STRATEGIC ADSORPTION/DESORPTION OF CELLULASES NS 50013 ONTO/FROM AVICEL PH 101 AND PROTOBIND 1000

by

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Abstract

Doctor of Philosophy in Chemical Engineering

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Desorption of active cellulases from lignocellulosic substrates is a potential technique to reuse cellulases for the production of bioethanol. For desorption studies, adsorption of cellulases had to be performed first. Adsorption of cellulases NS 50013 onto microcrystalline cellulose (Avicel PH 101) and wheat straw lignin (Protobind 1000) was studied. It was found that Protobind adsorbed twice the amount of cellulases than Avicel did. An adsorption strategy developed was to work at pH 5 and a temperature less than 323 K to get maximum adsorption on the cellulose component, less adsorption on the lignin component of lignocellulosic materials, and to harmonize adsorption temperature with the industrial hydrolysis situation. Desorption of cellulases from Avicel and Protobind over a range of 298 K to 343 K and a pH of 6 to 9 was studied. Desorption obtained at pH 9 and 333K was optimum for both Avicel and Protobind. Hence, desorption was enhanced by 21 % and 11% for Avicel and Protobind respectively. The cellulases activity for Avicel was 48 FPU mL^{-1} at pH 9, 333 K, 5% glycerol, representing 91 % of the initial activity and for Protobind, the activity was 33 FPU mL^{-1} which represents about 66 % of the initial activity. All of these values were higher than ever reported in literature. At pH 5 and 298 K the amount of cellulases desorbed from untreated wheat straw (WS) was 33 % of those initially used for the adsorption step. It was increased to 42 % when 30 % delignified WS was used, and was further increased to 48 % for 60 % delignified WS. Desorption obtained for 60 % delignified WS was 75 % at pH 9, 333K and 5%

glycerol. The desorption strategy recommended for bioethanol producing industries, is: 1) removal of lignin; 2) adsorption of cellulases at pH 5 and lower than 323K; 3) hydrolysis of lignocellulosic material; and 4) desorption of cellulases from non-hydrolyzed material at 333 K, pH 9, with 5-10 % glycerol. The proposed strategic desorption of cellulases may reduce the cost of Canadian bioethanol production by 26.5 % due to 75 % recyclability of active cellulases.

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Dedication

Dedicated to my beloved mother

Surrayia Baigum

who dreamed about my higher studies

Dream is not that which you see while sleeping it is something that does not let you sleep

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Nomenclature

a_{cel} ,	area occupied by cellulases molecules, 60 x 50 $Å^2$
BG	β-glucosidase
BSA	Bovine Serum Albumin
CBH I	Cellobiohydrolase I
CBH II	Cellobiohydrolase II
CBM	Carbohydrate Binding Module
Cel5A	Glycoside hydrolase family 5 endoglucanase
Cel6A	Glycoside hydrolase family 6 CBH II
Cel7B	Glycoside hydrolase family 7 CBH I
Cys	Cysteine at location 181
CD	Catalytic Domain
EG I	Endoglucanase I
EG II	Endoglucanase II
EG	Ethylene glycol
EnzHR	Enzymatic residual lignin

G	Glycerol
ΔG_a	Change in free energy of adsorption, $Jmol^{-1}K^{-1}$
ΔG_d	Change in free energy of desorption, $Jmol^{-1}K^{-1}$
ΔH_a	Change in enthalpy of adsorption, $kJmol^{-1}$
ΔH_d	Change in enthalpy of desorption, $kJmol^{-1}$
K_a	Constant, mL μg^{-1}
K_f	Freundlich constant for cellulases-substrate complex, distribution constant, $\mu g\ mg^{-1}$
K_N	Partition coefficient given by the slope, mL mg^{-1}
N_A	Avogadro's number 6.022E+23 mol^{-1}
$[P_a]$	Cellulases adsorbed on the substrate at equilibrium, $\mu g\ mL^{-1}$
P_e	Specific amount of cellulases adsorbed on substrate, $\mu g\ mg^{-1}$
$[P_f]$	Cellulases present in the solution at equilibrium, $\mu g\ mL^{-1}$
P_{am}	Maximum cellulases adsorbed (µg of cellulases) $(mg\ of\ substrate)^{-1}$
R	Universal gas constant 8.314 $JK^{-1}mol^{-1}$
SELP	Steam exploded lodgepole pine
SEAW	Steam exploded aspen wood
ΔS_a	Change in entropy of adsorption, $Jmol^{-1}K^{-1}$

ΔS_d	Change in entropy of desorption, $Jmol^{-1}K^{-1}$
STP	Standard temperature and pressure
T	Absolute temperature
V_m	Volume of gas adsorbed at STP to produce an apparent monolayer on the sample surface, mL
$\bar{y_c}$	Mean of the absorbance measured in replicates #1 and #2
σP_e	Variance in P_e

1.0 Introduction

Lignocellulosic materials are used as a source for production of bioethanol. These materials consist of lignin, cellulose and hemicellulose, which are interweaved in the cell walls. Cellulose component of biomass is insoluble and cellulases have to adsorb to cellulose to proceed for sugar release. Cost-effective liberation of fermentable sugars from lignocellulosic resource is still the largest obstacle to large-scale commercialization of this technique. There are two main problems associated with the process of liberating reducing sugars: the first one is presence of lignin that compete with cellulose for cellulases hence increased processing cost and the second problem is the cost of enzymes used in ethanol production process.

The solution to the first problem is to remove the lignin to prevent enzyme-lignin interaction and expose more and more cellulose. Several methods were suggested, such as performing enzyme-substrate reactions with surfactants because surfactants restrict interaction of cellulases with lignin. Another approach was to destroy and/or remove the lignin by pre-treatment. However, due to one or a combination of factors involved in, cost of some of pre-treatments, less amount of lignin removal, health and/or environmental concerns, the potential for bioethanol production is not promising. For example, to use steam explosion, high temperature and pressure resistant fabrication material for the reactor are required. Ammonia fiber expansion (AFEX) needs pressure resistant and corrosion resistant fabrication material for reactors and ammonia may trigger asthma in workers. Similarly, cellulose solvent and organic solvent-based lignocellulose fractionation (COSLIF), ionic liquids, or organosolv require expensive corrosion resistant fabrication materials and vapors of solvents may harm to workers. In our lab we have successfully removed 90 % of lignin by a novel technique which is a sequential use of water and ozone [Baig *et al*., 2015]. All reactions occurred at normal temperature and pressure therefore no expensive material would be required to construct a reactor. The half-life of ozone indoor is 7-10 min therefore it was considered less harmful (less health

concerns) than Cl_2, H_2O_2, etc. The cost of ozone is dependent on electricity price (less costly) because O_3 is produced when O_2 molecules are dissociated by an electricity source into oxygen atoms. Removal of lignin decrease process cost because lignin adsorbed more enzymes than cellulose, hence consume enzymes non-productively. The common features of all pretreatment methods were that they rearranged and removed some parts of the cell wall structure and hence, made cellulose more accessible for cellulases. Some of the lignin residues may be present even after pretreatment. However, such a pretreated biomass still offer much more exposed structures for cellulase to access cellulose. Since a pretreatment change the chemical composition and structure of a pretreated biomass, it is perceived that pretreatment might affect the adsorption of the enzymes during hydrolysis and fermentation and thereby probably also the recyclability of the enzymes.

The solution to second problem is to reduce the cost of enzymes. In 2001, the cost to produce enzymes from *Trichoderma reesei* (cellulases) was $0.8-1.32 U.S. per liter of ethanol [Novozyme, 2005]. Through collaboration between Novozymes and NREL the price was reduced to $ 0.03-0.05 per liter of ethanol in 2005 [Mathew *et al*., 2008]. The cost of cellulases is still far more expensive than the cost of starch-hydrolyzing enzymes used for grain based ethanol biorefineries (*i.e.*, $ 0.01-0.02 per liter of ethanol) [Zhu *et al*., 2009]. The reduction in cost of cellulases has been a main assignment for the researchers for production of bioethanol from waste lignocellulosic materials instead of food grains. To decrease the costs of cellulases some efforts were made by: (i) decreasing cellulases loading (*e.g.*, gram of cellulases used per gram of cellulose) by increasing cellulose interaction with enzymes which can be achieved by pretreating the biomass, (ii) recycling costly cellulases by adding fresh substrates, and (iii) increasing cellulase performance (activity) by using genetically modified cellulase (modified for thermal stability and binding ability). Some of these efforts were not practicable such as addition of substrate to reuse / readsorb enzymes from the

previously used substrate resulting in a build-up of lignin rich residues that increased capability of cellulases to adsorb but reduce the capability of cellulases to desorb. Other authors reported desorption agents to perform desorption of cellulases such as alkaline media [Otter *et al*., 1984; Zhu *et al*., 2009; Rodrigues *et al*., 2012], Tween [Seo *et al*., 2011], urea [Deshpande and Eriksson, 1984], glycerol [Beldman *et al*., 1985], and Triton X-100 [Bai *et al*., 2010]. In alkaline medium (beyond pH 10) enzymes were reported inactive due to denaturing. For reuse of enzymes in bioethanol production, desorption of cellulases alone is insufficient. Desorption of active cellulases is of utmost importance.

This study has a special focus on desorption of active cellulases. Previously, some work has been done on the effect of pH on cellulases adsorption, less attention has been given to the temperature dependence of the adsorption process which is still under continuing debates. The debate is to suggest a suitable temperature for adsorption and desorption of cellulases while maintaining their activity. Because researchers have conducted studies under different temperatures, various sources and type of enzymes. Therefore a study on wide range of temperature was required for adsorption and desorption. Furthermore, the enzymatic adsorption on insoluble substrates is complex. The complexity involves chemical composition, physical structure of substrates, and from the cellulases part, it's the transport and diffusion approach of cellulases to target sites. Some contrasting results were presented by researchers about cellulases showing optimal activity at various pH values. Correlations between structural changes in cellulases and changes in the activity of the enzymes were of great interest for desorption and reuse of cellulases in biofuel industry. Adsorption of cellulases was not strictly reversible [Hong *et al*., 2007; Shao *et al*., 2008]. For example, adsorption of all *Thermobifida fusca* cellulases to bacterial microcrystalline cellulose was irreversible [Jung *et al*., 2002]. On the other hand Palonen *et al*. (1999) reported that only 10 % of the adsorbed *Trichoderma*

cellobiohydrolase I and 30-40 % of the adsorbed *Trichoderma cellobiohydrolases II* were desorbed. Present study for adsorption and desorption of cellulases onto/from cellulose and lignin would help to estimate adsorption and desorption onto/from lignocellulosic substrates.

The main goal of present study was to develop a novel integrated desorption strategy by using environmental parameters and suitable chemical/s for cost-effective active desorption of cellulases from lignocellulosic substrates (the idea is to adapt this technique on exhausted solid residues from bioethanol producing industry for active desorption of cellulases for reuse) as well as optimize environmental parameters and the concentration of chemical/s used. During the course of meeting this goal some other objects were served, automatically. In this study, adsorption and desorption were conducted and evaluated through a series of the adsorption experiments under varying temperature, pH and concentration of cellulases, for Avicel PH 101 and Protobind 1000 to help settle the controversy (monolayer or multilayer) on type of adsorption occurred. The kind of adsorption play a key role in designing strategy for desorption of cellulases from lignocellulosic components and explore optimum conditions to get active desorption of cellulases. Although much has been accomplished in adsorption, less attention has been given to the change in enthalpy and entropy of adsorption / desorption processes, which is still under continuing debate (Medve, 1994; Radeva *et al*., 2011; Gan *et al*., 2012; Kumar and Wayman, 2013). It was difficult to evaluate desorption and/or reuse of cellulases, because of insufficient knowledge on the characteristics of cellulase adsorption /desorption onto/from cellulosic substrates. Therefore, adsorption /desorption of cellulases onto/from cellulose and lignin were studied under similar experimental conditions because cellulose and lignin exist together in lignocellulosic materials and undergo same operational conditions during bioethanol production. This study may help to decide whether lignin removal from lignocellulosic material for the conversion of lignocellulosic to biofuel should be conducted or not. Delignification of wheat

straw (a lignocellulosic material) is important for two reasons: first, to stop non-productive adsorption on lignin and second, to increase accessible surface area for cellulases to get adsorb and desorb. Further, getting all the insight for adsorption and desorption onto/from the pure components of lignocellulosic materials the knowledge was applied for desorption of cellulases from pre-adsorbed wheat straw.

2.0 Literature Review

The price of regular grade gasoline touched the peak of $4.25 per gallon in July 2008 [US Energy Administration, 2008] somehow it came back to its normal value i.e. $1.71, in January 2009 for a short time then it started rising again. In the May 2011 the price was $3.91 per gallon and it was $3.43 per gallon in 2016 [Knipping, 2016]. A decrease of $0.48 per gallon could not make any significant impact on the living cost of a Canadian individual. An average Canadian living in Toronto was paying $129.01 for 1000 kWh consumption of electricity in 2011, in 2013 the price fell down to $124.75 and in 2014 the prices rose to $ 137.84 without taxes and the individual was paying $143.72 in 2015 [Hydro Quebec, 2015]. An 11.4 % increase in electricity prices was observed with a 24% increase in food prices with reference to the prices in 2011. From the presented brief history, it can be figured out that the cost of living was affected by increased fuel price which made people to think about an alternative source of fuel. At the same time the desire for safe environment and clean energy motivated to demand for biofuel. Biofuel is a fuel produced from biomass. Biomass is a generic term for all animate organic matter (except fossil fuels) encircling crops, forestry and marine products but also organic wastes. Biomasses, as an energy source, have two prominent distinctiveness's:

- biomass is the only renewable organic resource
- biomass fixes carbon dioxide in the atmosphere by photosynthesis.

Among the various kinds of biomass, woody biomass was used traditionally as an energy source, for a long time and, even to this day, it is being used in the form of firewood or charcoal. It is, however, difficult to use firewood or charcoal as an alternative fuel for commercial equipment and industrial processes where fossil fuels, in particular oil, are used at present. It is necessary to develop technologies which could make possible conversion of biomass to a more useable form, such as liquid or gas.

Agricultural and forestry residues such as corn stover and wheat straw are inexpensive and readily available source of renewable lignocellulosic biomass [Mussato *et al*., 2008]. Exploitation of biomass as a carbohydrate source for glucose and ethanol production has been severely hindered by the low efficiency with which cellulose degrading organisms and enzymes are able to convert the polysaccharide portion of the residue into monomeric sugars. The production of biofuel from lignocellulosic biomass is facing a problem of not economically releasing cellulosic sugars [Lynd *et al*., 2008; Zhang, 2008; Yousuf, 2012; Limayem and Ricke, 2012]. The cellulosic sugars are protected by lignin in biomass. Therefore, a pretreatment is required to remove lignin. Various pretreatment techniques were tried for delignification, using physical, chemical, and physicochemical and /or biological methods. For example steam explosion, hot water extraction [Rosgaard *et al*., 2007], traditional chemicals like sulfuric acid, sulfur dioxide, sodium hydroxide and lime were studied. The new technologies explored were ozonolysis [Silverstein *et al*., 2007] ammonia fiber explosion (AFEX) [Sendich *et al*., 2008] and wet oxidation [Hendricks and Zeeman, 2009]. A novel technique has removed over 92 % of lignin from wheat straw by strategic use of water and ozone [Baig *et al*., 2015]. The obtained results demonstrated that ozonation of wheat straw using the proposed technique was practical, easy to manage for the removal of lignin and it may be considered as an alternative method for delignification of biomass in production of biofuel. Removal of lignin makes cellulose more accessible for enzymes to convert the cellulose into fermentable sugars [Mosier *et al*., 2005]. In next step, the fermentable sugars are converted to ethanol (CH_3CH_2OH) which is one of the biofuels. The achievability of bioethanol process depends either on the increase in yield of sugars or decrease in cost of process or both. These two influencing factors can be controlled by improving the existing pretreatment technologies or developing new technologies [Sathitsuksanoh *et al*., 2009; Ekwe, 2012] or developing enzyme of very precise performance [Heinzelman *et al*., 2009; Liu *et al*., 2009], or developing recyclable enzymes [Tu *et*

al., 2007; Zhu *et al*., 2009b, Weiss *et al*., 2013], or producing low cost enzyme [Himmel *et al*., 2007], or discovering easy to process bio-energy crops [Bomgardner, 2013]. The basic conversion process can be depicted in the following two reactions:

$$(C_6H_{10}O_5)_n + nH_2O \xrightarrow{Cellulases} nC_6H_{12}O_6 \quad \ldots \quad 2.1$$

Cellulose Glucose

$$C_6H_{12}O_6 \xrightarrow{microbes} 2C_2H_5OH + 2CO_2 \quad \ldots \quad 2.2$$

Glucose Ethanol

In Canada, since 1991, the grain-based ethanol production process exists, representing 92% of actual production capacity. Canadian ethanol plants are presently using corn 68%, wheat 29.9%, municipal waste 1.8 %, forestry waste 0.2%, agriculture waste 0.1 % [Quaiattini, 2010]. It was estimated that Canada will produced 1.745 billion liters of bioethanol in 2015 [Zimmerman and Dessureault, 2014]. For its need, Canada had to import 1.34 billion liters of bioethanol [Pratt, 2015]. Canada produced 26.8 million tons of wheat in the year 2009-2010 [Lyddon, 2011]. Depending upon the ethanol production process employed, commercial ethanol is yielded from 340 to over 500 liters per ton of wheat, depending on the type of wheat [Cheminfo Services Inc. *et al*., 2000]. Though Canada is among top ten producers of wheat in the world [Montane, 1998] yet the use of grain for fuel is not appreciated. A potential use of low cost ethanol production is to utilize waste lignocellulosic materials such as crops residues, grasses, saw dust, wood chips and animal waste. In last decade research has been done on the conversion of lignocellulosic materials to ethanol [Azzam, 1989, Cadoche and Lopez, 1989; Reshamwala *et al*., 1995, Bjerre *et al*., 1996]. The viability of wheat straw which is an agricultural waste and a renewable source was chosen to investigate in this study to produce a valuable product such as bioethanol. The average yield of straw is 1.3–1.4 kg per kg of

grain, which means that Canada has capability to produce 37.52 million tons of straw per year. The reactants (biomass, cellulases) and their interaction during adsorption and desorption steps of bioethanol production are discussed in following sections.

2.1 Lignocellulosic materials (biomass)

In recent years, there has been a great search for biomass as an energy source. In the biomass, energy of sunlight is stored in chemical bonds between adjacent carbon, hydrogen and oxygen molecules. When the bonds are broken, these substances release their stored chemical energy. The process by which biomass (chlorophyll containing organisms) capture energy in the form of light and convert to chemical energy is called, photosynthesis. This process is accompanied by the release of oxygen (O_2). The basic equation for photosynthesis can be written [Raven *et al.*, 2005] as

$$\boldsymbol{6CO_2 + 12H_2O + Light\ \ enrgy \quad \rightarrow \quad C_6H_{12}O_6 + 6H_2O + 6O_2} \qquad \ldots \ 2.3$$

The total energy captured from solar energy by plant photosynthesis is about 2 x10^{23} J $year^{-1}$ [Ito and Oikawa, 2004].

Biomass can be classified as four main types, namely; woody plants, herbaceous plants/grasses, aquatic plants and manures. Biomass contains varying amounts of cellulose, hemicellulose, lignin and a small amount of other extractives.

The properties of the material of interest, during subsequent processing as an energy source are related to

i. moisture contents
ii. calorific value
iii. proportions of fixed carbons and volatiles

iv. ash/residues contents

v. alkali metal contents

vi. cellulose /lignin ratio

The first five properties are of interest for dry conversion processes while the first and sixth properties are of concern for wet biomass conversion processes. High moisture contents in biomass such as herbaceous plant lends itself to a wet conversion process which involves biologically mediated reaction such as fermentation. Dry biomass such as wood chips is more economically suited to gasification, pyrolysis or combustion. Wet and dry pyrolysis are two extremes. Other factors such as ash, alkali and trace components impact adversely on thermal conversion processes and cellulose contents influences on biochemical fermentation processes. There are three main reasons to use biomass as an energy source [McKendry, 2002]:

1- The technical advancement for suitable crop production implies the use of biomass at lower cost and higher conversion efficiency. For example, low cost biomass residues can be used instead of fossil fuels for the production of electricity. A cost effective use of energy crops for production of methanol and hydrogen looks promising.
2- The agricultural sector in Western Europe, US and Canada is producing food surpluses. This situation has led to a policy in which land is set aside in order to reduce surpluses. On these spared lands, energy crops can be grown without effecting food producing crops.
3- The major stimulus is no potential threat to climatic change. Biomass emits roughly the same amount of carbon during conversion as is taken up during plant growth. Therefore, use of biomass will not contribute to a buildup of CO_2 in the atmosphere.

Table 1 shows the cellulose, hemicellulose and lignin contents of some of lignocellulosic materials.

Table 1: Average Composition of Various Biomass Materials

[Fan *et al*., 1980; Sun and Cheng, 2002; Galbe and Zacchi, 2002; Kim and Dale, 2004]

Materials	**Cellulose %**	**Hemicellulose %**	**Lignin %**
Hard wood stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Nut shells	25-30	25-30	30-40
Corn cobs	45	35	15
Corn stover	35-41	16-24	15-17
Coastal Bermuda grass	25	35-7	6.4
Leaves	15-20	80-85	0.0
Cotton Seed Hairs	80-95	5-20	0.0
Wheat straw	40-50	30-40	16-25
Barley straw	34-38	19-30	9-15
Newspaper	40-55	25-40	18-30
Waste papers from chemical pulps	60-70	10-20	5-10
Primary wastewater solid	8-15	NA	24-29
Solid cattle manure	2-5	2-4	2-6

Woody plant species have slow growth and are composed of tightly bound fibers. Herbaceous plants are more loosely bound fibers indicating lower portion of lignin. The relative proportion of cellulose and lignin is the determining factor of the suitable plant species.

2.2 Components of lignocellulosic materials

Cellulose

Cellulose is a polymer with an average molecular weight of around 100, 000. A single unit of glucose is shown in Figure 1**.** In the cellulose chain, the glucose units are in 6-membered rings, called

pyranoses. They are joined by single oxygen atoms (acetal linkages) between the C-1 of one pyranose ring and the C-4 of the next ring.

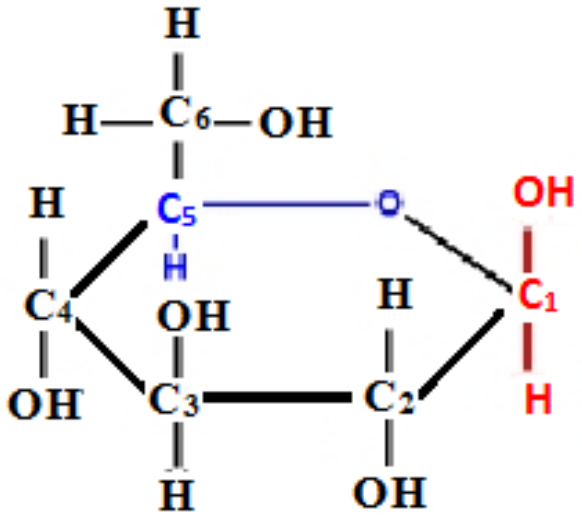


Figure 1: A glucose unit in cellulose chain

[Adapted from: Klemm *et al*., 2005; Netrabukkana, 1996]

The ends of cellulose chain are named as reducing and non-reducing end. The end where there is a C-1 carbon atom which is not involved in a glycosidic bond is the reducing end, while the other end, where the C-1 carbon atom is participating in a glycosidic bond, is called the non-reducing end as given in Figure 2. The approximate length of a cellulose molecule is about 50000 Å (5000 nm) whereas the average size of each crystalline region determined by x-ray diffraction analysis was only 600 Å in length, 50Å in width and 30Å in thickness. Approximately 36 – 42 of cellulosic chains arrange together (as a bundle) by complex hydrogen bonds network to form elementary fibrils [O'Sullivan, 1997; Himmel *et al*., 2007; Habibi *et al*., 2007].

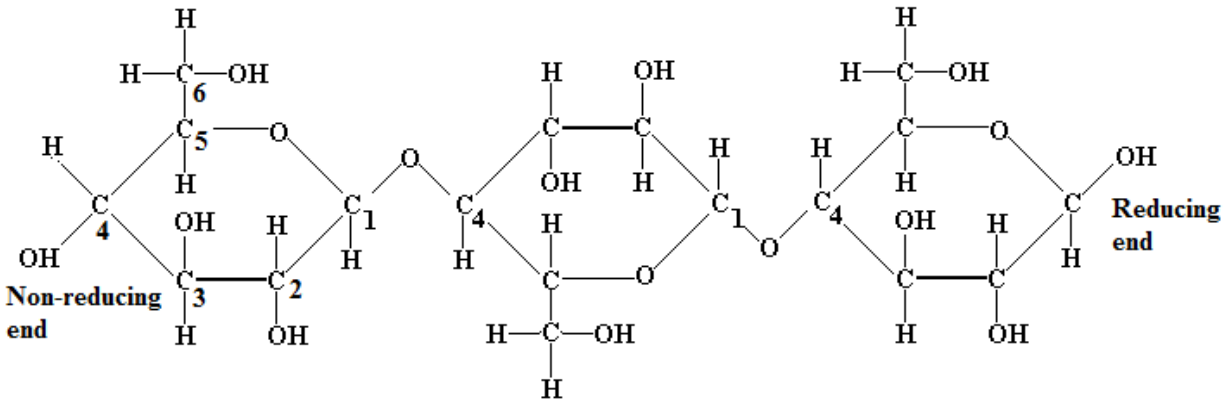


Figure 2: Glucose units in a cellulose chain (β-1, 4-D- linkages)

[Adapted from Morrison and Boyd, 1992; Klemm *et al*., 2005; Carey and Sundberg, 2007]

The hydrogen bonding is possible due to the equatorial positions of the hydroxyls on the cellulose chain. These hydrogen bonds cause the chains to group together in highly ordered (crystal-like) structures. Since the chains are usually longer than the crystalline regions, they are thought to pass through several different crystalline regions, with areas of disorder in between (the "fringed-micelle" model). The inter-chain hydrogen bonds in the crystalline regions are strong, giving the resultant fibers good strength and insolubility in most solvents. They also prevent cellulose from melting (i.e., non-thermoplastic). In the less ordered regions, the chains are further apart and more available for hydrogen bonding to other molecules. There are six possible conformations of cellulose chains to be held in the elementary fibrils depending on the polymerization conditions [O'Sullivan, 1997]. Native cellulose is composed of a heterogeneous mixture of at least two conformations [O'Sullivan, 1997]. Cellulose is highly crystalline in the center of its elementary fibrils, and insoluble in water [Pauly and Keegstra, 2008]. The crystalline structure of cellulose is difficult to disrupt such as cellulose must be heated to 320 °C at a pressure of 25 MPa [1 MPa = 9.87 atm] to remove its crystallinity [O'Sullivan, 1997]. By the action of enzymes, cellulose was broken down to cellobiose and which

was further broken down to glucose. Choi *et al*. (2002) has given the length and width of a glucose molecule as 5.6 Å and 6.5 Å. [Kuhtreiber *et al*., 1999 has given a glucose unit of 4.75 Å x 6.5 Å x 3.4 Å with β (1, 4) linkage based on the bond angle length. Cellobiose unit has dimensions of 9.0 Å x 6.5 Å x 3.4 Å [Kuhtreiber *et al*., 1999]. Cellobiose unit measure by x-ray diffraction is 10.4 Å x 5.3 Å x 3.4 Å [Lynd and Zang, 2004]. A cellulose molecule is a polytrihydric alcohol, containing one primary and two different secondary hydroxyls per unit of polymerization. Reaction of cellulose may occur by disrupting the linkages holding the units together, or it may be any of the usual reactions of hydroxyl groups. In most reactions, the distribution of hydroxyl groups along the cellulose chain provide excellent binding (adsorption) sites for enzymes.

Hemicellulose

Hemicellulose $(C_{31}H_{34}O_{11})_n$ is a mixture of polysaccharides [Buchala and Wilkie, 1970] with an average molecular weight of < 30,000. Hemicellulose is composed of sugars such as glucose, mannose, xylose and arabinose and methylglucuronic and galacturonic acids. Chemical structure of hemicellulose containing β-1, 3-D-linkage and β-1, 4-D-linkage are shown in Figure 3.

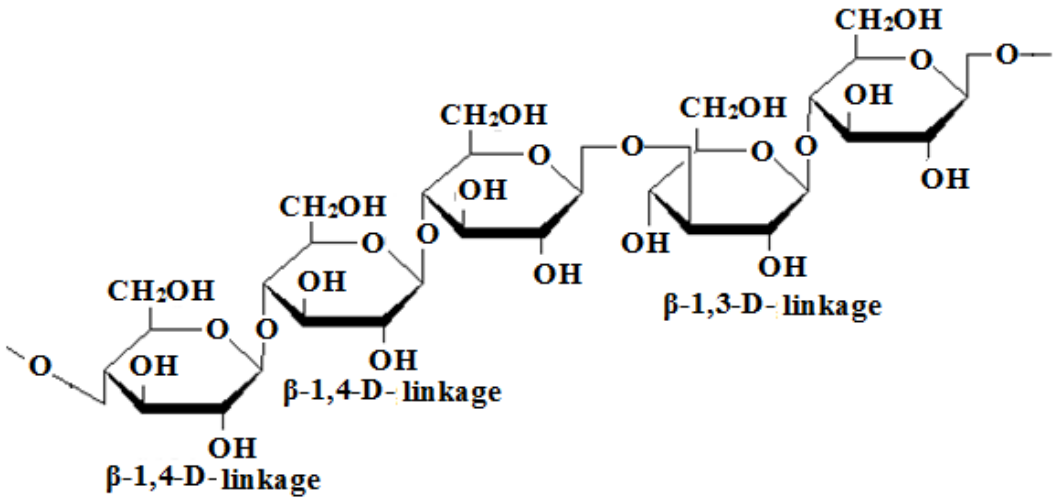


Figure 3: Chemical Structure of Hemicellulose

[Adapted from Klemm *et al*., 2005; Helmberger, 2009]

In contrast to cellulose, hemicellulose is a heterogeneous branched polysaccharide that binds to the surface of each cellulosic micro fibril [Hopkins, 1999].Cellulose is generally the largest fraction about 40-50 % by weight; and hemicellulose portion represents 20-40 % of the material by weight [Brigham *et al*., 1996]. Hemicellulose differs from cellulose, in consisting primarily of xylose and other five-carbon monosaccharides.

Lignin

Lignin can be regarded as a group of high molecular weight amorphous compounds. Lignin is believed to be a three carbon chain attached to rings of six carbon atoms, called phenyl propanes. A polymeric form of these molecules is shown in the Figure 4.

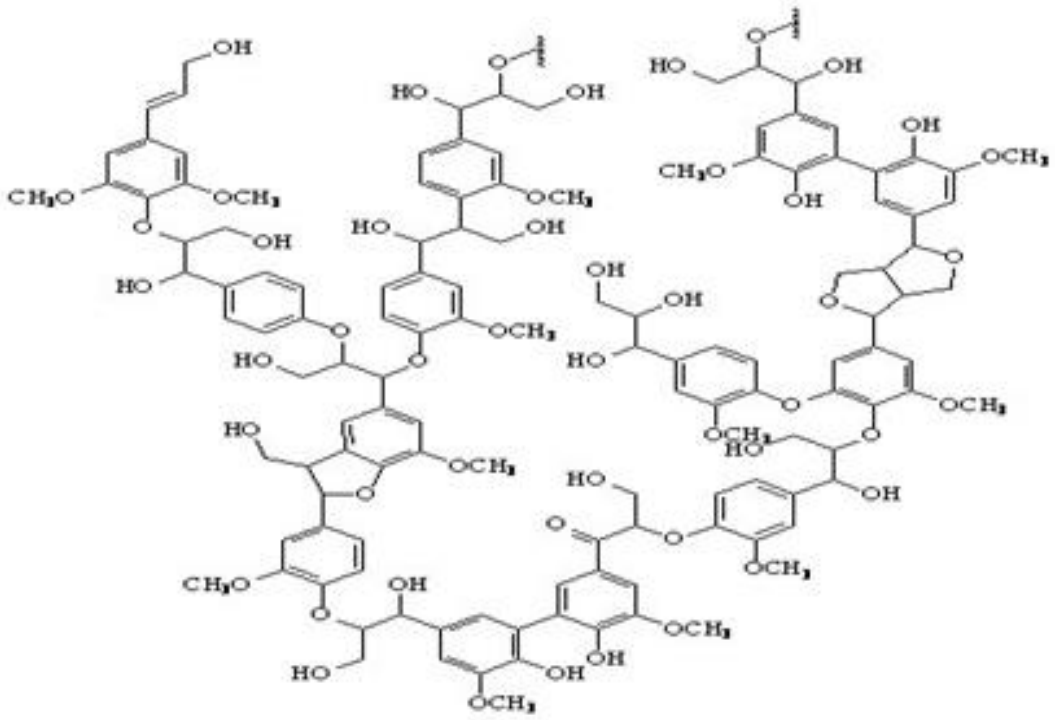


Figure 4: A lignin molecule

[Kirk *et al*., 1985]

There may be zero, one or two methoxyl groups attached to the rings giving rise to three structures, termed as I, II and III respectively. The structure-I is found in plants such as grasses, structure II in the wood of conifers and structure III is found in deciduous woods. Lignin strengthens the cell structures by stiffening and holding the fibers of polysaccharides together [Fan *et al.*, 1987]. Lignin contain several functional chemical groups, such as hydroxyl (phenolic or alcoholic), methoxyl, carbonyl and carboxyl, in various amounts, depending on origin and the applied isolation process [Gosselink *et al*., 2004; Sun *et al*., 2001].

Methoxyl groups (-CH_3) are found in lignin from all plants [Bykov, 2008]. The content of methoxyl groups found in softwood and hard wood are 0.92 and 0.94 per one phenyl propane unit respectively [Gosselink *et al*., 2004; Sun *et al*., 2001; Bykov, 2008]

Hydroxyl groups: There are aliphatic hydroxyl groups (bound to ϒ-carbon), secondary aliphatic hydroxyl groups (bound to α-carbon) and phenolic groups (bound to 4-C atom of aromatic rings. On average, lignin contain 0.2 primary aliphatic hydroxyl groups per 1 phenyl propane unit, 0.84 secondary aliphatic hydroxyl groups per 1 phenyl propane unit, 0.3-0.35 phenolic hydroxyl groups per 1 phenyl propane unit.

Carboxyl groups: Natural lignin contain 0.05 COOH-groups per 1 phenyl propane units. Further carboxy groups are produced during delignification as a result of oxidization of hydroxal and carbonyl groups. After alkaline delignification carboxyl groups increased to 0.15 to 0.16 per 1 phenyl propane unit. The increase of COOH increase result in increased hydrophobicity of lignin. Carboxyl groups are able to connect to other functional groups via H-bonds.

Carbonyl groups: The total carbonyl groups in lignin are 0.21 per 1 phenyl propane unit. However these can be of 4 different kinds, there are a few aldehyde groups at the ϒ-C atom (0.04 per 1 phenyl propane unit) and the rest of carbonyl groups are (0.17 per 1 phenyl propane unit) are ketone.

Hardwood lignin is easier to break down. The reason has to do with the monomers used for formation of lignin. Remember, hardwood lignins have sinapyl alcohol as a monomer. Since it has two methoxyl groups there is no site on the aryl ring where free radical coupling will occur. Therefore, hardwood lignin has more ether inter unit linkages than softwood lignin. Because of high reactivity of lignin, the presence of lignin is one of the major problems in bioethanol production [Dien *et al*., 2009].

Removal of lignin: In 1994, McMillan, recognized the importance of pretreatment of lignocellulosic materials. Problems in using lignocellulosic materials are: a) cellulose in nature is bound with lignin, b) lignin inhibit hydrolysis of cellulose. Pretreatment is not only necessary to remove lignin and hemicellulose but it also reduce cellulose crystallinity and increase the porosity of the materials. Pretreatment must meet the following requirements;

i. improve the formation of sugars
ii. avoid the degradation and loss of carbohydrate
iii. avoid byproduct formation which inhibit hydrolysis and fermentation processes
iv. be cost effective.

Practical efforts to utilize lignocelluloses as a source for glucose and other sugars have led to many studies of the effect. These have resulted in the development of various pretreatment to increase hydrolysis susceptibility. Pretreatment of lignocellulosic materials has been carried out by physical, physico-chemical, chemical and biological processes.

2.3 Cellulases

The term 'Cellulases' represents to a mixture of enzymes which contains CBHs, EGs and β-glucosidase (though in small quantity). Fungi and bacteria produce extracellular cellulases which degrade a number of wood and plants [Mandels and Weber, 1969]. A number of *Trichoderma* species such as *T. reesei, T. viride, T. kongii* and some other fungal species such as *Penicillium funiculosum, A. wentii* produce extracellular cellulases. *Cellulomonas* and *Clostridium thermocellum* are bacteria that produce extracellular species able to degrade cellulose. Literature review about the adsorption of cellulases onto cellulosic substrate is summarized in Table 2 where the type of enzyme (column 1) adsorbed on the type of substrate (column 2) is given.

Table 2: Summary of enzyme adsorption on cellulosic substrates

Enzyme	Substrate	Max Binding mg/g	Reference
Trichoderma reesei			
CBHI, 4 °C	BMCC	6.0	Reinikainen *et al*., 1995
CBHI, 4 °C	Avicel	48	Medve *et al*., 1994
CBHI, 25 °C	Avicel	63	Tomme *et al*., 1990
CBHII, 25 °C	Avicel	64	Tomme *et al*., 1990
CBHI, 50 °C	Avicel	25	Bothwell *et al*., 1997
Thermobifida fusca 50 °C			
EG III	Avicel	26	Bothwell *et al*., 1997
EG III	BMCC	741	Bothwell *et al*., 1997
EG IV, V	Avicel	31, 31	Bothwell *et al*., 1997
Trichoderma viride 30 °C			
EG I	Avicel	130	Beldman *et al*., 1987
EG III	Avicel	26	Beldman *et al*., 1987
CBHI	Avicel	6.6	Beldman *et al*., 1987
Cellulomonas fimi 30 °C			

Enzyme	Substrate	Max Binding mg/g	Reference
EGs, 30 °C	BMCC	144	Gilkes *et al*., 1992
CBHs, 30 °C	BMCC	184	Gilkes *et al*., 1992
CBH, 22 °C	Avicel	3	Ong *et al*., 1993
C. thermocellum 25 °C			
CBHs	Avicel	10	Morag *et al*., 1995
CBHs	PSAC	200	Morag *et al*., 1995
C. cellulovorans			
CBH, 37 °C	Avicel	2.1	Goldstein *et al*., 1993
Cellulases mixture			
T. reesei	Avicel	95.2	Lu *et al*., 2002
T. reesei	PSAC	1224	Lee *et al*., 1982
T. reesei, 4°C	Avicel	34.9	Zheng *et al*., 2013
T. reesei, 4°C	SPCL	1.9	Zheng *et al*., 2013
T.viride	Cotton	78-89	Beltrame *et al*., 1982
T. reesei, 40 °C	Avicel	56	Ooshima *et al*., 1983
T. reesei, 40 °C	A.P. wood	81	Ooshima *et al*., 1990
T. reesei, 50 °C	Avicel	5.3	Bommarius *et al*., 2008
T. reesei, 50 °C	Avicel	18.6	Zheng *et al*., 2013
T. reesei, 50 °C	SPCL	21.2	Zheng *et al*., 2013
C. thermocellum 60 °C	Avicel	17.5	Hayashi *et al*., 1997

BMCC: Bacterial modified crystalline Cellulose; A.P. Wood: Dilute acid pretreated wood;
PSAC: Phosphoric acid-swollen cellulose: SPCL: Steam explosion pretreated corn stover lignin;
Max Binding: Maximum binding achieved

Table 2 indicates that cellulases produced from *T. reesei* complex have excellent adsorption on crystalline cellulose. Therefore, cellulases from *T. reesei* were selected to study adsorption and desorption. *T. reesei* complex secretes endoglucanases (EGs, such as Cel7B, Cel5A) and cellobiohydrolases (CBHI (Cel7A) and CBHII (Cel6A)) (Vinzant *et al*., 2001). In addition to the

CBH and EG activity, *T. reesei* also produces β-glucosidase, BG (Chen *et al*., 1992). Table 3 represents main components of cellulases and their compositions in some available commercial enzyme complexes.

Table 3: Enzyme components from *T. reesei* cellulases complex

Enzyme	Lutzen *et al*., 1983	Rosgaard *et al*., 2007	Kang, 2011	Hilden and Johansson, 2004
	Celluclast	**Cellulases**	**Spezyme CP**	**NS 50013**
	%	%	%	%
Cel 7B, endoglucanase I	10	5-10	12	10
Cel 5A, endoglucanase II	10	1-10	9	10
Cel 7A, cellobiohydrolase I	55	40-60	50	60
Cel 6A, cellobiohydrolase II	10	12-20	14	15
β - glucosidase	1-2	N.D.	< 2	2

The modular structure of enzymes was deduced from the studies of *T. reesei* and *Cellulomonas fimi*. The modular structure features i) carbohydrate-binding domain (CBD), ii) catalytic domain (CD) or core, iii) linker which join CBD and CD [Srisodusk *et al*., 1993]. This structure has been confirmed by a number of researchers [Hefford *et al*., 1992; Ryu *et al*., 1991; Ramalingam *et al*., 1992; Wilson, 1992; Park *et al*., 1993]. Various instruments have been used in structure analysis such as Triple quadruple mass spectrometer with post-column splitter, reverse-phase HPLC on an Ultrafast Microprotein Analyzer, nuclear magnetic resonance, X-ray crystallography etc. Computer software's such as Multiview 1.0, DENZO, ROTAVATA and AGROVATA, RAVE, XPLOR and /or REFMAC can also help in structure analysis. CBD have shape like a wedge with dimensions approximately 30 x 18 x 10 Å [Linder and Teeri, 1997]. CBDs are thought to have three primary functions i) proximity effects, ii) substrate targeting and, iii) microcrystallite disruption [Kuutti *et*

al., 1991; Linder and Teeri, 1997; Boraston *et al*., 2004). CBMs promotes the association of the enzyme with the substrate (proximity effect) (Reinikainen *et al*., 1991; 1992].

CBDs are selective for substrates including crystalline and amorphous celluloses, and various soluble and non-soluble polysaccharides (substrate targeting) [Lamed *et al*., 1994; Tomme *et al*., 1995; Creagh *et al*., 1996; Tormo *et al*., 1996; Linder and Teeri, 1997; Henshaw *et al*., 2004]. In addition, CBDs disrupt the structures of the carbohydrate ligands to make the substrate more susceptible to enzymatic attack (microcrystalline disruption) [Din *et al*., 1994]. For the purpose of biomass conversion the CBDs with the function of substrate targeting (cellulose) are of interest [Phelps *et al*., 1995; Teeri, 1997; Boraston *et al*., 2004. The full catalytic domain consists of 89 to 450 amino acid residues. The active site of Cel6A (CBH II), as for the *T. reesei* CBH II, is enclosed by two extended surface loops (residues 174–196 and 407–435). This results in the formation of a long substrate-binding tunnel, over 20 Å long. The active-site tunnel of the *H. insolens* Cel6A (CBH II) constricts to a minimum dimension of approximately 3.5 Å. Both of these loops are stabilized by disulphide bridges (Cys181–Cys240, Cys372–Cys419. Two aspartic acid residues, located in the center of the tunnel are the probable catalytic residues [Rouvinen *et al*., 1990]. A mechanism outlined by Koshland (1953) requires the presence of two catalytic carboxylate groups: a proton donor to protonate the glycosidic bond and promote leaving-group departure, and a catalytic base to activate the hydrolytic water molecule for nucleophilic attack at the anomeric center.

Comparison of the primary structures of *T. reesei* cellulases revealed that a large catalytic domain joined by a flexible, 0-glycosylated linker peptide to a smaller cellulose-binding domain (CBD) [Penttila *et al*., 1986, Stahlberg *et al*.,1991]. The linkers identified in *T. reesei* cellulases may contain 30-44 amino acids in length [Fagerstam *et al*., 1984; Tomme *et al*., 1988; Claeyssens and Tomme,

1989; Srisodsuk *et al*., 1993]. Linkers are rich in proline, serine, threonine (Teeri *et al*., 1987) and they are negatively charged. The proline and hydroxyl amino acid content is different among the linkers. The length of linker from CBH I and CBH II observed was 5-15 nm [Lee and Brown, 1997; Liu *et al*., 2011]. Cellulase linkers represent extended, flexible hinges between the two domains facilitating their independent function (Burton, 1989; Bushuev *et al*., 1989). Figure 5 represents how CBHs, EGs and βGs approach cellulose. CBHs attack on the reducing (R) and nonreducing (NR) ends. Endoglucanase II (EG II) from *T. reesei* is able to hydrolyze the internal β-1,4-glycosidic bonds of soluble celluloses and amorphous regions of cellulose βGs converts tetrose, triose, cellobiose.

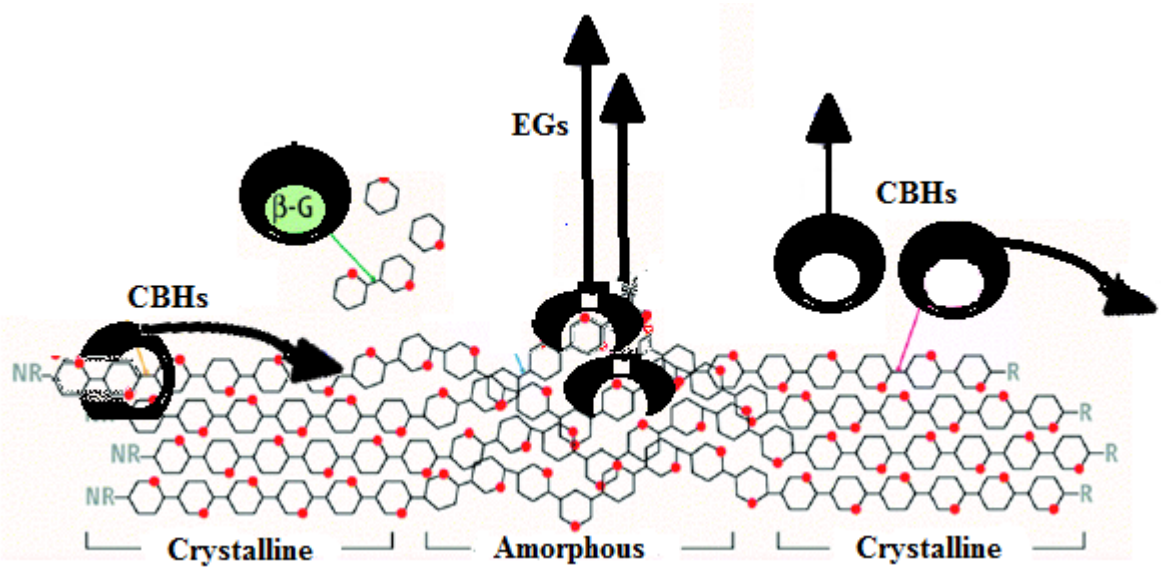


Figure 5: A conceptual diagram on functioning of cellulases onto cellulose

Structural difference between the EGs and CBHs is at the two loops which contains the active sites in the CBHs are absent in EGs. The one loop (407–435) present in Cel6A (CBH II) is absent from EGs while the other surface loop (174–196) is 'pulled up' in the EG structure in a way to expose the active site [Divne *et al*., 1994; Henriksson *et al*., 1996]. This results in an active-site tunnel for Cel6A (CBH II) and an open a cleft in EGs. The work pattern in both the enzymes is almost the same. The enzymes CBH I and CBH II have different structure and hydrolyze the cellulose from the reducing

end and the non-reducing end. They have tunnel shaped active sites. The enzymes CBH I, CBH II, EG I, and EG II shared a region of 36 amino acids well conserved [Terri *et al*., 1987; Divine *et al*. 1994]. From a structural point of view, adsorption of cellulase is through CBD [Creagh *et al*., 1996; Carrard *et al*., 2000; Fox *et al*., 2013]. Several papers have reviewed the roles and function of these binding domains [Shoseyov *et al*., 2006]. See further discussion on CBDs under the heading 'Cellulases adsorption'.

