome for experiment 8

# 8 RESULTS

Table 22 represents the summary of the best results obtained from all of the experiments. It shows the time needed to obtain the maximum yield of glucose in milligrams and in percentage from initial mass of fibre sludge as well as the maximum yield of total reducing sugars.

TABLE 22. The summary of the best results obtained from each of the experiment.

| | Max yield of TRS, mg | % from mass of FS | Max yield of glucose, mg | % from mass of FS | Time needed, min |
|---|---|---|---|---|---|
| Enzyme mix A | | | | | |
| Experiment 1 | 38.7 | 38.7 | 8.73 | 8.73 | 30 |
| Experiment 2 | 24.1 | 24.1 | 8.37 | 8.37 | 180 |
| | | | | | |
| Enzyme mix B | | | | | |
| Experiment 1 | 45.09 | 90.18 | 34.2 | 68.4 | 20 |
| Experiment 2 | 43.2 | 86.4 | 28.35 | 56.7 | 165 |
| Experiment 3 | 35.23 | 70.46 | 26.1 | 52.2 | 210 |
| Experiment 4 | 32.37 | 64.74 | 24.75 | 49.5 | 150 |
| Experiment 5 | 49.55 | 99.1 | 33.3 | 66.6 | 300 |
| Experiment 6 | 49.28 | 98.56 | 29.25 | 58.5 | 300 |
| Experiment 7 | 42.93 | 85.86 | 30.15 | 60.3 | 240 |
| Experiment 8 | 44.42 | 88.84 | 27.45 | 54.9 | 360 |

It can be clearly seen that the enzyme mix B was more effective in degrading cellulose than enzyme mix A. Even if the enzymatic loading of enzyme mix B was 4 times less than the loading of enzyme mix A, the outcome of glucose was 6 times higher.

Also, it can be observed that the best result was obtained in experiment 5 with enzyme mix B where the yield of total reducing sugars reached 99.1% and the yield of glucose reached 66.6% of the initial mass of dry fibre sludge with processing time 300 minutes. Another result with a good yield of glucose is from experiment 1 with enzyme mix B where the yield of glucose reached the level of 68.4% of the initial mass of dry fibre sludge with processing time 20 minutes. Comparing the conditions of these two experiments, it should be noted that the enzymatic loading in the first experiment was much higher than in experiment 5 which eventually led to a great difference in processing time. Also,

comparing results from experiments 5 and 6 that gave the same yield of total reducing sugars in the same processing time, though there is the difference in the yield of glucose. The enzymatic loading of enzyme mix B was decreased and enzymatic loading of β-glucosidase was increased in experiment 6 comparing to experiment 5. It did not result in the increase of the yield of glucose as expected.

In the last experiments 7 and 8 the level of glucose in sample 7 at processing time 180 minutes reached 88% from the initial mass of fibre sludge taken. The level of glucose in other samples taken during those experiments was of much lower concentration. It could be considered a mistake if it happened in one experiment only, but it was repeated in 2 completely separate experiments. However, the result was not systematic and was not taken into consideration.

Comparing results obtained from experiments where different pH of environment was used, it can be noted that the change in pH did not influence the rate or outcome of enzymatic hydrolysis significantly. All pH values, 5.0, 5.2 and 5.5, were taken within the range recommended by the manufacturer, and they seemed to have the same effect on the activity of enzymes.

# 9 DISCUSSION

The aim of the study was to determine the most favourable pH of the environment for the activity of the studied enzyme mix. Moreover, optimum enzyme concentration should have been determined in order to obtain maximum yield of glucose. Furthermore, the effectiveness of enzyme mixes from two manufactures in processing lignocellulose was compared. Lastly, the processing time of converting lignocellulose into glucose in different conditions is compared.

As a result of conducted experiments it was possible to reach maximum concentration of total reducing sugars which was equal to 49.55 mg of reducing sugars from initial substrate loading of 50 mg. However, the maximum level of glucose obtained was 33.3 mg which is equal to 66.6% of initial mass of substrate taken.

It can be possible to obtain higher yield of glucose as in the given experiments the maximum yield of total reducing sugars, which was equal to 99.1% of the initial mass of dry fibre sludge, was obtained. Therefore, further break down of TRS to glucose monomers could result in higher yield of glucose. Due to this fact, enzyme loading of β-glucosidase was increased after receiving 99.1% of TRS yield. However, the concentration of glucose did not increase as expected. Since β-glucosidase breaks down cellobiose to glucose, it could be possible that there was not enough of cellobiose units in TRS. For this reason, enzyme loading of enzyme mix B should be increased in order to break longer chains of cellulose down to cellobiose which can be degraded by β-glucosidase. Therefore, further research could be done in order to obtain a higher yield of glucose.

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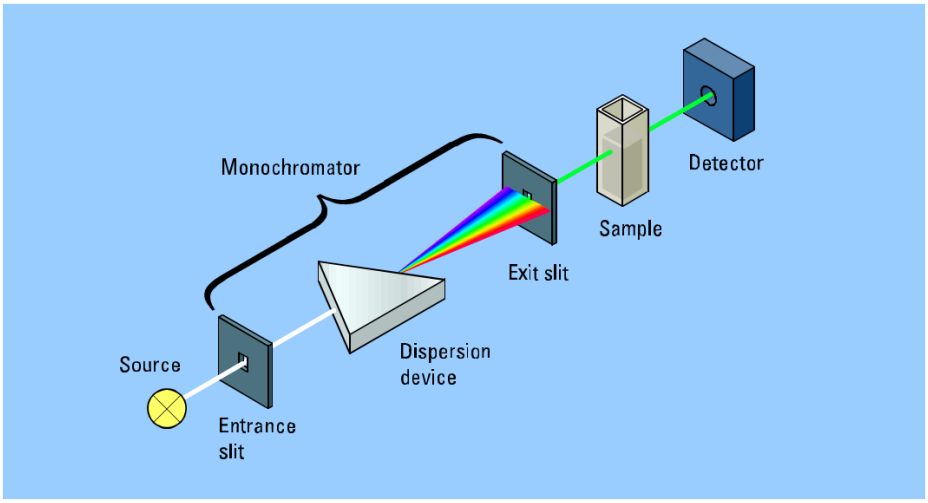
APPENDIX 1

# UV-SPECTROSCOPY

Absorption spectroscopic methods are among the most used analytical methods in quantitive analysis. The method involves the use of Ultraviolet-visible (UV) spectrophotometer which allows to determine the extent of absorption of a certain wavelength of UV light by a given solution and consequently to determine the concentration of a certain chemical in that solution. It is possible due to the fact that chemical compounds absorb light radiation at certain wavelength which is directly proportional to concentration of the compound in the solution.

The basis of the measurement procedure is the Beer-Lambert law. It states that the absorbance and transmission of light through a sample can be calculated by measuring the light intensity entering and exiting the sample. From this law it follows that as concentration of a chemical in a solution increases, light absorption increases linearly and light transmission decreases exponentially.

The function of the spectrophotometer is to provide a beam of monochromatic radiation to illuminate a sample and measure the intensity of light reaching the detector. The beam consists of photons. When photons reach the measured compound molecule (analyte), it will probably absorb the photon. Therefore, the number of photons reduces and consequently reducing the intensity of beam light. The work of UV-Vis spectrophotometer is illustrated in the graph below.




GRAPH 1. The principle of work of UV-Vis spectrophotometer (adapted from Davison college 2012)