Cellulases dimensions

Depending upon the substrates and the fermentation conditions, cellulases obtained from different sources are quite different in size and molecular weight. Using gel filtration Cowling (1975) estimated dimensions of cellulases complexes in two possible scenarios: 1) as hydrodynamic spheres, from 24 Å to 77 Å. 2) as ellipsoids, from 13 X 79 Å to 42 X 79 Å in width and length. Cel7A from *T. reesei* is composed of long loops, on one face of the sandwich, that form a cellulose-binding tunnel of ≈50 Å. The catalytic residues are glutamate 212 and 217, which are located on opposite sides of the active site, separated by an intervening distance consistent with a double-displacement retaining mechanism [Kleywegt *et al*., 1997]. Enzyme binding sites are regions on the surface of an enzyme specially designed to interact with other molecules. The binding site has the task of specifically recognizing the molecule upon which the enzyme acts [Shevelev and Hubscher, 2002] and develop binding. CBH I, II and EG I, II are two domain components of cellulases, consisting of a large catalytic core linked to a small cellulose-binding domain by a heavily glycosylated linked region [Abuja *et al*., 1988]. The catalytic domain of CBH (Cel7A) consists overall dimensions of approximately 60 Å x 40 Å x 50 Å [Grassic *et al*., 2004]. The third type of enzyme present in cellulases is β-glucosidase and it is a single domain enzyme. All the three type of enzymes act in synergy to convert cellulose to glucose.

Cellulases adsorption

EG II from *Trichoderma reesei* is able to hydrolyze: i) the internal β-1,4-glycosidic bonds of soluble celluloses and, ii) the amorphous regions of cellulose [Fukuda *et al*., 2006]. When EG II approach celluloses, its CBD binds to cellulose and its catalytic domain breaks the glycosidic bonds of cellulose (Nam *et al*., 2002; Ito *et al*., 2004; Linder *et al*., 1995). EG moves progressively along a crystalline cellulose chain, its CBD arranges the chain to feed to the catalytic domain where cellobiose is produced by hydrolyzing β-(1, 4) glycosidic linkages. CBD had a flat face composed of asparagine 29 (N29) and glutamine 34 (Q34) and three aromatic amino acid residues (W5), (Y31), and (Y32) on the flat face (Linder *et al*., 1995). The aromatic amino acid residues are the main part for the binding to crystalline cellulose, as shown by the alanine-substitution experiment of each aromatic amino acid residue (Linder *et al*., 1995). The conceptual diagram in Figure 6 is design by adapting published literature.

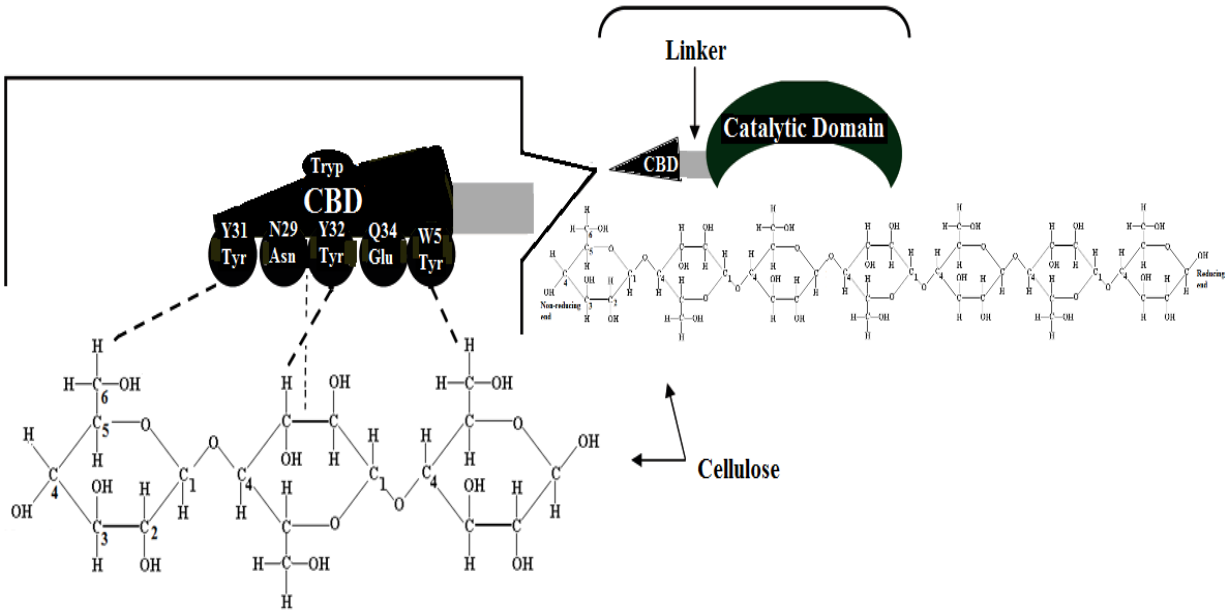


Figure 6: A conceptual diagram on action of CBDs on cellulose for adsorption

[Adapted from: Linder *et al.*, 1995; Nam *et al.*, 2002; Ito *et al.*, 2004; Fukuda *et al.*, 2006; Nimlos *et al*., 2007]

The distance between the groups is about the same as a cellobiose unit in the cellulose i.e. 11Å [Humphrey *et al*., 1996]. The 4th tyrosine residue in the CBD (Y13) moves from its internal position to form van der Waals interactions with the cellulose surface [Nimlos *et al*., 2007]. CBD because of the presence of aromatic residues behaves as hydrophobic and interact with the surface structure of crystalline cellulose [Lehtio *et al*., 2003]. In this conformation either 2 or 3 axial hydrogen atoms are exposed, while hydroxyl groups at the ring are in the equatorial position. As a result, the hydrophobic surfaces of CBDs bind to the hydrophobic surface of crystalline cellulose.

There are two hypotheses to explain adsorption of cellulases [Coughlan, 1989; Tomme *et al*., 1988; Zagursky *et al*., 1986]: i) the CBD simply binds to cellulose i.e. riding over the cellulose surface [Teeri, *et al*., 1992], ii) the CBD plays more active role in liberating cellulose chains from the crystal bundle [Knowles *et al*., 1987; Mulakala and Reilly, 2005]. The 3D structure of binding domain of CBH I was determined earlier [Kraulis *et al*., 1989]. The domain folds into a wedge-like structure with one face flat and hydrophilic and the other face is more hydrophobic. The flat face of CBD most probably adsorbs on the substrate. The amino acid sequences of the four cellulose-binding domains of *T. reesei* cellulases have similar 70 % amino acid character. The *tyrosines* Y492 and Y493 are strictly conserved in all *T. reesei* cellulases and the Y474 is substituted by *tryptophan* in CBH II and EG II. The spacing between the *tyrosines* (and the *tryptophans*) are almost the same as that the spacing between every second glucose unit in the cellulose crystal [Reinikainen *et al*., 1992].

Enzymes to substrate ratio

The number of studies that speak directly to this point is, however, limited, and thus extensive data are not available with respect to variables such as sources of enzymes, substrates, enzyme preparation method, enzyme/substrate ratios, and extent of reaction. In the Table 4 column 1 is for the enzymes

adsorbed on substrate (column 2), column 3 gives enzyme to substrate and column 4 shows enzyme concentration used in adsorption.

Table 4: Enzymes used for adsorption onto substrates

Enzyme	**Substrate**	**E/S**	**[Enzyme]** µg mL-1	**Reference**
Meicelase CEPB-5041 (*T. viride)*	MCC 2330	5.7-4.5	160-230	Converse *et al*., 1988
CBHI, CBHII	Avicel (M2331)	0.01, 0.03	N.D.	Medve *et al*., 1994
Cellulases *T. viride, T. reesei*	Avicel PH 101	0.02	40-500	Wang *et al*., 2011
cellulases QM 9414 (*T.uiride*)	Solkafloc 40	0.11, 0.22, 0.011 0.022, 0.044	1100 -2200	Peitersen *et al*., 1977
CBHI, CBHII	Avicel PH 101	0.008	100	Kim *et al*., 2001
CBHI, EG II	Avicel (M2331)	0.005,0.01,0.16	N.D.	Medve *et al.*, 1998
β-d-glucosidase, Mannanase, EGs, CBHs	Shirakamba wood	1, 0.1, 0.02,	N.D.	Ishihara *et al*., 1991
Tyrosine, Phenylalanine	Corn Cobs	0.01-0.002	20-100	Alves *et al.*, 2013

According to Lee and Fan (1982) a linear correlation exist between adsorption/hydrolysis rate and enzyme to substrate ratio. Bernardez *et al*. (1993) adsorbed cellulases (obtained from *Clostridium thermocellum*) on Avicel 105 and on pretreated mixed wood (90 % Birch wood, 10 % Maple) and examined the effect of enzyme to substrate ratio on adsorption. They concluded that at low E/S ratios, there was a linear trend of adsorbed enzyme with respect to the initial enzyme concentration. At higher E/S ratios, the results of their experiments showed that adsorbed enzyme leveled off due to the saturation of the cellulosic substrate with enzyme. A very crucial parameter is an enzyme/substrate (E/S) ratio, since a limitation of cellulase adsorption by substrate surface area may occur at high E/S ratios. Use as low a concentration of cellulase as possible in the experiments. High enzyme concentration may result in distorted inhibition patterns because of a possible lack of

cellulose surface area to bind the enzyme. For the same reason, our study used low cellulose concentrations and worked a fixed substrate concentrations, in other words, at low enzyme/substrate ratios.

Interactions of cellulases with cellulose

Cellulase adsorption onto crystalline cellulose was widely studied with a focus on CBD-cellulose interactions, CBDs adsorb more to cellulosic surfaces [Palonen *et al*., 1999]. The α- and β-structures of cellulose have hydrophilic and hydrophobic faces. The hydrophobic faces are only 38 % of surface area in crystals of 36 parallel cellulose chains [Nimlos *et al*., 2012]. Lehtio *et al*. (2003) reported that CBDs of family 1 and family 3 cellulases was preferentially adsorbed on the hydrophobic planes of Valonia cellulose. Enzymes interact with carbohydrates through charged and aromatic amino-acid residues and enzymes may form hydrogen bonds or van der Waals interactions with carbohydrates [Vyas, 1991]. There are contradicting results reported about the effect of pH on cellulase adsorption. For example, Reinikainen *et al*. (1995) showed that pH only had a modest effect on *T. reesei* Cel7A adsorption onto BMCC, while binding onto catalytic domain was unaffected by pH. Some other report suggested that at high substrate concentration the CBDs becomes less important for substrate recognition [Le Costaouec *et al*., 2013; Varnai *et al*., 2013].

Interactions of cellulases with lignin

Non-productive enzyme adsorption onto the lignin-rich components of biomass prevents efficient hydrolysis of feedstock [Chernoglazov *et al*., 1988; Sutcliffe and Saddler, 1986]. Adsorption onto lignin is disadvantageous for process economics: i) higher enzyme loadings are required to overcome the inhibitory effect, and ii) enzyme recycling is hindered after a completed reaction (Lee *et al*., 1995). Three types of interactions are involved in enzyme binding onto lignin: i) hydrophobic

(Eriksson *et al*., 2002), ii) electrostatic (Nakagame *et al*., 2011b), and iii) hydrogen-bonding interactions (Berlin *et al*., 2005). The CBDs of *T. reesei* cellulases, Cel7A and Cel5A were adsorbed onto lignin in significantly high amount [Palonen *et al*., 2004] due to hydrophobic interaction. Some isolated lignin have shown carboxylic acid, phenolic and aliphatic hydroxyl groups [Berlin *et al*., 2006]. Phenolic hydroxyls in lignin model compounds were found to be deprotonated in the pH range 6.2–11.3 [Ragnar *et al*., 2000]. Enzymatic hydrolysis of lignocellulosic materials is usually carried out at pH 5, when carboxylic acid groups present in lignin are deprotonated. Nakagame *et al*. (2011b) reported that at pH 4.8 the isolated lignin showed a negative charge, and *T. reesei* enzymes (Cel6A and Cel5A) were positively charged, resulting in better adsorption. Adsorption onto lignin was reduced at elevated pH values due to increased repulsive electrostatic forces between the enzymes and lignin (Lou *et al*., 2013). This finding has more practical value from adsorption and desorption point of view. Enzyme–substrate interactions are non-covalent type and the driving forces involve are hydrophobic and electrostatic interactions, with minor contribution from hydrogen bonding and dipolar interactions [Claesson *et al*., 1995; Norde, 1996].

Degradation of substrates during adsorption

Most of the reported results showed that the adsorbed cellulase reached a constant value in less than 90 min, and among these many were in less than 30 min whereas complete hydrolysis of cellulose took a day or more [Ooshima *et al*., 1983; Chernoglazov *et al*., 1988; Lee and Woodward, 1989; Singh *et al*., 1991; Boussaid and Saddler, 1999]. Andersen and co-workers reported that Avicel PH-101 was hydrolyzed up to 7 % in 24 hours by using a commercial enzyme mixture (EGs, CBHs and β-glucosidase). Most of this hydrolysis (5 %) was completed after 5 hours of the adsorption. Kyriacou *et al*. (1988) studied adsorption of EG I, EG II, EG III and CBH I components of cellulases on Solkafloc (purified wood cellulose) at temperature 5 ^{o}C, 30 ^{o}C and 50 ^{o}C. The degree of hydrolysis

of Solkafloc was examined by each cellulases component during the adsorption process by monitoring the reducing sugars produced. At 5.0 °C, cellulases EG I, EG II, and EG III hydrolyzed less than 1.0 % of Solkafloc and CBH I did 1.5 %. At 50.0 °C, EG I could degrade 2.0 %, EG II and EG III, did by 2.4 %, while CBHI degraded Solkafloc by 3.3 %. In our cellulases system there were 10 % EG I, 10 % EG II and 60 % CBH I, therefore the expected degradation could be 2.24 %, which is negligible and we were not working with Solkafloc also. Hu *et al.* (2011) adsorbed Celluclast 1.5L on steam pretreated corn stover and found that the substrate was 5 % hydrolysed after 24 hours. Lee and Woodward (1989) adsorbed cellulases *T. reesei* C30 on Avicel at pH 5, 5.6 and 9.6. Adsorption equilibrium was achieved in 30 minutes and after 4 hours hydrolysis just started. Similarly, Gao *et al.* (2014) studied hydrolysis of Avicel by using a mixture of cellulases and β-glucosidase and 0.4 % hydrolysis occurred at 24 hours. Notice another point here that Gao *et al*. (2004) were also using β-glucosidase which helps hydrolysis. Therefore, during the period of adsorption/desorption no hydrolysis of Avicel was expected by using cellulases NS50013 alone.

Sahoo *et al.* (2011) reported that the thermal degradation onset temperature for Protobind 1000 was 155 ^{o}C. Onset temperature is the temperature at which the weight loss begins. The initial (small) degradation in Protobind 1000 was observed at 55 ^{o}C which was negligible because it was due to dehydration of xylose. Protobind 1000 contains < 4 % xylose which degrade around 200-270 ^{o}C. The thermal decomposition reactions of polysaccharides occur through: i) dehydration, decarboxylation, decarbonylation, ii) cleavage of glucoside bonds, C-H, C-O and C-C bonds [Hoareau *et al*., 2004]. Xylose undergoes dehydration during the initial stage of heating (55 ^{o}C) and depolymerization of xylose take place on increasing the temperature above 200 ^{o}C [Lappalainen *et al*., 2006]. The major degradation (52.7 %) occurs between temperatures 200 to 600 ^{o}C. At 800 ^{o}C

char residues obtained were about 38 %. Therefore, in this study the degradation of Protobind during adsorption/desorption studies was not expected.

2.3.1 Cellulases adsorption isotherms

The first step in cellulolysis is binding (adsorption) of the cellulase onto the substrate. Equilibrium relationships between cellulases and substrates are described by adsorption isotherms. Adsorption isotherms give the capacity of the adsorbent based on the ratio between the quantity adsorbed and the remaining in solution at fixed temperature at equilibrium [Dhir and Kumar, 2010]. The adsorption isotherms have history for being used in representing adsorption of gases on liquids [Wilkins, 1934; Wylie, 1952; Dabarowski, 2001] and liquids on solid surfaces [Lee, 1999; Berti, *et al*., 2000]. Paper pulp, silica or mica were used to model the behaviour of cellulose due to the presence of hydroxyl groups [Bogdanovic *et al*., 2001; Lingstrom *et al*., 2007], but the mineral surfaces cannot be considered as very good representatives of cellulosic surfaces. Therefore various ultrathin films have instead been developed to represent more precisely surface of cellulosic fibres. They were introduced in detail in a review article (Kontturi *et al*., 2006) and were considered for studies of adsorption and swelling of materials (Falt *et al*., 2003; Tammelin *et al*., 2006) or direct surface force measurements (Notley and Wagberg, 2005). Recently, some reports were available on cellulases adsorption on various lignocellulosic substrates, such as microcrystalline cellulose [Medve *et al*., 1994; Hong *et al*., 2007], newspaper pulp, bagasse, and treated wood chips [Yu *et al*., 1995; Goel and Ramchandran, 1983], corn stover [Xu *et al*., 2008], steam-exploded Douglas fir [Lu *et al*., 2002], pretreated hardwood [Bernardez *et al*., 1993], isolated lignin from softwood [Palonen *et al*., 2004] and lignin preparations from Lodgepole pine [Tu *et al*., 2009].

The information about adsorption of cellulases onto lignin is very rare and for wheat straw lignin none could be found. Adsorption of cellulases on cellulose and lignin under similar experimental conditions was never studied although both remained present on lignocellulosic substrate at the same time. The aim of the present work was to investigate the adsorption of cellulases on cellulose (Avicel PH 101) and wheat straw lignin (Protobind 1000) under similar experimental conditions.

Peitersen *et al*. (1977) and Kim *et al*. (1995a; 1998) determined that adsorption of cellulases onto microcrystalline cellulose could be represented by Langmuir model. Some of the researchers have suggested Freundlich isotherm for adsorption of cellulases [Carrard and Linder, 1999; Tu *et al*., 2009; Sun *et al*., 2011;]. Few reports used both Langmuir and Freundlich isotherms to represent adsorption of cellulases [Kanchagar, 2003; Vadi *et al*., 2010; Singh and Kaur, 2014]. The present work will elucidate adsorption pattern of cellulases whether it follow Langmuir or Freundlich or another type of isotherm. Furthermore, this is the first report on the study of cellulases adsorption on Protobind 1000.

Linear adsorption isotherm

Linear adsorption isotherm is also known as Nernst adsorption isotherm. According to Nernst isotherm equation an enzyme (cellulases) irrespective of its total amount, distributes itself between two layers (adsorbed or bulk) in a constant concentration ratio, at constant temperature; the ratio, equal to the constant in Equation 2.4, is referred to as the distribution or partition ratio/coefficient (K_N) [Oscik and Cooper, 1982]:

$$P_e = K_N \left[P_f\right] \quad \ldots \quad 2.4$$

where, P_e = specific amount of cellulases adsorbed on substrate, µg mg^{-1}, [P_f] = concentration of cellulases present in the solution, µg mL^{-1}, K_N = Partition coefficient given by the slope, (mL mg^{-1}).

Langmuir adsorption isotherm

The Langmuir adsorption model assumes:1) The substrate is composed of uniform binding sites and, 2) There is no interaction between the adsorbing molecules. A monolayer of adsorbents is formed and that the free energy of adsorption remains constant. The amount of adsorbed cellulases were obtained by the following Equation 2.5 [Langmuir, 1918]:

$$P_e = [P_f] \frac{K_a P_{am}}{(1+ K_a[P_f])} \quad \ldots \quad 2.5$$

where,

P_{am} = maximum cellulases adsorbed (µg of cellulases/mg of substrate), K_a = constant $(mL/\mu g)^{-1}$

Equation 2.5 can be rearranged to get a linear form:

$$\frac{[P_f]}{P_e} = \frac{1}{K_a P_{am}} + \frac{1}{P_{am}} [P_f] \quad \ldots \quad 2.6$$

Pietersen *et al.* (1977) used equation 2.6 for the first time. The Equation 2.5 can also be rearranged in another way called 'Scatchard' type, used for the first time in 1949:

$$\frac{P_e}{[P_f]} = K_a P_{am} - K_a P_e \quad \ldots \quad 2.7$$

Freundlich adsorption isotherm

The Freundlich isotherm has a place in colloid chemistry for characterising the adsorption of molecules onto an interface [Reed and Matsumoto 1993; Proctor and Toro-Vazquez, 1996]. Freundlich isotherm was used to describe adsorption of α-amylase to starch granules. Freundlich isotherm for adsorption to heterogeneous surfaces is given as follows [Freundlich, 1906]:

$$P_e = K_f \left[P_f\right]^{\frac{1}{m}} \quad \ldots \quad 2.8$$

Where, K_f = Freundlich constant for cellulases-substrate complex, distribution constant, µg mg^{-1}, m (µg mg^{-1}), power term of the Freundlich isotherm (m>1), m and K_f are empirical parameters.

The linear form of Equation 2.8 can be written as:

$$lnP_e = \frac{1}{m} ln[P_f] + lnK_f \quad \ldots \quad 2.9$$

Cellulases adsorption on cellulose is a prerequisite to desorption. Adsorption of cellulases generally reached to its equilibrium within a short time. The adsorption pattern was commonly explained using Langmuir isotherm [Hong *et al*., 2007; Zhang and Lynd, 2004]. The Langmuir isotherm assumed that the adsorption was a single layer phenomenon and it maintained a balance between rates of adsorption and desorption, binding affinity and adsorption capacity. The values of parameters in Langmuir isotherms are available through a number of reports. However, the reported data have a wide variation, due to different methods and experimental conditions used. Some studies reported contrasting hypothesis [Zhang and Lynd, 2004] such as cellulases bound reversibly to cellulosic materials while some claimed partially irreversible [Kyriacou *et al*., 1989; Palonen *et al*., 2004]. Cellulases have different binding preferences (Pinto *et al*., 2004; Boraston, 2005; Ding *et al*., 2006). Therefore, a dedicated study on the adsorption of cellulases on cellulose and lignin was required and present study fulfill these needs. Adsorption is the ability of cellulases to stay on a substrate. The more tightly adsorbed cellulases to a substrate, the less likely cellulases will desorb [Azevedo *et al*., 2000]. The adsorption studies also provide insight about the structure of adsorbed layers and the type of interaction of cellulases with the substrates hence about desorption because desorption is dependent on surface coverage [Wang *et al*., 2013] and adsorption pattern.

2.3.2 Adsorption of mixtures of endoglucanases and exoglucanases

Kanchagar (2003) studied adsorption of mixtures of endoglucanase or exoglucanases in different combinations. Some components of endoglucanase and exoglucanases showed synergism in hydrolytic action while others do not show any synergism CBH II showed synergistic action in the presence of EG II but in presence of EG I, CBH II showed preferential adsorption on microcrystalline cellulose. CBH II was found to be inactive on microcrystalline cellulose when used alone [Kanchagar, 2003]. Some other research groups reported that CBH II showed synergistic action when present in the ratio of 1:1 (w/w) with EG I or EG II [Kim *et al*., 1995a, Kim *et al*., 1995b, Kim *et al*., 1995c].

Beldman (1987) studied adsorption of endoglucanase and exoglucanases from *Tricoderma viride* and reported that some enzymes adsorbed strongly (EG I, III and CBH I) and the others adsorbed moderately on crystalline cellulose (EG II, IV, CBH II) and Ryu *et al*. (1984) observed that a part of endoglucanase do not adsorbed at all. The competitive adsorption of CBH I (Cel7A, an exoglucanase and endoglucanase I (Cel7B) from *T. longibrachiatum* was studied by Maurer *et al*. (2012) on cellulose and it was observed that Cel7A (CBH I) has a higher adsorptive affinity for cellulose than does Cel7B (EG I). The rate at which each surface enzyme bound (when they are present alone) was identical. Because of the higher affinity of Cel7A for the cellulose surface, when Cel7A and Cel7B compete for surface sites. A significantly higher bulk concentration of Cel7B is required to achieve comparable surface enzyme concentrations. The choice of bulk composition (initial composition) of enzymes would be designed by considering nature (composition) of substrate. According to Linder *et al*. (1995) the cellulose binding modules of CBH I and EG I show single amino acid substitutions leading to differences in binding affinity. In addition, catalytic domains of cellulases are known to specifically adsorb to cellulose binding sites independently of

cellulose binding modules [Lynd *et al*., 2002]. The maximum cellulase adsorption at equilibrium was higher for EG I than for CBH I, indicating more accessible cellulose binding sites for EG I, which was also observed by other researchers [Beldman *et al*., 1987, Nidetzky *et al*., 1994]. Besides the aforesaid differences in cellulases structure and binding affinity, these maximum adsorption differences could be explained by the lower molecular mass of EG I and, therefore, a better access to internal binding sites (pores) as described for other proteins and materials [Hunter and Carta, 2002; Oberholzer and Lenhoff, 1999].

On the other hand, it was suggested that the substrate to enzyme ratio may not be maintained because of a decrease in number of sites for endoglucanases and increase in number of sites for exoglucanases and individual nature of adsorption of endoglucanases and exoglucanases [Ooshima *et al*., 1983; Kim *et al*., 1992]. Under various tested conditions the results obtained by the mixed enzymes exceeds the sum of the individual enzymes [Qi *et al*., 2007; Zhang *et al*., 2010; Boutard *et al*., 2014]. Therefore, the adsorption of cellulases mixture (as planned in this study) could be a representative adsorption of a complete enzyme system. Some contrasting results were presented by researchers about cellulases adsorption and optimal activity because these researches were conducted under different temperatures, pH and with enzymes obtained from various sources. Therefore, our study is using a commercial mixture of cellulases on wide range of temperature (same conditions for both cellulose and lignin) to help estimate adsorption and desorption on/from lignocellulosic substrates

Time taken for adsorption of cellulases

The adsorption profile of cellulases on microcrystalline cellulose (Avicel), and potato pulp was determined by Singh *et al*. (1991). It was observed that most of the enzymes were rapidly adsorbed onto Avicel and pulp within the first 10 min of contact at 30 ^{o}C. No significant adsorption was

observed thereafter. Steiner *et al*. (1988) reported that half of the maximally adsorbed enzyme was bound within 1-2 min of contact with microcrystalline cellulose, however, adsorption equilibrium was established less than 30 min. Pulp exhibited higher enzyme adsorption than Avicel. An appreciable amount (about 18%) of cellulase was also found to be adsorbed onto xylan substrate within 10-20 min of contact. Jager *et al*. (2010) reported that cellulase adsorption on α-cellulose was rapid, cellobiohydrolase CBH I reached equilibrium in 20 minutes and endoglucanase EG I in 30 min. Pareek *et al*. (2013) adsorbed cellulases on spruce lignin (SP) and black cotton wood lignin (BCWL) and found that more than 60% of the protein had adsorbed within 30 min of incubation, while it took more than 2 h for the BCWL to reach the same value. Tu *et al*. (2009) reported that the time taken by cellulases to adsorb on ethanol pretreated lodgepole pine wood and steam exploded Lodgepole Pine was 30-60 minutes. These examples indicated that adsorption of cellulases onto cellulose was considerably different from that of lignin.

Effect of temperature on adsorption of cellulases

Controversial results were reported for the effect of temperature on cellulase adsorption process. Some of the researchers suggested that cellulases adsorption on lignocellulose was an exothermic and enthalpy-controlled reaction. They found that the amount of cellulases adsorption decreased as the temperature increased [Medve *et al*., 1994; Kim *et al*., 2000 and Ooshima *et al*., 1983]. On the contrary, Hoshino *et al*. (1992) and Creagh *et al*. (1996) proposed that cellulases adsorption on cellulose was an endothermic and entropy-driven reaction, means increased with temperature. Tu, *et al*. [2009] investigated reaction of cellulases on a soft wood Lodgepole pine, *Pinus contorta,* (cellulose, 47.6 %; hemicellulose, 22.9 %; lignin, 26.3 %; and extractives, 4.7 %). The lignin derived from steam exploded Lodgepole pine (L-SELP), and lignin derived from ethanol pretreated Lodgepole pine (L-EPLP) was used for the experiments. The results showed a higher level of

cellulase adsorption onto lignin at 45 ^{o}C than at 4 and 25 ^{o}C, indicating that cellulases adsorption on L-SELP and L-EPLP probably was an endothermic reaction [Eriksson *et al*., 2002; Tu *et al*., 2009; Zheng *et al*., 2013]. This most likely is true for the cellulases adsorption onto lignin, because hydrophobic interaction is the main force between lignin and cellulase enzymes [Eriksson *et al*., 2002; Tu *et al*., 2007], and the entropy could be the predominant driving force in the adsorption process through hydrophobic interactions [Eriksson *et al*., 2002; Tu *et al*., 2009; Wang *et al*., 2010]. The adsorption process which is entropy-driven was established by experimental data obtained from titration micro calorimetry [Creagh *et al*., 1996]. It means that lignin adsorbs more cellulases and with increase in temperature the adsorption on lignin increased. The results also implied that delignification of lignocellulosic substrates for cellulases desorption was necessary. Through literature survey it was observed that effect of temperature is a complex phenomenon because at one hand it influence adsorption, desorption and on the other hand it affect activity of cellulases.

Denaturation and loss of activity of cellulases with temperatures

There could be two ways to determine feasible/optimal temperatures for enzymes to work with, where enzyme do not lose activity or do not denature:

i- Considering denaturing temperature of each fundamental component of an enzyme

ii- Searching examples from literature

Table 5 presents the temperatures at which amino acids in a cellulase enzyme denatured. The fundamental amino acids in enzymes are listed in first column and their denaturing temperature are given in the second column.

Table 5: Denaturing temperatures of amino acids present in cellulases

Amino acid	Temperature °C	Reference
Aspartate	60	Eze and Echetebu, 1979
Glutamate	70	Kang *et al*., 1981; Consalavi *et al*., 1991
Arginine	60	"Brenda", 2016
Lysine	60	"Brenda", 2016
Tryptophan	50-60	Neurath *et al*., 1944
Tyrosine	55	Lazar *et al*., 1981
Isoleucine	60	"Brenda", 2016
Histidine	50, 60	Westmoreland and Matthews, 1973; Savany and Cronenberger, 1982
Phenylalanine	55	"Brenda", 2016
Alanine	60	Eze and Echetebu, 1979
Cysteine	65	"Brenda", 2016
Leucine	60	Berman and Boyer, 1969

Arginine is one of the amino acids which perform anionic bonding with the substrate, other one is lysine. Phenylalanine starts denaturing at 55 °C but this amino acid is located in the inner core of enzymes therefore temperature around 55 °C start affecting on cellulases structure. Srinivas and Balasubramanian (1995) have reported that the presence of proline delays the thermal unfolding of the enzymes by approximately 10 °C. It means that over all denaturing temperature of enzymes have higher values than that of individual participating amino acid.

Literature provided examples of activities of cellulases components as a function of temperature such as Baker *et al*. (1992) investigated thermal denaturation of four purified *Trichoderma reesei* cellulase components, cellobiohydrolases CBH I, CBH II, endoglucanase EG I, and EG II. The cellulases components were monitored using a combination of differential scanning calorimetric (DSC), and

thermal scanning fluorescence emission spectrometry. The substrates studied were cellulose (Avicel), carboxymethyl cellulose (CMC). In Table 6 first column contains cellulase components and the second column comments on their interaction with substrate under the influence of temperature.

Table 6: Denaturing temperatures of the components of the cellulases system

Cellulase	**Influence on temperature on enzyme activity**
CBH I	Increases with increase in temperature from 20 to 55 ^{o}C, maintains up to 60 ^{o}C and had a rapid fall and completely denatured at 70 ^{o}C
EG I	Increases with increase in temperature from 20 to 50 ^{o}C, maintains up to 60 ^{o}C and had a rapid fall and had complete denatured at 70 ^{o}C
EG II	For CMC, Increases with increase in temperature from 20 to 50 ^{o}C, maintains up to 65 ^{o}C and had a rapid fall and had complete denatured at 70 ^{o}C For Avicel max was achieved at 60 ^{o}C and complete denaturing at 75 ^{o}C
CBH II	Increases with increase in temperature from 20 to 50 ^{o}C, maintains up to 60 ^{o}C and had a rapid fall and had complete denatured at 70 ^{o}C

Both Differential Scanning Calorimetry (DSC) and the activity measurements showed that EG II had maximum temperature 75 ^{o}C to be denatured. It means that EG II to be the most stable by 10-11 ^{o}C than the other three enzymes (Table 6). Few example will explain complexity of the influence of temperature. *T. versicolor* laccase, was adsorbed on pre-silanized silica beads by Dehghanifard *et al*. (2013). The results demonstrated that the maximum activity for both free and adsorbed *lassase* was at 40 ^{o}C. At 60 ^{o}C 70 % of the activity of adsorbed *laccase* was retained while free *laccase* were completely inactive. Because inactivation of a protein on a surface is a slower process and it is dependent on temperature. Andreaus *et al*. (1999) during studying adsorption of cellulases on Avicel found that the activity of the enzymes, was not affected until 37 ^{o}C, it was slightly reduced (-20 %) between 37 and 50 ^{o}C and it decreased sharply above 50 ^{o}C. Above 70 ^{o}C almost no activity was

found. Rahikainen *et al*. (2011) worked on lignins prepared from softwood and reported that activity of endoglucanases was reduced to 16 % and that of exoglucanases to 35 % of the original activity at 45 ^{o}C. It could be that enzymes with less compact structure are more likely to undergo structural changes during binding (Billsten *et al*., 1995; Haynes and Norde, 1995). The loss in activity could be due to conformational changes (or denaturing) in enzymes. Possible means to prevent enzyme denaturation on lignin surfaces would be to decrease the process temperature.

Other reported activities of endoglucanase and exoglucanase (Cel7A) after adsorption to enzymatic residual lignin (EnzHR). Such as, at 45 °C, 29 % of the initially added TeCel7A-CBM3 enzymes were free in solution, whereas at 55 °C only 9 % were free after 4 h of incubation. Furthermore, at high temperature TeCel7A-CBM3 was found to bind stronger on spruce EnzHR lignin, indicated by a steeper decrease in the amount of bound enzyme at 55 °C than at 45 °C [Rahikainen *et al*., 2011]. Increased enzyme binding onto lignin and increased surface denaturation are possible explanations for the negative effect of lignin at elevated temperatures [Rahikainen *et al*., 2011; Borjesson *et al*., 2007; Viikari *et al*., 2007]. Bonomo *et al*. (2006) investigated stability in the temperature range of 10-40 ^{o}C for Bovine serum albumin (BSA) and β-lactoglobulin (β-lg)/Hydrophobic adsorbent (Streamline Phenyl, packed in a column HR 5/5) and suggested that over 30 ^{o}C a great conformational change occur. Cellulases from *T. reesei* (Celluclast 1.5 L, Spezyme CP), cellulases from *Penicillium* sp. (MSUBC) on cellulolytic enzyme lignin (CEL) from steam-exploded Lodgepole pine (SELP) and on ethanol (organosolv)-pretreated Lodgepole pine (EPLP) showed that the activity of Celluclast 1.5L increased from 25 ^{o}C to 45 ^{o}C and enzymes got denatured from 55 ^{o}C to 75 ^{o}C [Tu *et al*., 2009b]. Therefore, in order to find the optimum desorption temperature it is quite tempting to the study of desorption of cellulases from substrate at temperatures 40 ^{o}C, 50 ^{o}C, and 60 ^{o}C, though 70 ^{o}C was added in desorption studies but it was not promising.

Accessibility of surface area for adsorption

A specific surface area (SSA) may play a great role in accessibility of substrate to cellulases. Fan *et al*. (1980) investigated the influence of surface type on hydrolysis by using Sigmacell 50 (50 μm), Avicel 101 (50 μm), and Avicel 105 (20 μm) as substrate. Through literature survey, it was known that the rate of hydrolysis was proportional to the concentration of the active adsorbed enzymes [Converse *et al*., 1988; Ucar *et al*., 1989], and rate limiting step is adsorption. It was expected that the adsorption of cellulases would increase with an increasing surface area. However, the published data showed that the specific surface area may not significantly affect the adsorption rate. It appeared that the extent of adsorption was determined by specific surface area (SSA) and the surface area showed no clear correlation with the adsorption in these reports and created a confusion. The effect of the SSA of cellulose to enzymatic hydrolysis was discussed in some other researches (Cowling, 1975; Gharpuray *et al*., 1983; Stone *et al.*, 1967; Burns *et al*., 1989) as well. Shewale and Sadana (1979) found no difference between the adsorption rates for two samples of microcrystalline cellulose with average particle size 38 and 90 μm. Similarly, Rivers and Emert (1988) also reported that the particle size was not the major factor for determining accessibility of lignocellulosics to cellulolytic enzymes. Some researchers [Welmer and Weston, 1985; Lin *et al*., 1985; Burns *et al*., 1989] developed a relationship between pore size (pore volume) distribution and hydrolysis rate. It was shown that the rate-limiting size of pores was equal or greater than the size of cellulase, which was estimated to be 40-90 Å [Welmer and Weston, 1985; Stone *et al.*, 1967; Burns *et al*., 1989]. Therefore, in the determination of surface area accessible to cellulases a molecular probes with similar size must be used. Clementi and Palade (1969) used *peroxidase* or *chymotrypsin* because of their inertness to cellulose and fairly close molecular sizes and weights to the cellulases. The diameter of *peroxidase* molecule was estimated to be 50 Å [Clementi and Palade, 1969], the size of

chymotrypsin was estimated as 40 x 40 x 50 Å [Squire and Himmel, 1979]. SSA measured by peroxidase was 0.30 m^2/g. The SSA of cellulose measured by Fan *et al*. using nitrogen adsorption gave a value of 2.2 m^2/g. This discrepancy resulted from structural non-uniformity of cellulose in respect to adsorption of low-mol-wt gases, and of high-mol-wt proteins. In the present work, the effect of surface area of Avicel PH 101 and Protobind 1000 on adsorption of cellulases was discussed.

2.4 Thermodynamics of enzymatic adsorption and desorption

Thermodynamics study could indicate feasibility of the adsorption and desorption from the obtained values of the thermodynamic parameters. The van't Hoff equation can be adapted to represent the adsorption and desorption processes of cellulases to/from adsorbents like Avicel PH 101 and Protobind 1000. It relates the main thermodynamics parameters such as enthalpy (ΔH) and entropy (ΔS) to the equilibrium distribution coefficient (K) of the adsorbed or desorbed species between an aqueous solution and an adsorbent, as shown in Equation 2.10:

$$\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \qquad \ldots \qquad 2.10$$

K is the ratio of cellulases adsorbed in equilibrium to the cellulases present in supernatant in equilibrium, R is the universal gas constant (8.314 $JK^{-1}mol^{-1}$), and T is the absolute temperature. The other thermodynamic parameter Gibbs free energy (ΔG) is given by Equation 2.11:

$$\Delta G = \Delta H - T\,\Delta S \qquad \ldots \qquad 2.11$$

The values of ΔH and ΔS were calculated from slope and intercept of the plot of lnK vs 1/T and ΔG was calculated from the obtained of ΔH and ΔS values.

Enthalpy

There were conflicting reports in literature on the effect of temperature on the adsorption of cellulases and hence calculated ΔH values both decreased and increased (positive or negative) with increasing temperature were observed. For one system of an enzyme and a substrate it can have a positive and negative value depending upon experimental conditions. For example, Ooshima *et al.* (1985) studied thermodynamic parameters for adsorption of EGs and CBHs on cellulose for a temperature range of 5 ^{o}C to 50 ^{o}C. ΔH values obtained at 10 ^{o}C were positive due to endothermic adsorption process. It was suggested that the increase in ΔH may be produced by the release of the water molecules which was structurally adjusted on the surface of cellulose and/or around the cellulases. The adsorption was entropy driven. On the other hand, the ΔH values reported at 40 ^{o}C were negative. At 40 ^{o}C the adsorption process was exothermic and enthalpy-driven. It was observed through literature survey that ΔH values also changed with the structure of substrates, for more porous structure (activated carbon and AFEX treated wheat straw) there was more ΔH. Table 7 is a brief presentation of ΔH values, reported in literature for various sets of enzymes and substrates, measured at given temperature ranges.

ΔH values reported in Table 7 for Avicel up to 30 ^{o}C were positive and for the cases when it was reported for temp 30-50 ^{o}C and it was negative. ΔH values reported for activated carbon were positive. ΔH is a state function. Being a state function means that ΔH is independent of the processes between initial and final states. In other words, it does not matter what steps we take to get from initial reactants to final products—the ΔH will always be the same. ΔH can have a positive value and a negative value. A positive sign means that the adsorption system (cellulases solution + substrate) uses heat and is endothermic.

Table 7: ΔH values for adsorption for various enzyme and substrate systems

Enzyme	Substrate	ΔH, kJ mol $^{-1}$	Conditions	Reference
CBH I from *I. lacteus*	Avicel Cotton	0. 18 0. 267	5 ^{o}C to15 ^{o}C	Hoshino *et al*., 1992
Endo 1 from *I. lacteus*	Avicel Cotton	0. 467 0. 2	5 ^{o}C to 15 ^{o}C	Hoshino *et al*., 1992
d-glucono-ξ-lactone hydrolase	lignocellulosic	-5.1	25 ^{o}C	Murphy, 2010
T. reesei mix	AFEX-treated wheat straw.	-118	35 ^{o}C to 49 ^{o}C	Brown *et al*., 2010
Trichoderma viride mix	Sigmacell 20, 50 (20 µm, 50 µm)	-29.930 -24.942	5 ^{o}C to 50 ^{o}C	Kim *et al*., 1988
CBHs from *C. fimi*	BMCC	-5.6	25,30, 35 ^{o}C	Creagh *et al*., 1996
EGs	Avicel	8.20 -0.142	5 ^{o}C to 30 ^{o}C 30 ^{o}C to 50 ^{o}C	Ooshima *et al*., 1983
CBHI , CBHII: *T. reesei*	Avicel	-16.71, -3.35	20 ^{o}C to 35 ^{o}C	Kim and Hong, 2000
CBHI, CBHII	Avicel	-18.0, -6.2	15 ^{o}C to 30 ^{o}C	Kim *et al*., 2001
Cr (VI)	Kraft lignin	-20.68	20 ^{o}C to 40 ^{o}C	Tazrouti and Amrani, 2009
Malachite green	Lignin sulfonate	131.58	10 ^{o}C to 60 ^{o}C	Tang *et al*., 2015
Rhodamine B	Aerobic granular wastewater sludge	-2.20	5 ^{o}C to 45 ^{o}C	Zheng *et al*., 2005
T viride (EGs)	Cotton fibers	-48.9		Beltram *et al*., 1984
Malachite Green $C_{50}H_{52}N_4O_8$, FW = 927.03 g/mol	Aerobic granules wastewater sludge	0.03	30 ^{o}C to 50 ^{o}C	Sun *et al*., 2008
Cr (III)	PAm-g-RWF	105.6	30,50,70 ^{o}C	Mahdavi *et al*., 2011
Aspergillus niger cellulase	Activated carbon powder	11.12	20 ^{o}C to 60 ^{o}C	Daoud *et al*., 2010
Cadmium	Goethite	12.6	10 ^{o}C to 70 ^{o}C	Johnson, 1990
Safranin	NaOH treated rice husk	40.92	30 ^{o}C to 50 ^{o}C	Chowdhury *et al*., 2010

1= considering Avicel has a CrI of 0.64; PAm-g-RWF: Acrylamide grafted on Rubberwood fibers; ITC: isothermal titration calorimetry was used to measure the thermodynamics of the binding

The negative ΔH value means that the reactants have greater enthalpy than the products, or there is a removal of energy from the reaction (and addition into the surroundings).

Entropy

Some examples of ΔS values for adsorption of enzymes and big molecules on lignocellulosic, activated carbon and rubber are given in Table 8.

Table 8: ΔS values for various adsorption systems

Enzyme	Substrate	ΔS, $Jmol^{-1} K^{-1}$	Conditions	Reference
CBH I from *I. lacteus*	Avicel Cotton	0.01, 0.02	5-15 ^oC	Hoshino *et al*., 1992
EG I from *I. lacteus*	Avicel Cotton	0.011 0.013	5-15 ^oC	Hoshino *et al*., 1992
EGs	Avicel	167.36 -21.34	5-30 ^oC 30-50 ^oC	Ooshima *et al*., 1983
Safranin	NaOH treated rice husk	153.95	30 ^oC to 50 ^oC	Chowdhury *et al.*, 2010
CBH I, CBH II: *T. reesei*	Avicel	54.4, 86.2	20-35 ^oC	Kim and Hong, 2000
Trichoderma viride cellulase	Sigmacell 20, 50 (20 µm, 50 µm)	16.731 31.236	5-50 ^oC	Kim *et al*., 1988
Trypsin	CuS nanoparticles	-137.1	10-40 ^oC	Saha *et al*., 2014
Cr (VI)	Kraft lignin	86.0	20 ^oC to 40 ^oC	Tazrouti and Amrani, 2009
Malachite green	lignin sulfonate	131.58	10 ^oC to 60 ^oC	Tang *et al*., 2015
Aspergillus niger cellulase	activated carbon powder	84.0	20 ^oC to 60 ^oC	Daoud *et al*., 2010
EGs from Bacillus species	regenerated cellulose	15^oC= -174.47 25^oC= -119.66	Measured by ITC	Boraston, 2005
Glucoamylase from *A. niger*	acarbose	-11.67	Measured by ITC, 27 ^oC	Christensen *et al*., 1999
Glucoamylase from *A. niger*	β-cyclodextrin	-68.0	Measured by ITC, 27 ^oC	Christensen *et al*., 1999
Rhodamine B	aerobic granules wastewater sludge	100	5 ^oC to 45 ^oC	Zheng *et al*., 2005
Malachite Green $C_{50}H_{52}N_4O_8$, FW = 927.03 g/mol	aerobic granules wastewater sludge	110	30 ^oC to 50 ^oC	Sun *et al*., 2008
Cr (III)	PAm-g-RWF	12.64	30,50,70 ^oC	Mahdavi *et al*., 2011

Enzyme	Substrate	ΔS, $Jmol^{-1}$ K^{-1}	Conditions	Reference
EGs from *Clostridium cellulovorans*	regenerated cellulose	15 ^{o}C= -198.74 25 ^{o}C= -230 35 ^{o}C = -181.59	Measured by ITC	Boraston, 2005
Cu(II)	Rubber (*Hevea brasiliensis*) leaf powder	-95.94	27 ^{o}C to 47 ^{o}C	Ngah and Hanafiah, 2008
Cd(II)	Red algae (*Ceramium virgatum*)	-42.4	20 ^{o}C to 50 ^{o}C	Sari and Tuzen, 2008
Ni(II)	Baker's yeast	-23.66	27 ^{o}C to 60 ^{o}C	Padmavathy, 2008
Ni(II), Pb(II)	Lichen (*Cladonia furcata*) biomass	-71.5, -57.6	20 ^{o}C to 50 ^{o}C	Sari *et al*., 2007
Malachite Green	Neem sawdust	-169.57	25 ^{o}C to 45 ^{o}C	Khattri and Singh, 2009
Acid Yellow 17	Non-living aerobic granular sludge	-15.79	20 ^{o}C to 50 ^{o}C	Gao *et al*., 2010
Acid Orange 52	*Paulownia tomentosa* Steud. leaf powder	-17.00	25 ^{o}C to 45 ^{o}C	Deniz and Saygideger, 2010
Benzoic Acid	Amberlite XAD-4	18^{o}C= -77.99 32^{o}C= -74.44 46^{o}C = -70.63	Decreased with increasing temperature	Liu *et al.*, 2003

1= considering Avicel has a CrI of 0.64., PAm-g-RWF: Acrylamide grafted on Rubberwood fibers;
ITC: isothermal titration calorimetry was used to measure the thermodynamics of the binding

ΔS values reported for lignin, activated carbon, aerobic granule and rubber was positive for all range of temperatures. For other lignocellulosic materials it was negative. For cellulose ΔS values were positive for low temperatures (5 ^{o}C to 30 ^{o}C) and negative when reported for temperatures 30 ^{o}C and above. It is considered that the reason which holds for positive value its opposite would be a reason for negative value, in fact, this is not true.

The positive ΔS value could be due to one or more indicated reasons: i) increased randomness at the solid/solution interface with some structural changes in the adsorbate and the adsorbent [Rincon-Silva *et al*., 2015], ii) The adsorbed solvent molecules, which are displaced by the adsorbate species,

gain more translational entropy than is lost by the adsorbate ions/molecules, thus allowing for the prevalence of randomness in the system [Ateya *et al*., 2010], iii) an increase in the degree of freedom of the adsorbed species [Zhao *et al*., 2012], vi) the total entropy change of the adsorption process (ΔS) is the sum of the above entropy increase and the entropy reduction and the net ΔS is positive [Shalabi *et al*., 2014]. v) Substrate is degrading, releasing small molecules. A negative value of ΔS suggests that the process is enthalpy driven and it could be due to: i) a decreased disorder at the solid/liquid [Liu *et al*., 2003]. ii) The mobility of the adsorbate was more restricted on the adsorbent surface than in solution [Freundlich, 1906], iii) structural changes during adsorbate-adsorbent interaction [Srivasta *et al*., 2011].

In this study ΔH and ΔS values for adsorption onto cellulose and lignin were calculated under similar experimental conditions and reported for the first time. For desorption investigations, ΔH and ΔS values were calculated for a wide range of temperature (25 ^{o}C to 70 ^{o}C) and pH (6 to 9) under similar experimental conditions.

2.5 Desorption of cellulases from substrates

In contrast to adsorption, attempts to desorb cellulases from substrate and recycle it in active form were not successful. Otter *et al*. (1984) studied desorption of cellulase on Avicel at 50-55 ^{o}C at two pH values of 5 and 10. At pH 5, the desorbed amount was 40 % of initial amount and 90 % of this 40 % was active only while at pH 10 desorption was 45 % with 0 % activity of the cellulase recovered. Deshpande and Eriksson (1984) investigated desorption of cellulases from Avicel and steam exploded wheat straw (SEWS). The enzymes were recovered by suspending the residues (3 % suspensions) in a number of eluents at 40 ^{o}C for 15 minutes. The most effective elution was, obtained with phosphate buffer which gave 40 % endo, 38 % exo and 30 % β-glucosidase of the original amount of the cellulases used. Jackson *et al*. (1996) studied desorption of cellulase from hardwood

and soft wood samples they could desorb 40 % and 55 % of cellulases, respectively, at 25 ^{o}C by using a surfactant, Tween 80, and the cellulase activity reported for both the substrates was 27 %. They pointed out that all the cellulase became inactive under the alkaline washing conditions. Therefore, high alkaline condition deactivates cellulases.

Other attempts to desorb cellulases include the use of surfactants, alkali, urea and buffers of varying pH. Otter *et al*. (1989) used variety of surfactants to study desorption of cellulase from Avicel and steam exploded aspen wood (SEAW). The amount desorbed from SEAW and Avicel at pH 10 and 50 ^{o}C by using Triton X-100 was 25 % and 69 % respectively but the activity of the enzymes was not reported. Yang *et al*. (2010) investigated on the desorbed cellulases from filter paper Whatman No.1, in the presence of a surfactant Tween 80 and found that the activity of the enzymes was declined. The compounds like Tween 80, Triton X-100 and urea react with enzymes and may instigate conformational changes in their structures which may cause denaturing of enzymes [Horton *et al*., 1996]. Therefore, choice of surfactants to help desorption should be made with care.

Some researchers reported desorption of cellulases from solid residues by contact with fresh substrate [Vallander and Eriksson, 1987; Lee *et al*., 1995; Qi *et al*., 2011]. But they could not prove, i) how much enzymes were desorbed and, ii) how much transferred to the fresh substrate surface. Even, due to the repeated recycling, the solid residue became lignin-rich. Girard and Converse (1993) and Lee *et al*. (1995) used similar technique of desorbing by fresh substrate and reported that the hydrolytic ability of the recovered cellulase was decreases.

There is a lack of specific research on cellulases desorption from lignin rich substrates or lignin [Tu *et al*., 2009]. Berlin *et al*. (2005) worked on delignified Douglas fir (*Pseudotsuga menziesii*) they could desorb 50 % of cellulases with 35 % of activity. Rahikainen *et al*. (2011) prepared lignin rich substrate from steam treated spruce wood chips to estimate capacity of lignin to interact with

cellulases and reported that the cellulases were not desorbed due to the strong interaction. The studies, in general, on desorption of cellulases (though they are few) and reuse for the production of ethanol have shown the following limitations:

1- The number of tested samples were small, reducing the predictive ability of the conducted tests.
2- A narrow spectrum of controlling factors was studied. A detailed strategic study of the effects of pH, temperature was not conducted, for example working only on two temperatures or pH values and then generalizing results.
3- Cellulases desorption and activity was not investigated systematically.
4- Most of the studied were based on action of cellulases from the pure substrates such as crystalline cellulose or filter paper, a few studies have covered forestry lignocellulosic materials and the study on agricultural material is extremely rare.

Our work is planned on predicted range of pH and temperature to find optimum active desorption. Desorption of cellulases is difficult to represent by Langmuir though Langmuir equation provides a good data fitting in most cases. Because, Langmuir assumed that there will be a reversible desorption of cellulases. Actually, more complicated situation exists, such as partially irreversible adsorption occur due to entrapment by substrate matrix (Lee *et al*., 1983; Palonen *et al*., 1999), and/or inactivity of enzymes.

2.5.1 Time taken for desorption of cellulases

Reese (1982), demonstrated that 90 % of cellulase with 60 % activity could be recovered (desorbed) using 6M urea at pH 4.9 and 40 °C for 30 min. The research showed that activity of the desorbed cellulases could be increased to 72 % when desorption time was shortened to 5 min. It was also

reported a combination of high pH and urea was ineffective for desorption. Nutor and Converse (1991) studied the hydrolysis of pretreated poplar wood with GC123 *Trichoderma reesei* cellulase at pH 4.8 and 40 ^{o}C. The enzyme rapidly adsorbed initially, reaching a maximum in about 30 min and about 50 % of the cellulase desorbed. Zhu *et al*. (2009) studied adsorption of cellulase on Avicel and dilute acid (DA)-pretreated corn stover at 4 ^{o}C. The adsorption time was taken 10 minutes and with no desorption time, desorption was 55-57 % at pH 8 and pH 9. Otter *et al*. (1984) demonstrated that desorption by NaOH was essentially complete within 30 seconds and was not affected by increases in desorption time to 15 min. A desorption step in a cellulose hydrolysis process can therefore be designed with a short contact time from 30 second to 15 min.

2.5.2 Effect of temperature on desorption

The only one direct example for desorption found from literature was given by Otter *et al*. (1984). They discovered that desorption of cellulase from Avicel was effective in the range of 22–55 ^{o}C, and a further increase of temperature reduced enzyme stability. The other examples provided indirect information for desorption temperature because they were discussing feasibility of reaction temperature in terms of activity and stability of cellulases. Some researchers studied interaction of *laccase* and/or *tyrosinase* on different substrates (porous glass beads, aqueous phenol and bisphenols). Though glass beads are different from cellulose yet they provide analogy to design a study. Their results demonstrate that the activity of adsorbed enzyme at 60 °C retained about 70 % of its maximal activity, while the free enzyme were inactivated [Lante *et al*., 2000; Champagne *et al*., 2007; Nicolucci *et al*., 2011]. Pardo and Forchiassin (1999) investigated influence of temperature on cellulase activity and stability in *Nectria catalinensis* and found that optimal temperature for the activity of the cellulase system ranged from 50 to 55 ^{o}C. Fungsin *et al*. (2007) carried out bioconversion of cassava waste into sugar by using microbial enzyme. The fungal cellulase was

produced from *Trichoderma reesei.* The optimum temperature found for bioconversion was 60 ^{o}C. Acharya (2010) studied saccharification of wheat bran by cellulases and concluded that endoglucanase and *β-glucosidase* retained 55 and 65 % activity at 60 ^{o}C. Increase in the temperature more than 60 ^{o}C resulted in a sharp decrease in the activity which can be attributed to thermal inactivation of the enzymes. Getting maximum activity at 60 ^{o}C at saccharification provided information that enzymes were performing their maximum during adsorption, desorption and hydrolysis at 60 ^{o}C. The stability of cellulases reported as the activity of cellulases which starts falling after 55 ^{o}C [Otter *et al*., 1984, Andreaus *et al*., 1999 and Tu *et al*., 2009]. The loss of activity of enzymes was due to thermal denaturation of the enzymes [Peterson *et al*., 2007; Daniel *et al*., 2013]. Heat can disrupt hydrogen bonds and non-polar hydrophobic interactions between amino acids in cellulases. Hydrogen bonding is very important in cellulosic structure. Hydrogen bonding occurs between amide groups in the secondary chain structure. Hydrogen bonding between "side chains" occurs in tertiary protein structure in a variety of amino acid combinations. This occurs because the increase in heat increases the kinetic energy which makes the molecules to vibrate so speedily and intensely that the bonds are disrupted [Ophardt, 2003; Velisek, 2014]. There is a lack of documentation in reporting of desorption temperatures.

2.5.3 Effect of pH on desorption

There are some examples of desorption of cellulases demonstrating effect of pH on desorption from various materials. Otter *et al*. (1989) achieved maximum desorption from Avicel at pH 11.5, and pH 12 but the cellulase activity was 9 % and 6 % of the original activity. In another study they used cellulases on steam exploded Aspen and Lucerne wood at pH 10 and achieved desorption of 50 % and 70 % respectively. In the discussion they reported that at alkaline pH cellulases were probably inactivated. Andreaus *et al*. (1999) studied desorption of cellulases on cotton fabric for pH 5 to pH

10. They discovered that desorption of cellulases from cotton fabrics increased from pH 5 to pH 10, and only 11 % of protein were desorbed at pH 10. During desorption experiments the activity of desorbed cellulases was determined by measuring reducing soluble sugars. The amounts of reducing soluble sugars found in desorption supernatant might be ascribed to enzymatic degradation of cotton fabric during preparation and storage of the cellulase adsorbed fabrics. The adsorption and desorption time was 7 hours while storage time was not reported. The amount of reducing sugars formed at pH 10 was the least of all pH values. Therefore, alkaline pH destroyed enzymes. Seo *et al.* (2011) reported that desorption of cellulases from delignified pine wood chips were almost doubled by increasing pH from 4.8 to pH 7.2 but the presented data was not telling about activity of desorbed cellulases. Du *et al*., 2012 examined the adsorption and stabilities of cellulase at various pH values. The acidic condition (pH 4.8) favored adsorption, whereas alkaline condition (pH 7 and 10) favored desorption. It was found that the influence of pH on the activity of cellulases was reliant on temperature such as under severe conditions (pH 10, 50 ^{o}C), irreversible inactivation took place. Alkaline pH value affects the activity of cellulases and pH also effects on desorption of cellulases. Since this research is on the reuse of cellulases, therefore, the effect of pH on desorption as well as on the activity of cellulases (desorption of active cellulases) was studied.

Table 9 shows the effect of pH on amino acids. The first column in Table 9 displays amino acids which are components of cellulases as indicated by Kleywegt *et al.* (1997). The second column is for optimum working pH values for amino acids and third column is dedicated for the pH at which an amino acid denatured. Data for second and third columns are taken from Brenda's website. From Table 9 it is clear that most of the amino acids denatured above pH 9 while most of the desorption studies reported at pH 10 and above. Therefore, desorption of deactivated cellulases is quite evident. Hence, a detailed strategic study of pH is required to get desorption of active cellulases from lignocellulosic substrate because desorption of cellulase has never been studied with a complete plan.

Table 9: Denaturing pH of component amino acids

Amino acid	Optimum pH	Denaturing pH
Aspartate	N.A.	>9.5
Glutamate	9-9.5	9.5
Arginine	5.2-9.5	9.5
Leucine	6-9.5	10
Tyrosine	6-9.5	9.5
Tryptophan	6-9.5	7.0- 9.5
Phenylalanine	8.4-10.1	N/A
Histidine	8-9.5	N/A
Methionine	8.9-11.5	N/A
Lysine	9.59	10
Valine	6-8	9
Serine	7-10	N/A
Threonine	7-10.3	10.5
Alanine	6.4-9.0	10.0

["Brenda", 2016]

The interaction between amino acids is changed as pH alters as given in Table 10. The change in interaction in amino acids inside enzymes change the structure of enzyme. The change in structure results in the change in behavior of enzyme i.e. a change in interaction with substrate. The change in the charges of amino acids as a change in pH is given in Table 10. The contents for Table 10 are retrieved from: Amino acids available in cellulases, [Divne *et al*., 1994; Kleywegt *et al.*, 1997; Abuja *et al.,* 1988; Belaich *et al*.,1992. ; Mackenzie *et al*., 1998]; charges on cellulases are from the work of Cameselle *et al*. (1986); functions of amino acids are taken from Molecular Biology [Horton *et al*., 1996]. As pH increases from 5 to 10 the negativity of charges on aspartate (Asp) and glutamate (Glu) increases 2.5 and 5 times respectively, positive charge on arginine and lysine decreases. Tryptophan and Tyrosine have positive charge and they hold substrate in the active sites so that Asp

and Glu can act on substrate [Linder *et al*., 1995: Fukuda *et al*., 2006], also a decrease in positive charge will decrease their capability to make hydrogen bonds with substrate. Alanine, leucine, isoleucine, valine, methionine, phenylaline and proline are non-polar. At pH 10, all of the amino acids are negatively charged, this situation brings structural problems and the enzyme goes through conformational changes. From Table 10 glutamate, arginine, methionine, tyrosine, and tryptophan start denaturing above pH 9.

Table 10: Charges on component amino acids at various pH levels of desorption system

Amino Acid Residues	**pH**					
	5	**6**	**7**	**8**	**9**	**10**
Aspartate (Asp) Cation binding, H^+ transfer,	-0.96	-1	-1	-1.02	-1.2,	-1.72
Glutamate (Glu) Cation binding, H^+ transfer,	-0.85	-0.98	-1	-1.02	-1.18	-1.68
Arginine (Arg) Anionic binding,	1	1	0.99	0.91	0.49	0.09
Lysine (Lys) Anionic binding,	1	1	0.99	0.94	0.59	-0.01
Tryptophan (Trp) H-bond with ligands[a],	0	0	0	-0.04	-0.27	-0.78
Tyrosine (Tyr) H-bond with ligands[a],	0	0	0	-0.08	-0.51	-1.31
Threonine (Thr)[bb] Covalent bond with acyl-,	0	0	0	-0.07	-0.44	-0.89
Serine (Ser)[bb] Covalent bond with acyl-,	0	0	0	-0.06	-0.38	-0.98
Histidine (His) H^+ transfer	0.91	0.5	0.08	-0.05	-0.4	-0.87

Amino Acid Residues	5	6	7	8	9	10
Cysteine (Cys)	0	0	-0	-0.31	-0.85	-1.24
Alanine (Ala)	0	0	0	-0.2	-0.17	-0.67
Leucine (Leu)	0	0	0	-0.02	-0.2	-0.72
Isoleucine (Ile)	0	0	0	-0.02	-0.17	-0.68
Valine (Val)	0	0	0	-0.02	-0.19	-0.71
Methionine (Met)	0	0	-0	-0.06	-0.38	-0.86
Phenylalanine (Phe)	0	0	-0	-0.06	-0.4	-0.87
Proline (Pro)	0	0	0	0	-0.02	-0.19

a = hydrophobic; b = hydrophillic, polar; bb=hydrophillic no charge
[Cameselle *et al*., 1986; Horton *et al*., 1996]

Therefore, reading Table 9 and Table 10 together gave information that the pH suitable for desorption is 6-9. From this literature survey and the discussion given above, it was decided to use pH of 6, 7, 8 and 9 to get desorption of active cellulases where pH 8 and pH 9 look more promising.

2.5.4 Decrease in desorption due to entrapment of cellulases

Jung *et al*. (2002) found that the cellulases were entrapped in the pores of substrate during adsorption of enzymes from *T. fusca* Cel5A, Cel6B, Cel48A onto BMCC and resulted in irreversible desorption. Tanaka *et al.* (1988) found that one cause of the rapid decrease in the hydrolysis rate was the diffusion of cellulases into small pores of cellulose. When cellulases enter the small pores they get trapped and reduce the rate of hydrolysis.

There were certain requirements of the internal geometry of a substrate if high enzyme activity retention was to be achieved. That is, the enzyme should be able to enter into pore to be adsorbed.

The pore size of the selected substrate should meet three requirements.

i. pore size should be at least of the same order as enzyme size [Ferreira *et al*., 2002]

ii. enzyme-conformation mobility should be not significantly reduced compared with that of the free enzyme, thus necessitating the presence of larger pore size than the size of the enzyme molecules [Hwang *et al*., 2004].

iii. diffusion constraints should be mitigated to retain high apparent activity [Tischer and Kasche, 1999]

Hwang *et al*. (2004) investigated on adsorption of papain on porous silica with pores in the range 30–90 nm, through the series of experimentation they concluded that pore size should be 5–10 times the size of the enzyme regarding the high retention of activity. If the pore sizes are less than suggested enzymes size there are 60 to 70 % chances of entrapment of enzymes which will result in reduction in desorption. The average pore size of Avicel is around 30Å [Rodriguez, 1997] and the average size of CBDs of CBHs and EGs is around 28.5 Å [Gilkes *et al*., 1992]. CBD can get trapped in the pore, resulting in a decrease of desorption of cellulases.

2.6 Enhancement of Desorption

After strategic use of pH and temperature, the question that remains is: can this desorption be enhanced to get new maxima, if at all possible? Chemical such as glycerol, polyethylene glycol and enzyme β-glucosidase were selected to be used for the reasons given in the respective sections below. Simplicity and cost effectiveness are among the key strengths of this enzyme recycling strategy.

Glycerol

Glycerol was observed as an effective desorbent [Otter *et al*., 1989]. The maximum desorption (67 % with 56 % activity) of cellulases from Avicel was achieved at pH 10 with 10 % glycerol while 57 % desorption with 88% activity was achieved at pH 5 and 60 % glycerol [Otter *et al*., 1989]. Similarly, Wang *et al*. (2012) used 40 % glycerol to desorb endoglucanases from sulfite treated poplar and they could desorb 10% endoglucanases and activity was not reported. Therefore, I noticed that high concentrations for glycerol (>40%) can help in desorption. Literature survey gave me insight that high concentration of glycerol denature enzymes [Wenbin *et al.*, 2002] and low concentration of glycerol (<20%) increase stability of proteins. Therefore, I decided to use glycerol in low concentration mixtures with distilled water to enhance stability of enzymes and desorption. Moreover, in aqueous medium, the amino acid chains on the surface of cellulases preferentially excluded glycerol from the core of cellulases and made it more compact thus forming a thermodynamically unfavorable situation [Gekko and Timasheff, 1981b]. To minimize the effect of glycerol, the cellulases fold themselves more and became more compact, hence, stability was increased [Gekko and Timasheff, 1981a]. Glycerol could increase cellulases stability during desorption at high pH. Glycerol competed with the enzyme for hydrogen bond at sites on the cellulose and as the glycerol concentration increased cellulases was desorbed [Gekko and Timasheff, 1981b]. In most cases, binding of proteins to insoluble substrates is weakened at high temperatures because of increased kinetic energy. Therefore it helped desorption. At elevated pH values due to increased repulsive electrostatic forces between the enzyme and lignin [Lou *et al*., 2013]. Enzyme-lignin interaction are non-covalent type and the driving forces involve are hydrophobic and electrostatic interaction, with minor contribution from hydrogen bonding and dipolar interactions [Claesson *et al*., 1995; Norde, 1996]. Therefore, I expect that elevated temperature and pH will help desorbing

cellulases from wheat straw (lignocellulosic materials) and glycerol will stabilize cellulases at elevated temperature and alkaline pH. A concentration larger than 10 % was resulted in instability of cellulases [Nagayama *et al*., 2012] either by cellulases precipitation or by the penetration of glycerol into core of the cellulases which result in conformational changes of cellulases. Hence, decrease in activity of cellulases.

Ethylene glycol

Ethylene glycol was used to pre-treat substrates to prevent substrates from adsorption of enzymes [Alstine *et al*., 1996; Borjesson *et al*., 2007; Hofs *et al*., 2008]. The mechanism of ethylene glycol in rejection of enzymes is that steric repulsion where surface attached ethylene glycol competes with the hydrophobic interaction between the enzymes and the surface [Jeon *et al*., 1991]. I used the same property of ethylene glycol to remove adsorbed enzymes (cellulases) from the substrates.

The electrons within O-H bonds in ethylene glycol have a higher probability of appearance around the Oxygen atom than their probability of appearance around the Hydrogen atom, which makes the Hydrogen atom slightly electropositive. The electropositive hydrogen atom develop an affinity with the substrate-cellulase complex, providing H^+ to cellulases attached with cellulose and let it desorb from the substrate (cellulose, lignin, wheat straw). Ethylene glycol is oxidized by the enzymes [Eder *et al*., 1998]. The cellulases-ethylene glycol complex is not stable because H-bond of ethylene glycol molecule have strong affinity with H-O-H. Ethylene glycol satisfy its charges from water molecule and release/ desorb cellulases. Elevated temperatures increase cellulases desorption from the surface of Protobind. Ethylene glycol may attach to the lignin in one of two ways (1) terminally; or (2) by interaction between the Protobind and ethylene glycol. The first interaction with the surface of

Protobind require high EG dosage to cover the whole surface, while the latter require a low concentration to make a very thin ethylene glycol layer on the surface.

It can suggest that the interaction is formed between ethylene glycol and the phenolic hydroxyl groups [Matsushita, 2015] of Protobind. The importance of phenolic hydroxyl groups in lignin and cellulase interaction has already been hypothesized [Sewalt *et al*., 1997]. Phenolic hydroxyl groups exposed on the lignin surface can easily form hydrogen bonds with the oxygen atoms of the ethylene glycol polymer. This would mean that ethylene glycol is accepting a hydrogen bond, and free phenolic hydroxyl group of lignin is donating the hydrogen bond [Borjesson *et al*., 2007]. The accepted hydrogen bond balance the charge requirement of cellulases and cellulases desorbed from the substrate (cellulose, lignin wheat straw).

β-glucosidase

Cellulases are a family of enzymes having three members endoglucanase (EC 3.2.1.4), cellobiohydrolase (CBH) (EC 3.2.1.91), and β-Glucosidase (EC 3.2.1.21). End-product inhibition has shown to play a major role in hindering a continuously fast desorption/hydrolysis rate. In the cellulase family of multiple enzymes, endoglucanases (EG) act on amorphous regions or regions of low crystallinity in the cellulose. Exoglucanases (CBH I; Cel7A) progressively adsorb and hydrolyze the cellulose chain from the reducing end through a retaining mechanism to produce β-cellobiose, whereas exoglucanases (CBH II; Cel6A) preferentially acts from the nonreducing end of the chain via an inverting mechanism to produce α-cellobiose. After the action of endoglucanase and exoglucanases, cellobiose, cellotriose and cellotetraose are formed. Cellulases (EGs and CBHs) adsorbed on cellobiose/cellotriose/ cellotetraose cannot desorb [Xiao *et al*., 2004; Gruno *et al*., 2004], called fixation of cellulases. Working dimensions of a cellulases were studied on Cel45. Cel45 are

endoglucanases (EC 3.2.1.4) from *Humicola insolens* endoglucanase V. Cel45 have a (maximum stretch of a molecule) D_{max} of 125 Å which results in a maximum distance between the centers of the constitutive modules of 85 ± 10 Å (CBM and CM or CD). From this, one can conclude that from a fixed binding site of the CBM onto a cellulose chain, Cel45 has a maximum operation range of about 40 Å, e.g. may operate only a maximum of four glycosidic bonds of similar orientation on a single chain. [Receveur *et al*., 2002]. That is why some times cellobiose/cellotriose/ cellotetraose are obtained in products. The presence of *β-glucosidase* decrease the chances of fixation of cellulases with adsorption products of EGs and CBHs [Levine *et al*., 2010; Manara, 2012]. To decrease the chances of fixation of cellulases *β-glucosidase* for desorption enhancement were used. There is also a lack of information on whether *β-glucosidase* favor desorption and effects on cellulase activity during cellulose conversion, hence it is need to be addressed.

3.0 Objectives

Objectives of thesis

Bioethanol has received a tremendous interest of research community as an alternative fuel because of the climate change effect and the fear of depletion of fossil fuels. My extensive literature survey identified huge gaps in the previously reported studies on bioethanol production. The detected gaps may be the reasons for non-commercialization of enzymatic hydrolysis in the bioethanol producing industry. The gaps are summarized as: i) narrow spectrum of factors influencing desorption was studied. A detailed strategic study of the effects of pH, temperature was not conducted, for example working only on two temperatures or pH values and then results were generalized, ii) cellulases desorption and activity was not investigated systematically, iii) most of the studies were based on action of cellulases from the pure chemicals such as crystalline cellulose or filter paper, a few studies have covered forestry lignocellulosic materials and the study on agricultural material was very rare.

This Ph. D. thesis deals with the active desorption of cellulases from the lignocellulosic materials being used in producing bioethanol in a cost-effective and environmental friendly way. It is hypothesized that: i) the poor desorption of cellulase from lignocellulosic substrates would be enhanced by using a high pH (weakening of H-bonding). Similarly, the use of high temperature may give more desorbed cellulases but the desorbed cellulases may not be active cellulases (thermal denaturing), ii) desorption of cellulases can be enhanced by competing adsorption of glycerol, ethylene glycol and controlling entrapment of cellulases by reaction by-products (fixation of cellulases) by using β-glucosidase, iii) the techniques used for desorption of cellulases from the components of lignocellulosic materials can be used for lignocellulosic materials used in bioethanol producing industry.

The main objective of this work has been to gain a better understanding of adsorption and desorption of cellulases from a lignocellulosic material, more specifically:

1. Determine adsorption isotherms for adsorption onto Avicel PH 101 to resolve the conflicts in literature about adsorption patterns of cellulases and determine adsorption pattern of cellulases on Protobind 1000 for the first time.
2. Determine adsorption capacity of Avicel PH 101 and Protobind 1000 under similar experimental conditions such as temperature, pH, dynamics and type of enzymes. Quantify the adsorption for a range of initial cellulases concentration. Determine the changes in enthalpy (ΔH_a) and entropy (ΔS_a) for adsorption of cellulases on Avicel PH 101 and Protobind 1000 to predict the feasibility of adsorption and the optimum adsorption conditions.
3. Determine active desorption of cellulases from adsorbed cellulases from both substrates, Avicel PH 101 and Protobind 1000. Determine the feasibility of desorption through changes in enthalpy (ΔH_d) and entropy (ΔS_d) for desorption of cellulases from Avicel pH 101 and Protobind 1000. Estimate optimum conditions for active desorption of cellulases, using a wide range of temperature and pH, to fill the gaps in literature.
4. Enhance the active desorption of cellulases by strategic use of pH, temperatures, as suggested by last 3 steps. Clarify the use of additives (such as desorbing agents) to enhance desorption of active cellulases. Quantify desorption of cellulases for a range of initial cellulases concentration, temperature, pH and additives to patch the holes in the fundamental knowledge of desorbing cellulases from lignocellulosic materials in the biofuel business.
5. Apply the strategy developed till the step 4 for desorption of active cellulases from wheat straw and delignified wheat straw and make recommendations for the bioethanol producing industry.

4.0 Material and Methods

4.1 Materials

Avicel PH 101

Avicel PH 101 (analytical grade, 100% solids, Sigma-Aldrich Corp., Missouri, and USA) is a white powder cotton-source microcrystalline cellulose of approximately 50 µm particle size, 78% porosity, and 56.7% crystallinity. Avicel was purchased from Sigma-Aldrich Corp. It has a molecular weight 370.35 g/mol and density 0.600 kg L^{-1}. The functional groups of Avicel PH 101 participating in adsorption are –OH group, the –C–O–C– group of the β-1, 4- glycosidic linkage. Stored at room temperature.

Protobind 1000

Protobind 1000 (analytical grade, 100% solid) is an aromatic polyphenolic material derived from wheat straw lignin during its soda pulping is generously donated by Dr. J. Lora from Green Value Enterprises LLC. It is a dark brown solid with an average particle size of 200 µm, and 64% crystallinity. Its molecular weight 3000 and density of 0.550 kg L^{-1}. The Protobind 1000 was found to be composed of *p*-coumaric and ferulic acid, acetosyringone, syringaldehyde and vanillin types of lignin. Stored at room temperature.

Wheat straw

Wheat straw (*Tritium sativum*) is an agricultural waste. For this study two bales of wheat straw were gifted from a farm north of Toronto. The straw was dedusted by gentle tapping and milled by a machine (Type SM100 Comfort, Retsch Inc. Philadelphia, USA). The milled wheat straw was sieved separately to the required sizes (0.5 mm), wheat straw has cellulose 46.81%, and lignin 18-22% and the crystallinity of the cellulose is 55.57%. Wheat straw used was 30% and 60% delignified for studies while delignification was conducted by ozonation.

Chemicals

Cellulases, a commercial cellulase mixture, brown liquid, (NS50013, batch # CCN03129) from *Trichoderma reesei* was generously donated by Novozymes (Denmark). According to the manufacturer, the solution mostly contains exocellulases (cellobiohydrolases: CBHI and CBHII). CBHI is responsible for hydrolysis of both amorphous and crystalline cellulose. Endocellulases (Endoglucanases: EGI and EGII), are known to attack and adsorb preferentially on amorphous cellulose. The approximately composition of commercial enzyme mixture was: CBH I (60 %), CBH II (15 %), EG I and II (20 %), and BG (2 %) [Wenger, 2006]. Molecular weights estimated were 52000 for EGs [Li *et al* 1965], 23000-58000 for EGs [Beldman *et al*., 1985], and 60500 - 62000 for CBHs [Beldman *et al*., 1985]. To simplify calculations the molecular weight of cellulases was taken as 60000. It was stored at 4 °C until used. Every time before use mother solutions (varying concentrations) of cellulases were prepared form the original stock solution. Protein concentration as well as total activity (FPU) of the mixtures were determined according the procedures described in Analyses. It was used as additive to desorption studies.

A commercial β-glucosidase (BG, NS50010, batch # DCN00218) from *Trichoderma reesei* was generously donated by Novozymes (Denmark). The β-glucosidase has a molecular weight of 76000 [Beldman *et al*., 1985].

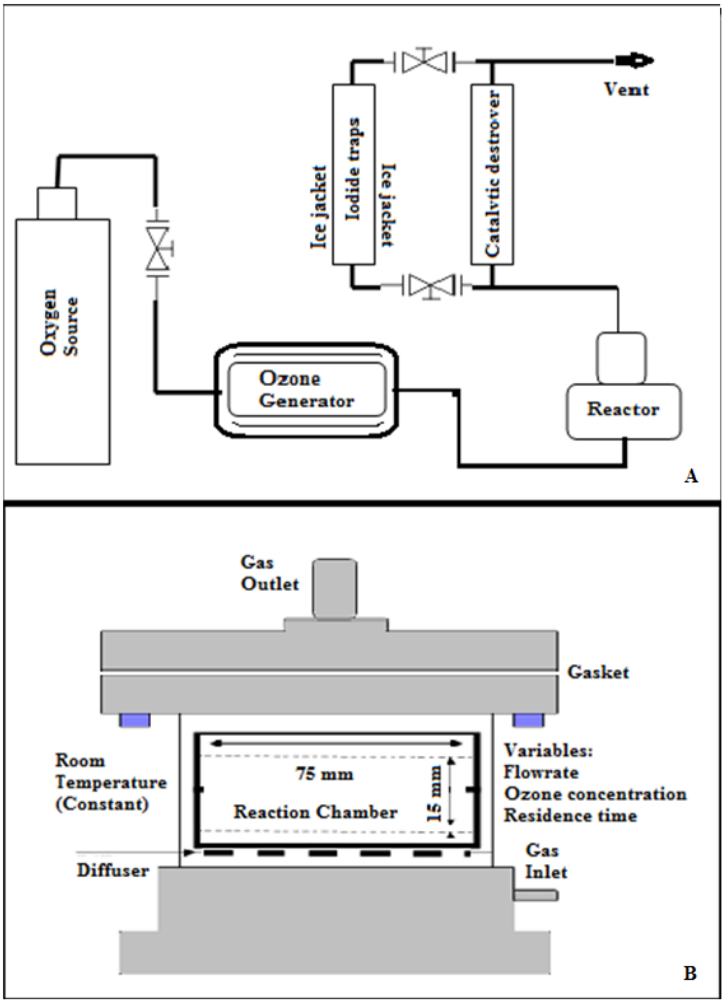
Glycerol (G, electrophoresis reagent grade; 99 % pure) was a product of Sigma-Aldrich. It has molecular formula $C_3H_8O_3$ with a formula weight of 92.09. It was stored at room temperature, and used as specified.

Ethylene glycol (EG, Analyzed reagent grade; 99.7 % pure) was a product of J.T. Baker. It has molecular formula $C_2H_6O_2$ with a formula weight of 62.07. It was stored at room temperature

4.2 Instruments

Delignification

Ozone at concentration of ca. 6.5 to 65.5 mg/L was produced from pure oxygen using an ozone generator (PC1-WEDCO, Model GL-1, USA) as shown in part A of Figure 7. Oxygen was supplied in cylinders by Linde Canada Limited. Ozone is made by passing pure oxygen gas through a tube though which energy is directed. The energy breaks apart the molecules as described above, and what emerges from the other side is ozone.



[Baig *et al*. 2015]

Figure 7: Delignification by ozone. Figure A) Experimental set up for delignification, B) Delignification reactor

Wheat straw samples were exposed to the flowing ozone streams using a 2 phase solid-gas fluidization reactor as shown in Figure 7 (b), which had a stainless steel reaction chamber. At the entrance and exit of reactor ozone flow is maintained and controlled by control valves. The concentration of ozone entering was measured automatically by an online spectrophotometer (at 257 nm). After entering in the reactor, ozone passed through a diffuser which distribute ozone homogenously in the reaction chamber to react with wheat straw. The unreacted ozone left the reactor from an outlet located at the top and a catalytic destroyer convert it to oxygen before it was sent to the vent.

Incubation for Adsorption and Desorption

New Brunswick Shakers Innova 40 (Figure 8) was used for adsorption and desorption studies for incubation of samples. It's an orbital shaker with controllable rpm and temperature. Its temperature was monitored by a glass thermometer for the temperatures of 25 ^{o}C and 60 ^{o}C and no difference in temperature readings were found.

Figure 8: Incubator used for Adsorption and desorption studies

Spectrophotometer for measurement of activity and concentration of enzymes

The Biochrom Libra S50 model is a low maintenance UV/Visible spectrophotometry. Spectrophotometers use a monochromator containing a diffraction grating to produce the analytical spectrum. The grating can either be movable or fixed. The Biochrom Libra S50 model, the grating is fixed and the intensity of each wavelength of light is measured by a different detector in the array. After amplification of the energy received the display gives out the readings as shown in Figure 9.

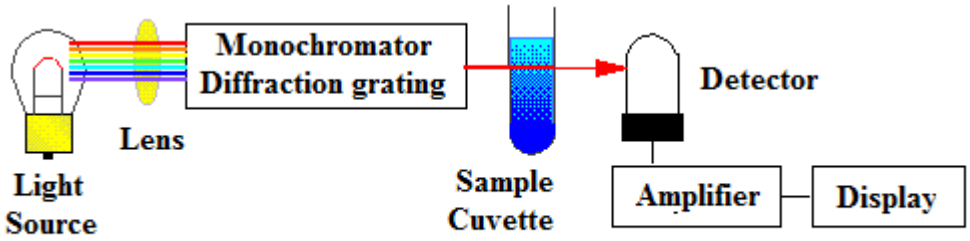


Figure 9: Detection mechanism of a spectrophotometer

Spectrophotometry is an important technique used in enzymology experiments that involve protein isolation, enzyme kinetics and biochemical analyses. [Trumbo *et al*., 2013]. A brief explanation of the procedure of spectrophotometry includes comparing the absorbance of a blank sample that contain everything of the enzyme solution but not the substance (or enzyme) of interest to a sample that contains the substance. The spectrophotometer is used to measure colored compounds in the visible region of light (between 350 nm and 800 nm), [Ninfa *et al*., 2010]. In enzymology experiments, a chemical and/or physical property is chosen and the procedure that is used is specific to that property in order to derive more information about the sample, such as the enzyme purity, enzyme quantity, enzyme activity, etc. Because a spectrophotometer measures the wavelength of a compound through its color, an enzyme binding substance can be added so that it can undergo a color change and be measured. Spectrophotometers have been developed and improved over decades and

have been widely used among chemists. It is considered to be a highly accurate instrument that is also very sensitive and therefore extremely precise, especially in determining color change. [Mavrodineanu *et al*., 1973]

4.3 Procedures

Proposed treatment for delignification

The novel proposed pretreatment for delignification of wheat straw by using ozone is a three-step procedure:

Step 1: Wheat straw was oven dried and soaked in 1% NaOH for 12 hours. The resulting aqueous mixture was filtered using binder-free, glass micro fiber filter grade 691, 9.0 cm diameter with pore size of 1.5µm. The filtered residues were washed with deionized water until the pH value was around 7.0. The washed residue were dried at 105 ^{o}C in an oven (Catalogue No. 3605, 1200watts, Labline Inc., USA) until a constant weight of dry wheat straw was obtained.

Step 2: The dried sample was sprayed with 3 times of the dry weight of wheat straw by a spray bottle. Then, the moistened sample was wrapped in aluminum foil, placed in covered Petri-dishes, further wrapped in cellophane and placed in a fridge at 4 ^{o}C to avoid water loss for 24 hours. The time was provided to let the water absorb in the wheat straw sample completely.

Step 3: The moistened sample was unwrapped and quickly subjected to ozonation.

Adsorption and desorption

An initial concentration of commercial cellulases [P_0] was added to substrate. The concentration of free cellulases present in the supernatant after adsorption was labelled [P_{fa}]. The concentration which remained adsorbed [P_a] calculated by the difference between initial concentration and free non-adsorbed cellulases. Figure 10 shows that the supernatant obtained from the centrifugation of

adsorption sample after incubation were used for estimation of cellulases concentration and activity. The solid residues obtained from the adsorption step was used for desorption studies. Similarly, supernatant obtained from desorption sample after the centrifugation was used for the concentration of the cellulases desorbed and activity of the cellulases desorbed. The solid residues obtained from desorption were discarded.

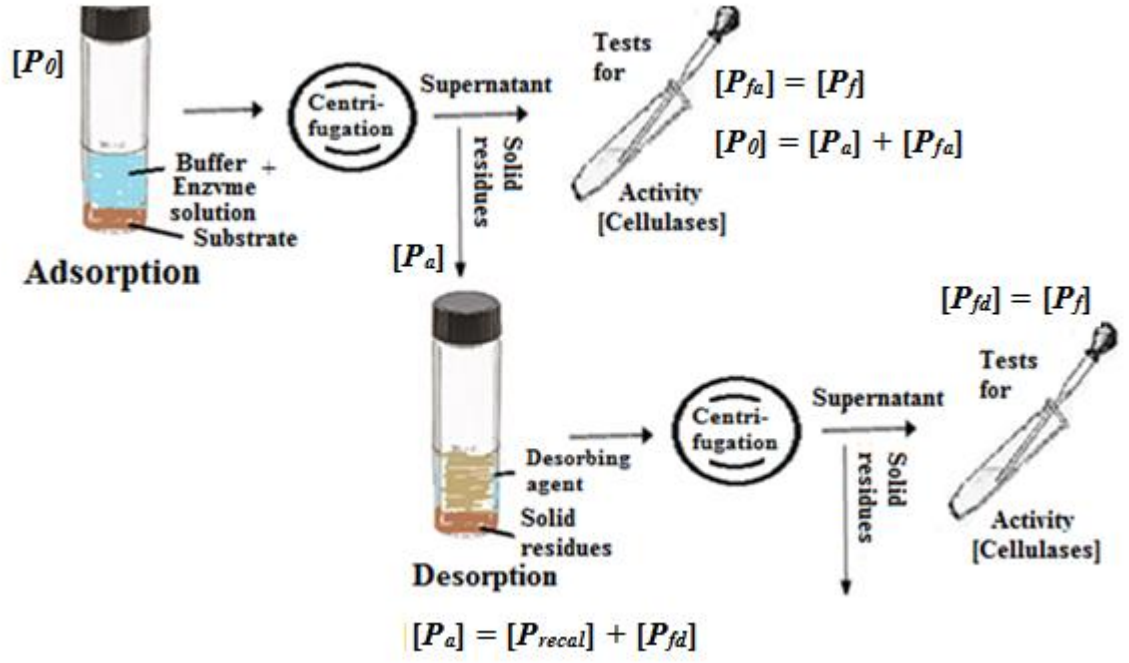


Figure 10: Procedure for adsorption and desorption

The concentration of cellulases that could not desorbed from substrates was [P_{recal}].

Adsorption of cellulases onto substrates

Thirty milliliters of distilled water were placed in a 250-mL Erlenmeyer flask. Then 70 mL of 0.05mM sodium citrate buffer at pH 5 was added followed by addition of the brownish commercial cellulases mixture NS50013 (in predefined amounts). The solution was hand mixed to uniformly disperse the cellulases (observed by dispersion of color). The pH was adjusted to 5 using distilled water or NaOH 0.1M. The solution was hand-mixed again, and placed at 25°C in an Innova 40

Incubator Shaker (New Brunswick Scientific, USA) at 100 rpm for 2 hours (values chosen by literature survey). Performed this step to get a homogenous mixture. Five 1-mL samples of the solution were drawn to measure the initial concentration of cellulases, [P_0], as described in the section Analyses, and 5 mL of the solution were added into each of 10-mL screw cap glass test tubes already containing 100 mg of Avicel PH 101. The test tubes were placed in the incubator shaker at 25 °C and 100 rpm for 5, 10, 20, 30, 40, 50, 60, and 90 min of contact time (as pre-designed through literature survey). The experiment was triplicated. Each test tube was centrifuged in a Clinical 100 centrifuge (VWR USA) at 4000 rpm for 4 minutes. The concentration of non-adsorbed free cellulases, [P_f], in the supernatant was determined in triplicate, and the solid residue of adsorbed cellulases on Avicel was discarded. The concentration of adsorbed cellulases on Avicel was calculated as [P_a] = [P_0] – [P_f]. This procedure was repeated using 0.077, 0.096, 0.107, 0.123, 0.138, 0.169, 0.192, and 0.199 µL of commercial cellulase mixture, resulting in initial [P_0] of about 100, 125, 140, 160, 180, 220, 250 and 260 µg of protein per mL of solution.

Using about 180 µg protein per mL of solution^{-1} on 100 mg of Avicel with a contact time of up to 60 minutes, the temperature of the experiment was maintained at 25, 40, 50, 60 or 70 °C, and the concentration of non-adsorbed free cellulases was determined as before. Experiments were done in triplicate.

The above procedure for adsorption and for effect of T was repeated in the Protobind 1000, also in the triplicate.

Wheat straw (0.5 mm) was used as substrate for adsorption at 25 ^{o}C, pH 5, and 100 rpm over 120 minutes of contact time.

Desorption of cellulases from substrate

Adsorption with an initial cellulase concentration protein per ml of solution, at 25 °C, pH 5 and after 30 minutes of contact, the supernatant was discarded after measurement of its concentration of non-adsorbed free cellulases, which was transformed into amount of adsorbed cellulases available on Avicel for future release by desorption. The centrifuged solid residue, with its adsorbed cellulases, was immediately re-suspended in 5 mL of distilled water already equilibrated at 25 °C and at pH 5. Preliminary desorption studies were conducted for Avicel for a time range of 5 minutes to 60 minutes by placing 11 pre-adsorbed reaction tubes, (including water blank, substrate blank) in the incubator. Fresh reaction tubes were used as data points for each of the taken out supernatant initially at 5 minute interval for first 40 minutes and then after 10 minutes for 50 to 60 minutes. Each experiment was conducted in triplicate. Triplicates of results were obtained in this fashion. Each test tube was centrifuged in the same manner as for adsorption samples. The concentration of the desorbed and activity of desorbed free cellulases in supernatants were determined as before. The activity of the desorbed enzymes was determined using filter paper assay described in the analysis section. Solid residues were discarded.

To investigate the effect of temperature and of pH, the same initial cellulase concentration as above was used. Through literature survey it was decided to conduct desorption at temperatures of 40, 50, 60 and 70 °C while desorption at 25 ^{o}C was taken as a reference. For a similar reason desorption was evaluated at a pH of 6, 7, 8 and 9, along with the 40, 50, 60 and 70 °C. All experiments were in triplicates.

To enhance desorption, glycerol or ethylene glycol were added to the distilled water used to re-suspend the solid residues with their adsorbed cellulases, at 1, 5, and 10 v/v %. Another additive β-glucosidase, was also used in distilled water 5, 10 and 18 v/v %. After 20 minutes of contact time at

40, 50 and 60 °C, at pH 8 and 9, the concentration and activity of desorbed free cellulases in supernatants were determined as before while the solid residues were discarded.

The whole desorption process was repeated using Protobind 1000 within an adsorption time of 45 minutes and desorption time of 20 minutes, as determined by preliminary adsorption and desorption profiles. Similarly, desorption of cellulases from wheat straw (0.5 mm) was performed at 25ºC, pH 5, 100 rpm for up to 100 minutes of contact (adsorption) time, after adsorption in conditions similar to those of Avicel PH 101, in triplicates. Conditions at 60 ºC, pH 9 and 40 minutes of desorption were compared to those of Avicel PH 101 and Protobind 1000.

Wheat straw (30 % and 60 % delignified) triplicate experiment were also performed at 60 ºC, pH 9 and 40 min. They were compared with glycerol (G) added and with conditions of 25 ºC and pH 5.

4.4 Analyses

Detection of Lignin

The contents of lignin in lignocellulosic material (Wheat Straw, WS) can be analyzed gravimetrically after hydrolyzing the cellulose and hemicellulose fractions with sulfuric acid. Acid insoluble lignin present in test samples of WS were measured as Laboratory Analytical Procedure #003 entitled as Determination of Acid-Insoluble Lignin in Biomass used by the National Renewable Energy Laboratory (NREL), which is operated by the Midwest Research Institute (MRI) for the Department Of Energy, USA. The procedure is following:

A pretreated sample of 1.0 g was weight and placed in a 20x150 mm test tube. Record as W_1, the initial sample weight. Each sample was ran in triplicate. For reference the sample taken was natural wheat straw. Added 15.00 mL of 72 % H_2SO_4, chilled to 4 ºC in the refrigerator. Used a glass stirring

rod to mix for 1 minute, or until the sample was thoroughly wetted. Hydrolyzed the sample for 2 hours at room temperature (approximately 23 ^{o}C), stirred every 15 minutes to assure complete mixing and wetting. Transferred the hydrolysate to a 1000 mL Erlenmeyer flask and dilute to a 3 % acid concentration with 560 mL of deionized water. Placed the flask on the heating manifold and attach to the reflux condenser as shown in Figure 11 for hydrolysis of wheat straw for measurement of acid insoluble lignin.

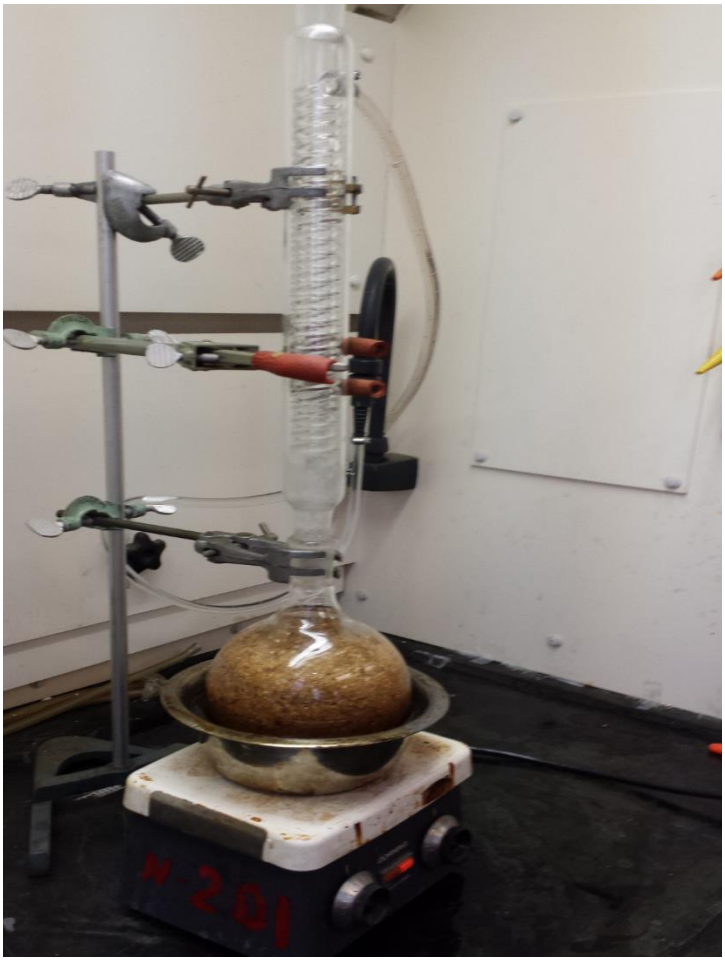


Figure 11: Hydrolysis of wheat straw for measurement of acid insoluble lignin

Heated the liquid to a gentle boil. Started timing at the onset of boiling, and set on reflux for 4 hours minutes. At the end of 4 hours, rinse the condenser with a small amount of deionized water before

disassembling reflux apparatus. Vacuum filtered the hydrolysis solution through one of the previously ignited filtering crucibles. To determine acid-soluble lignin determination (LAP-004), recorded the weight of the collected filtrate. Decanted 15-25 mL of filtrate into a resealable container, aliquot was store in refrigerator at 4 ^{o}C for further analysis. Used hot deionized water to wash any particles clinging to the glass bottle into the crucible and to wash the filtered residue free of acid using vacuum filtration. Dried the crucible and contents at 105 ^{o}C for 2 hours or until constant weight was achieved. Cooled in desiccator and record the weight, W_2, the weight of the crucible, acid-insoluble lignin, and acid-insoluble ash. Placed the crucible and contents in the muffle furnace and ignite at 575^{o}C for a minimum of 3 hours, or until all the carbon is eliminated. Heat at a rate of 10 ^{o}C/min to avoid flaming. If the sample tends to flare up, the container should be partially covered during this step. Avoid heating above the maximum stated temperature. Cooled in desiccator and record the weight, W_3, the weight of the crucible and acid insoluble

Ash Contents of WS for delignification

The ash contents are approximate measure of the mineral content and other inorganic matter in lignocellulosic material. The ash contents are used with other assays to determine the total composition of lignocellulosic samples. It proceeded was adapted from Laboratory Analytical Procedure #005 of NREL, as:

A crucible was marked with a unique identification using a porcelain marker, placed it in the muffle furnace, and brought to constant weight by igniting at 575 ^{o}C. The crucible was removed from the furnace, cooled to room temperature in a desiccator, weighed and recorded this weight as the tare weight. The test sample taken into the tared crucible was 1.0 gram. The initial weight (container plus sample minus tare weight of container) of the test specimen, W_2, was recorded. The container and contents were placed in the muffle furnace and ignite at 575 ^{o}C for a minimum of three hours. At the

start to avoid flaming, heat slowly, and to avoid flare up of WS samples, the container was partially covered during this step. The crucible was placed in a desiccator, cooled to room and the final weight of the ash, W_1 was recorded, as the container plus ash weight minus container tare weight.

Reducing sugars formed during adsorption

The reducing sugars formed during adsorption process were detected by DNS method. A standard curve of the absorbance at 540 nm was developed by using 50 to 800 µg mL glucose standard/mL solution in triplicates, shown in Figure 12.

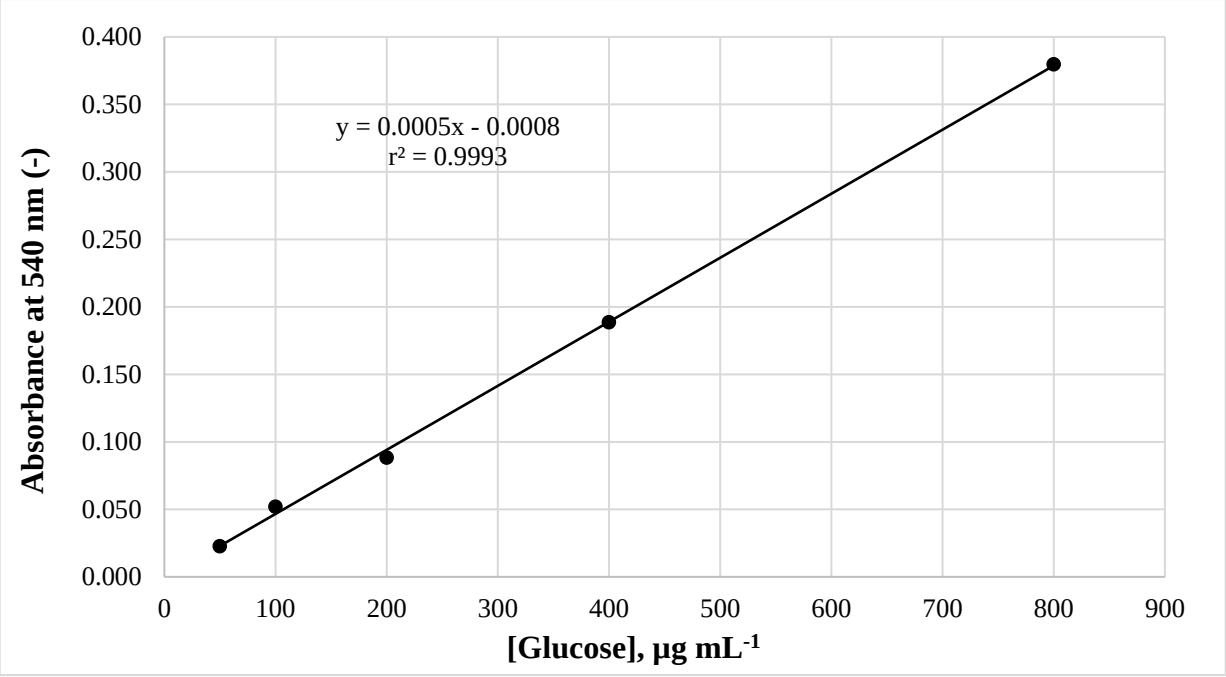


Figure 12: Glucose calibration curve (DNS method) used for the measurement of reducing sugars formed during adsorption process.

The supernatants obtained after adsorption of 180 µg mL^{-1} of cellulases NS 50013 on Avicel, at 25 ^oC, pH 5, for 60 minutes was used to determine the concentration of any reducing sugar formed during the process. After adding buffer to the supernatant as given in the 'Activity of cellulases solutions (Filter Paper Assay)', the mixture was placed in boiling water for 5 minutes in a water bath**.** The least squares regression line resulted in an r^2 value of 0.9993 was:

$$A_{540nm} = 0.0005[Glucose]-0.0008.$$

Protein concentration of cellulases solutions

The concentration of cellulases in solution (commercial mixture; P_0; P_f) for adsorption and desorption was determined according to the Lowry method [Waterberg, 2002] using Bovine Serum Albumin (BSA) as the reference protein. One test tube for water blank and one other was a substrate control added the samples to the appropriately labeled test tube. One milliliter of the Lowry Reagent Solution to control, blank, and each samples (0.5 mL) tubes. Mix well. Allow solutions to stand at room temperature for 20 minutes. With rapid and immediate mixing, add 0.5 ml of the Folin and Ciocalteu's Phenol Reagent Working solution to each tube. Allow color to develop for 30 minutes. Transfer solutions to cuvette and measure the absorbance of the control and sample tubes vs. blank and control at a wavelength of 750 nm. The absorbance of the color developed for triplicate solutions of 0 to 400 µg BSA mL^{-1} as shown as a standard curve in Figure 13.

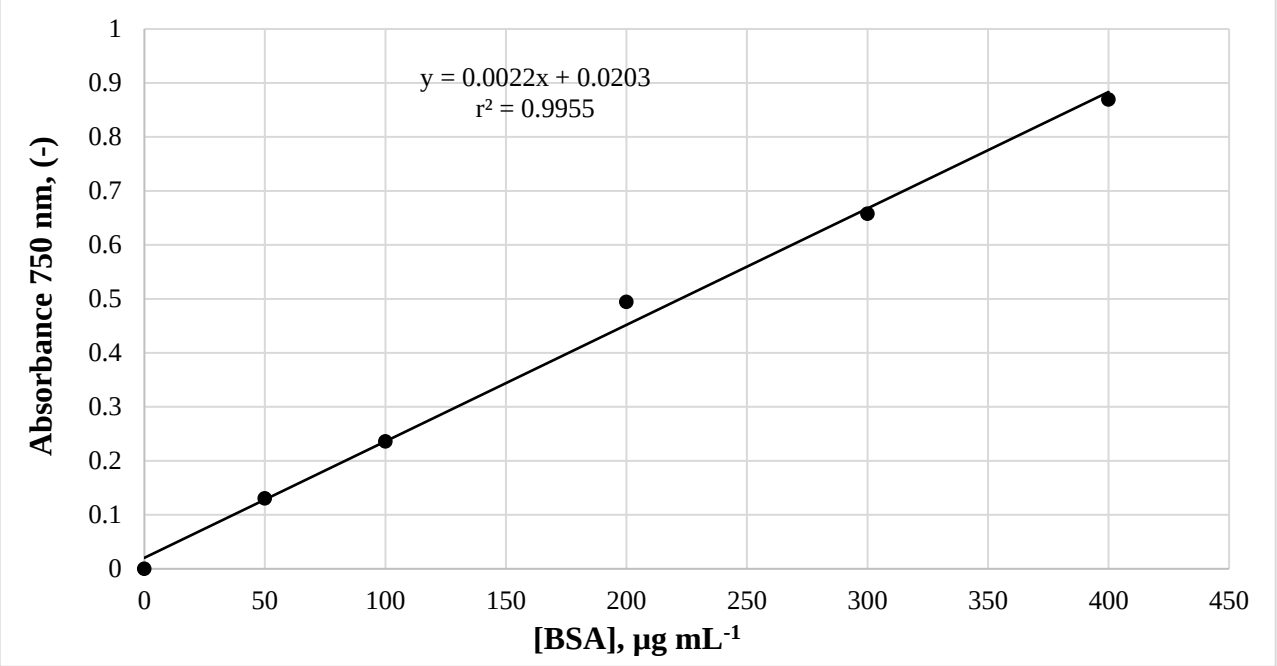


Figure 13: Calibration curve for the determination of cellulases concentration

A least squares regression line with an r^2 value of 0.9955 was obtained:

$$A_{750nm} = 0.0022\ [BSA] + 0.0203.$$

The concentration of an approximately diluted enzyme solution (commercial mixture, P_0,) will be read off the regression line, including error in the standard data. The procedure for measuring this error is shown in Appendix B. Sample calculation for activity of cellulases from supernatants (i.e. [P_{fa}], [P_{fd}]) is also given in Appendix D.

Activity measurement procedure (Filter Paper Assay)

The Filter Paper Activity (FPA) of cellulases was determined by the National Renewable Energy Laboratory Analytical Procedure (Technical Report NREL/TP-510-42628 2008. Measurement of Cellulase Activities (Adney and Baker, 1996). Strips of 50-mg Whatman No. 1 filter paper (1.0 x 6.0 cm) were placed in contact with appropriately diluted cellulase solutions for 60 minutes at 50°C. Reducing sugars released from this limited hydrolysis reaction (due to activity of enzymes on filter paper) were quantified using dinitrosalicylic (DNS) method. In this method, the enzyme action is immediately stopped adding 3.0 ml DNS reagent and mixing. Blanks, controls and glucose standards should be incubated at 50 ºC along with the enzyme assay tubes. Boil all tubes for exactly 5.0 minutes in a vigorously boiling water bath containing sufficient water to cover the portions of the tubes occupied by the reaction mixture plus reagent. All samples, controls, blanks, and glucose standards should be boiled together. After boiling, transfer to a cold ice-water bath. Let the tubes sit until any suspension was settled. Prepared dilution of 0.2 mL into 2.5 mL of distilled water, homogenized the mixture. Transfer each dilution (from all blanks and samples) into the cuvettes to read from the standard curve as shown in Figure 14. This procedure was repeated in triplicate.

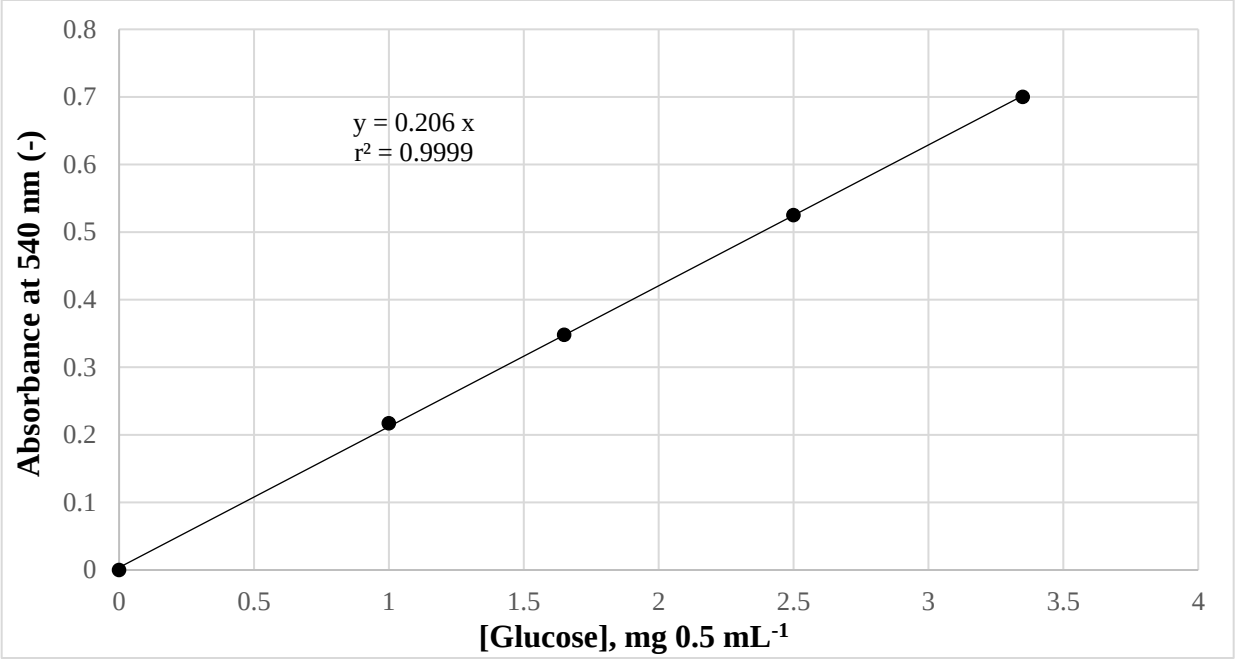


Figure 14: Glucose calibration curve (DNS method) used for cellulase enzyme activity measurement

The least squares regression line resulted in an r^2 value of 0.9999 was obtained: A_{540nm} = 0.206 [Glucose]. The unknown samples were transferred in cuvettes to read at 540 nm using UV/Visible Spectrophotometer Biochrome 50 which gives an absorbance reading in comparison with the standard curve. A set of sample calculations for cellulases NS 50013 enzyme activity is given in Appendix A.

Activity of unknown enzymes from the supernatant of adsorption which contain non-adsorbed cellulases [P_{fa}] and similarly from the supernatant of desorption which contain desorbed enzymes [P_{fd}] can be found from by using Figure 14. All other sample calculations for cellulases adsorbed, % desorbed, lnK_a and lnK_d and the calculation for activity of cellulases from supernatants are given in Appendix D.

5.0 Results and Discussion

5.1 Delignification

The goal of delignification of wheat straw was to degrade and remove lignin that was an obstacle to get the best accessibility to cellulose of wheat straw. To evaluate lignin degradation/removal Klason lignin determination method was applied. It was well known that ozone remove lignin by degrading carbon-carbon double bonds present in lignin [Kratzl *et al.*, 1974; Kaneko *et al.*, 1981]. In preliminary experiments, no reaction was observed between wheat straw brought in contact with low concentration of ozone. On the other hand, only 11- 13 % of lignin was removed at vigorous conditions of ozone flow rate of 4 L min^{-1} with a concentration of 40 mg L^{-1} for 120 minutes. Next thought was to introduce a reaction medium, hence, water was used as a reaction medium which increased the lignin removal up to 30 % of the original lignin present in the wheat straw. Now we can share our best results and used techniques for delignification.

Effect of water contents on delignification

The removal of lignin by using ozone from wheat straw was strongly influenced by the water contents. Wetting of the wheat straw surface causes water to absorb into the pores of wheat straw resulting in the swelling of pores and increasing the surface area for reaction with ozone. A range of water contents from 0 to 300 % (3 times the weight of dry wheat straw) was tried. Various water absorption times, and the styles of applying water (dropping water on surface, spraying, spraying and mixing) were attempted. The pretreatment where water was sprayed on wheat straw and then mixed with it until homogeneity, gave the best results. When the water contents were 30 % the dry mass of wheat straw, the lignin removed was 73 %. As water contents were raised to 100 % (1:1), amount of lignin removal obtained was also increased to 85 %. Further increase in the ratio of water contents to dry weight of wheat straw from 1:1 to 1:3, could only increase 3-6 % lignin removal. The

effect of water contents on lignin removal was given in Figure 15 while other experimental conditions were fixed as: ozone flow rate 4 L min^{-1}, ozone concentration in ozone-oxygen gas stream was 2 % wt/ wt, and reaction time as mentioned by each experimental value i.e., 120 min. Each data point was measured independently and it was an average of triplicate results. The optimum amount of water contents observed was 300 % (3 times) of the amount of dry mass of wheat straw; hence, this amount was used in this study. It was noticed that increase of water contents beyond 1:3 resulted in decrease of lignin removal. For example in the slurry of wheat straw in water (1:100) the amount of lignin removed was 30.1 % only [Baig *et al*., 2015]. .

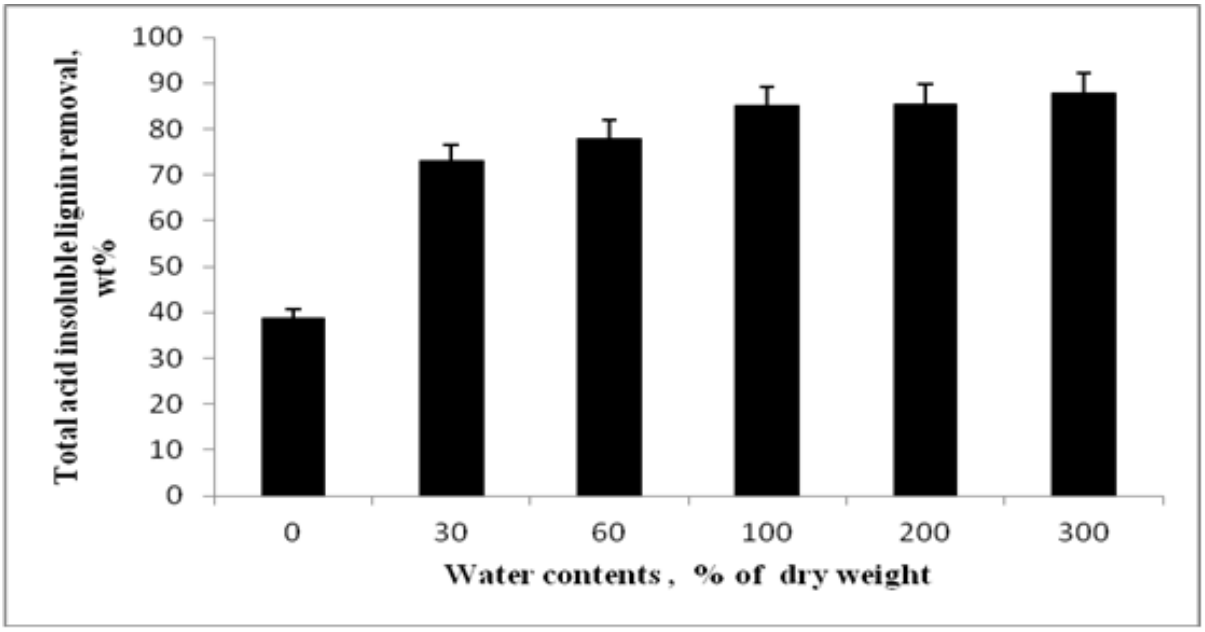


Figure 15: Effect of water contents on lignin removal. Ozone flow rate 4 L min^{-1}, ozone concentration in ozone-oxygen gas stream was 2 % wt/ wt

[Baig *et al*., 2015]

At 300 % water content and spraying and mixing, the amount of lignin removed was increased from 84 % to 88 % for a 60 minute of reaction time while all other reaction conditions remained the same.

Effect of flow rate of ozone through the sample, the concentration of ozone in the flowing ozone stream and the particle size of wheat straw was also investigated. The range of flow rates investigated the experiments was 1- 6 L minutes^{-1}. Percent of lignin removal increased with increasing flow rate of ozone up to 4 L min^{-1}. Further increase in ozone flow rate showed no increase in percent of lignin removed. In fact, the percent of lignin removal was decreased while going beyond 4 L min^{-1} to 6 L min^{-1} which was attributed to the lignin condensation. It was expected that ozone will react more with wheat straw if the concentration of ozone in the supplied ozone-oxygen gas mixture stream was increased. The effect of concentration of ozone was studied over a range from the 0.5 % wt/wt to 5 % wt/wt (6.55 to 65.5 mg L^{-1}) with a reaction time of 60 minute. The increase in concentration from 13.1 to 52.4 mg L^{-1}, gave an increase in the acid insoluble lignin (AIL) removed which was 77.3 % to 89.6 % and further increase in concentration from 52.4 mg L^{-1} to 65.5 mg L^{-1}, AIL remained unchanged. For the higher concentrations (52.4 mg L^{-1} and above) all available lignin was consumed quickly and an equilibrium is established between depolymerized and repolymerized lignin radicals hence, no significant change in lignin removal was observed [Baig *et al*., 2015].

The particle size of wheat straw could affect the reaction due to the contact and diffusion of ozone into the complex internal structure of lignocellulosic materials. It was observed that with the increase in particle size the AIL removed was decreased. The AIL removed was 35 % less when the particle size was increased to 20 times (i.e. from 0.5 mm to 10 mm). It was observed that reducing the particle size less than 500 microns resulted in loss of 20 % of material during handling, reaction, and filtration and drying [Baig *et al*., 2015]. Through the course of our experiments 0.5 mm particle size, 52.4 mg L^{-1} ozone concentration and 300 % water contents were selected as optimum parameter values for the delignification and the contact time was evaluated in next step.

Effect of reaction time

In Figure16, AIL removed during delignification of wheat straw was plotted against time of reaction with ozone. Three flow rates of ozone 4 L min^{-1}, 2 L min^{-1}, 1 L min^{-1}, containing 2 % ozone by weight, at room temperature were studied for 1 min to 180 minutes. Each data point was an average of triplicate of the percent of acid insoluble lignin removed and each data point was measured independently. In the start the lignin removal was fast because it was easy for ozone to access lignin freely. For example at the flow rate of 4 L min^{-1} around 86 % of lignin was removed at 60 min. There was only 4-8 % change in % lignin removed from 60 min to 120 min [Baig *et al*., 2015]. At 90 minutes of reaction with ozone, the AIL removed was 90 % of the total lignin present in untreated wheat straw.

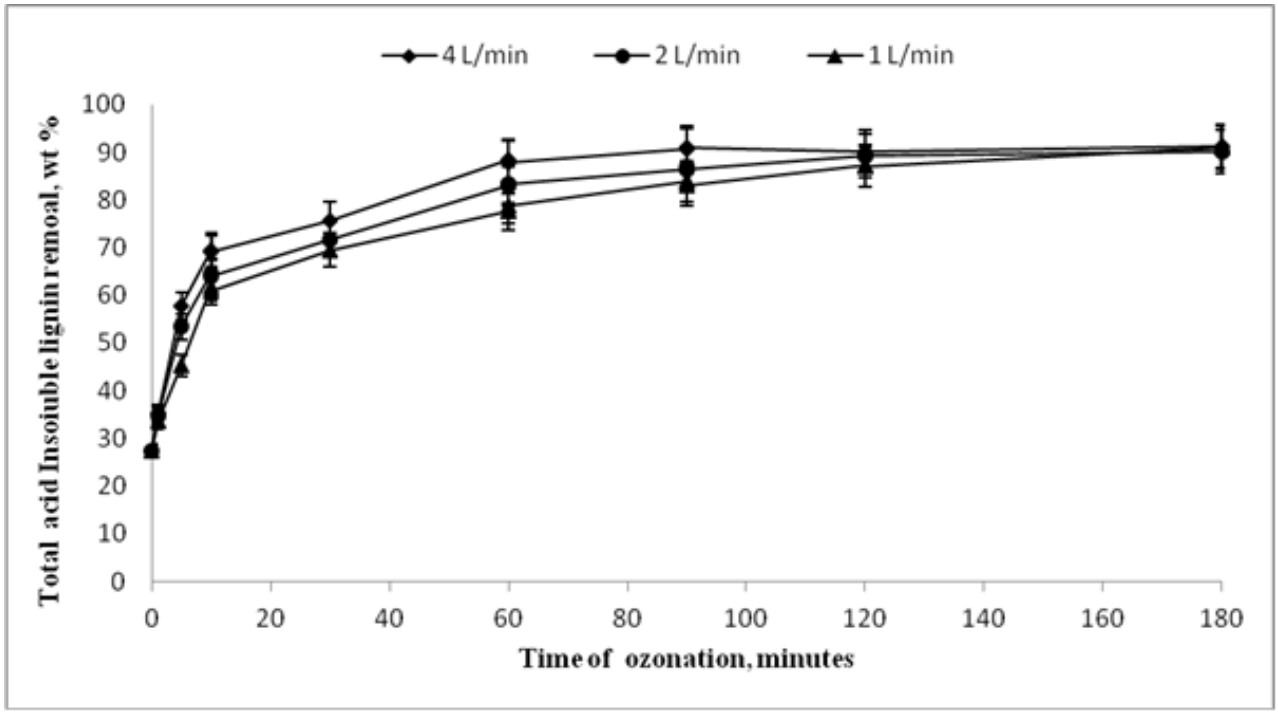


Figure 16: Lignin removal with various ozone flow rates, containing 2 wt % of ozone in ozone-oxygen gas stream, at 22 ºC
[Baig *et al*., 2015]

Samples treated with 4 L min^{-1} maintained more or less the same AIL value which they have attained at 90 min. Samples treated with ozone at flow rates of 2 L min^{-1} showed the same AIL value at about 120 minutes as that of 4 L min^{-1} at 90 minutes of ozonation. It was also observed that all flow rates (1 L min^{-1} to 4 L min^{-1}) showed almost the same value for AIL removed at 180 minutes of ozonation.

Tock *et al*. (1982) could only remove 40 % of lignin from Mesquite wood with ozone for a reaction time of 300 minute. Vidal and Molinier (1988) ozonated popular saw dust and 66 % of lignin was removed for a 360 minute of reaction time in 45 % acetic acid as reaction medium while our proposed technique removed 90 % of AIL with 90 minutes of ozonation. It is worth mentioning here that 88.3 % AIL was removed at 60 minutes of ozonation. García-Cubero *et al.* (2009) reported AIL content decreased from 22.1 % (w/w) for non-ozonated material to 12.3 % which is only 43.96 % removal of lignin for 150 min of ozonation reaction. They also observed that the lignin removed remained constant for reaction times above 150 minute. Similar observation was also noticed in our study where no change in the amount of lignin removed after 120 minutes. It can be explained as that there was no accessible lignin available for ozone to react.

It has been demonstrated that the novel proposed technique was suitable for lignin removal from wheat straw. Since the proposed technique was used at room temperature and ambient pressure, therefore, fabrication of reactor would not need expensive construction material. Ozone can be generated on site hence no stock of chemicals would be required for industrial scale production of ozone. Excessive ozone can be decomposed to oxygen before sending it into the atmosphere; therefore, the technique results in no environmental impact. Treatment of wheat straw needs surface preparation for an effective pretreatment with ozone. It was found that soaking in 1 % NaOH and then washing to neutral pH was sufficient to proceed for reaction with ozone. The highest value of lignin removal was achieved when the water content was 3 times of the weight of oven dried wheat

straw, ozone concentration was 2 L min^{-1}, with ozone concentration 52.4 mg L^{-1} and particle size of wheat straw was 0.5 mm. 30 % and 60 % delignified wheat straw samples were prepared for the evaluation of novel active desorption strategy in coming experiments.

5.2 Adsorption of cellulases

5.2.1 Adsorption of cellulases on Avicel and Protobind

For the study of desorption, adsorption was a prerequisite. In Figure 17 amount of cellulases adsorbed [P_{fa}] on Avicel at room temperature, 100 rpm, was for various contact times, for eight initial cellulases concentration [P_0] was studied for a time range between 0-90 minutes. Each data point was measured separately in triplicate except for 163 µg mL^{-1} when 9 replicates were done. Hence, the plots in Figure 17 are obtained by 189 experiments. Entries given in the legend bar are initial concentrations of cellulases P_0 s' with the units' µg mL^{-1}.

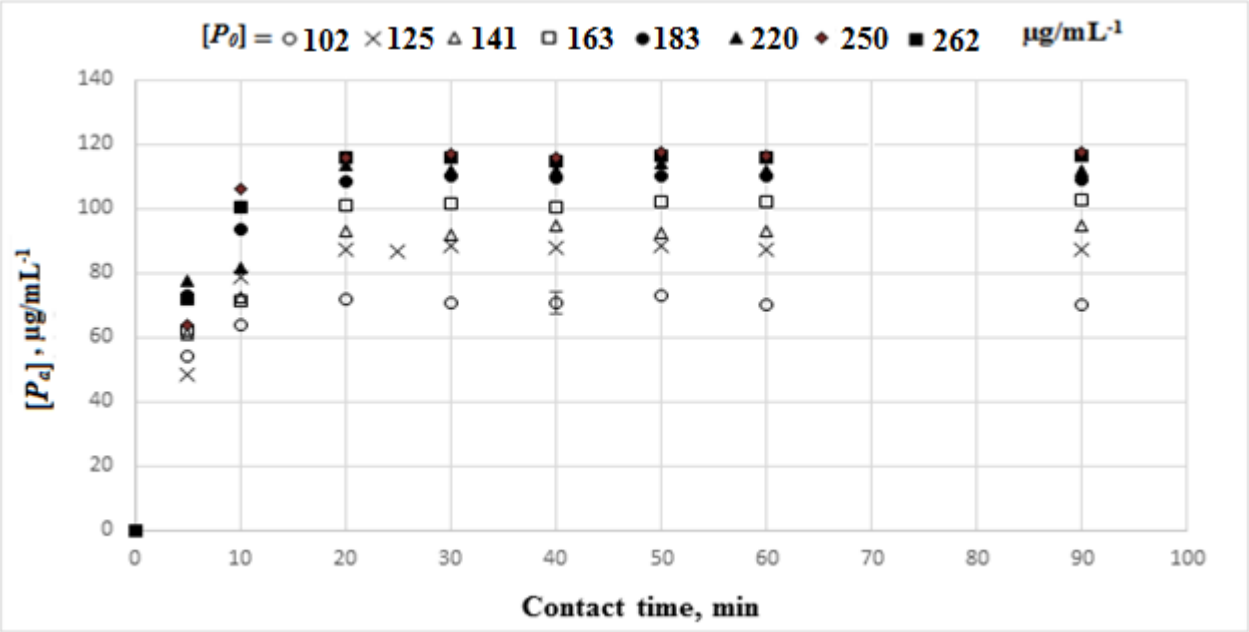


Figure 17: Adsorption of cellulases on Avicel PH 101 at 100 rpm, pH 5, 25 ºC, [P_0] varied from 102, 125, 141, 163, 183, 220, 250, to 262 µg mL^{-1} with error bars at 95 % probability
[Baig *et al*., 2016]

In the start increasing contact time increased the cellulases adsorbed and adsorption achieved its maximum almost at 20 minutes after of contact time, then it seemed to level off. Therefore, the time used for adsorption studies was set 30 minutes. In the study of enzymatic adsorption/hydrolysis, it was a tradition that the enzyme contents in the supernatant were determined for the free cellulases [P_f] and the adsorbed cellulases [P_a] were calculated from the difference between the initial cellulases concentration and the free cellulases [Stuart and Ristroph, 1984; Tu *et al*., 2009] which was carried out. In this set experiments it was found that increasing initial cellulases concentration [P_0] from 100 μg mL^{-1} to 183 μg mL^{-1}, adsorption increased from 68.45 μg mL^{-1} to 109.3 μg mL^{-1} for 100 mg of Avicel. Further increase in [P_0] from 183 μg mL^{-1} to 262 μg mL^{-1} cannot bring any significant change in [P_a] as shown in Figure 11 (i.e. from 109.3 μg mL^{-1} to 116.2 μg mL^{-1}). The value of maximum cellulases adsorbed was similar to that determined by other researches on various lignocellulosic substrates. [Singh *et al*., 1991, Nutor and Converse, 1991, Nidetzky and Steiner, 1993]. The supernatant obtained after adsorption of 183 μg mL^{-1}, at 25 ^{o}C, pH 5 for a contact time of 60 minutes showed no reducing sugars were formed. So, no noticeable hydrolysis took place during any of the adsorption experiments. This was expected since, Andersen and co-workers reported that Avicel PH-101 was only hydrolyzed up to 7 % in 24 hours and that most of this hydrolysis (5 %) was occurred after 5 hours of contact [Anderson *et al*., 2008].

Similarly adsorption of cellulases on Protobind 1000 was studied for seven initial concentrations of cellulases NS 50013 as given in the legend bar of Figure 18, the units of [P_0]s' were μg mL^{-1}. Increasing [P_0] from 100 μg mL^{-1} to 263 μg mL^{-1} adsorption increased from 45.62 μg mL^{-1} to 199.0 μg mL^{-1} for 100 mg of Protobind as shown in Figure 18 as well. The adsorption profiles were in agreement with that of published literature [Lu *et al*., 2002; Chen *et al*., 2006; Jagar *et al*., 2010; Li *et al*., 2011; Das *et al*., 2012; Zheng *et al*., 2013; Machado *et al*., 2014; Karmakar and Ray, 2015]

for adsorption of enzymes on various substrates.

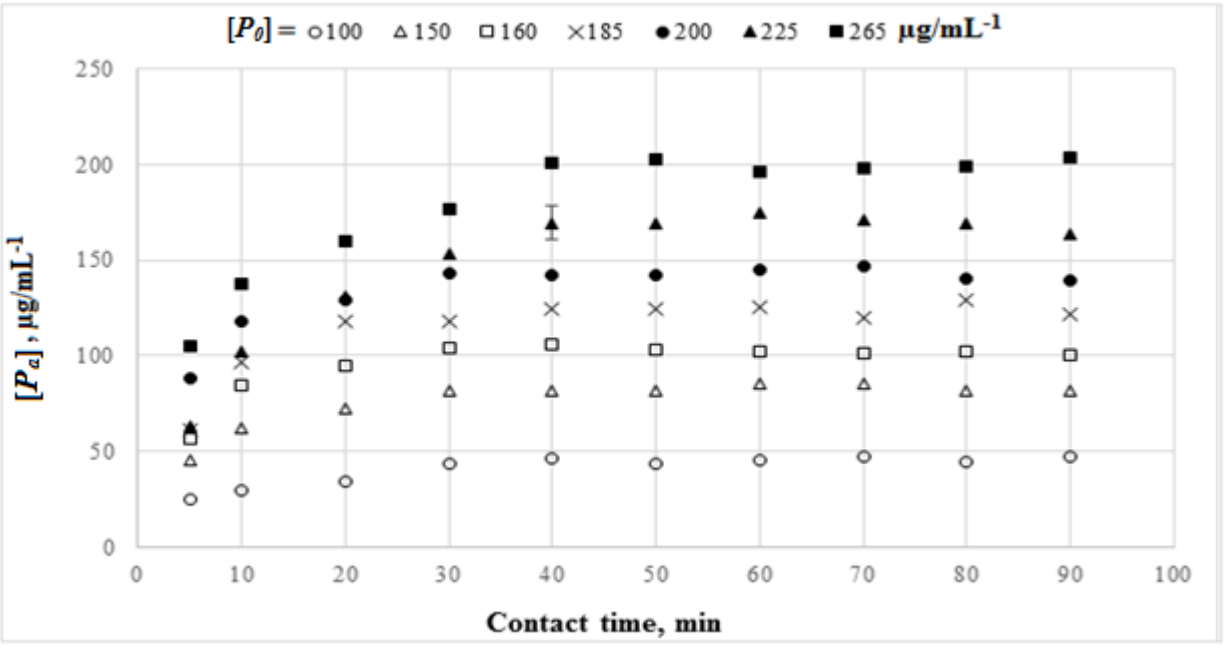


Figure 18: Adsorption of cellulases on Protobind 1000 at 100 rpm, pH 5, 25 ºC, [P_0] varied from 100, 150, 160, 185, 200, 225, to 265 µg mL^{-1} with error bars at 95% probability
[Baig *et al*., 2016]

The maximum adsorption was achieved at 40 minutes of contact time. Therefore, future adsorption experiments were done at 45 minutes of contact time. Long adsorption time of cellulases experienced for lignins versus cellulose can be explained by considering the functional groups taking part in this interaction. Cellulose contains a large number of hydroxyl groups [Sjostrom, 1993] to interact with cellulases. Cellulases adsorption on lignin is affected by functional groups, such as carboxylic, carbonyl, aliphatic hydroxyl, and phenolic hydroxyl groups [Berlin *et al*., 2006; Pan, 2008; Pareek *et al*., 2013; Rahikainen *et al*., 2013].

The cellulase adsorption ability of lignin involved more complex factors other than just the interaction with the hydroxyl groups as in case of cellulose [Kawamoto *et al*., 1992; Berlin *et al*., 2006]. More functional groups in lignin offers more attraction to cellulases. When a number of

cellulases approaches to lignin molecule, electrostatic repulsion [Norde, 1986; Nakagame, 2010] came into play between the cellulases themselves and they need time to rearrange themselves for adsorption. Another cause of delayed interaction could be a repulsion between amino acids tyrosine and histamine from cellulases and carbonyl from lignin may allow cellulase to rearrange in order to adsorb on lignin. Therefore, involvement of a number of positive and negative functional groups make adsorption on lignin complex phenomena which takes longer time. Detection of reducing sugars from the contact of cellulases with Protobind was not evaluated since lignin does not contain any polymeric sugars.

5.2.2 Effect of temperature on adsorption of cellulases

Adsorption of cellulases on Avicel PH 101 and Protobind 1000 under the influence of temperature is shown in Figure 19 and Figure 20 respectively.

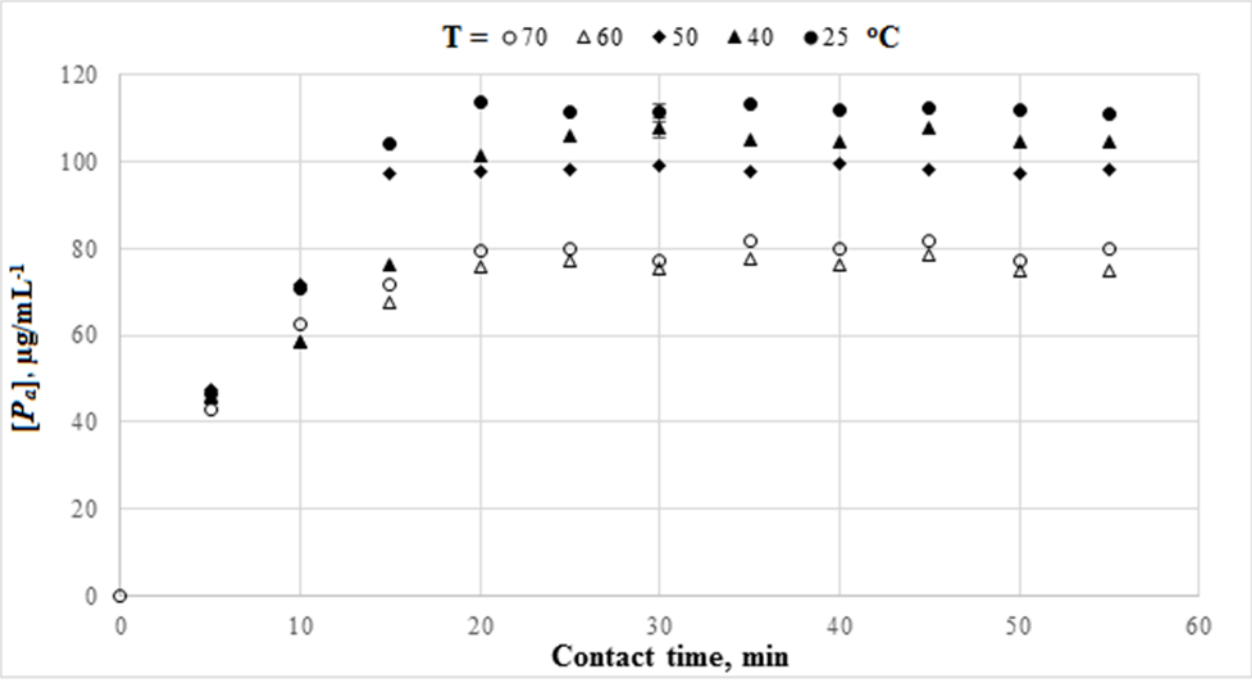


Figure 19: Adsorption of cellulases on Avicel PH 101 at varying temperature at pH 5, 100 rpm and maximum error bar at 95 % probability

Adsorption of cellulases varied with varying temperatures from 25 to 60 ^{o}C and adsorption decreased with further increase in temperature. Lee *et al.* (1982) studied adsorption of cellulases on pure cellulosic materials (absorbent cotton, Avicel PH 102, Solkafloc SW-40 hammer milled, Solkafloc BW-200 ball milled, and hammer-milled newspaper which passed through a 40-mesh) at only two temperatures 4 ^{o}C and 50 ^{o}C and concluded that the cellulases adsorbed at 4 ^{o}C was more than 50 ^{o}C though the difference was very small. Tomme *et al*. (1990) reported only a small effect on the adsorption by varying the temperature from 4 to 50 °C. Our results for adsorption of cellulases on Avicel PH 101 for temperature range of 25 to 50 ^{o}C were in agreement with the results of Lee (1999) and Tomme *et al.* (1990). For example, Tomme *et al*. (1990) reported that the fall in adsorption was less than 10%. Our adsorption on Avicel at 25 ^{o}C was 59.67 %, it was fallen to 52.94 % at 50 ^{o}C, which is also less than 10%.

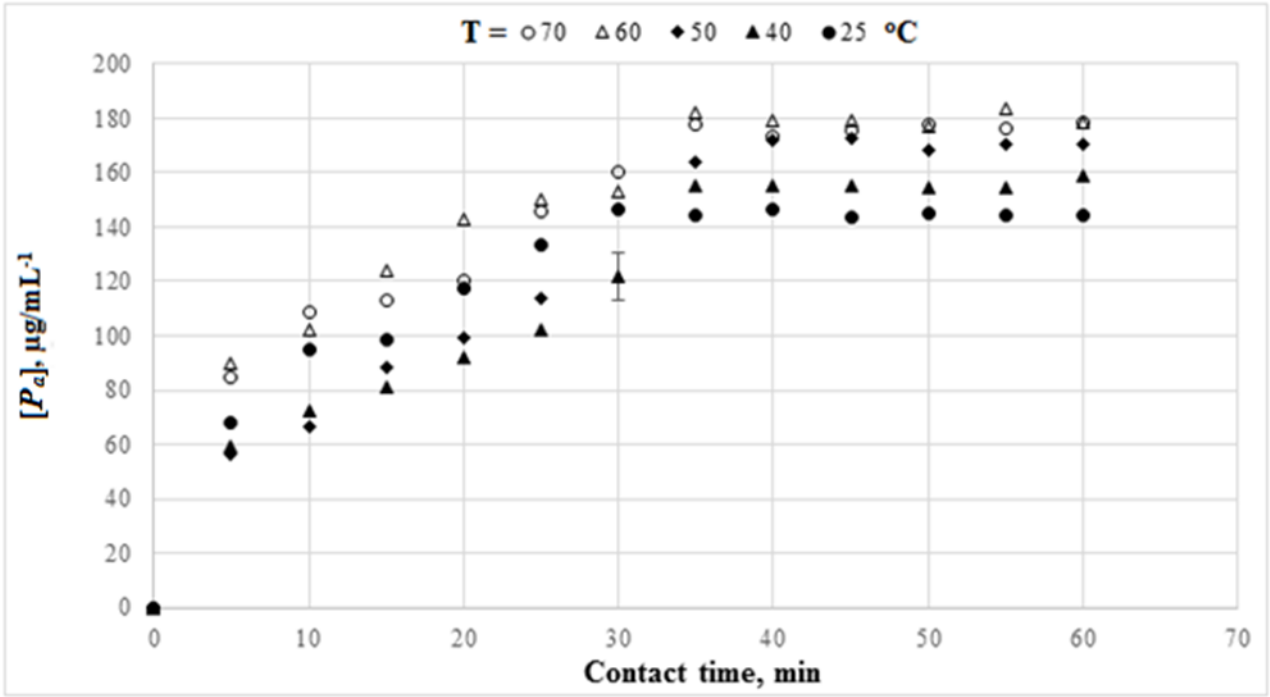


Figure 20: Adsorption of cellulases on Protobind 1000 at varying temperature at pH 5, 100 rpm and maximum error bar at 95 % probability

The most of researchers investigated adsorption of metal ions, dyes and enzymes on various lignocellulosic substrates at varying temperatures and found that by increasing temperature, the amount adsorbed increased but the time to reach equilibrium remains the same [Medve, 1994; Sethi *et al.*, 1998; Kim *et al*., 2001; Qadeer, 2005; Alzaydien and Manasreh, 2009; Daoud *et al*., 2010; Zheng et *al*., 2013; Du *et al*., 2014]. Our results are in agreement with their findings.

Both the substrates (Avicel PH 101 and Protobind 1000) behave almost opposite to each other. For Protobind E_a % increased up to 60 ^oC and further increase in temperature it was almost the same. Our hypothesis was that the increase in temperature increase adsorption of cellulases onto Protobind for a temperature range of 25 ^oC to 60 ^oC, which proved right. Tu *et al*. (2009) studied adsorption of cellulase on isolated lignins from Enzyme pretreated Lodgepole pine (EPLP) and steam pretreated Lodgepole pine (SELP) for temperatures 4, 25 and 45 ^oC and concluded that increase in temperature from 4 ^oC to 45 ^oC increased the adsorption, i.e. for EPLP adsorption raised from 1.85 µg mg^{-1} to 2.99 µg mg^{-1} and for SELP from 4.25 µg mg^{-1} to 6.10 µg mg^{-1}. Rahikainen (2013) stated similar results of adsorption of cellulases on two types of lignin (enzymatic mild acidolysis lignin: EMAL and lignin-rich hydrolysis residues: EnzHR) for 4 and 45 ^oC. Our results on adsorption of cellulases on Protobind for temperature 25 to 50 ^oC were in agreement with those from Rahikainen (2013) and Tu *et al*. (2009). Increase in temperature beyond 50 ^oC create some chemical changes in lignin due to dissociation of lignin phenolic groups [Evstigneev, 2011] and expose more substrate for cellulases. These changes could result in increasing adsorption on lignin [Kawamoto *et al*., 1992]. The adsorption of cellulases NS 50013 onto Protobind 1000 was conducted and reported for the first time in our study. It is also for the first time that the study was conducted systematically and reported for the temperature range of 25 ^oC to 70 ^oC. On the other hand this increase in temperature brings no changes in chemical structure of cellulose and the increased temperature create conformational

changes in cellulases folding, consequently, cellulases cannot remained adsorbed due to these conformational changes on the Avicel PH 101 and the adsorption decreased. Therefore, it can be concluded that the increase in temperature effects on adsorption one way or other.

In an adsorption process, [P_a] is a function of time. The equilibrium quantity of cellulases adsorbed on the substrates (cellulose, lignin) are described by adsorption isotherms at constant temperature. The adsorption isotherm also explains adsorption pattern of cellulases and adsorption capacity of substrates.

5.3 Cellulases Adsorption Isotherms

In this study, four isotherms (Nernst, Langmuir (2 linear types), and Freundlich) were evaluated for Avicel PH 101 and Protobind 1000.

5.3.1 Nernst adsorption isotherm

In Figure 21, amount of cellulases adsorbed per unit mass of Avicel PH 101 P_e is plotted against the concentration of free cellulases [P_{fa}] according to Equation 2.4. Every data points is an average of at least triplicate experiment conducted with initial enzyme concentration [P_0] at 102 µg mL^{-1} to 262 µg mL^{-1}. First data point on right had side is at [P_0] = 262 µg mL^{-1} where [P_{fa}] was 135 µg mL^{-1}. [P_0] and [P_{fa}] were measured, P_e was calculated. The concentration of adsorbed [P_a] is equal to the total enzyme concentration [P_0] minus the concentration of the enzymes in the solution [P_{fa}] [Stuart and Ristroph, 1984; Tu *et al*., 2009]. Error bars were obtained for 95 % probability and reside within the points being so small. Each data point was measured separately. According to the Nernst theory, irrespective of the initial (total) cellulases concentration [P_0], cellulases distribute themselves in P_e and [P_{fa}] in a constant ratio.

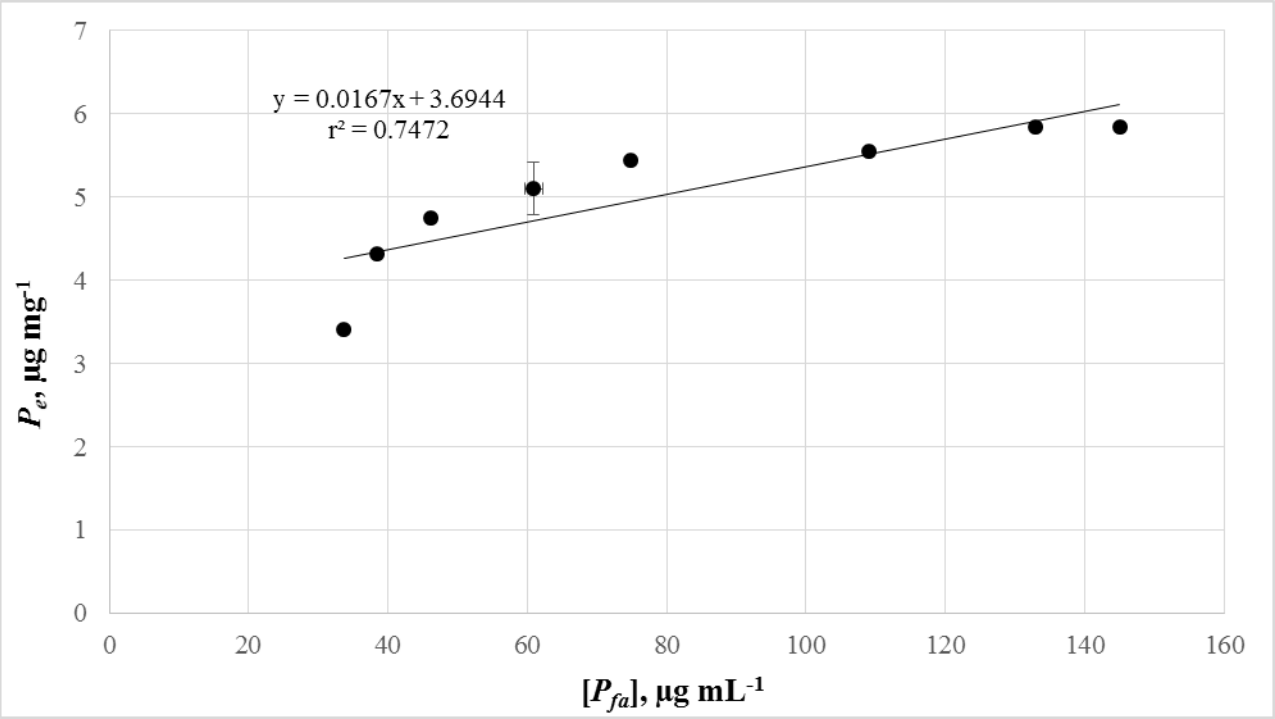


Figure 21: Adsorption of cellulases on Avicel PH 101 according to Nernst isotherm model, at pH 5, 25 ºC, and 100 rpm also shown with maximum error bar at 95 % probability
[Baig *et al*., 2016]

Apparently the average of data point do not flows a straight line but with considering error. The model equation obtained by plotting P_e against P_{fa} according to the Nernst theory was: P_e = 0.016 (P_{fa}) + 3.694 with an r^2 of 0.7472. In contrary to expectations, the linear curve did not pass through origin. Therefore, Nernst isotherm will not be able to represent adsorption of cellulases on Avicel PH 101.

Nernst isotherm for Protobind 1000 is shown in Figure 22. All data points are obtained by conducting experiments under same experimental conditions as Avicel PH 101. The data is clustered between 50 µg/mL to 70 µg mL^{-1} indicated that the increase in specific adsorption P_e is much more than the corresponding increase in [P_{fa}]. The r^2 value obtained was 0.210 which suggest that Nernst adsorption model was not a good representation of adsorption of cellulases on Protobind 1000.

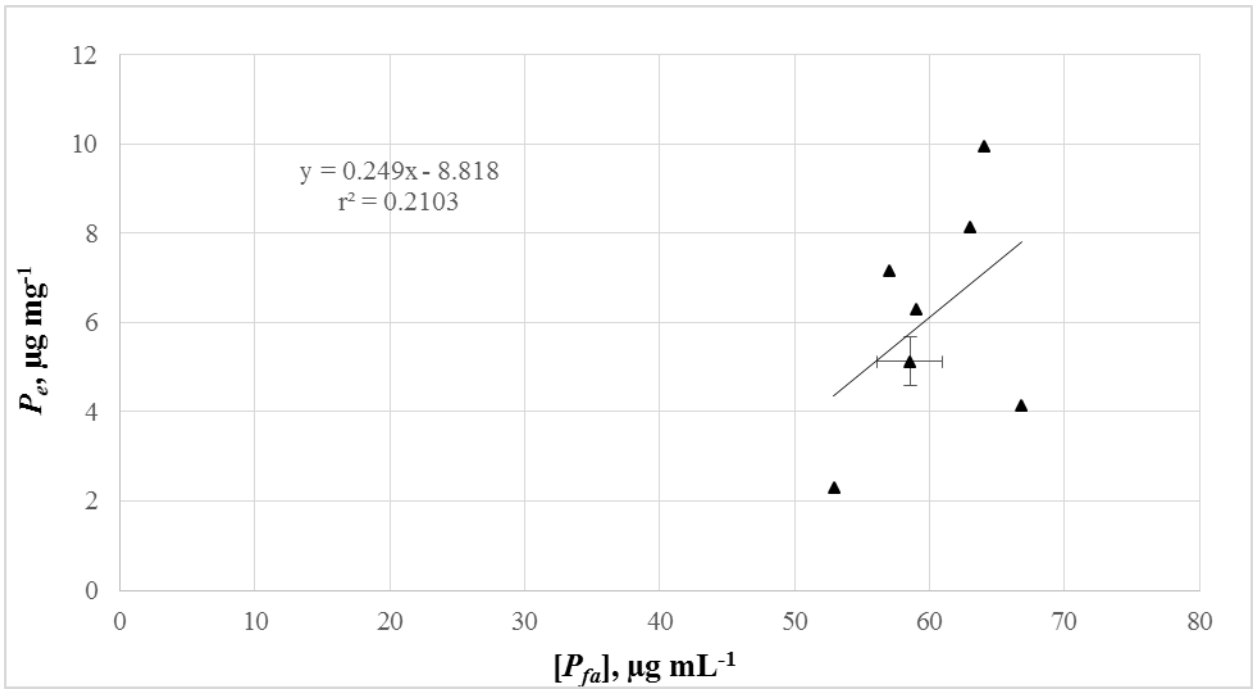


Figure 22: Adsorption of cellulases on Protobind 1000 according to Nernst adsorption, data was obtained at pH 5, 25 ºC, and 100 rpm with maximum error bar at 95 % probability
[Baig *et al*., 2016]

5.3.2 Langmuir, adsorption isotherm

Adsorption data for Avicel according to Langmuir isotherm is shown in Figure 23. The ratio of specific adsorbed cellulases to non-adsorbed cellulases (y-axis) versus the specific adsorbed cellulases (x-axis).

The plot gave a straight line with a slope of (K_a , (µg/mL)$^{-1}$) and an intercept of (K_a P_{am}, ((µg/mL)$^{-1}$* (µg/mg of substrate)). As [P_e] increased the corresponding (P_e/[P_f]) decreased. Eliminating one outlier, rest of the seven data points gave the existing plot with r^2 value equal to 0.9572. Thus, 96 % of the specific adsorbed cellulases were directly accounted for specific adsorbed cellulases per cellulases present in the bulk of solution. The model equation obtained from the plotted data was: P_e/ [P_f] = -0.049 E_e + 0.3302.

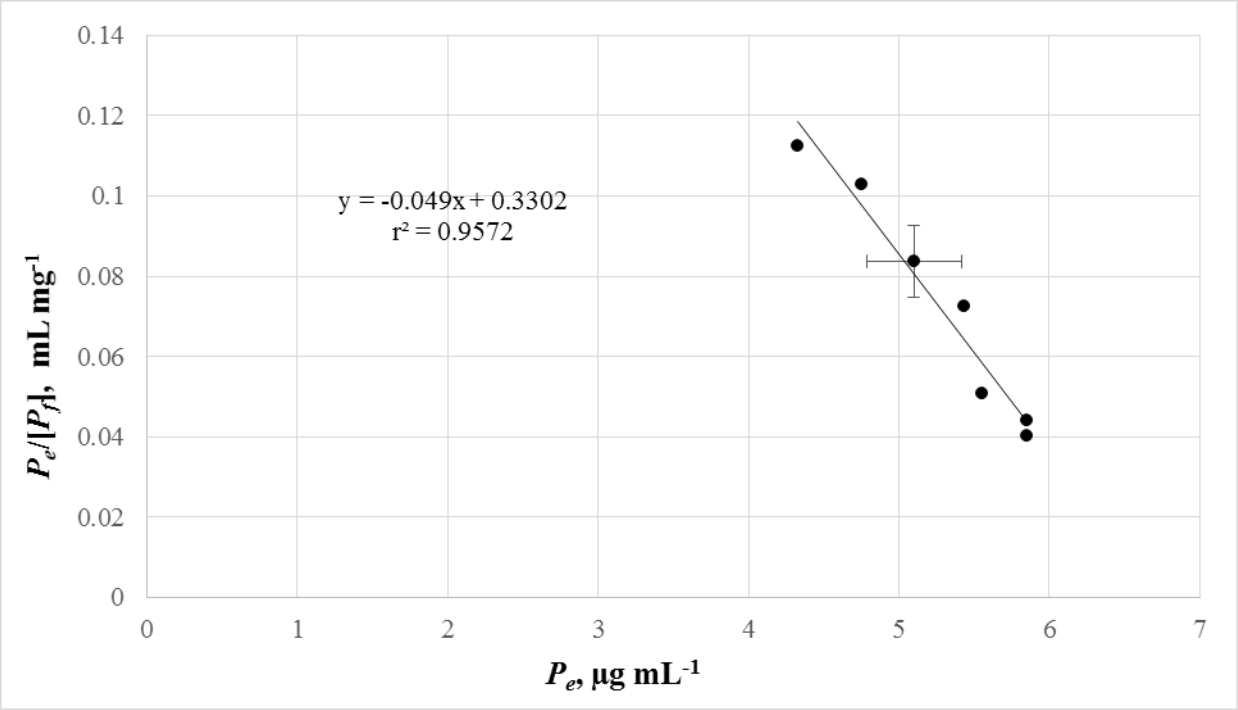


Figure 23: Langmuir adsorption isotherm (linear form as Equation 2.7) for Avicel PH 101 data was obtained at pH 5, 25 ºC, and 100 rpm and maximum error bar at 95 % probability
[Baig *et al*., 2016]

The non-adsorbed cellulases [P_f] was found to be directly proportional to the initial cellulases concentration [P_0]. The results also indicated that the cellulases adsorbed on the surface of Avicel PH 101 homogenously and all adsorption is monolayer type. Similar results were observed by other researchers as well [Hoshino *et al*., 1992; Kim *et al.,* 2001].

Langmuir adsorption isotherm for adsorption of cellulases on Protobind 1000 is shown in Figure 24. The ratio of specific adsorbed cellulases to non-adsorbed cellulases (y-axis) versus the specific adsorbed cellulases structures this plot. The plot gave a straight line with a slope of (0.0151, K_a) and an intercept of (K_a P_{am}).

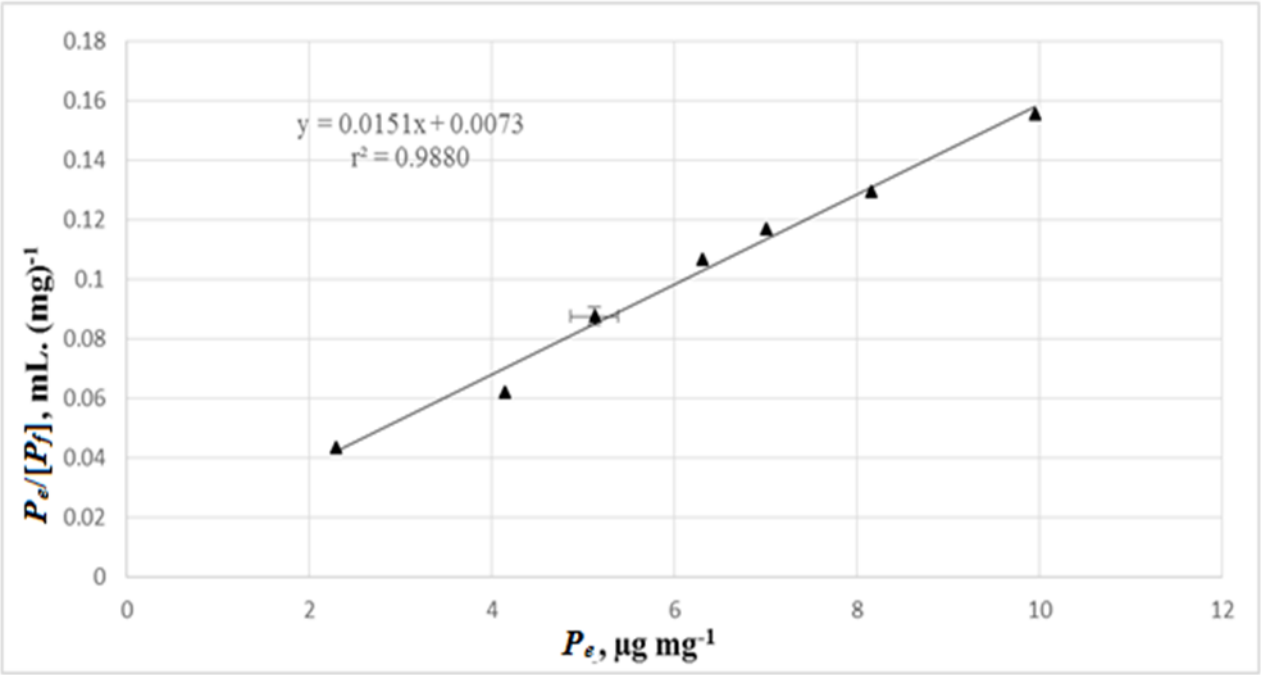


Figure 24: Langmuir isotherm (linear form as Equation 2.7) for adsorption on Protobind 1000 data was obtained at pH 5, 25 ºC, 100 rpm and error bar at 95 % Probability
[Baig *et al*., 2016]

The curve line obtained from the plotted data-points follow a model equation as P_e/P_{fa} = 0.015 (P_e) + 0.007 with an r^2 value of 0.9880. The plotted data showed a positive correlation between P_e vs $P_e/[P_{fa}]$. It was indicated that ratio of specific amount of cellulases adsorbed on Protobind to the concentration of cellulases in the solution follow a smooth increase with the increase in P_e. The Scatchard plot with a positive slope is contrary to generally accepted wisdom derived from studies of cellulases on lignocellulosic materials. In life sciences, the positive slope was recognized by a number of researchers while studying, binding of DNA to ligands, binding of oxygen to hemoglobin or drugs to nucleic acids etc. [Dahlquist, 1978; Nesbitt *et al*., 1982; Elmore *et al*., 1988; Ofek and Doyle, 1994; Gupta, 2011]. The positive slopes cooperative adsorption. Cooperative adsorption is adsorption where an adsorbed cellulases has a positive effect on the adsorption of ‘‘new’’ adsorbing cellulases. Positive cooperative binding of enzymes was never noticed before. This study noticed

this positive cooperative adsorption because it is first thorough study of adsorption of cellulases (enzymes) to Protobind (Protobind 1000).

5.3.3 Freundlich adsorption isotherm

Freundlich adsorption isotherm of cellulases on Avicel is shown in Figure 25, it was obtained by plotting natural logarithm of specific adsorbed cellulases ($\ln P_e$) against natural logarithm of cellulases present in the bulk of solution $\ln P_f$.

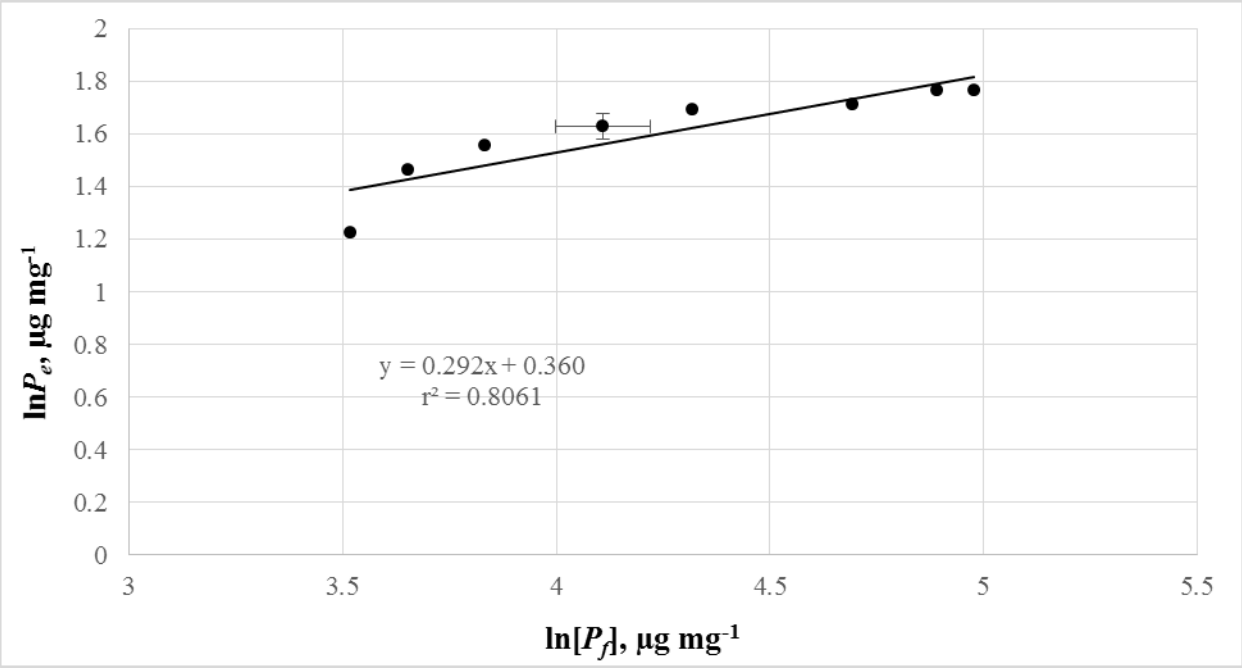


Figure 25 : Freundlich isotherm for adsorption of cellulases on Avicel PH 101, data was obtained at pH 5, 25 ºC, 100 rpm and maximum error bar at 95 % probability
[Baig *et al*., 2016]

The model equation obtained after plotting the experimental data was: $\ln P_e = 0.292\ (\ln P_{fa}) + 0.360$. Plotting the graph a straight line with value of slope (1/m) equal to 0.292 and $\ln K_f$ as y-axis intercept was 0.360. The K_f value obtained was 1.43 µg mg^{-1}. According to Freundlich isotherm this intercept was expected. The slope is called heterogeneity factor and ranges between 0 and 1. A system is considered to be more heterogeneous when slope is closer to 0. The slope value 1.43 is closer to 1,

indicated that adsorption of cellulases on Avicel showed some uneven distribution of cellulases on the adsorbent surface. Freundlich isotherm was used for adsorption on heterogeneous surfaces or surface supporting sites of varied affinities [Dabrowski, 2001]. Since r^2 value is equal to 0.8061 which means that Avicel surface was not having different affinities and we cannot expect any multilayer adsorption. The [P_e] value predicted was in around 3.0% error with respect to the corresponding experimental value.

Figure 26 shows Freundlich adsorption isotherm for adsorption of cellulases on Protobind 1000. The plot was obtained by plotting of lnP_e (y-axis) vs lnP_{fa} was a straight line with r^2 = 0.2813 and its intercept giving lnK_f. (-11.71).

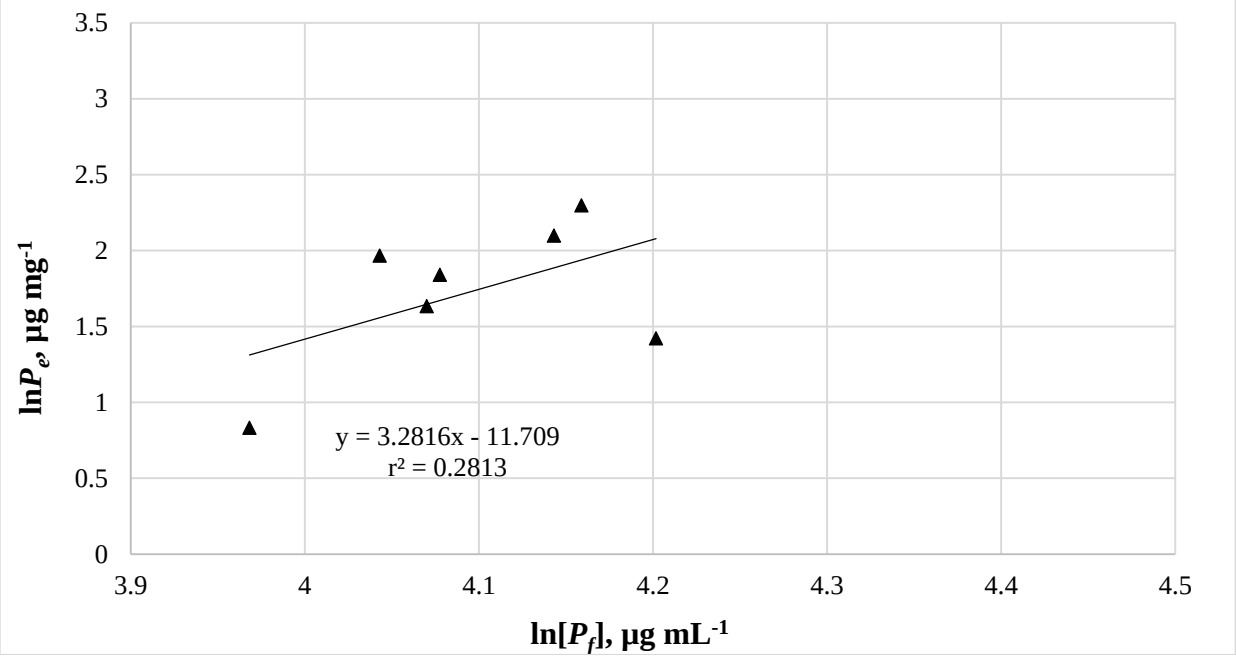


Figure 26: Freundlich isotherm for adsorption of cellulases on Protobind 1000, data was obtained at pH 5, 25 °C, and 100 rpm and with maximum error bar at 95 % probability
[Baig *et al*., 2016]

The K_f calculated for wheat straw lignin (Protobind 1000) was 8.21x 10-6 µg mg^{-1} while K_f for Avicel PH 101 was 1.43 µg mg^{-1}. Reading the value of K_f with r^2 value showed not to use Freundlich

isotherm as representative for adsorption on Protobind 1000. The correlation factor obtained for Freundlich adsorption isotherms from Avicel and Protobind were 0.8679 and 0.2813 respectively. By removing one outlier at lnP_{fa} = 4.2 µg/mg (Figure 22), the shape of the trend line obtained was: lnP_e = 6.683 (ln P_{fa}) - 25.46, with r^2 became equal to 0.8120 with remaining 6 data points. The r^2 = 0.8120 (for 6 data points) was still not better than r^2= 0.9880 (for 7 data point for Langmuir isotherms as shown in Figure 24).Hence Freundlich isotherm was not a good representative of cellulases adsorption on the studied substrates. Therefore, it can be stated that during adsorption, cellulases did not make multilayers adsorption on Avicel as well as on Protobind.

5.3.4 Choice of a model

The plots in Figure 21 and 22 were used to test whether the linear model was a good fit for the adsorption data of the cellulases. Plots in Figure 23 and Figure 24 were used to determine whether the adsorption data of the cellulases onto Avicel PH 101 and Protobind 100 was explained by Langmuir adsorption isotherm. The plot in Figures 25 and 26 tested the goodness of fit for the Freundlich model. Langmuir adsorption isotherm appear to be a good selection for the representation of adsorption of cellulases on Avicel PH 101 as well as for Protobind. Among other adsorption methods it stand out because of correlation coefficient for both substrates.

Therefore, adsorption of cellulases on Avicel PH 101 and Protobind 1000 followed Langmuir's adsorption pattern. This adsorption pattern suggested that the adsorption of cellulases in monolayer and interaction between cellulases do not form multilayer of enzymes as suggested by some others [Vadi *et al*., 2010; Sun *et al*., 2011]. The amount of cellulases distributed on substrate and in bulk solution is not directly proportional. The amount adsorbed increased and decreased with increase in or decrease in cellulases taken as initial amount but not in the same proportion. For maximum concentration of initial cellulases taken (265 µg mL^{-1}) the maximum amount of cellulases adsorbed

on same mass (100 mg) of Avicel and Protobind was 108-115 µg mL^{-1} and 180-199 µg mL^{-1} which means Protobind showed almost 1.5 to 1.8 times more adsorption capacity than that of Avicel. Amount of cellulases need for the conversion of wheat straw to ethanol can be adjusted accordingly by the cellulose and lignin contents in the wheat straw. Lignin adsorbed almost twice the amount of cellulases as that of cellulose and adsorption of cellulases was faster on lignin that on cellulose. Disagreement of data with Freundlich isotherm indicated that cellulases do not interacts to form multilayer on the substrates. Therefore all cellulases adsorbed was on the surface of substrates. Langmuir isotherm give us maximum amount of cellulases adsorbed, P_{am}, which can be a lead to find accessible surface area of a substrate. The equilibrium concentrations of cellulases adsorbed on the substrate [P_a] and remaining in the bulk [P_f] can be used for the study of enthalpy and entropy. The study of adsorption isotherms told us that cellulases adsorb on the substrate in monolayer. Monolayers (cellulases-substrate) are stronger than the multilayer (cellulases-cellulases) [Qin, *et al*., 2014]. Since this study is about desorption strategies, therefore studying desorption from a monolayer adsorption system would be the real job.

5.3.5 Pore diffusion of cellulases

In general, the quantity of an adsorbed enzyme on to a substrates to a large extent related to the pore size rather than the surface [Dong *et al*., 2012; Du *et al*., 2013]. For example, in a study of adsorption of papain in porous glass it was found that little or no protein was adsorbed in the pores when glass pores approached the molecular dimensions of the protein. Thus, it was suggested to achieve maximum adsorption, the pores in substrates should be 3–9 times larger than the size of the enzymes [Stone *et al.*, 1969]. It means that the pore sizes play an important role in the enzyme adsorption [Deere *et al.*, 2002; Li *et al*., 2010; Bayne *et al.*, 2013]. Bayne *et al.* (2013) reported that for an enzyme of 5 nm to adsorb on a substrate having pore diameters less than 10 nm in general there will

be a decrease in enzyme adsorption because of the physical restrictions faced by enzymes in accessing surface offered. For substrates pore sizes above 100 nm the adsorption of enzymes (5 nm) increased due to an associated increase in available surface area. For the pores sizes of 10–100 nm a decrease in protein adsorption level was expected with increasing pore diameter. It was observed that the enzymes adsorption in this range of pore diameter remained almost constant. Bayne *et al.* (2013) concluded that decrease in pore diameter restricted access to the available surface area. Monsan (1978) found that the adsorption of trypsin (an enzyme) on amino silica was strong due to the pore size but not due to the surface area [Datta *et al*., 2013]. It suggested that the pore size usually dictated the loading of the enzymes. Similarly, it was found that penicillin G acylase was 0.01 mg g^{-1} adsorbed on triacrylate substrate having surface area 245 m^2g^{-1} and pore diameter 12.64 nm. The amount of penicillin G acylase adsorbed on the Eupergit C was one tenth (~0.1) which has almost similar surface area (180 m^2g^{-1}) but much greater pore diameter size (20–40 nm). As expected in the former example the enzyme faced restriction to enter the pores of triacrylate (5 nm was the size of) and apparently, all the adsorption was on the external surface [Thygesen *et al*., 2010]. From this discussion it was derived that cellulases not only adsorb on the external surface area, they may adsorb on the pores of substrates. Again, not at all the pores but the pores with diameter more than 10 times of the enzymes diameter.

The experimental adsorption data of cellulases adsorbed onto test substrates was modeled by using Bangham's Equation 5.2 to evaluate pore diffusion [Aharoni and Ungarish, 1977]. Bangham model studied to uncover the mechanism of cellulases adsorption/binding onto the surfaces of Avicel and Protobind. The goodness of fit will be used to estimate the role of pore diffusion of cellulases in the adsorption process.

$$log\left[log\left\{\frac{P_0}{(P_0-P_eM)}\right\}\right] = \log\left[\frac{k_0}{2.303V}\right] + \propto \log(t) \quad \ldots \quad 5.2$$

Where, E_0 is the initial concentration of the cellulases in solution (mg L^{-1}),

V is the volume of the solution (mL),

M is the mass of substrate (g),

$\propto$ is the constant of Bangham Model

P_e (mg g^{-1}) is the amount of adsorbate retained at time t and α (less than 1) and k_0 are the constants.

As such log [log {P_0/(P_0-P_e M)}] was plotted against log(t) in Figures 27, and 28 the two substrates. The plot was found to be linear for each adsorbent with good correlation co-efficient, such as 0.9034, and 0.9027, for Avicel, Protobind respectively. Since r^2 value (>0.90) which was a positive linear association indicated that experimental data confirmed to Bangham's equation and therefore it can be deduced that pore diffusion has some control on the adsorption of cellulases on the substrates.

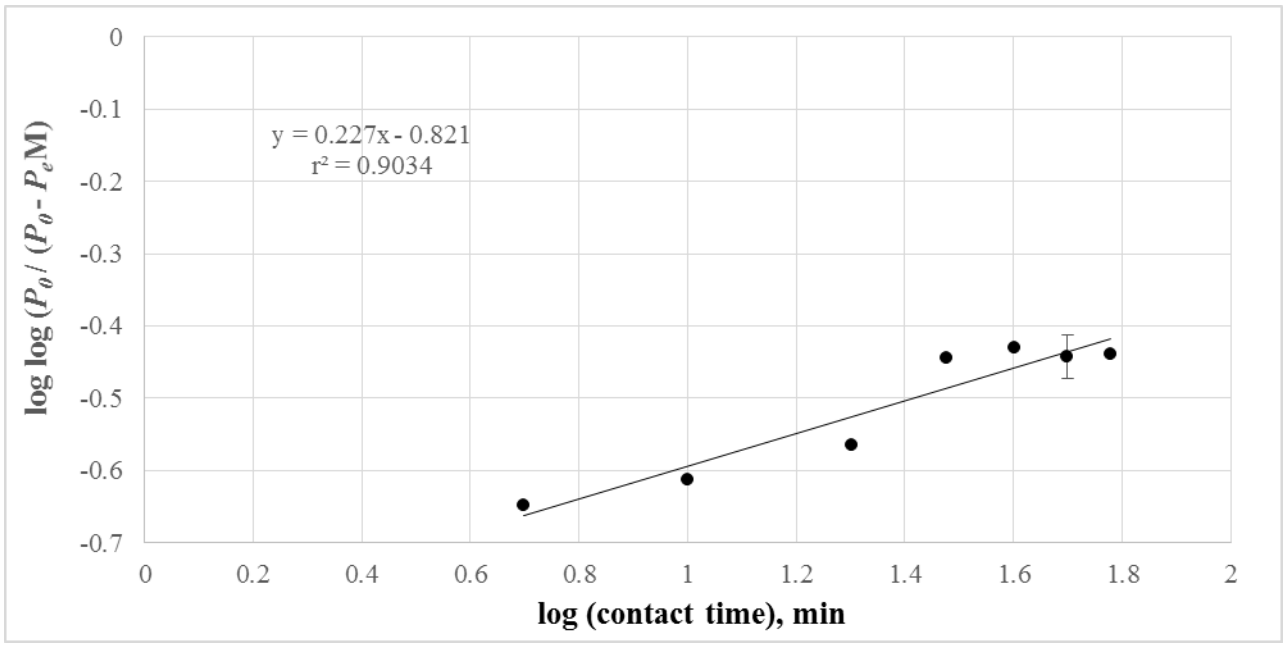


Figure 27 : Pore diffusion plot for Avicel PH 101, with maximum error bar at 95 % probability

The plots for Avicel and Protobind obtained by Bangham model have almost same intercepts, i.e. 0.821 and 0.972 respectively. In the case of slopes, the slope of Avicel (0.227) was almost two times of the slope of Protobind (0.401). As, it could be noticed that log [log {P_0/ (P_0-P_e M)}] was increasing with log (t), it indicated that {P_0/ (P_0-Pe M)} is increasing with contact time. If P_0 is consistent, then (P_0-P_e M) is decreasing to make this term large, hence adsorption is increasing. Therefore, adsorption in the pores of Protobind was 2 times of that of Avicel.

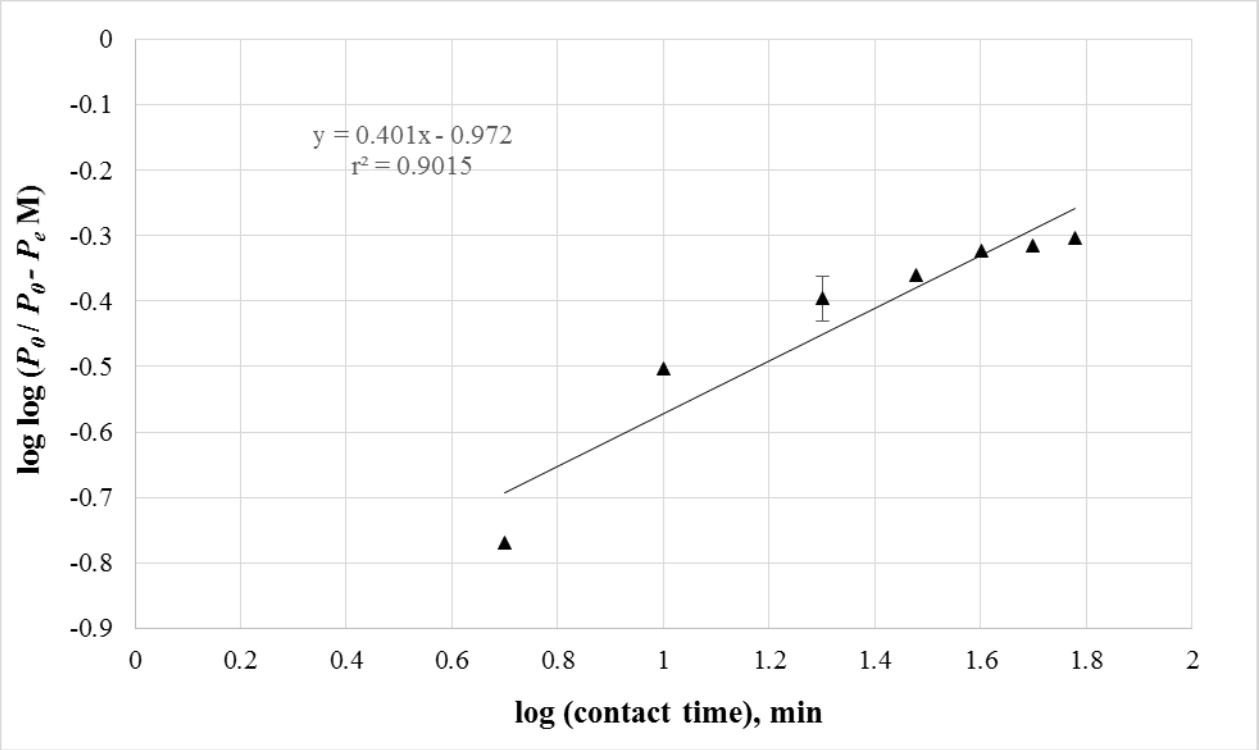


Figure 28 : Pore diffusion for Protobind 1000, with maximum error bar at 95 % probability

Pore diffusion may have share in adsorption of cellulases on to Protobind 1000 as the correlation coefficient r^2 was 0.9015. This indicated that the adsorption is dependent on surface area which in turn is dependent on the pore size because pores increase surface area.

5.4 Enthalpic and entropic changes for adsorption under the influence of temperature

The temperature dependency and thermodynamics of desorption, however, was rarely reported in the literature. The van't Hoff equation was adapted to represent the adsorption processes of cellulases to Avicel PH 101 and Protobind 1000. Plotting the equilibrium adsorption results for both solid substrates as a van't Hoff relation (Equation 2.10) linearity of adsorption is observed over the temperature range of 298 K ($1/T = 3.35 \times 10^{-3}\ K^{-1}$) to 343 K ($1/T = 2.92 \times 10^{-3}\ K^{-1}$) (Figure 29). The left side of the plot represents higher temperature.

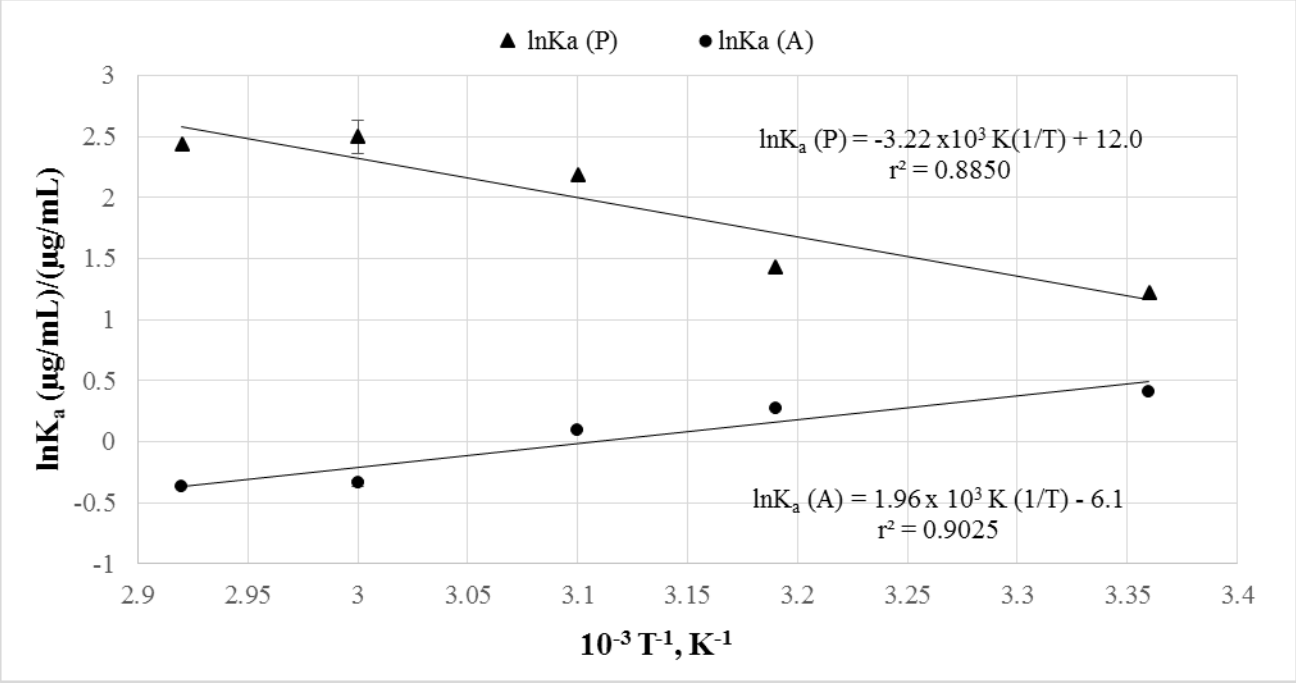


Figure 29: Van't Hoff plots for adsorption on Avicel PH 101 and on Protobind 1000 at pH 5 with maximum error bar at 95 % probability
[Baig, 2016]

For Avicel, lnK_a decreased with an increase in temperature, there was a small decrease (10-12 %) in adsorption at 323 K compared to 298 K, and at 343 K the adsorption decreased by 33 %, as was expected from published literature [Andreaus *et al*., 1999; Kim *et al*., 2001; Tu *et al*., 2009b; Rahikainen *et al*., 2013]. Other research groups reported that some cellulases started denaturing

around 323 K [Otter *et al*., 1984; Andreaus *et al*., 1999; Tu *et al*., 2009a; Rahikainen *et al*., 2011]. The Novozymes cellulases used in this study surpassed this limit by 10 K. On the other hand, Baker's group [Baker *et al*., 1992] proved that individual cellulases maintain their activities up to 333 K. The observed $\ln K_a$ at lower temperature may be associated to a reduced translational energy and rotational energy (vibrational energy is negligible) of cellulases and cellulose allowing opposite charges on the cellulose-binding domain (CBD) of cellulases and on the Avicel to align themselves. The van't Hoff model gave $\ln K_a = 1.96 \times 10^3 \ (1/T) - 6.10$. The change in enthalpy (ΔH_a) value obtained from the slope of the equation was found to be 15.95 $kJmol^{-1}$. The adsorption process sometimes is a arrangement of two processes: (a) desorption of the solvent (water) molecules formerly adsorbed on it or a neighbor molecule, and (b) the adsorption of the cellulases. In an endothermic process, the cellulases has to displace more than one water molecule for their adsorption and this result in the endothermicity of the adsorption process. Therefore, ΔH_0 will be positive. The positive ΔH_a of adsorption means that non-covalent interactions (such as electrostatic, van der Waals, hydrogen bonding, etc.) are significant [Ooshima *et al*., 1983; Hoshino *et al*., 1992] between cellulases and Avicel. The ΔS_a value obtained from intercept of the plot was -50 $Jmol^{-1}K^{-1}$. The negative entropy indicated that the mobility of the adsorbed cellulases on the surface of Avicel was restricted. Negative values of ΔS_a were also observed for adsorbed enzymes during other studies [Ooshima *et al*., 1983; Boraston, 2005; Saha *et al*., 2014] and for ionic adsorption [Ngah and Hanafiah, 2008; Padmavathy, 2008; Ramdane *et al*., 2014]. Another reason for negative ΔS_a value could be unfolding (conformational changes) of cellulases. The unfolding of endoglucanases from *Aspergillus niger* and α-amylase from *Bacillus licheniformis*, DNA ligase and xylanase were reported where the ΔS value was negative [Violet and Meunier, 1989; D'Amico *et al*., 2003; Collins *et al*., 2003]. Violet and Meunier (1989) presented the formation of an intermediate (X) on the pathway between the native (N) and the denatured (D) enzyme forms (i.e. $N \rightarrow X \rightarrow D$). They noticed that the intermediate state

(adsorption of enzymes on substrate) is more ordered structure than the starting state i.e. ΔS_a is negative.

According to the second law of thermodynamics for any spontaneous process, the overall $\Delta S \geq 0$. The negative change in entropy does not contradict the second law, because the adsorption of cellulases on Avicel have a sufficiently large negative ΔH_a (over 320 times of that of ΔS_a) results in a sufficiently large increase in entropy that overall the change in entropy is positive. That is, the ΔS of the liquid increases enough because of the exothermicity of the reaction that it overcompensates for the negative ΔS of the solid-liquid interface, and since the overall $\Delta S_a = \Delta S_{surroundings} + \Delta S_{system}$, the overall change in entropy is still positive. The Gibbs free energy (ΔG_a) value increased from -1170 $Jmol^{-1}$ to 1080 $Jmol^{-1}$ as temperature increased from 298 K to 343 K. A negative ΔG_a, expected for spontaneous adsorption occurred only from 298 K to 323 K. Consequently, only this temperature range is recommended for adsorption of cellulases on Avicel PH 101. For bioethanol producing industries, using wheat straw where cellulose is a component, adsorption can be performed on delignified wheat straw between the temperature ranges of 298 K to 323 K. Since fermentations are conducted in most of the industries around 313 K therefore, adsorption can also be performed at the same temperature for the ease of process.

In the case of Protobind, the experimental data points plotted between 298 K and 343 K showed $\ln K_a$ increased with an increase in temperature. The trend line equation (regression equation) was $\ln K_a = -3.22 \times 10^3 (1/T) + 12.0$. Therefore, ΔH_a was 26 $kJmol^{-1}$, which indicates an endothermic reaction. The amount of cellulases adsorbed increased from 75 % to 94 % of the initial cellulases concentration when temperature increased from 298 K to 333 K. The ΔS_a for the adsorption of cellulases on Protobind 1000 was positive (100 $jmol^{-1}K^{-1}$) which means that disorder of the system was increased [Saha and Chowdhury, 2011]. In accordance with the second law of thermodynamics since $\Delta S_a > 0$,

the adsorption of cellulases on lignin appears to be an irreversible process. The ΔG_a decreased from $-2.90x10^3$ $Jmol^{-1}$ to $-7.40x10^3$ $Jmol^{-1}$ as temperature increased from 298 K to 343 K, which becomes the suitable adsorption temperature range for Protobind 1000. By choosing 298 K, 20 % less cellulase would be adsorb on ligneous component than at 343 K and 30 % more cellulases will adsorb [delignified wheat straw].

The negative ΔH_a, ΔS_a and ΔG_a indicated that maximum temperature suitable for adsorption is 298 K to 320 K. In depth study showed that maximum adsorption on Avicel was between temperatures 298 K to 313 K and maximum adsorption on Protobind was on temperature 323K to 343K. To avoid adsorption on lignin (in addition to optimum removal of lignin) low temperatures such as 298 K to 313 K should be used for adsorption because at these temperature thermodynamic conditions give less support for adsorption on lignin. Therefore, a temperature closer to 298 K is recommended for adsorption part of bioethanol production process. Reuse of adsorbed enzymes to save cost of enzymatic hydrolysis is important, therefore, entropy and enthalpy of desorbed cellulases from was studied next.

5.5 Desorption of cellulases

Desorption of adsorbed cellulases (adsorbed on Avicel PH 101) remaining on the solids residues after centrifugation is shown Figure 30. Desorption was conducted with distilled water at 25 ºC. Cellulase desorbed over time, found their way in the supernatant and reported as a percentage of the concentration. Half of the % desorbed was obtained in first 5 minutes then desorption progressed slowly till it reached to its equilibrium around at 20 minutes. Variance in measurement were estimated by error propagation technique and finalized to get 95 % probability of desorption. Therefore, desorption time in further studies for Avicel was taken as 20 minutes.

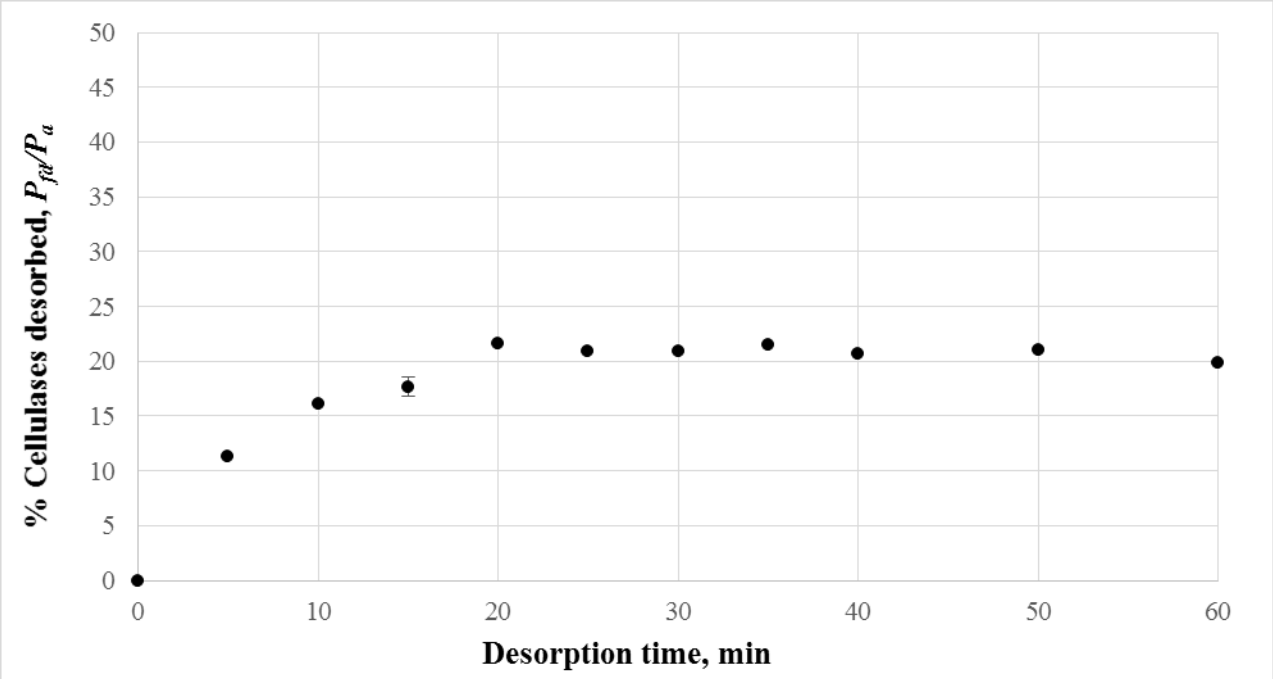


Figure 30: Desorption of cellulases from Avicel PH 101 using distilled water, at pH 5, 25 ºC, E_0 at 165.2 μg mL^{-1} and maximum error bar at 95 % probability

Desorption time 20 minutes is closer to the reported time in literature. For example Otter *et al.* (1984) demonstrated that desorption from steam exploded aspen was completed in 30 seconds and was not affected by increases in desorption time to 15 min. Similarly, Nidetzky and Steiner (1993)

reported achievement of desorption from microcrystalline cellulose within 15 minutes, which is closer to 20 minutes. The existing hypothesis was evaluated and found that desorption from Avicel by using distilled water was only 22% which means that adsorption was not completely reversible.

The plots in Figure 31 showed % of cellulases desorbed at temperatures varying from 25 to 70 ^{o}C at pH from 6 to 9. Every data point was measured individually for an average of 5 replicates. All the curves seem to follow a similar trend leading to a maximum concentration of desorbed enzymes at 60 ^{o}C. Maximum desorption of cellulases from Avicel achieved was 74 % at pH 9 and 60 ^{o}C. The second maximum cellulases desorbed achieved was 62 % at pH 8 and 60 ^{o}C. At pH 9, 50 ^{o}C desorption was around 52 %. Increasing temperature over 60 ^{o}C decreased desorption.

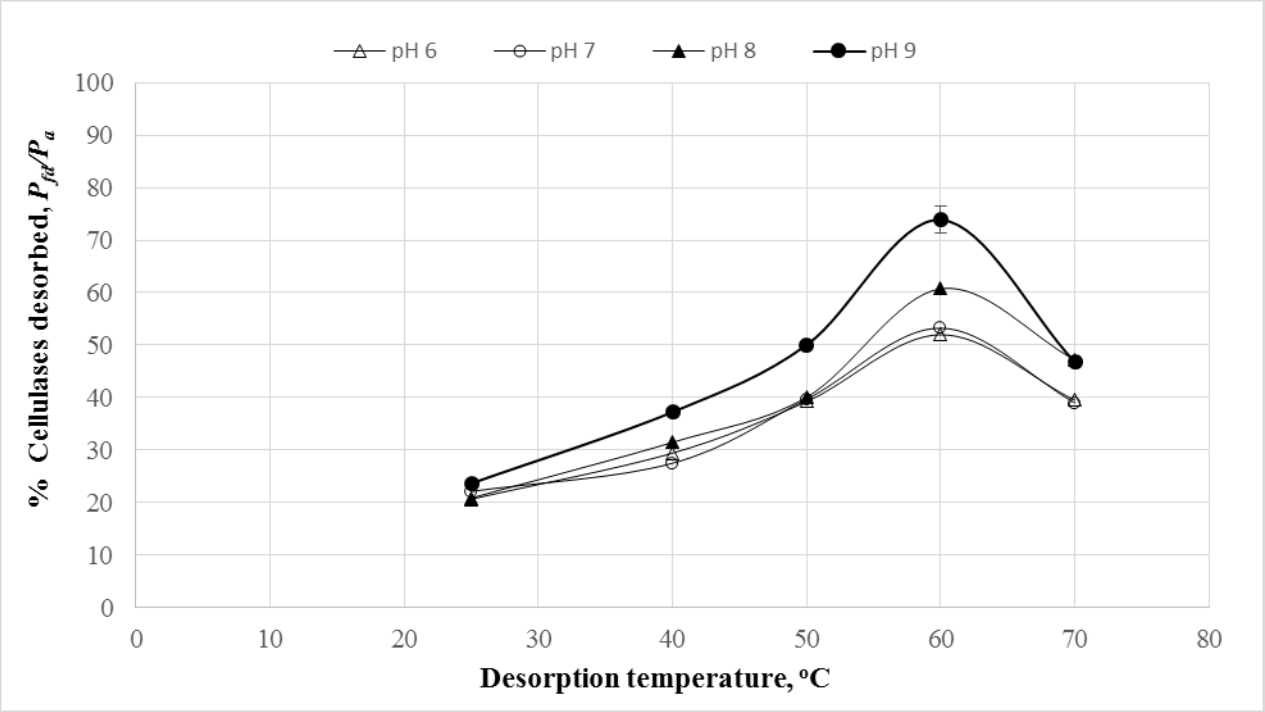


Figure 31 : Desorption of cellulases from Avicel with varying temperature (from 25 ^{o}C to 70 ^{o}C) and pH (from pH 6 to pH 7), 100 rpm and maximum error bar at 95 % probability
[Baig *et al.*, 2013]

Ye (2012) studied desorption of cellobiohydrolases (CBH I) at 0 ^{o}C and 50 ^{o}C from Avicel and reported that 25 % of total enzymes desorbed at 50 ^{o}C while desorption at 0 ^{o}C was less than 10 %.

Implied that increase in temperature from 0 ^{o}C to 50 ^{o}C increased desorption. Our results showed that desorption of Avicel was increased from 20 % to 75 % with respect to the initial cellulases concentration when temperature was increased from 25 ^{o}C to 60 ^{o}C at pH 9. Hong *et al*. (2007) reported that increase in pH from 5 to 10 increased desorption to 61 %. Our results have similar trends as presented in literature and have better values while increasing from pH 6 to pH 9 at 60 ^{o}C, desorption increased from 51 % to 75 %. Hong *et al*. (2007) also reported that desorption was 84 % from Avicel and 94 % from dilute acid pretreated corn stover at pH 13 but desorbed enzymes were not active. Desorption of all non-active enzymes was useless as enzymes could not be reused.

The relative activities of the cellulases desorbed are shown in Figure 32. All the data points were measured separately and were averages of 5 replicates.

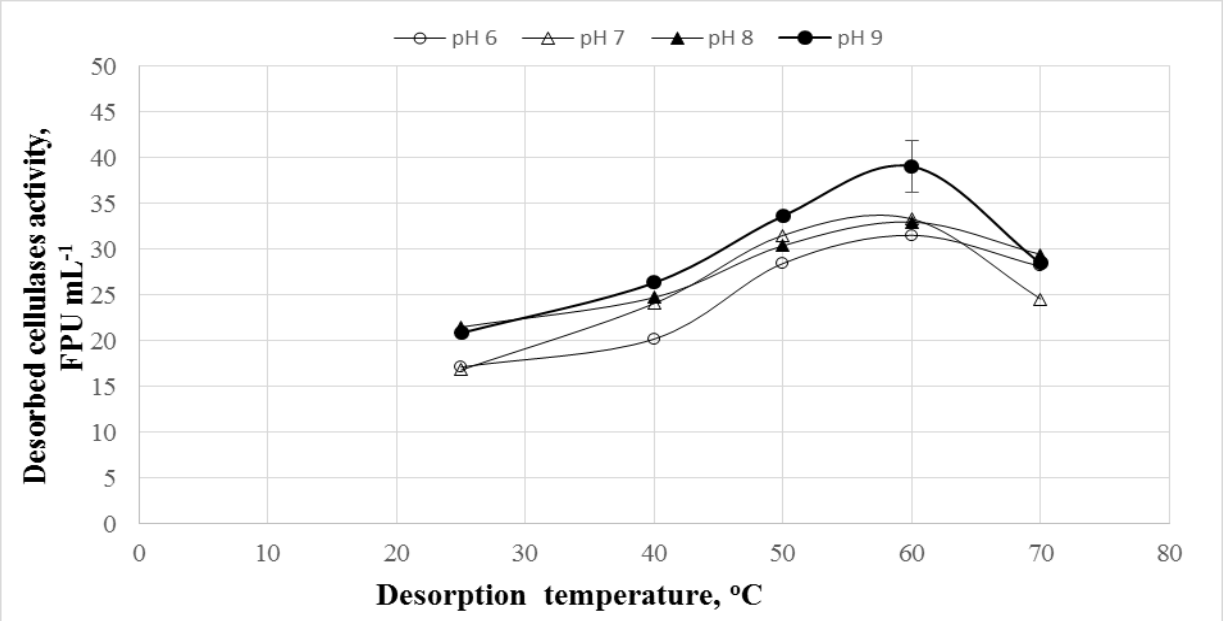


Figure 32 : Activity of desorbed cellulases from Avicel with varying temperature (from 25 ^{o}C to 70 ^{o}C) and pH (from pH 6 to pH 9), 100 rpm and maximum error bar shown at 95 % probability

All curves seem to follow a similar trend and obtained their maximum at 60 ^{o}C. At pH 9, beyond 60 ^{o}C cellulases lost around 30 % of their activity which may be an attribute of denaturing. The

maximum activity achieved at pH 8 and 9 at 60 ^{o}C were 33.01 and 39.07 FPU ml^{-1} respectively. The activity values obtained at pH 8 and pH 9 are almost the same up to 50 ^{o}C. Otter *et al*. (1989) desorbed 68 % of cellulases at pH 10 with zero activity, the amount was there maximum reported. Our result indicated that pH 8 and pH 9 are suitable to desorb active cellulases from cellulosic substrates. We achieved maximum active desorption at pH 9 at 60 ^{o}C from Avicel. Another test was also conducted by heating Avicel PH 101 at 70 ^{o}C, pH 9, and 100 rpm for 60 minutes which showed that no reducing sugars were formed. Therefore no degradation or self-hydrolysis of Avicel occurred at the conditions of desorption. In Figure 33 each data point plotted was measured individually and was an average of triplicates. The desorption trend is similar to that of Avicel desorption.

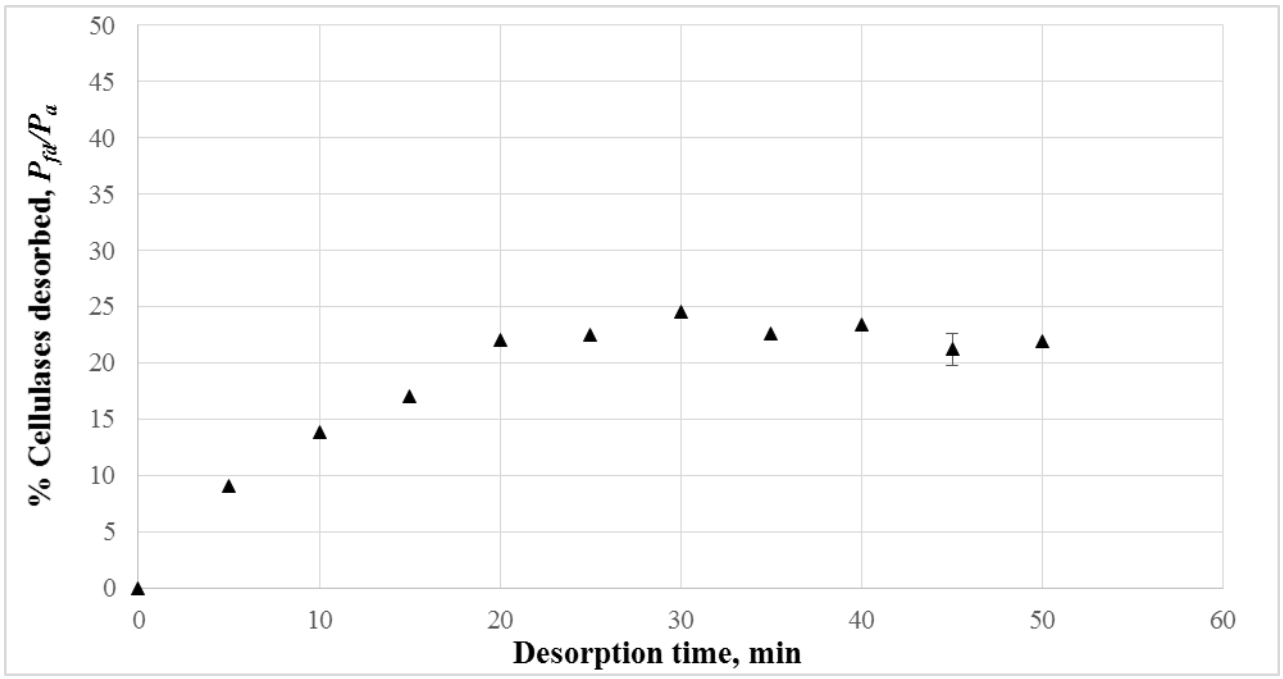


Figure 33 : Desorption of cellulases from Protobind 1000 using distilled water, at pH 5, 25 ^{o}C, E_0 at 182.7 µg mL^{-1} and maximum error bar at 95 % probability

It was surprizing to experience that desorption equilibrium for Protobind was established around 20 min which is the same as that of Avicel. It needs further research to determine why desorption time from Protobind and Avicel is the same. For further desorption experiments desorption time was

selected as 20 minutes. The existing hypothesis was evaluated for desorption from Protobind by using distilled water and 25 % cellulases were desorbed which means that adsorption was not completely irreversible. Therefore, existing hypothesis that adsorption on lignin is irreversible was proven wrong.

Figure 34 contain plots of cellulases desorbed from Protobind 1000 under similar conditions as studied during desorption from Avicel.

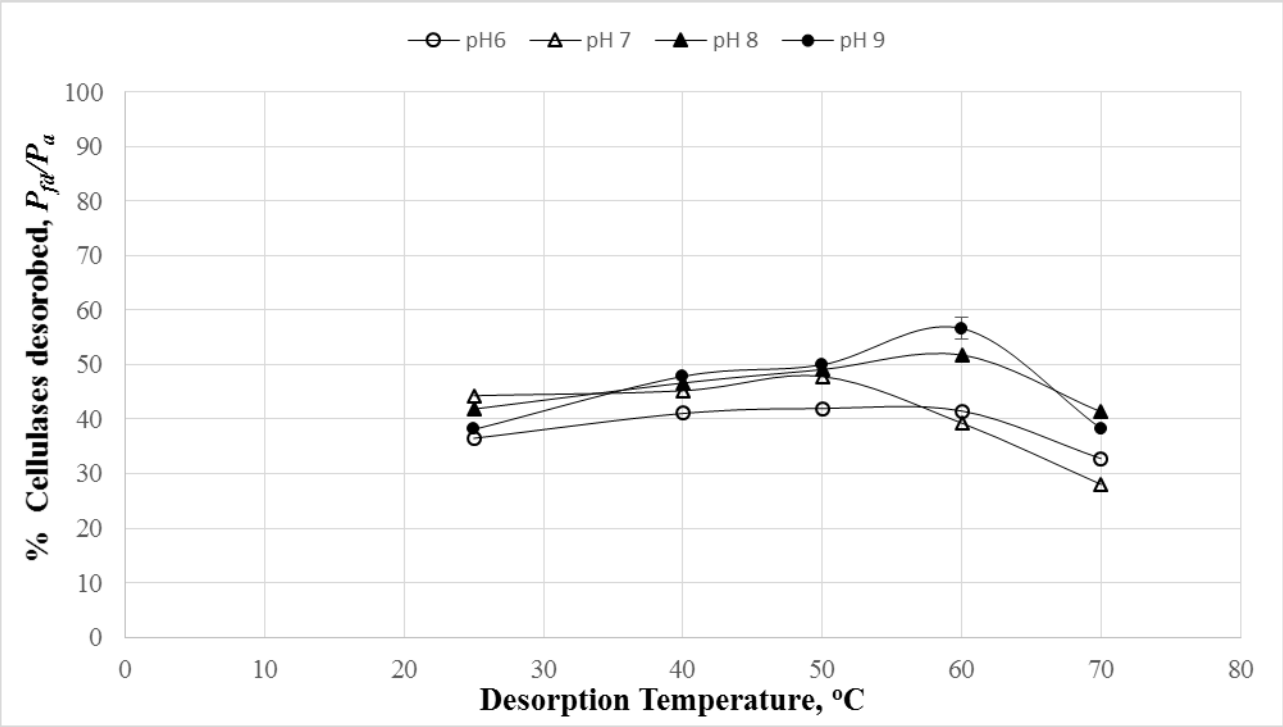


Figure 34 : Desorption of cellulases from Protobind with varying temperature from (25 ºC to 70 ºC), pH (from pH 6 to pH 9), 100 rpm and maximum error bar at 95 % probability
[Baig *et al.*, 2013]

For pH 9, the maximum desorption (55 %) was achieved at 60 ºC. Desorption at pH 9 and 50 ºC was around 50 %. Desorption increased from 42 % to 55 % with increase in temperature from 25 ºC to 60 ºC at pH 9. Beyond 60 ºC, a fall in activity was observed. At 70 ºC there was almost 15 % decrease in desorption than of 60 ºC. A pH 8 has shown similar % cellulases desorbed at 50 ºC and 60 ºC as that of pH 9. Results of Protobind also supported that pH 8 and pH 9 with 50 ºC and 60 ºC are good

desorption conditions. The % of cellulases desorbed from Protobind were less than % of cellulases desorbed from Avicel. The presence of multiple functional groups on the surface of lignin make the strong interactions between lignin and cellulases and hence make it difficult for cellulases to desorb. Wang *et al*. (2012) prepared artificial lignocellulosic substrates with lignin concentration of 29.6 % and 29.1 % of lignin by mass. These percentages of lignin were closer to the concentration of lignin in wheat straw (i.e. 23 %). Figure 35 showed activity of the cellulases desorbed plotted along y-axis with varying desorption temperatures plotted along x-axis. All the data points were measured separately and were average of 5 replicates.

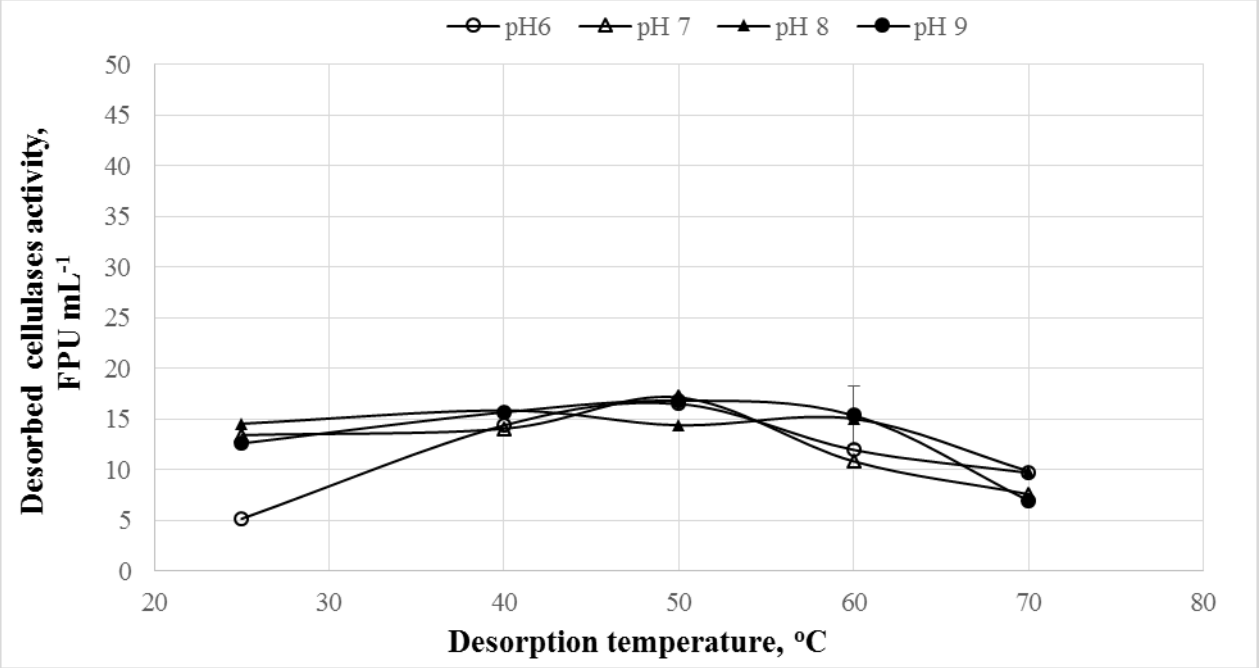


Figure 35 : Activity of desorbed cellulases from Protobind 1000 with varying temperature (from 25 ºC to 70 ºC), pH (from pH 6 to pH 9), 100 rpm and maximum error bar at 95% probability

Contrary to results with Avicel, activity of cellulases after desorption from Protobind reached to its maximum at temperature 50 ºC and pH 9 around 17 FPU. The activity measured at pH 9 and 60 ºC was around 17 FPU (close to 50 ºC). At 70 ºC the activity of the desorbed cellulases decreased to 65

% of their value at 60 ^{o}C for PH 8 and 45 % to their value at pH 9 (almost to its half than its value at 60 ^{o}C). The activities at pH 8 and pH 9 were almost the same at 40 ^{o}C to 60 ^{o}C. The thermo-stability of enzymes was studied by Rodrigues *et al*. (2012) at temperatures 30, 37, 40, 45 and 50 ^{o}C for one artificially prepared substrate, 4-methylumbelliferyl-β-D-cellobioside (not a lignin). They found that at 45 ^{o}C the desorbed cellulases was 89.7 % active while at 50 ^{o}C there was a sharp decline in activity i.e. only 37.5 % active and at pH 10 the structure was partially lost. These results were almost the same as ours. For the substrates containing cellulose and lignin pH 8 and pH 9 with temperatures 50 ^{o}C and 60 ^{o}C are suitable for desorption of cellulases. Through literature survey we hypothesized that alkaline pH and elevated temperature can desorb active cellulases in significant amount. The change in temperature dictate the change in enthalpy and entropy of the system.

5.6 Enthalpic and entropic changes for desorption under the influence of temperature

The temperature dependency of desorption, however is reported and discussed here. The results could indicate feasibility of the desorption reactions from the obtained values of the thermodynamic parameters. It also give insight whether desorption is enthalpy driven or entropy driven. The equilibrium adsorption results for both solid substrates (Avicel PH 101 and Protobind 1000) were plotted as a van't Hoff relation (Equation 2.10) for linearity of adsorption over the temperature range of 298 K to 343 K (Figure 30).

5.6.1 Desorption from Avicel PH 101 and from Protobind 1000

Van't Hoff plots were constructed for the desorption of cellulases from Avicel over a temperature range of 298 K to 343 K and a pH range of 6 to 9 (Figure 36) for desorption time 20 minutes (data not shown). The curves were drawn up to $T \leq 333$ K because there appears to be denaturing of

cellulases occurring after 333 K [Dehghanifard *et al*., 2013; Tavares *et al*., 2013]. The denaturing of cellulases was similar to what happened for adsorption but was much more pronounced.

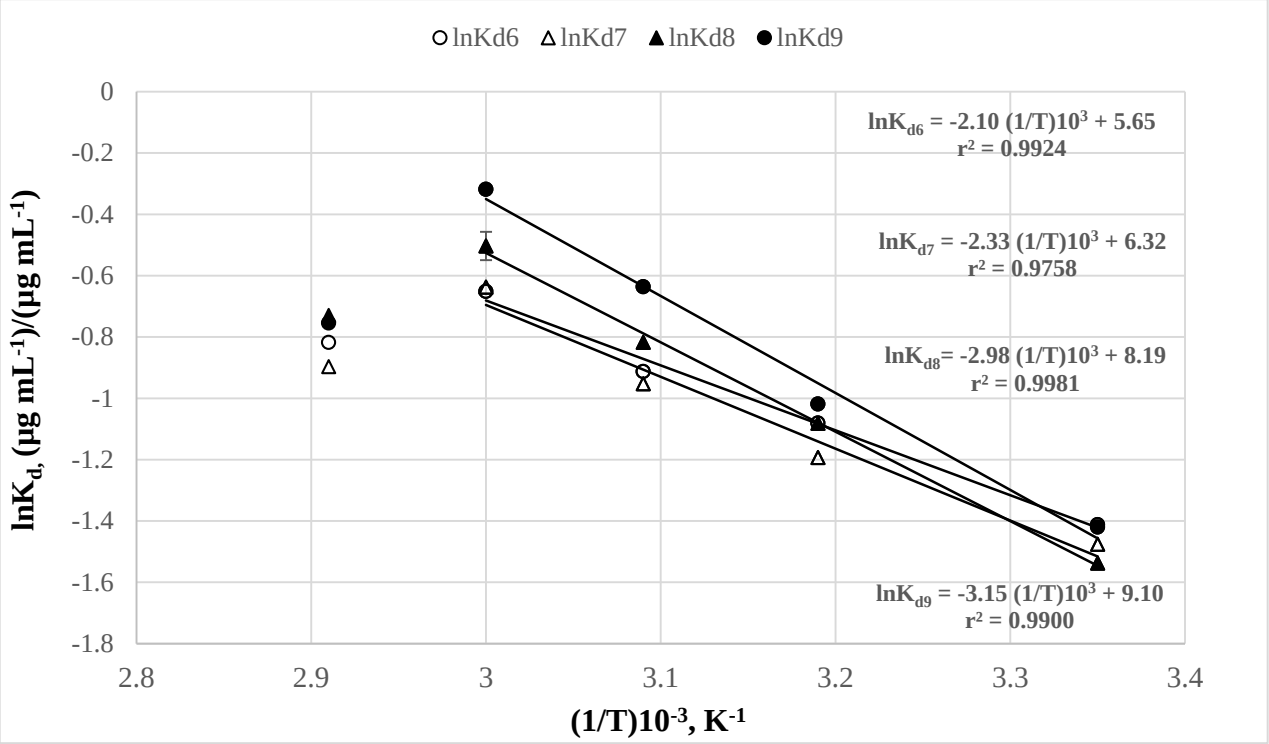


Figure 36: Desorption of cellulases NS 50013 from Avicel PH101 at varying pH (from pH 6 to pH 9), temperature (from 298 K to 343 K), 100 rpm and maximum error bar at 95 % probability

Desorption regression equations are given below:

$$lnK_{d6} = (-2.10)\frac{1}{T}10^3 + 5.65 \quad \ldots \quad 5.3$$

$$lnK_{d7} = (-2.33)\frac{1}{T}\ 10^3 + 6.32 \quad \ldots \quad 5.4$$

$$lnK_{d8} = (-2.98)\frac{1}{T}10^3 + 8.19 \quad \ldots \quad 5.5$$

$$lnK_{d9} = (-3.15)\frac{1}{T}10^3 + 9.10 \quad \ldots \quad 5.6$$

Since all slopes of the van't Hoff equations were negative, all ΔH_d values were positive. There was a slight increasing trend in ΔH_d from pH 6 to pH 9: 17.5, 19.4, 24.1, 26.2 $kJmol^{-1}$, respectively. This meant that heat energy was gained by the cellulases desorption system, which resulted in the decrease in van der Waals interactions and hydrogen bonding between cellulases and cellulose. The ΔH_d values obtained from the last two point 333 K to 343 K for all pH values were negative as -15.8, -24.6, -21.5, and -41.5 $kJmol^{-1}$ respectively. The negative signs of enthalpy usually mean that the heat is being released by the occurring reaction. The changes in entropy, ΔS_d, for all pH values were (46 to 75 $Jmol^{-1}K^{-1}$) positive. [Entropy of adsorption was ΔS_a was -50] This indicated decreased randomness which favored desorption. At 333 K to 343 K the ΔS_d was -52, -79, -68, and -127 $Jmol^{-1}K^{-1}$ for pH 6 to pH 9 which indicated further decreased desorption. The values of ΔG_d obtained from Equation 2.11 are given in Table 11.

Table 11: ΔG_d obtained for desorption of cellulases from Avicel PH 101 for pH 6 to pH 9 at temperatures 298K to 343K

Temperature K	ΔG_d			
	pH 6 $Jmol^{-1}K^{-1}$	pH 7 $Jmol^{-1}K^{-1}$	pH 8 $Jmol^{-1}K^{-1}$	pH 9 $Jmol^{-1}K^{-1}$
298	3.54×10^3	3.77×10^3	3.80×10^3	3.60×10^3
313	2.84×10^3	2.98×10^3	2.80×10^3	2.48×10^3
323	2.37×10^3	2.12×10^3	2.10×10^3	1.72×10^3
333	1.90×10^3	1.94×10^3	1.45×10^3	0.970×10^3
343	2.31×10^3	2.52×10^3	2.05×10^3	2.10×10^3

ΔG_d values were all positive and decreased with increase in temperature. Gibbs free energy (ΔG_d) decreased with the increase in temperature which means at pH 9 and 333 K the cellulases showed minimum affinity (adsorption interest) for the substrate. The maximum desorption of cellulases was

achieved at almost 333 K for all pH values. Theoretically, the maximum desorption achieved should be at 343 K since high temperature support desorption. The decrease in desorption while moving from 333 K to 343 K can be attributed to the configurational changes of cellulases which render the cellulases to desorb. The ΔG_d values calculated for temperature 343 K were large and positive as compare to the values at 333 K represented a less desorption the temperature (last row of Table 12). The positive and large ΔH_d, small ΔS_d value, with positive ΔG_d values indicated that desorption process was favorable for pH ranging from 6 to 9 and the temperature ranging from 298 K to 333 K only. Beyond $T \geq 333$ K, ΔH_d was negative ΔS_d was negative and ΔG_d was very large which resulted in decrease in desorption due to denaturing of cellulases.

Desorption of cellulases NS 500013 from Protobind, in similar conditions as that for Avicel is shown in Figure 36. The maximum error bar of all individualized experiments is at pH 8 and 313 K (3.19×10^{-3} K^{-1}). Again data for $T \geq 333$ K showed incompatibility with rest of the data because of very low $\ln K_d$ values observed (low desorption from the Protobind surface). The $\ln K_d$ increased with an increase in temperature from 298 K to 333 K and the corresponding regression equations are given below:

$$lnK_{d6} = (-1.17)\frac{1}{T}10^3 + 2.60 \quad \ldots \quad 5.7$$

$$lnK_{d7} = (-0.610)\frac{1}{T}10^3 + 1.00 \quad \ldots \quad 5.8$$

$$lnK_{d8} = (-0.830)\frac{1}{T}10^3 + 1.80 \quad \ldots \quad 5.9$$

$$lnK_{d9} = (-0.920)\frac{1}{T}10^3 + 2.20 \quad \ldots \quad 5.10$$

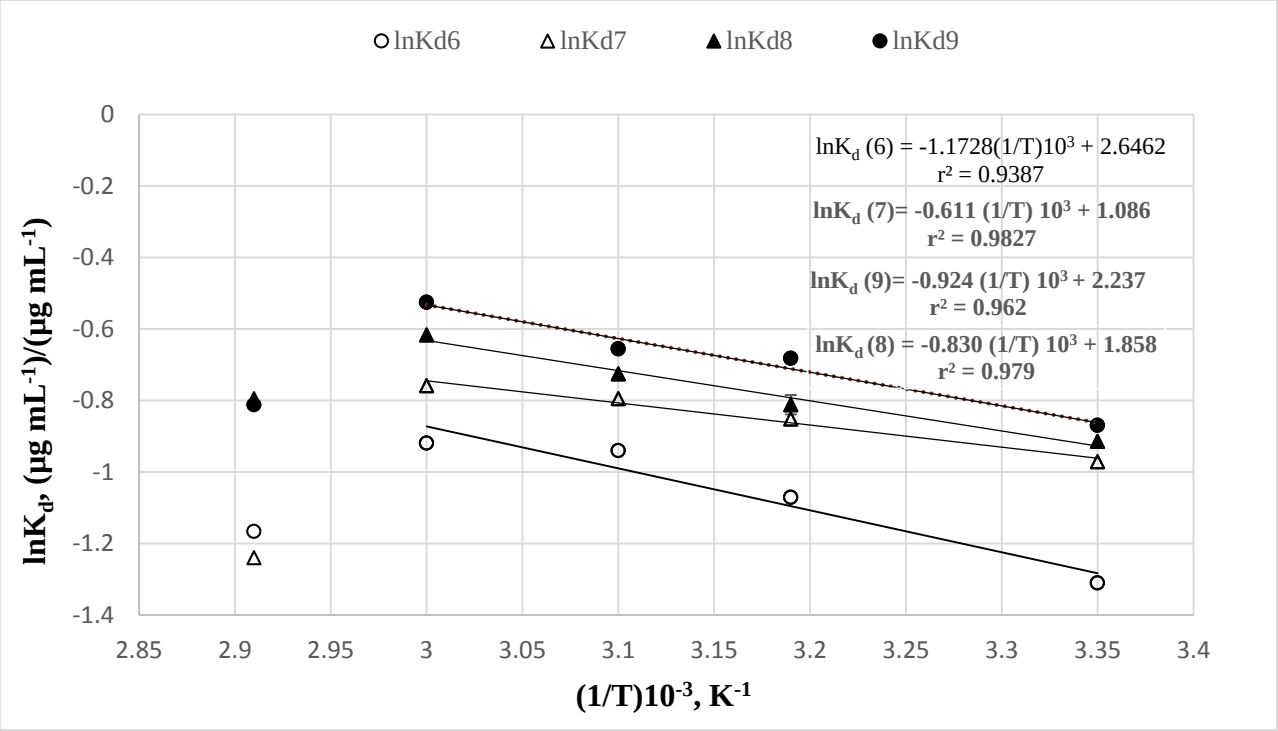


Figure 37: Desorption of cellulases NS 50013 from Protobind 1000 at varying pH (from pH 6 to pH 9), temperature (from 298 K to 343 K), 100 rpm and maximum error bar at 95% probability.

As with Avicel, all slopes for desorption from Protobind had negative values, although their values for Protobind were in the range of 0.610 K to 1.160 K, 2-4 times less than Avicel at the respective pH values, leading to positive ΔH_d values of 9.60, 5.10, 6.90 and 7.70 kJmol^{-1} respectively. The positive ΔH_d values indicated that the supplied heat energy was consumed in weakening the interactions between cellulases and Protobind. The large negative ΔH values were observed between 333 K to 343 K and was attributed to a conformational change in enzymes. The small positive ΔS_d values of 21.70, 9.00, 15.40, and 18.60 Jmol^{-1}K^{-1} for all desorption from Protobind signify that all reactions are not entropy driven rather enthalpy-driven. This small ΔS_d in case of desorption could be the result of an indirect decrease in entropy due to the influence of water molecules in the vicinity of non-polar cellulases residues which are broken down at higher temperatures [Rezus and Bakker, 2007; Mukhergee and Gupta, 2015]. The decrease in the entropy of desorption may be due to the decrease in the conformational flexibility of cellulases [Privalov, and Gil, 1988]. The small ΔS_d

implying that the cellulases-substrate complex has restricted flexibility (highly ordered), therefore less desorption. For a process that involves a decrease in entropy and a small change in enthalpy, a positive free energy change, ΔG, means that will not occur spontaneously [Rout *et al*., 2003]. ΔG_d values calculated for pH 6, 7, 8 and 9 over the range of temperature 298 K to 333 K are presented in Table 12. They remained positive and almost the same throughout the range of temperature and pH.

Table 12: ΔG_d obtained for desorption of cellulases from Protobind 1000 for pH 6 to pH 9 at temperatures 298 K to 343 K

Temperature K	ΔG_d			
	pH 6 $Jmol^{-1}K^{-1}$	pH 7 $Jmol^{-1}K^{-1}$	pH 8 $Jmol^{-1}K^{-1}$	pH 9 $Jmol^{-1}K^{-1}$
298	3.18×10^3	2.38×10^3	2.30×10^3	2.14×10^3
313	2.86×10^3	2.25×10^3	2.08×10^3	1.86×10^3
323	2.64×10^3	2.16×10^3	1.93×10^3	1.67×10^3
333	2.40×10^3	2.07×10^3	1.78×10^3	1.48×10^3
343	3.29×10^3	3.47×10^3	2.24×10^3	2.28×10^3

ΔG_d values decreased from 2.40, 2.07, 1.78 and 1.48 $kJmol^{-1}K^{-1}$ as pH increased from pH 6, 7, 8, and 9 at 333 K and it also decreased with increase in temperature up to 333 K. Minimum energy is required to desorb at pH 9 and 333 K. ΔG_d was increased for all pH at 343 K. The ΔG_d values calculated for temperature 343 K were very large and positive as compare to the values at 333 K represented a less desorption the temperature (last row of Table 12). The maximum energy to desorb cellulases from Protobind was obtained at 343 K for all pH values as given in the last row of Table 12. Desorption from the cellulases-substrate complex was restricted due to unfolding of cellulases. The positive ΔH_d for both Avicel and Protobind meant that heat energy was gained by the system by

increasing temperature from 298 K to 333 K favored desorption. The positive ΔS_d for both Avicel and Protobind indicated that the increased randomness also favored desorption for 298 K to 333 K. The minimum ΔG_d obtained at pH 9 and 333 K indicated that cellulases have less affinity for substrate at this point in another words cellulases are maximum on desorption. Since wheat straw is a combination of cellulose and lignin, the thermodynamic study offers a strategy for using wheat starw for ethanol production. For desorption of cellulases from wheat straw, the positive ΔH_d, ΔS_d and ΔG_d indicated favored desorption at pH 9 temperatures 323 K and 333 K.

5.6.2 Effect of pore diameter of substrates on desorption of cellulases

Average pore diameter in the mesopore and macropore ranges using Barrett-Joyner-Halenda analysis (BJH) was measured. The model was based on the assumption that pores had a cylindrical shape and that pore radius was equal to the sum of the radius and the thickness of the film adsorbed on the pore wall [Barrett, 1951; Kalliopi, 2005]. The pore diameter measured by a single set of experiment (average diameter from mesopores and macropores) for Avicel PH 101 was 47.93 nm and for Protobind 1000 was 28.89 nm. The reported diameter size of cellulases was 6 nm (60 Å) [Grassick *et al.*, 2004]. Stone *et al*. (1969) suggested to achieve maximum adsorption, the pores in substrates should be 5–9 times larger than the size of the enzymes [Stone *et al.*, 1969]. Since the pore diameter of Protobind 1000 was smaller than the 5 times of the size of cellulases (*i.e.*, 30 nm). The cellulases enter into the pores, get adsorbed and then, they get trapped. The cellulases those are entrapped are unable to function, cannot get desorbed. Therefore, one of the reasons that desorption from Protobind is less than that of Avicel is entrapment of cellulases into the pores.

5.7 Enhancement of desorption of cellulases

Temperature facilitates in desorption but at 70 ^{o}C and beyond temperature participated in deactivation of cellulases which was resulted in less desorption. Through desorption studies, it was concluded that maximum concentration of the cellulases were desorbed at pH 60 ^{o}C from both Avicel and Protobind. The maximum activities of desorbed cellulases from Avicel were obtained at 60 ^{o}C and Protobind have almost the same activities at 40, 50 and 60 ^{o}C. It was also observed that desorbed cellulases behaved similar at pH 8 and pH 9. Therefore, pH range from 8 to 9 and temperature 40 ^{o}C to 60 ^{o}C were selected for enhancement studies. Some chemicals (Glycerol, Ethylene Glycol) and β-glucosidase were used in this enhancement studies.

5.7.1 Glycerol

The consequence of adding glycerol on desorption of cellulases from Avicel PH 101 was studied at 40, 50, 60 ^{o}C, and pH 8 and pH 9.

Avicel PH 101

In Figure 38, concentration of cellulases desorbed under various conditions of temperature and pH was plotted. All the data points were measured separately and were average of 5 replicates.

It seems from the plots that the maximum was achieved at 50 ^{o}C to 60 ^{o}C. Maximum cellulases desorbed was at pH 9 at 60 ^{o}C with 5 % glycerol was around 96 %. The next closest values for same conditions with 10 % glycerol were around 91 % while the maximum desorbed at pH 9 and 60 ^{o}C without glycerol was around 75 %. Therefore, there was a 16-26 % enhanced desorption of cellulases from Avicel using glycerol. It seems from the plots that the maximum was achieved at 50 ^{o}C to 60 ^{o}C. Maximum cellulases desorbed was at pH 9 at 60 ^{o}C with 5 % glycerol was around 96 %.

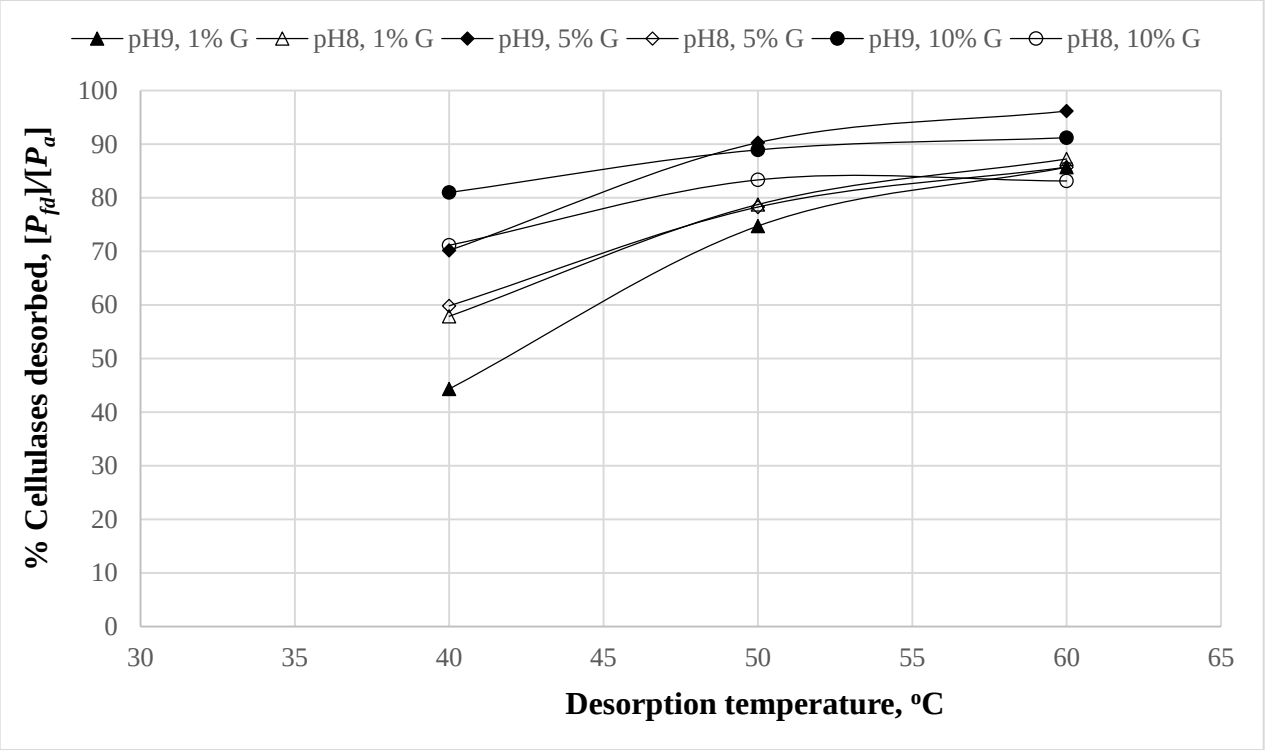


Figure 38 : Desorption of cellulases from Avicel by using glycerol (1 %, 5 % and 10 %), pH 8 and pH 9, temperature (40 ºC, 50 ºC and 60 ºC), 100 rpm and maximum error shown at 95 % probability

The next closest values for same conditions with 10 % glycerol were around 91 % while the maximum desorbed at pH 9 and 60 ºC without glycerol was around 75 %. Therefore, there was a 16-26 % enhanced desorption of cellulases from Avicel using glycerol.

Activity of the cellulases desorbed are given in Figure 39. All data points were measured individually and were average of 5 replicates. The maximum activity was achieved was around 47 FPU mL^{-1} at pH 9 with 5 % and 10 % glycerol 60 ºC which is around 91 % of the activity of the adsorbed cellulases. Otter *et al*. (1988) observed that glycerol was an effective desorbent to desorb cellulases. The maximum desorption (68 %) of cellulases from Avicel was achieved at pH 10 with 10 % glycerol while 57 % desorption was achieved at pH 5 and 60 % glycerol [Otter *et al*., 1988].

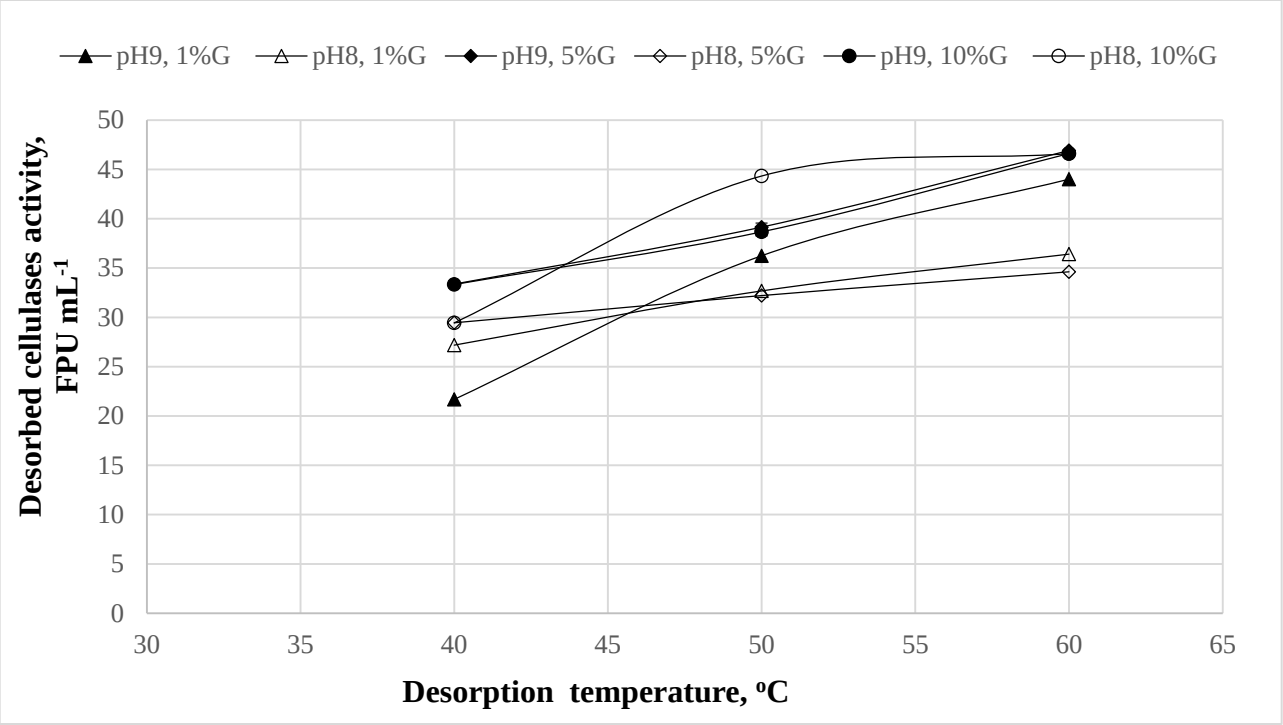


Figure 39 : Activity of cellulases desorbed from Avicel PH 101 by using glycerol (G 1 %, 5 % and 10 %), pH 8 and pH 9, temperature (40 ºC, 50 ºC and 60 ºC), 100 rpm and maximum error shown at 95 % probability

Activities at pH 10 and pH 5 were not reported but it was stated that at pH 10.5 all enzymes lost activity. Under vigorous industrial operational conditions it would be difficult to maintained the fine line (ΔpH = 0.5 pH) of pH control, therefore, our suggested technique is giving a safe pH to work with. This study also obtained more desorption than ever reported in literature, almost no loss of activity.

Protobind 1000

Figure 40 and Figure 41 shows desorption of cellulases from Protobind in the presence and varying concentrations of glycerol (1 %, 5 %, and 10 %) and their activity, respectively. The maximum cellulases desorbed were around 66 %. Considering error bars it seems that at temperature 50 ºC to 60 ºC for both pH 8 and pH 9 almost similar '% desorbed' of cellulases for all concentrations of glycerol.

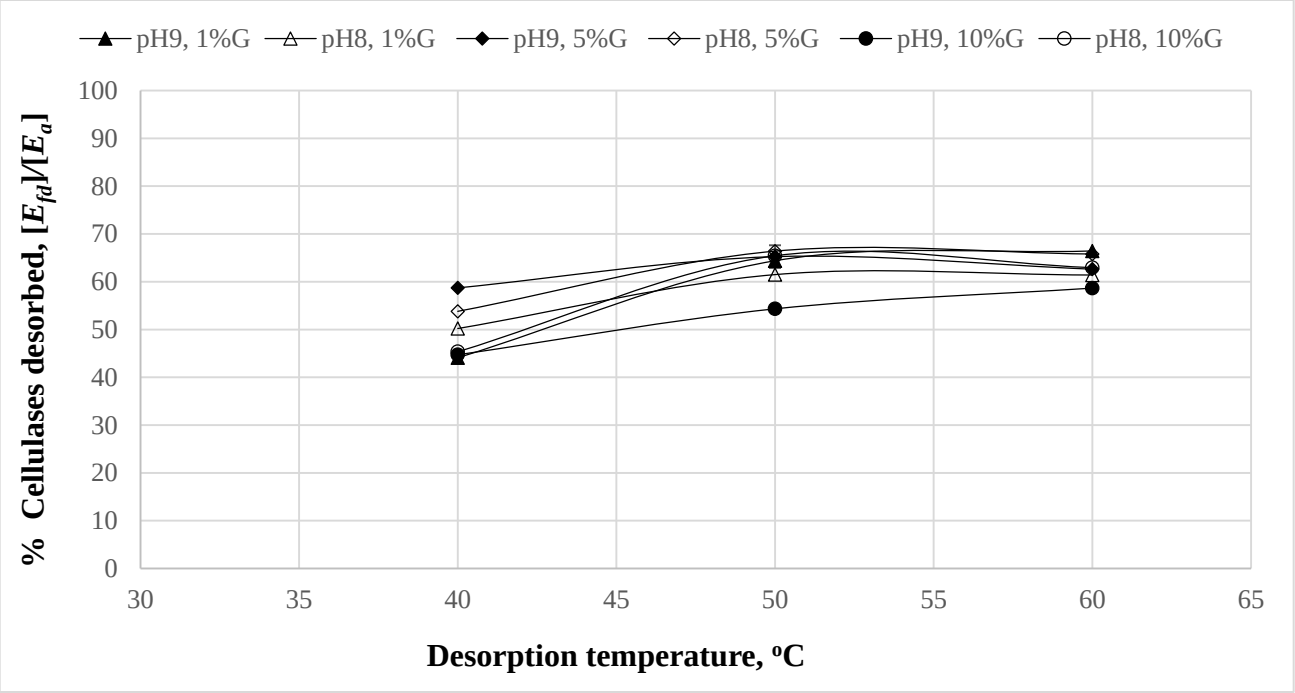


Figure 40 : Desorption of cellulases from Protobind 1000 by using glycerol (G 1 %, 5 % and 10 %), pH 8 and pH 9, temperature (40 ºC, 50 ºC and 60 ºC), 100 rpm and maximum error shown at 95 % probability

Maximum % desorption from Protobind without glycerol achieved in our studies was around 55 %, therefore a 10 % more desorption was achieved with glycerol. Lu *et al*. (2002) reported that desorption of cellulases from lignin containing substrates were difficult. They could remove 30 % and 65 % of cellulases from steam exploded Douglas fir (lignin 46.1 %) hot alkali peroxide-treated Douglas fir (8.2 % lignin) substrate. It is to be noted that the more lignin content (46.1 %) in a substrate the lesser was desorption (30 %) while Protobind 1000 has 100 % lignin and with our techniques 65 % lignin was desorbed. The maximum activity of % desorbed cellulases achieved at 50 ºC was 37 FPU mL^{-1} and 33 FPU mL^{-1} at pH 8 (1% glycerol) and pH 9 (1% glycerol) respectively. At 60 ºC activity values was 33 FPU at pH 9, 5% glycerol which is almost the same as values reported for 50 ºC.

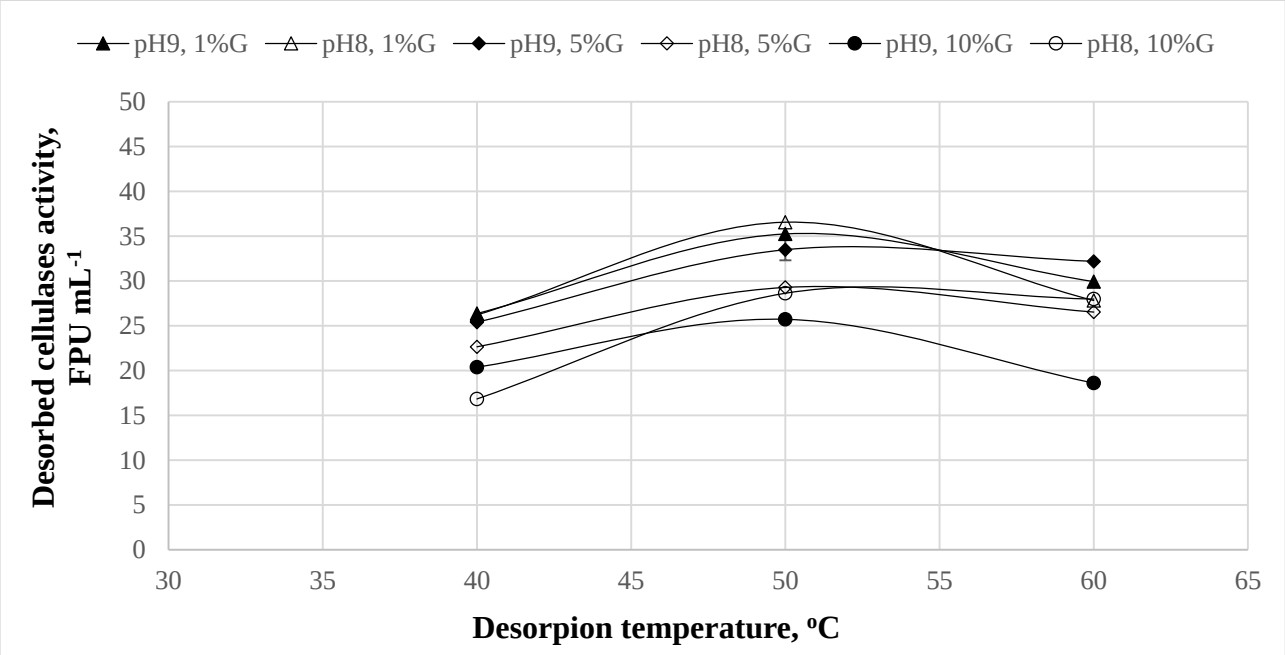


Figure 41 : Activity of desorbed of cellulases from Protobind 1000 by using glycerol (G 1 %, 5 % and 10 %), pH 8 and pH 9, temperature (40 ºC, 50 ºC and 60 ºC), 100 rpm and maximum error shown at 95 % probability

The maximum activity of desorbed cellulases achieved from Protobind without glycerol was around 17 FPU. It was also observed that 10 % glycerol affect desorbed activity. Tu *et al*. (2007) find that the activity in the desorbed cellulases from ethanol-pretreated mixed softwood substrate (EPMS, 6% lignin) could not exceed from 35 % even though they increased initial cellulases concentration from 25 % to 51 % (almost twice). The increased initial cellulases concentration to get better results were based on the assumption that there was product inhibition [Berlin *et al*., 2005] which did not worked. In our technique addition of 5 % glycerol increased activity of desorbed cellulases from 17 FPU mL^{-1} to 37 FPUmL^{-1} which is approximately double.

5.7.2 Ethylene Glycol

The impact of addition of ethylene glycol on desorption of cellulases from Avicel PH 101 and Protobind 1000 was studied. The concentration of glycerol used was 1 %, 5 %, and 10 %

Avicel PH 101

Figure 42 shows amount of cellulases desorbed from Avicel PH 101 in the presence of ethylene glycol. All the data points were average of 5 replicates and measured separately.

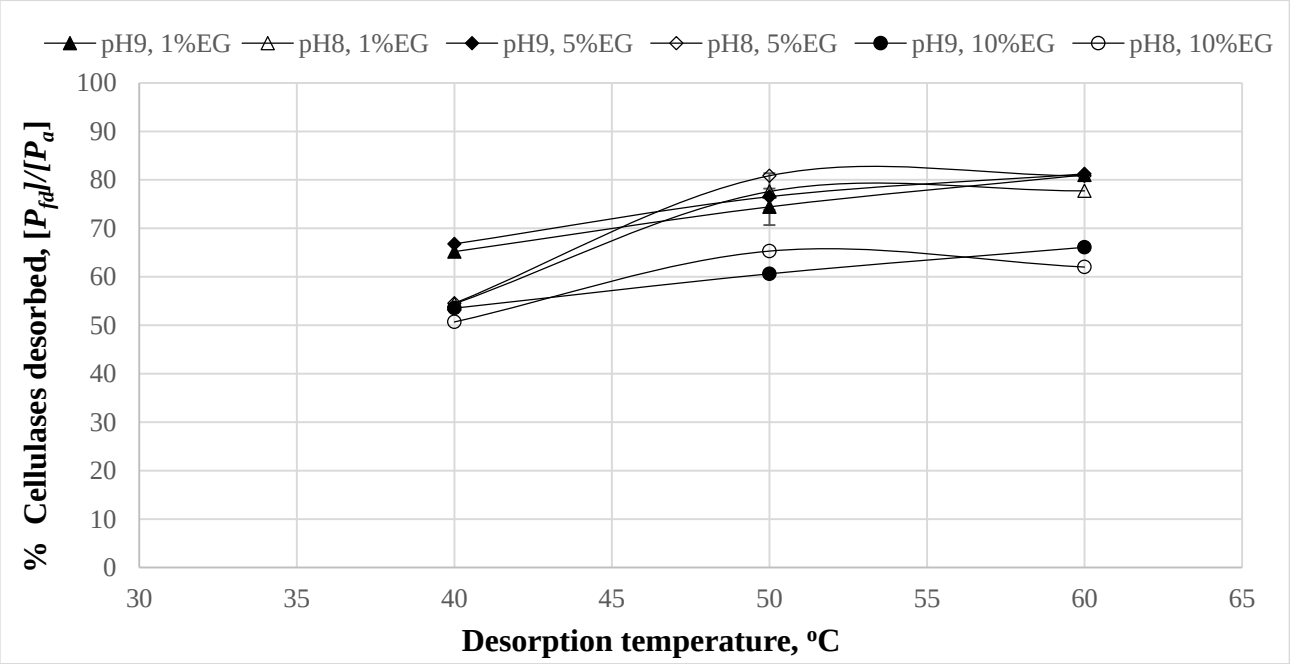


Figure 42: Desorption of cellulases from Avicel PH 101by using ethylene glycol (EG 1 %, 5 % and 10 %), pH 8 and pH 9, temperature (40 ºC, 50 ºC and 60 ºC), 100 rpm and maximum error shown at 95 % probability

Maximum % desorbed cellulases were achieved at 60 ºC for pH 9, 1% EG and 5 % EG and the increase in % desorbed cellulases was 35 % with no glycerol. Addition of 10% ethylene glycerol had a small increase of 15 % desorption. The maximum % desorbed from EG (80%) was 10 % less than maximum % desorbed from glycerol. Figure 43 shows activity of cellulases desorbed from Avicel PH. All the data points were average of 5 replicates and measured separately. At 60 ºC with pH 8, 1% EG and pH 9 5% EG have activity of 37 FPU mL^{-1} and 38 FPU mL^{-1} respectively. The activity values were which is 2-5% lower than the activity achieved without addition of EG. The minimum activity was achieved with pH 9 with 10% EG at 60 ºC which is 34 FPU mL^{-1} which was about 12

% less than the maximum activity achieved with the addition of glycerol. Therefore addition of EG did not produced any significant increase in activity for desorbed cellulases from Avicel.

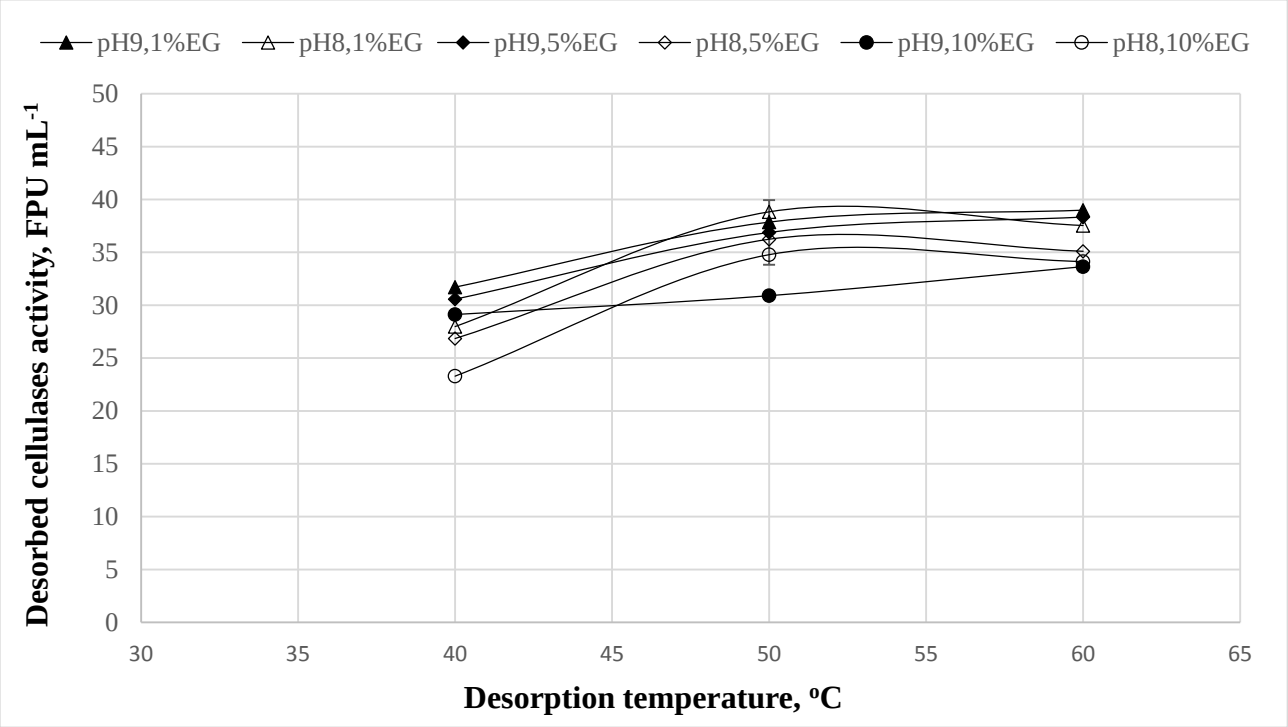


Figure 43 : Activity of desorbed cellulases from Avicel by using ethylene glycol (EG, 1 %, 5 % and 10 %), pH 8 and pH 9, temperature (40 ºC, 50 ºC and 60 ºC), 100 rpm and maximum error shown at 95 % probability

Protobind 1000

Figure 44 shows % desorbed from Protobind in the presence of ethylene glycol. All the data points were average of 5 replicates and measured by separately conducted experiments. The maximum of % desorbed was achieved at pH 8 and 9 at 50 ºC to 60 ºC with 1 % EG i.e. around 62 % of the cellulases adsorbed on Protobind. In comparison with the cellulases desorbed from Protobind without use of EG it was 14 % higher.

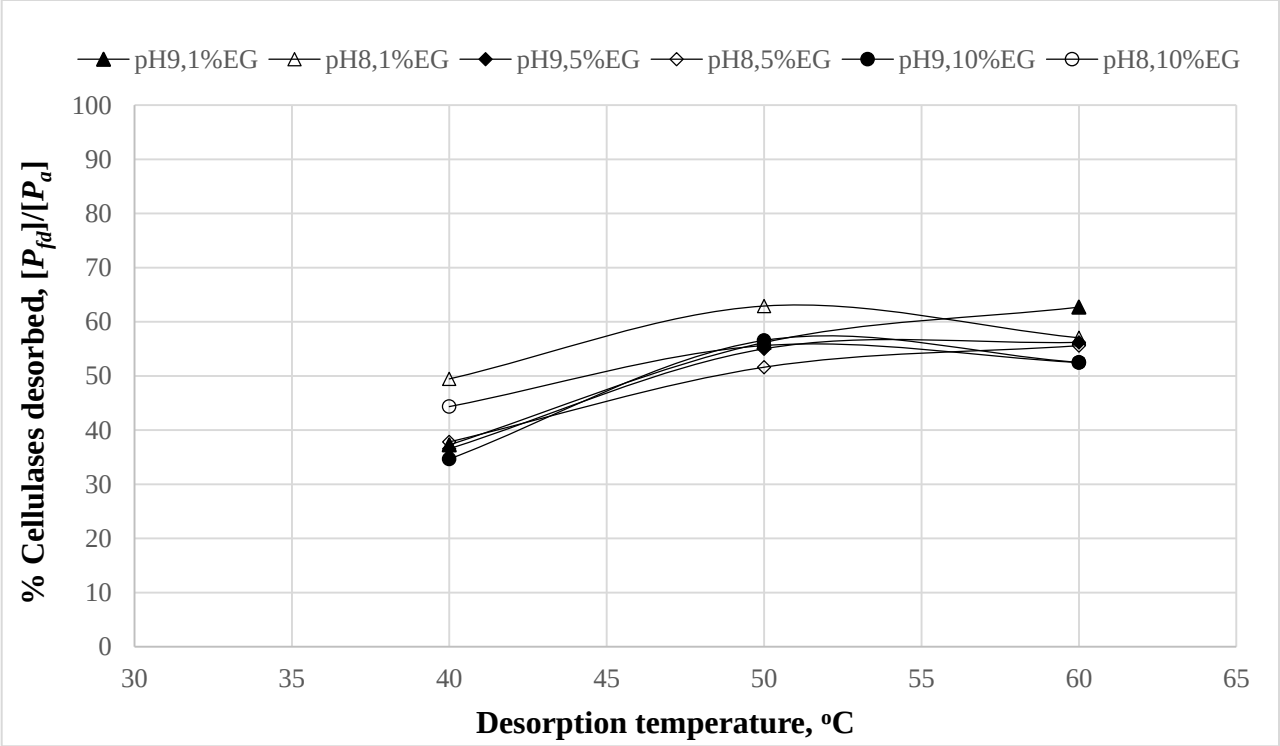


Figure 44 : Desorption of cellulases from Protobind 1000 by using ethylene glycol (EG 1 %, 5 % and 10 %), pH 8 and pH 9, temperature (40 °C, 50 °C and 60 °C), 100 rpm and maximum error shown at 95 % probability

Zhu *et al*. (2009) reported desorption of cellulases (Spezyme CP) from Avicel and dilute acid treated corn stover as 81 % and 74 % respectively, with using 72 % EG. The activity of the desorbed cellulases was not reported either. Since the aim of this research was to reuse active desorbed cellulase economically, the use of 72 % of EG was not feasible. In Figure 45 activity of the cellulases desorbed from Protobind is given. Maximum desorption was achieved at 50 °C than other temperatures with 5 % glycerol at pH 9 i.e., 24 FPU mL^{-1} which is 48 % of the activity of the cellulases adsorbed. The activity of the maximum desorbed was only 7 units higher than the activity archived without using EG.

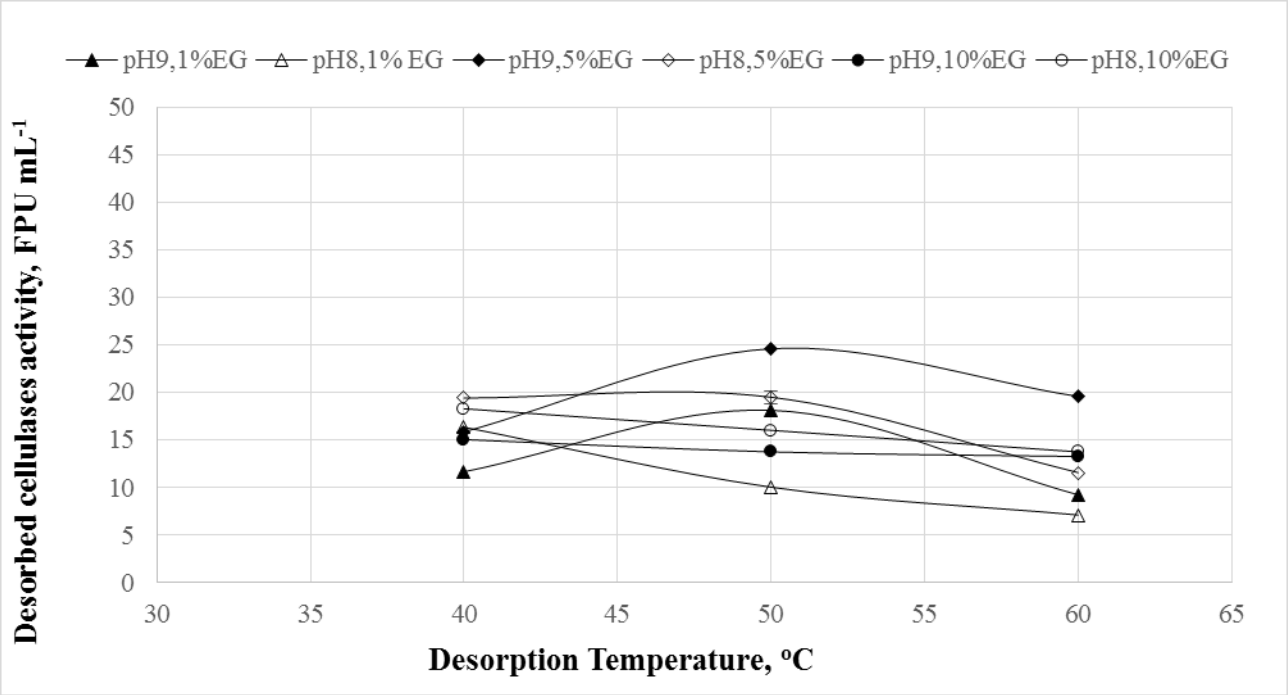


Figure 45: Activity of desorbed cellulases from Protobind 1000 by using ethylene glycol (EG 1 %, 5 % and 10 %), pH 8 and pH 9, temperature (40 ºC, 50 ºC and 60 ºC), 100 rpm and maximum error shown at 95 % probability

The activity of the desorbed with EG from pH 9 at 10 % EG and from pH 8 at 5 % and 10 % EG showed no improvement in the % desorption of cellulases from Protobind.

5.7.3 β-Glucosidase

Avicel PH 101

The amount of cellulases desorbed in the presence of 5 %, 10 % and 18 % β-glucosidase (BG), of the concentration of cellulases used, from Avicel PH 101 is shown Figure 46 and Figure 47 gave % of cellulases desorbed and the activity of the desorbed cellulases at temperature of 40 ºC, 50 ºC and 60 ºC, respectively. All the data points were average of 5 replicates and measured by separately conducted experiments.

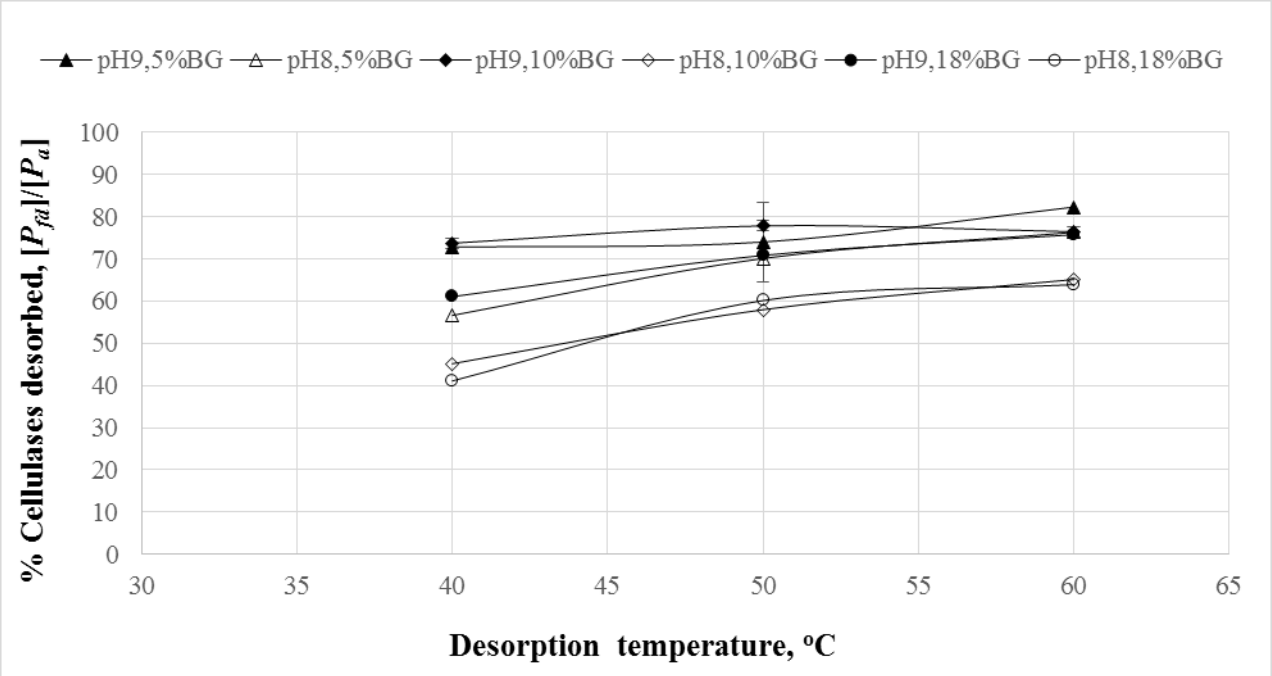


Figure 46 : Desorption of cellulases from Avicel by using β-glucosidases (BG 5 %, 10 % and 18 %), pH 8 and pH 9, temperature (40 ºC, 50 ºC and 60 ºC), 100 rpm and maximum error shown at 95 % probability

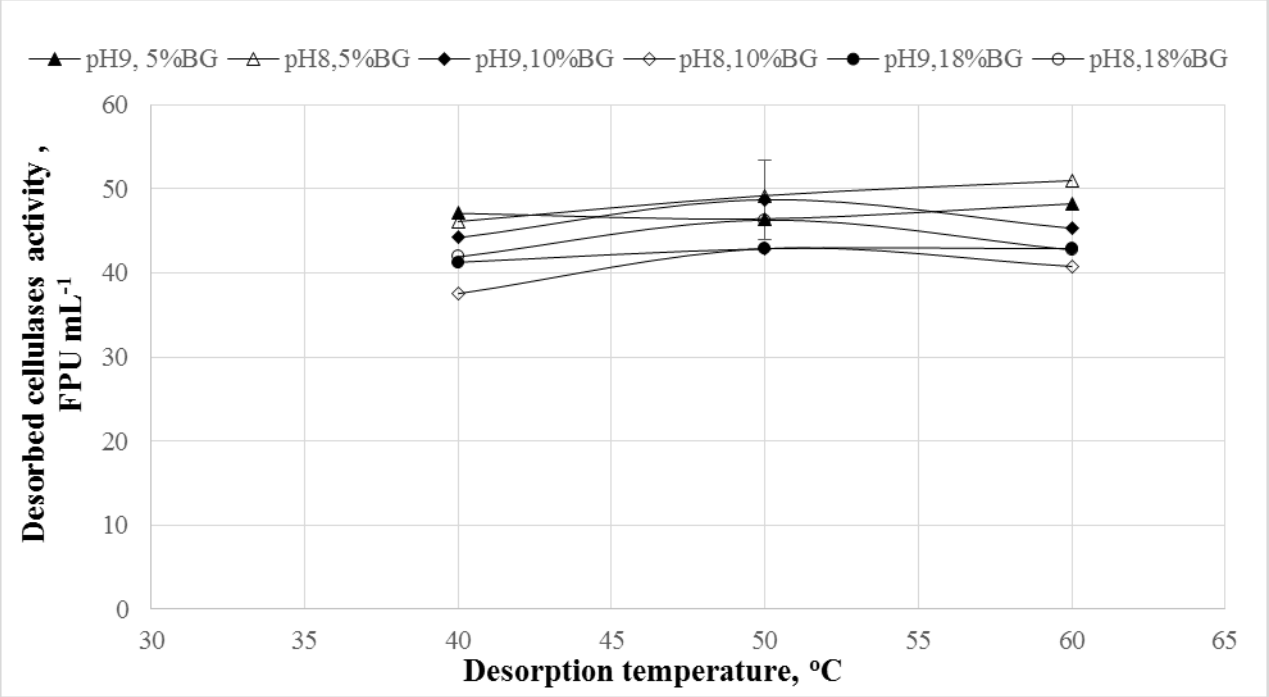


Figure 47: Activity of the desorbed cellulases from Avicel by using β-glucosidases (BG, 5 %, 10 % and 18 %), pH 8 and pH 9, temperature (40 ºC, 50 ºC and 60 ºC), 100 rpm and maximum error shown at 95 % probability

The maximum cellulases desorbed 82 % at pH 9, 60 ^{o}C and 5% β-glucosidases (BG). Desorption at pH 9, with 10 % and was almost the same for all temperature range from 40 ^{o}C to 60 ^{o}C (73 % to 77 %). The maximum desorption with BG 9 % higher than that obtained without β-glucosidase. Figure 42 gave activity of the desorbed cellulases from Avicel. All the data points were average of 5 replicates and measured by separately conducted experiments. The maximum activity obtained was 50 FPU mL^{-1} for 5 % of β-glucosidase used at 60 ^{o}C, pH 8 which is almost the same as at pH 9 (48 FPU mL^{-1}) under same conditions. The maximum activity obtained is almost the same as that obtained by using glycerol.

Protobind 1000

Figure 48 shows the % of cellulases desorbed from Protobind under the influence of BG. All the data points were average of 5 replicates and measured by separate experiments.

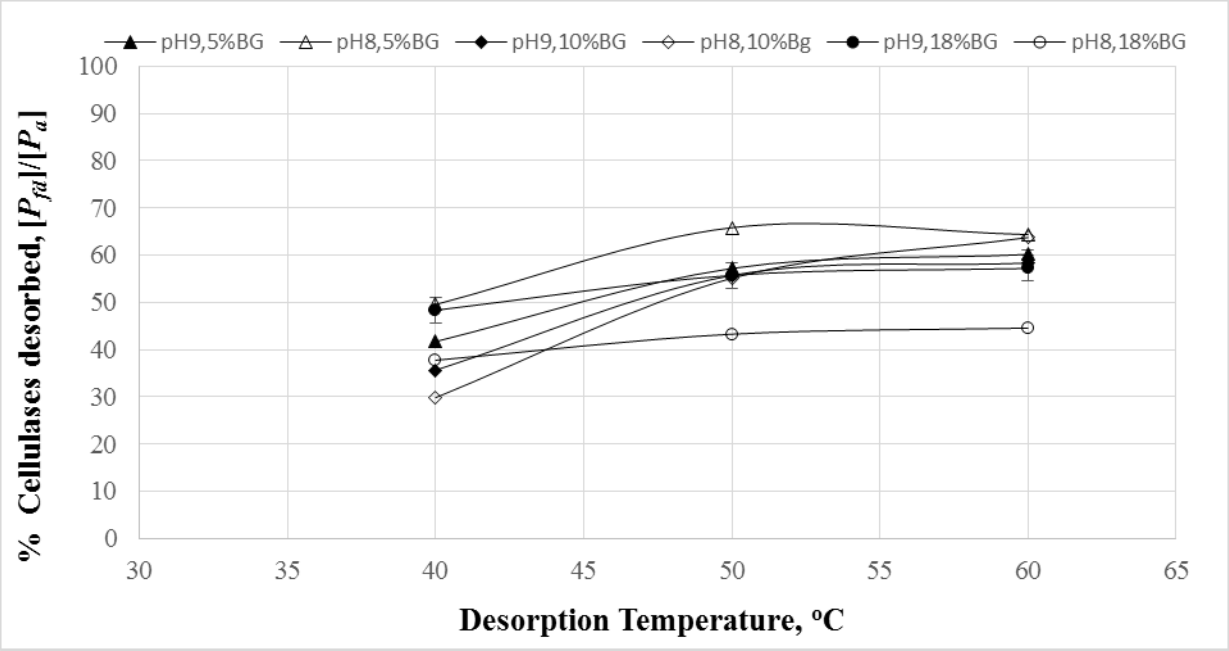


Figure 48 : Desorption of cellulases NS 50013 from Protobind 1000 by using β-glucosidases (BG, 5 %, 10 % and 18 %), pH 8 and pH 9, temperature (40 ^{o}C, 50 ^{o}C and 60 ^{o}C), and 100 rpm and maximum error shown at 95 % probability

The plots showed the trend that desorption increased from 40 ^{o}C to 50 ^{o}C and somehow it remained the same till 60 ^{o}C. The maximum cellulases desorbed 65 % for pH 8 and pH 9 for 5 % addition of BG.

Figure 49 shows the activity of cellulases desorbed from Protobind under the influence of BG. All the data points were average of 5 replicates and measured by separately. The maximum activity of desorbed cellulases achieved at pH 9, 5 % BG 50 ^{o}C was 29 FPU mL^{-1}. The activity of desorbed cellulases by pH 8 and pH 9 with 5 % BG in the temperature range from 40 ^{o}C to 60 ^{o}C appear to be similar.

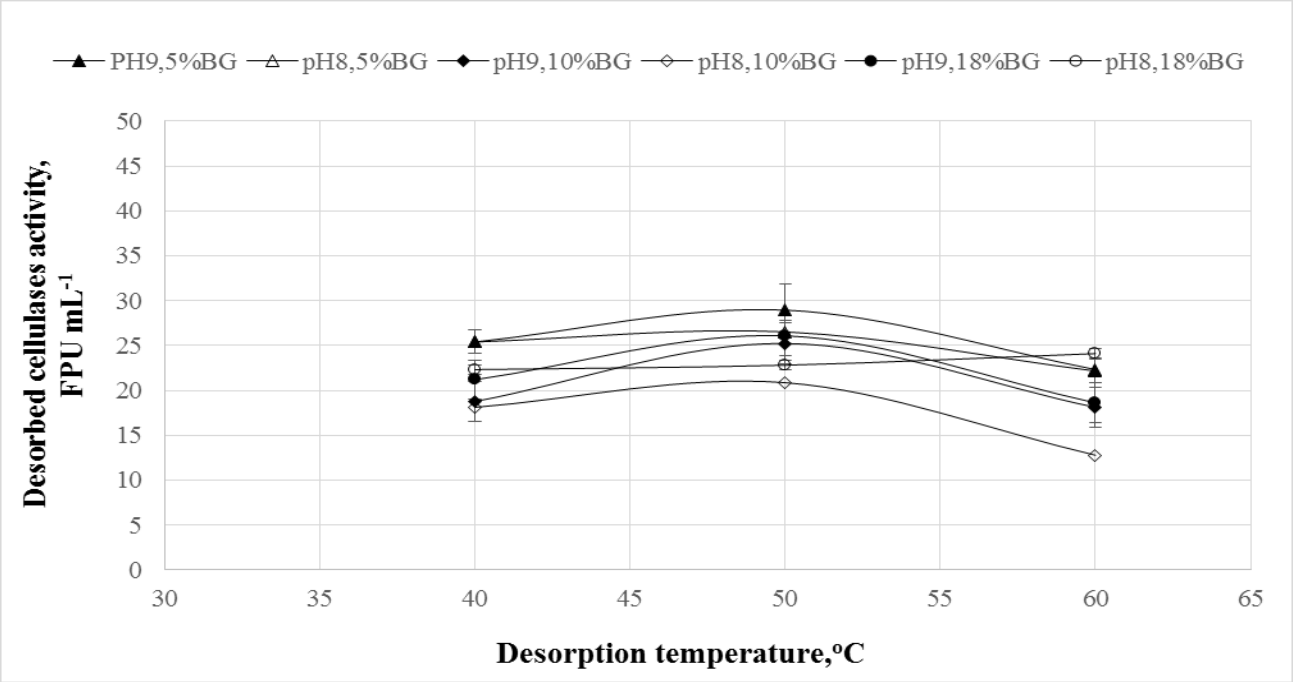


Figure 49 : Activity of the desorbed cellulases NS 500113 from Avicel PH 101 using β-glucosidases (BG, 5 %, 10 % and 18 %), pH 8 and pH 9, temperature (40 ^{o}C, 50 ^{o}C and 60 ^{o}C), 100 rpm and maximum error shown at 95 % probability

The maximum % desorbed from Avicel at pH 8 or pH 9 at temperature 60 ^{o}C by using 5 % glycerol was 85 % and 96 % with activity of 35 and 47 FPU mL^{-1}. The maximum % desorbed from Protobind at pH 8 or pH 9 at temperature 60 ^{o}C by using 5 % glycerol was 66 % and 63 % with activity of 32

FPU mL^{-1} and 26 FPU mL^{-1}. Using 5 % β-glucosidase showed 82 % desorption from Avicel and from Protobind it was 60 % at pH 9 almost the same at pH 8. The activity with 5 % β-glucosidase was 22 FPU mL^{-1} and 28 FPU mL^{-1} at 60 ^{o}C and 50 ^{o}C. Glycerol provided 6 % more cellulases desorbed from Protobind than that of β-glucosidase and 45 % more activity at 60 ^{o}C and 15 % more activity at 50 ^{o}C. Therefore, from the exhausted feed stock from bioethanol producing industry, desorption of cellulases should be achieved by using 5 % glycerol at pH 8 or pH 9 and temp 50 ^{o}C to 60 ^{o}C. Therefore, desorption of active cellulases can be enhanced by use of 5 % glycerol.

5.8 Application of novel desorption strategy on Lignocellulosic material

Adsorption of cellulases on wheat straw

Figure 50 shows the concentration of cellulases adsorbed [P_a] on wheat straw at pH 5, 25 ^oC temperature, 100 rpm is given along y-axis for varying adsorption time from 0 to 120 minutes. Each data point was measured individually. All the results were triplicates. In the start adsorption was rapid and increased with increasing time. Almost half of the cellulases adsorbed at equilibrium was adsorbed in first 5 minutes. Similar adsorption pattern is reported in literature [Steiner *et al*., 1988; Sing *et al*., 1991; Zeng *et al*., 2013]. The increase in adsorption time brought adsorption to an equilibrium at 40 minutes after that further increasing adsorption time the amount of cellulases adsorbed almost remained the same. The equilibrium time was important for desorption studies.

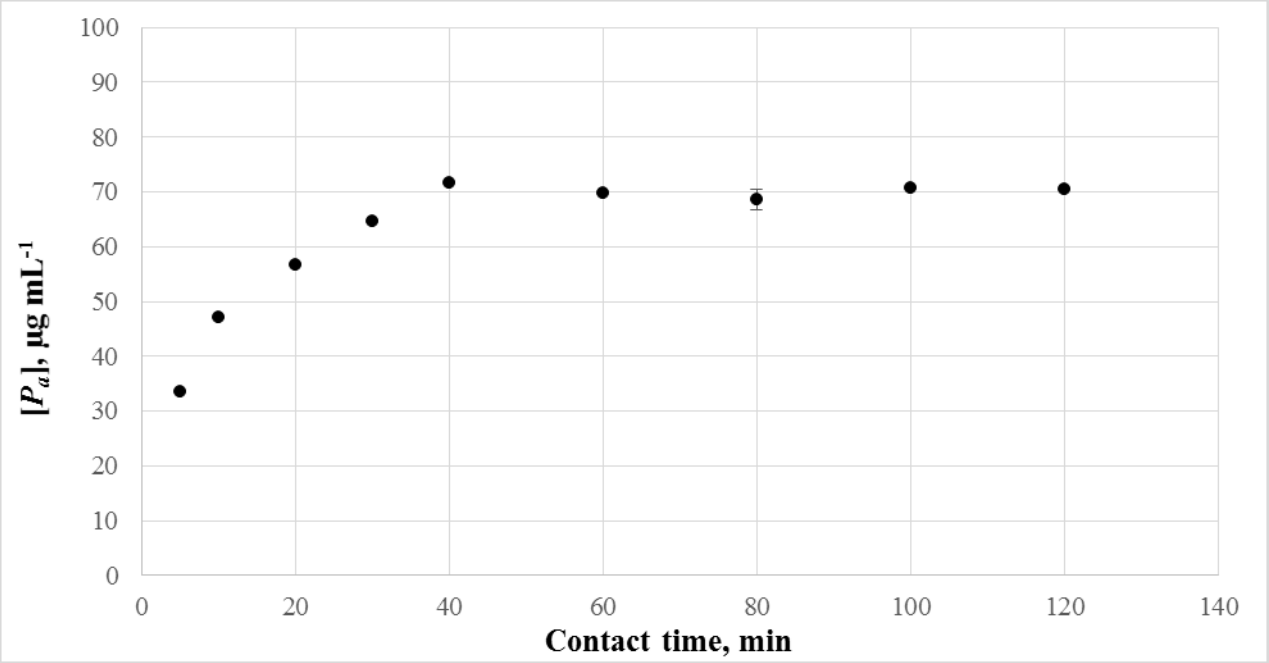


Figure 50 : Adsorption of cellulases NS 50013 onto wheat straw (0.5 mm) at pH 5, 25 ^oC, 100 rpm, E_0 = 250 µg mL^{-1} and maximum error shown at 95 % probability

Adsorption equilibrium time depends on substrate type, enzymes type and adsorption conditions. For example, Singh *et al.* (1991) has reported adsorption equilibrium time for Avicel as 30 minutes while in our studies it was 20 minutes for Avicel and 45 minutes for Protobind. Tu *et al*. (2009) reported

adsorption equilibrium time of cellulase onto enzyme pretreated lignin obtained from ethanol-pretreated Lodgepole pine (EPLP), but not on steam-exploded Lodgepole pine (SELP) as 3 hours. The equilibrium adsorption time on EPLP and SEPLP substrates was 30-60 minutes. Adsorption of pure cellulases components CBH I, EG I, EG II, and EG III on SolkaFloc was reported as 60 min [Kyriacos et al., 1988]. Li *et al*. (2011) showed that the equilibrium time for adsorption of Accelerase 1000 to lignocellulosic substrate was reported to be 45 min for delignified corncobs and 60 min for isolated lignin. Adsorption equilibrium time on raw biomass was not available in literature therefore it was not compared. But it was observed that the untreated lignocellulosic substrates even with high lignin content had slower adsorption than that onto cellulose. The equilibrium time of adsorption was used to set adsorption time for the desorption studies. Figure 51 shows results 7 pre-adsorbed samples and blanks a subjected to desorption at 25 ^{o}C at pH 5.

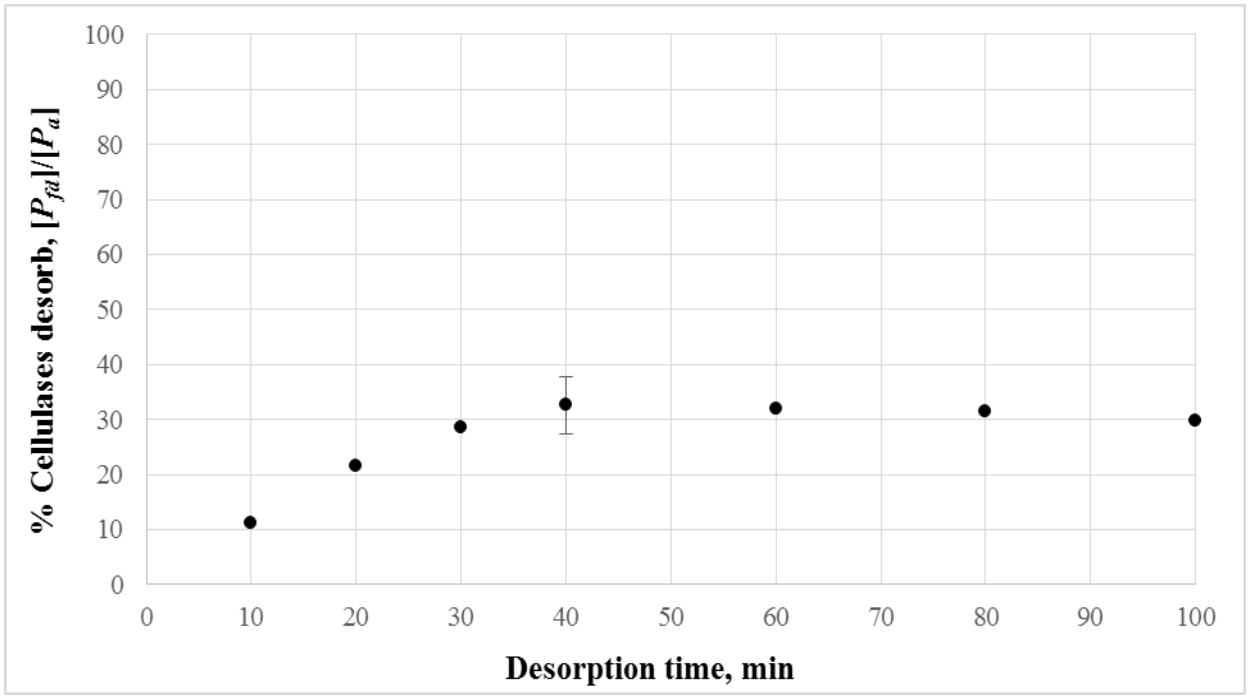


Figure 51 : Desorption of cellulases at pH 5, 25 ^{o}C, and 100 rpm, from wheat straw and maximum error shown at 95 % probability

Each data point is an average of triplicate measured individually. The cellulases desorption started maintaining its maximum desorption at 30 min. For further desorption studies 40 minutes were taken as desorption equilibrium time for cellulases from wheat straw samples.

After knowing adsorption equilibrium time and desorption equilibrium time, experiments were conducted at pH 9 and 60 ^{o}C to determine % desorbed cellulases. Figure 52 shows % cellulases desorption and activity (FPU mL^{-1}) of desorbed cellulase from wheat straw. The results from wheat straw were further compared with the same parameters from Avicel PH 101 and Protobind 1000 at the same conditions.

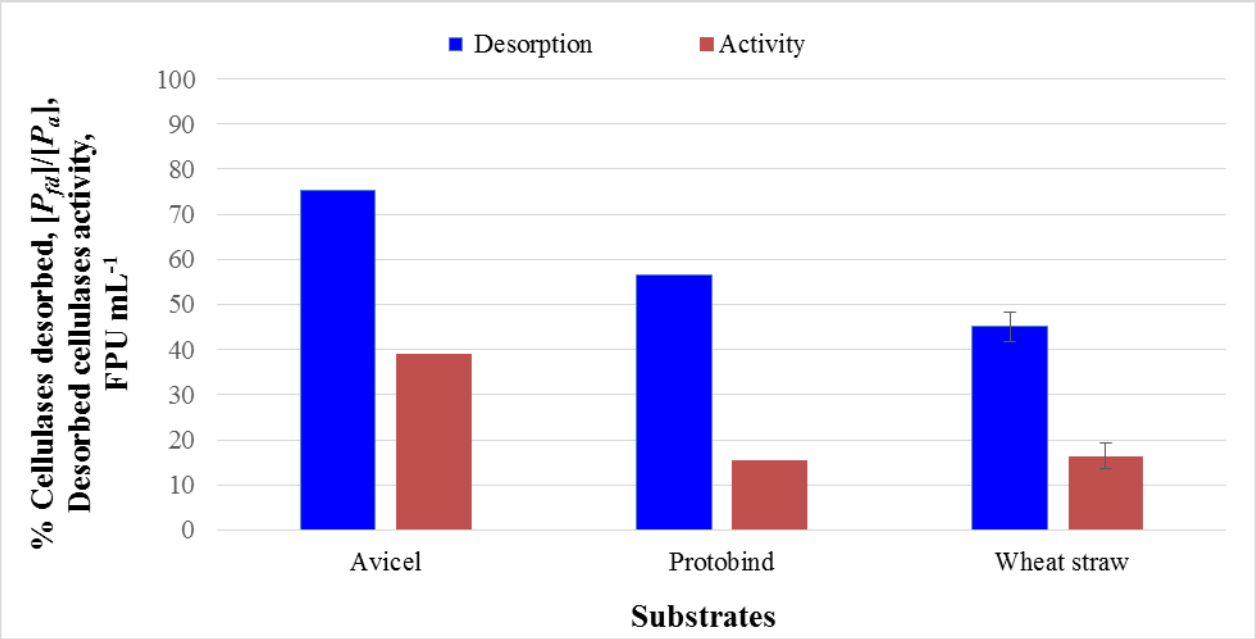


Figure 52 : Comparison of desorbed cellulases and activity of the desorbed cellulases among substrates at pH 9, 60 ^{o}C, 100 rpm and maximum error bar shown at 95 % probability

The % desorbed cellulases from wheat straw were 30 % less than those obtained from pure cellulose (*i.e.* Avicel). The activity of the desorbed cellulases was about 58 % less than that of Avicel. The % desorbed cellulases from Protobind were 10 % higher than that of wheat straw which could be due to the structure of raw wheat straw which is more porous that than that of Protobind. In porous

substrates enzymes get trapped in some of the pores. The activity of cellulases desorbed is almost the same as that of Protobind. Desorption from untreated lignocellulosic material (wheat straw) gives less desorption and activity than the pure cellulose and lignin.

Desorption from 30% delignified wheat straw (DWS)

Figure 53 shows % desorption and activity (FPU) of desorbed cellulase from 30 % delignified wheat straw. The results from 30 % DWS were further compared with the % desorbed and activity of the desorbed cellulases at different experimental conditions. Each data point was measured separately and was an average of triplicates of a set of 5 tests (3 x 5=15).

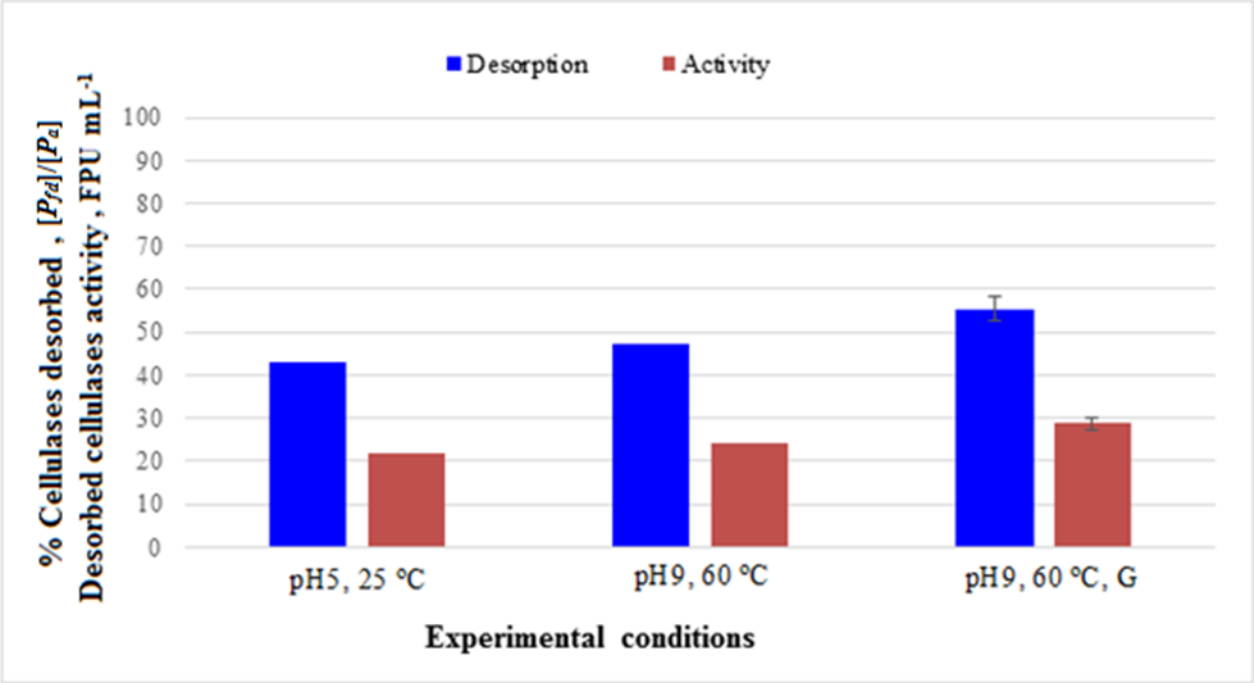


Figure 53 : Comparison of % desorbed and activity of the desorbed cellulases from 30 % delignified wheat straw and different experimental conditions, and maximum error bar shown at 95 % probability

The maximum cellulases were desorbed were around 55 % with activity of around 29 FPU at pH 9, 60 ^{o}C with 5 % glycerol. A 5 % improvement in % desorption and 48 % in activity was observed in

comparison with that of raw wheat straw for pH 5 and 60 ^{o}C. Adding 5 % glycerol to conditions of pH 9 and 60 ^{o}C, there was significant improve net in desorption (23 %) and activity (48 %) from 30 % DWS with respect to raw wheat straw.

Desorption from 60 % delignified wheat straw (DWS)

Figure 54 shows % desorption and activity (FPU) of desorbed cellulase from 60 % delignified wheat straw. The results from 60 % DWS were further compared with the % desorbed and activity of the desorbed cellulases at different experimental conditions. Each data point was measured separately and was an average of triplicates of a set of 5 tests (3 x 5=15).

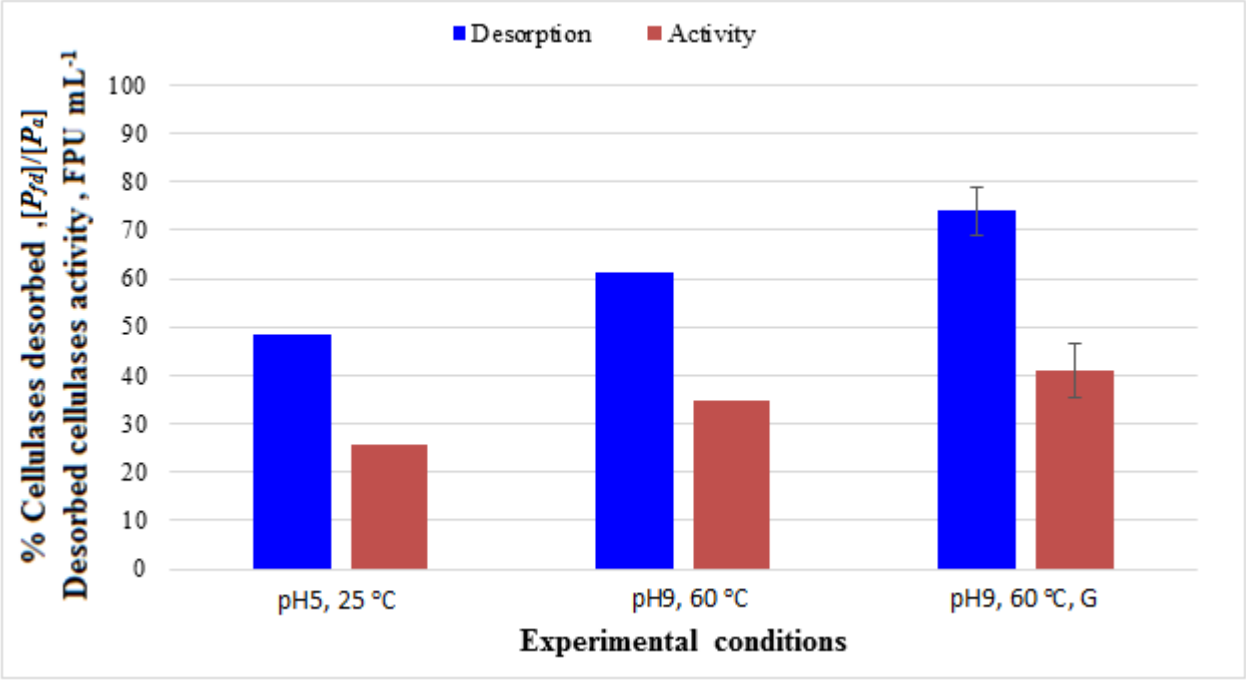


Figure 54: Comparison of %desorbed and activity of the desorbed cellulases from 60 % delignified wheat straw and different experimental conditions, and maximum error bar shown at 95 % probability

The maximum cellulases were desorbed were around 75 % with activity of around 41 FPU at pH 9, 60 ^{o}C with 5 % glycerol. A 36 % improvement in % desorption and 112 % in activity was observed

in comparison with that of raw wheat straw for pH 5 and 60 ^{o}C. Adding 5 % glycerol to conditions of pH 9 and 60 ^{o}C there was significant improve net in desorption (64 %) and activity (150 %) from 60 % DWS with respect to raw wheat straw. Isaacs (1984) conducted a simulation model to estimate enzymatic hydrolysis of corn stover and aspen wood and concluded that 60 % of enzyme reuse can reduce 21.2 % of the cost of the ethanol production process. At this stage of our research (60 % delignified wheat straw, pH 9, 60 ^{o}C, 5 % glycerol) our study is offering a 75 % reuse of active cellulases which may reduce 26.5 % of the cost of the ethanol production.

6.0 Conclusion

1. The concentration of cellulases adsorbed increased depending upon the amount and type of substrate
2. No hydrolysis/self-hydrolysis occurred during the time span of adsorption or desorption under suggested working conditions.
3. The adsorption of cellulases on Avicel PH 101 follow Langmuir isotherm while following Langmuir isotherm, Protobind 1000 has indicated cooperative adsorption. The results indicated that cellulases adsorb on the surface of substrates, one at one site, giving monolayer adsorption. Disagreement of experimental data with Freundlich isotherm indicated that cellulases do not interacts with each other to form multilayer on the substrates. Therefore all cellulases adsorbed on the substrates in monolayer.
4. Protobind (lignin) showed almost twice more adsorption capacity for cellulases than that of Avicel (cellulose). Therefore, delignification of lignocellulosic materials is important to avoid loss of cellulases by adsorption on lignin.
5. It was observed that cellulases adsorption appeared to be strongly influenced by the overall cellulases accessibility to substrates. Therefore, a substrate with porous structure (wheat straw) would have a more accessible surface (unless its natural wax coatings and lignin is removed) and hence more adsorption.
6. Thermodynamics parameters obtained from data could be used to indicate spontaneity of the adsorption and hence feasibility of adsorption. ΔG_a for Avicel is negative for 298 K to 323 K and for Protobind it is negative for all temperature range (298 K to 343 K). In normal ethanol production operation the adsorption on lignin is least required, therefore, a working temperature between the ranges of 298 K to 323 K would have less loss of enzymes by adsorption on lignin.

7. The solid residues obtained from bioethanol industry needs to be treated at the same time to desorb cellulases because desorption equilibrium for both cellulose and lignin reached at same time).
8. The minimum value of free energy change of desorption (ΔG_d) was obtained at pH 9 and 333 K. Hence, pH 9 and 333K was suggested to use for production of bioethanol.
9. Desorption with the help of 5% glycerol would improve desorption and activity of the desorbed cellulases from lignocellulosic material. Therefore, a pH 8 or 9, temperature 323K with 5% glycerol would give maximum desorption and activity (active desorption) of cellulases from wheat straw.
10. Delignification of natural lignocellulosic materials is important for active desorption of cellulases. Desorption increased as delignification increased. The complete delignification would be expensive, therefore, delignification of lignocellulosic material to an optimum level is recommended.

7.0 Future work

The results reported in this dissertation may have generated many questions, some of which may be important for further studies.

- In future, research needs to be focused on adaptation of results obtained from the components of lignocellulosic materials and delignified (ozonated) wheat straw to other lignocellulosic materials and then scaling-up these lab-results for large reactors. The problems foreseen for large scale production would include: handling of biomass at high solids concentrations, large particle sizes and high amounts of silica (coming from agricultural residues). Solutions for these identified problem have to be tested and verified to ensure long-term stability of a future bioethanol production.
- Cellulases with higher activity ranges should be developed and tested for wide ranges of pH and temperature.
- For further improvements of the enzymatic bioethanol production process, recyclability of desorbed cellulases (again and again) should be investigated.
- Because the addition of glycerol for desorption increased cellulase activity, it is desirable to determine the effect of glycerol on adsorption of cellulases because desorbed cellulases may contain glycerol with them when recycled.
- Addition of some chemicals that have preferential adsorption on lignin may inhibit all adsorption of cellulases on lignin and prevent non-productive adsorption on lignin. This will be due to the masking of adsorption sites on lignin through a process of competitive interactions. This research will save cost and time for delignification of lignocellulosic materials.
- Further investigations on the pore size of the pretreated lignocellulosic material is likely to be conducted to improve both pretreatment and enzymatic hydrolysis.

Appendices

Appendix A

Measurement of the Activity of cellulases
Modified Laboratory Analytical Procedure #006 (NREL, 1995)

Reagents and Materials

DNS Reagent

Dissolve 10.6 g of 3, 5 Dinitrosalicylic acid and 19.8 g of Sodium hydroxide then add 306 g of Rochelle salts (sodium potassium tartrate), 7.6 ml of Phenol and 8.3 g of Sodium metabisulfite. Titrate 3 ml sample with 0.1 N HCl to the phenolphthalein endpoint. It should take 5-6 ml of HCl. Add NaOH if required.

Citrate Buffer

Novozymes Cellulase enzyme (NS 50013) assays are carried out in 0.05 M citrate buffer pH 5.0. To prepare 1.0 M citrate buffer, dissolve 210 g of Citric acid monohydrate in 750 ml of distilled water. Adjust the pH of the solution to 4.3 by adding NaOH (50-60 g). To prepare 0.05 M citrate buffer, dilute 50 ml of 1 M citrate buffer stock to 1 liter by adding 950 ml distilled water. Adjust the pH of the solution to 5.0 by adding NaOH.

Blank and controls

Reagent blank: 1.5 ml of 0.05 M citrate buffer.

Enzyme control: 1.0 ml of 0.05 M citrate buffer + 0.5 ml enzyme dilutions (prepare a separate control for each dilution tested).

Substrate control: 1.5 ml of 0.05 M citrate buffer + filter-paper strip.

Glucose standards

A working stock solution of anhydrous glucose (10 mg mL^{-1}) should be made up. Aliquots of this working stock should be tightly sealed and stored frozen. The standard should be vortexed after thawing to ensure adequate mixing. Dilutions are made from the working stock in the following manner:

1.0 ml + 0.5 ml buffer = 1:1.5 (3.35 mg 0.5 mL^{-1}).

1.0 ml + 1.0 ml buffer = 1:2 (2.5 mg 0.5 mL^{-1}).

1.0 ml + 2.0 ml buffer = 1:3 (1.65 mg 0.5 mL^{-1}).

1.0 ml + 4.0 ml buffer = 1:5 (1.0 mg 0.5 mL^{-1}).

Glucose standard tubes should be prepared by adding 0.5 mL of each of the above glucose dilutions to 1.0 m of 0.05 M citrate buffer in a 13 x 100 mm test tube.

Blanks, controls and glucose standards should be incubated at 50 ºC along with the enzyme assay tubes, and then "stopped" at the end of 60 minutes by addition of 3.0 ml of DNS reagent.

Color development

1) Boil all tubes for exactly 5.0 minutes in a vigorously boiling water bath containing sufficient water to cover the portions of the tubes occupied by the reaction mixture plus reagent. All samples, controls, blanks, and glucose standards should be boiled together. After boiling, transfer to a cold ice-water bath.

2) Let the tubes sit until all the pulp has settled, or centrifuge briefly. Dilute all tubes (assays, blanks, standards and controls) in water (0.200 mL of color-developed reaction mixture plus 2.5 mL of water

in a spectrophotometer cuvette works well, use the pipette to mix by drawing the mixture into the pipette tip repeatedly). Determine color formation by measuring absorbance against the reagent blank at 540 nm. With this dilution the glucose standards described above should give absorbance in the range of 0.1 to 1.0 A.

Calculations

1) Construct a linear glucose standard curve using A 540 plotted against its concentration of glucose (mg 0.5 mL^{-1}). Verify the standard curve by running a calibration verification standard, an independently prepared solution containing a known amount of glucose which falls about midpoint on the standard curve.

2) Using this standard curve determine the glucose released for each sample tube after subtraction of enzyme blank.

3) Estimate the concentration of enzyme which would have released exactly 2.0 mg of glucose by means of interpolation. To find the required enzyme concentration take two data points that are very close to 2.0 mg as in Table 14

Table 13: Dilution of Cellulase enzyme NS50013 from enzyme stock that had been diluted 1:15 in citrate buffer

Dilution #	**Citrate buffer**	**1:10 Enzyme**	**Dilution factor**	**Absorbance At 540 nm**	**[Glucose]**
	mL	mL	mL mL^{-1}	(-)	(mg 0.5 mL^{-1})
1	1.65	0.35	0.0166	0.789	-
2	1.70	0.30	0.0100	0.713	3.278
3	1.80	0.20	0.0067	0.545	2.505
4	1.85	0.15	0.0050	0.438	2.013
5	1.90	0.10	0.0033	0.346	1.59

The term "Dilution factor" is used to represent the proportion of the original enzyme solution present in the dilution added to the assay mixture. For example a 1:15 dilution (Dilution # 4) of the 1:15 working stock of enzyme will have:

$$Dilution\ Factor = \frac{\frac{0.15}{0.15+1.85}}{15} = 0.005$$

In another word, the original enzyme was diluted two times. In the first dilution, it was diluted (1:15) and in the second dilution (1:10). Therefore, after performing the hydrolysis and the DNS test described in Appendix A, glucose concentrations of the five cellulase enzyme assays were determined using glucose standard curve of Figure 8.

$$Cellulase\ activity\ \left(\frac{(FPU}{mL}\right) = \frac{0.37}{[enzymes]releasing\ 2.0\ mg\ glucose}$$

[Enzyme] represents the proportion of original enzyme solution present in the directly tested enzyme dilution (that dilution of which 0.5 ml is added to the assay mixture).

The numerator 0.37 in the equation is derived from the factor for converting the 2.0 mg of glucose equivalents generated in the assay to µmoles of glucose

$$\frac{\frac{2.0\ mg\ glucose}{0.180\ mg\ glucose/\mu mol}}{0.5\ mL\ enzyme\ dilution\ x\ 60\ min} = 0.37\ \frac{\mu mol}{\min mL}$$

Table 14 shows the raw data for the change of absorbance with glucose concentration variation. These data were used to plot the glucose standard curve as shown in Figure 8. The absorbance values were measured using Biochrom UV spectrophotometer, model number: Ultraspec 50, England.

Table 14: Data for glucose standard curve (DNS method)

[Glucose] X_i	**Absorbance Replicate #1**	**Absorbance Replicate #1**	**Absorbance mean, (Yi)**	**$(X_i)^2$**	**$(Y_i)^2$**	**X_iY_i**
mg/0.5 mL	(-)	(-)	(-)	mg/0.5 mL	(-)	mg/0.5 mL
1.00	0.215	0.219	0.217	1	0.047	0.217
1.65	0.346	0.350	0.348	2.722	0.121	0.574
2.50	0.519	0.531	0.531	6.250	0.275	0.1312
3.35	0.692	0.707	0.700	11.222	0.490	0.234
8.500			1.790	21.195	0.934	4.448

Columns 3, 4, and 5 of Table 14 contain computed values for x_i, y_i and x_iy_i, with their sums appearing as the last entry in each column which were used to analyze the glucose measurements by the least square method (Skoog *et al*., 2007). The calculation of the slope and intercept is simplified by defining three quantities S_{xx}, S_{yy} and S_{xy} as follows:

$$S_{xx} = \sum X_i^2 - \frac{(\sum x_i)^2}{N} = 21.195 - \frac{(8.5)^2}{4} = 3.1325 \left(\frac{mg}{0.5ml}\right)^2$$

$$S_{yy} = \sum y_i^2 - \frac{(\sum y_i)^2}{N} = 0.934 - \frac{(1.790)^2}{4} = 0.133\ (-)$$

$$S_{xy} = \sum x_i\, y_i - \frac{\sum x_i \sum y_i}{N} = 4.448 - \frac{(8.500\ X\ 1.790)}{4} = 0.645 \left(\frac{mg}{0.5ml}\right)^2$$

The slope of the line, m:

$$m = \frac{S_{xy}}{S_{xx}} = \frac{0.645}{3.13} = 0.206 \left(\frac{0.5ml}{mg}\right)$$

The means (averages) for x and y values:

$$\bar{x} = \frac{\sum x_i}{N} = \frac{8.500}{4} = 2.125 \left(\frac{mg}{0.5\ ml}\right)$$

$$\bar{y} = \frac{\sum y_i}{N} = \frac{1.790}{4} = 0.4475\ (-)$$

The intercept, b:

$$b = \bar{y} - m\bar{x} = 0.4475 - 0.206\ X\ 2.125 = 0.009\ \approx 0.000$$

Thus the equation for the least square line of the standard curve is:

$$Absorbance = 0.206\ X\ [Glucose]$$

The standard deviation about regression:

$$S_r = \sqrt{\frac{S_{yy} - m^2 S_{xx}}{N-2}} = \sqrt{\frac{0.133 - (0.206)^2\ X\ 3.132}{4-2}} = 0.00093(-)$$

The standard deviation of the slope:

$$S_r = \sqrt{\frac{S_{yy}}{S_{xx}}} = \sqrt{\frac{(0.00093\ )^2}{3.132}} = 0.00526\ \left(\frac{0.5mL}{mg}\right)$$

The standard deviation of the intercept:

$$S_b = S_r \sqrt{\frac{1}{N - \frac{(\sum X_i)^2}{\sum x_i^2}}} = \quad 0.00093\ X\ \sqrt{\frac{1}{7 - \frac{(8.500)^2}{21.195}}} = 0.00049\ (-)$$

The standard deviation for results obtained from the standard curve:

$$S_c = \frac{S_r}{m} \sqrt{\frac{1}{M} + \frac{1}{N} + \frac{(\overline{y_c} - \bar{y})^2}{m^2\ S_{xx}}}$$

$$S_c = \frac{0.00093}{0.206} \sqrt{\frac{1}{2} + \frac{1}{4} + \frac{(\overline{y_c} - \ 0.4475)^2}{(0.206)^2\ X 3.132}}$$

Where:

M = number of replicates

N= number of points used in the standard curve

$\bar{y_c}$ = mean of the absorbance measured in replicates #1 and #2

The Confidence Function (CF) was calculated at 95 % probability using the following equation:

$$CF = \pm 1.96\, X \left(\frac{S_c}{\sqrt{M}}\right)$$

Tables 15 show the glucose concentration measured using DNS method for five dilutions of cellulase enzymes NS50013. The glucose concentration data was used to find the dilution that will release 2.000 gm/ 0.5 ml of glucose sugar. Then this dilution was used to calculate the cellulase enzymes activity. A sample of the unknown enzyme activity measurement is shown in Appendix D.

Table 15: Dilution and glucose concentration of cellulase enzyme NS50013 stock that had been diluted in citrate buffer

Run #	Dilution factor	Replicate # 1		Replicate # 2		Glucose conc. Mean ±CF[1]	Standard deviation (S_c)
		Abs. at 540nm	Glucose conc.	Abs. at 540nm	Glucose conc.		
	mL/mL	(-)	mg/0.5mL	(-)	mg/0.5mL	mg/0.5mL	mg/0.5mL
1	0.0250	0.740	-	0.789	-		
2	0.0150	0.658	3.185	0.675	3.277	3.213±0.029	0.03
3	0.0100	0.509	2.473	0.517	2.509	2.491±0.029	0.03
4	0.0075	0.438	2.128	0.416	2.018	2.073±0.019	0.02
5	0.0050	0.317	1.540	0.327	1.590	1.565±0.009	0.01

[1] = Confidence Function at 95 % probability

A standard deviation of 0.03 is negligible will not be considered in calculations of FPU.

Table 16 shows cellulase enzymes NS50013 activities measured by using the filter paper unit method. The absorbance for the duplicate sets with the means, standard deviations and the confidence functions at 95 % probability level are shown in Table 16.

Table 16: Activity of commercial cellulases NS 50013 measured by filter paper unit method.

	[Enzymes]	**Cellulases, Activity**
Replicate #1	0.00695	53
Replicate #2	0.00739	50
Mean		51.5
STDEV		2.12

$\sigma(S_c)$ = Max. Standard deviation from the curve =0.03 (negligible)

σ (Samples) = 2.12

$$\sigma_{Total} = \sqrt{(\sigma S_c)^2 + \left(\sigma S_{sample}\right)^2}$$

$$95\%\ Probablity = \pm 1.96\ X\ \left(\frac{\sigma(Total)}{\sqrt{N}}\right)$$

N = Number of replicates

$$95\%\ Probablity = \pm 1.96\ X\ \left(\frac{2.12}{\sqrt{2}}\right) = 2.94$$

Therefore, activity of cellulases = 51.5 ± 2.94

Appendix B

Measurement of error in readings by using standard curve for concentration of cellulases

Table 17: Data for absorbance of standard samples

Stand. Conc., X, µg mL^{-1}	**Absorbance**			**Mean**
	Replicate 1	Replicate 2	Replicate 3	Yi
0	0.000	0.000	0.000	0.000
50	0.130	0.133	0.128	0.130
100	0.233	0.236	0.238	0.235
200	0.525	0.450	0.508	0.500
300	0.610	0.725	0.638	0.664
400	0.920	0.845	0.843	0.869
Sum				
1050				2.387

Data showing absorbance for varying concentrations of cellulases was plotted in Figure 13. There appeared to be a linear relationship between the absorbance and the concentration of standards. Therefore, the method of least squares was applied.

Table 18: Statistical manipulation of data given in Table 17

X_i^2	Y_i^2	X_iY_i	$(X_iY_i)^2$
0	0.0000	0.0000	0.0000
2500	0.0169	6.5500	42.902
10000	0.0557	23.600	556.96
40000	0.2500	100.00	10000
90000	0.4413	199.29	39716
160000	0.7600	348.00	121104
Sum			
302500	1.5239	677.39	171419

Columns 1, 2, and 5 of Table 18 contain computed values for X_i^2, Y_i^2 and X_iY_i, $XiYi^2$ and their sums appearing as the last entry in each column which were used to analyze the cellulases measurements by the least square method (Skoog *et al*., 2007). Xi and Yi the coordinated of the

individual data points, N is the number of pairs of data used in preparation of the calibration curve. The calculation of the slope and intercept are simplified by defining three quantities S_{xx}, S_{yy} and S_{xy} as follows

$$S_{xx} = \sum X_i^2 - \frac{(\sum X_i)^2}{N}$$

$$S_{yy} = \sum Y_i^2 - \frac{(\sum Y_i)^2}{N}$$

$$S_{xy} = \sum X_i Y_i - \frac{\sum X_i \sum Y_i}{N}$$

$$S_{xx} = 302500 - \frac{(1050)^2}{5} = 82000$$

$$S_{YY} = 1.524 - \frac{(2.387)^2}{5} = 0.3844$$

$$S_{xy} = 677.39 - \frac{1050 * 2.387}{N5} = 177.59$$

Symbols $\dot{X}$ and $\dot{Y}$ are average values of the variables

$$\dot{X} = \frac{\sum X_i}{N} = \frac{1050}{5} = 210$$

$$\dot{Y} = \frac{\sum Y_i}{N} = \frac{2.387}{5} = 0.476$$

The standard deviation of residuals S_y, [the vertical deviation of each point from the straight line is called a residual], is given by:

$$S_y = \sqrt{\frac{S_{yy} - m^2 S_{xx}}{N-2}}$$

$$S_y = \sqrt{\frac{0.3844 - (0.0216)^2 * 82000}{5-2}} =$$

$$S_y = \sqrt{\frac{0.3844 - 0.38438}{3}} = 0.0025$$

The standard deviation of slope S_m, is given by:

$$S_m = \frac{S_y}{\sqrt{S_{xx}}}$$

$$S_m = \frac{0.0025}{\sqrt{82000}} = 0.00017$$

The standard deviation of intercept S_b, is given by:

$$S_b = S_y * \sqrt{\frac{1}{N - \frac{(\sum X_i)^2}{\sum X_i^2}}}$$

$$S_b = 0.0025 * \sqrt{\frac{1}{5 - \frac{(1050)^2}{302500}}} = 0.00214$$

The standard deviation of S_c for analytical results obtained with the calibration curve:

$$S_c = \frac{S_r}{m}\left(\sqrt{\frac{1}{L} + \frac{1}{N} + \frac{(Y_c - y)^2}{m^2 S_{xx}}}\right)$$

In this case, 1/L= 0.33, 1/N= 0.2, m^2S_{xx} = 0.3845, Y_c is the average (i.e. Y_c-Y_i), Y represent values of absorbance in one set of data in Appendix B.

Table 19: Standard deviation of one set of data from Table 17 against Standard Curve

Y_c	Y	$(Y_c-Y)^2$	S_c
0.13	0.13	0	0.8426
0.236	0.233	9.00E-06	0.8426
0.5	0.525	0.0006	0.8426
0.6643	0.61	0.0029	0.8426
0.869	0.845	0.0005	0.8426
0.869	0.92	0.0026	0.8426

The value of 0.84 will be used to make up an error of cellulases concentration measurements.

Appendix C

Measurement of error in readings by using standard curve for reducing sugars

Data in Table 20 showing absorbance for varying concentrations of cellulases was plotted in Figure 12.

Table 20: Data for absorbance of standard samples of glucose

Stand. Conc., X, µg mL^{-1}	**Absorbance**			**Mean**
	Replicate 1	Replicate 2	Replicate 3	Y_i
0	0.000	0.000	0.000	0.0000
50	0.024	0.022	0.022	0.0227
100	0.048	0.052	0.056	0.0520
200	0.086	0.089	0.090	0.0880
400	0.184	0.189	0.193	0.1890
800	0.0.384	0.381	0.374	0.3790
Sum				
1550				0.7307

There appeared to be a linear relationship between the absorbance and the concentration of glucose standards. Therefore, the method of least squares was applied.

Table 21: Statistical manipulation of data given in Table 20

$\mathbf{X_i^2}$	$\mathbf{Y_i^2}$	$\mathbf{X_iY_i}$	$\mathbf{(X_iY_i)^2}$
0	0.0000	0.0000	0.0000
2500	0.0005	1.135	1.2880
10000	0.0027	5.200	27.040
40000	0.0077	17.60	309.76
160000	0.0357	75.60	5715.3
640000	0.1436	303.2	91930
Sum			
852500	0.1902	402.73	97983

Columns 1, 2, 3 and 4 of Table 21 contain computed values for X_i^2, Y_i^2 and X_iY_i, $X_iY_i^2$ and their sums appearing as the last entry in each column which were used to analyze the reducing sugars measurements by the least square method (Skoog *et al*., 2007). X_i and Y_i the coordinated of the

individual data points, N is the number of pairs of data used in preparation of the calibration curve. The calculation of the slope and intercept are simplified by defining three quantities S_{xx}, S_{yy} and S_{xy} as follows

$$S_{xx} = \sum X_i^2 - \frac{(\sum X_i)^2}{N}$$

$$S_{yy} = \sum Y_i^2 - \frac{(\sum Y_i)^2}{N}$$

$$S_{xy} = \sum X_i Y_i - \frac{\sum X_i \sum Y_i}{N}$$

$$S_{xx} = 852500 - \frac{(1550)^2}{5} = 372000$$

$$S_{YY} = 0.1902 - \frac{(0.7307)^2}{5} = 0.0834$$

$$S_{xy} = 402.73 - \frac{1550 * 0.7303}{5} = 176.22$$

Symbols $\dot{X}$ and $\dot{Y}$ are average values of the variables

$$\dot{X} = \frac{\sum X_i}{N} = \frac{1550}{5} = 310$$

$$\dot{Y} = \frac{\sum Y_i}{N} = \frac{0.7307}{5} = 0.1461$$

The standard deviation of residuals S_y, [the vertical deviation of each point from the straight line is called a residual], is given by:

$$S_y = \sqrt{\frac{S_{yy} - m^2 S_{xx}}{N-2}}$$

$$S_y = \sqrt{\frac{0.08341 - (0.000473)^2 * 372000}{5-2}} =$$

$$S_y = \sqrt{\frac{0.08341 - 0.08328}{3}} = 0.0065$$

The standard deviation of slope S_m, is given by:

$$S_m = \frac{S_y}{\sqrt{S_{xx}}}$$

$$S_m = \frac{0.0065}{\sqrt{372000}} = 0.0000106$$

The standard deviation of intercept S_b, is given by:

$$S_b = S_y * \sqrt{\frac{1}{N - \frac{(\sum X_i)^2}{\sum X_i^2}}}$$

$$S_b = 0.0065 * \sqrt{\frac{1}{5 - \frac{(1550)^2}{852500}}} = 0.0044$$

The standard deviation of S_c for analytical results obtained with the calibration curve:

$$S_c = \frac{S_y}{m}\left(\sqrt{\frac{1}{L} + \frac{1}{N} + \frac{(Y_c - y)^2}{m^2 S_{xx}}}\right)$$

In this case, 1/L= 0.33, 1/N= 0.2, m^2S_{xx} = 0.08328, Y_c is the average (i.e. Y_c-Y_i), Y represent values of absorbance in one set of data in Appendix C.

Table 22: Standard deviation of one set of data from Table 20 against Standard Curve

Y_c	Y	$(Y_c-Y)^2$	S_c
0.0000	0.000	0.000	0.000
0.0227	0.022	4.9E-07	12.012
0.0520	0.052	0	12.012
0.0880	0.089	0.000001	12.012
0.1890	0.189	0	12.012
0.3790	0.381	4E-06	12.012

A standard deviation of 12.012 will be used to make up a propagation error for reducing sugars measurement

Appendix D
Error analysis

Error bars for Adsorption

Cellulases adsorbed is calculated by the following formula.

$$\boldsymbol{P_a = E_0 - P_f}$$

Values of P_0 and P_f were calculated from the standard calibration curve

[P_0]: Initial concentration of cellulases, µg mL^{-1}

[P_f]: Cellulases in solution at equilibrium, µg mL^{-1}

[P_a]: Cellulases adsorbed at equilibrium, µg mL^{-1}

Values of P_0 and P_f were calculated from the standard calibration curve as given in Figure 17.

Probability of in the value of [P_a] was calculated by the following formula using error propagation method. σ_{Sc} is the standard deviation of results from the standard curve.

$$\sigma_{Pa} = \sqrt{(\sigma_{Sc})^2 + (\sigma_{P0})^2 + \left(\sigma_{Pf}\right)^2}$$

Some parameters used in adsorption uncertainty calculation

Sample 1, P_0	Sample 2, P_0	Sample 3, P_0	Sample 1, P_f	Sample 2, P_f	Sample 3, P_f
103.1	98.99	105.8	30.42	36.59	33.17

Average		Standard deviation		
P_0	P_f	σ_{Sc}	σ_{P0}	σ_{Pf}
102.0	34.00	0.84	3.420	3.09

$$\sigma_{Pa} = \sqrt{(0.84)^2 + (3.42)^2 + (3.09)^2}$$

$$= 4.68$$

$$95\%\ Probability = 1.96\ \frac{\sigma}{\sqrt{n}} = 1.96\ \frac{4.68}{\sqrt{3}} = 5.30$$

Errors for all [P_a] values were calculated in this way. Particularly, this error value was used in Figure 11.

Error Bar for an adsorption parameter (P_f/P_e) of Langmuir plot

Error bar for use in Langmuir plot was calculated and used the Figure 23. The plot was constructed by plotting $[P_f]/P_e$ Vs $[P_f]$. where

P_e: Cellulases adsorbed per unit substrate, μg mg^{-1}

Calculation for uncertainty in an adsorption parameter (E_f/E_e) of Langmuir plot

P_f	σP_e	σP_f	A = $\sigma P_e / P_e$	B = $\sigma P_f / P_f$	C= (S_c)	$(A)^2$	$(B)^2$	$(C)^2$	$\sqrt{A^2 + B^2 + C^2}$
34	0.171	5.82	0.050	0.171	0.84	0.0025	0.0292	0.7056	

$$\sigma\left(\frac{P_e}{P_f}\right) = \left|\frac{P_e}{P_f}\right| \sqrt{\left(\left(\frac{\sigma P}{P_f}\right)^2 + \left(\frac{\sigma P_e}{P_e}\right)^2 + (S_C)^2\right)4}$$

$$\sigma\left(\frac{P_f}{P_e}\right) = \left|\frac{3.4}{34}\right| * 0.85 = 0.085$$

$$95\ \%\ probability = 1.96 * \frac{0.085}{\sqrt{5}} = 0.075$$

Error Bar for an adsorption parameter (E_e/E_f) of Langmuir plot in Figure 23

Sample calculations for % desorbed

Step -1, calculations for adsorption, P_a

E_0 was measured in the same way as given below as P_f

Amount remaining in solution at equilibrium, P_f

Sample 1 = 88.99 µg mL^{-1}

Sample 2 = 90.81 µg mL^{-1}

Sample 3 = 92.62 µg mL^{-1}

Sample 4 = 91.26 µg mL^{-1}

Sample 5 = 89.44 µg mL^{-1}

Average mean

$$\overline{X} = \frac{1}{N} \sum_{i=1}^{N} X_i \quad \ldots \quad \text{A-10}$$

Where Xi = X_1, X_2, X_3, X_4, X_5

$$\overline{X} = 90.62$$

S_c (0.84) is the standard deviation from the standard curve

Standard deviation is:

$$\sigma = \sqrt{\frac{1}{N}} \left(\sum_{i=1}^{5} \left(X_i - \overline{X}\right)^2 + (Sc)^2\right) \quad \ldots \quad \text{A-11}$$

$$= 1.61$$

$$P_a = P_0 - P_f \quad \ldots \quad \text{A-12}$$

$$P_a = 202 - 88.99 = 113.01$$

For 5 samples P_a was measured using Equation 3

E_d, from 5 adsorbed supernatants, average of 5 samples = 111.38

Standard deviation of 5 samples = 1.61

Therefore, $P_a = 111.38 \pm 1.61$

Step 2, calculations for desorption

P_d, from 5 desorbed supernatants, average of 5 samples = 44.016

Standard deviation for P_d from desorbed supernatant, average of 5 samples = 4.13

% desorbed

$$\%\ Desorbed = \frac{amount\ desorbed}{amount\ adsorbed} * 100 \quad \ldots \quad \text{A-14}$$

$$\%\ Desorbed = \frac{44.016}{111.38} * 100$$

$$\%\ Desorbed = 39.52$$

Step 3 Propagation error

$$\sigma\ Desorbed = \sqrt{\left(\frac{\sigma P_d}{P_d}\right)^2 + \left(\frac{\sigma P_a}{P_a}\right)^2 + (S_c)^2} = \quad \ldots \quad 5$$

$$\sigma\ Desorbed = \sqrt{\left(\frac{4.13}{44.01}\right)^2 + \left(\frac{1.46}{111.38}\right)^2 + (0.84)^2} = \ldots$$

$$\sigma\ Desorbed = \sqrt{0.00881 + 0.00017 + 0.7056} = 0.845$$

Therefore,

$$\%\ Desorbed = 39.52 \pm 0.845$$

A 95 % error bar would be

$$95\ \%\ probability = 1.96 * \frac{0.845}{\sqrt{5}} = 0.743$$

Error bars added to all % desorption curves including Figure 32, 34,36, 38,40 , 42, 44 etc were calculated in this way.

Sample calculations for lnK_a and lnK_d

$$K_a = \frac{P_a}{P_f} \quad \ldots \quad \text{A-15}$$

Step 1

Table 18 shows basic data to calculate lnK_a values and the corresponding error used in Figure 23 and other similar valuations of lnK_d

Table 23: Calculation for $\ln K_a$ from [E_a] and [E_f]

Sample 1	Sample 2	Sample 3	Average	StdevA
P_a				
113.42	113.51	109.17	112.03	2.48
P_f				
75.42	74.92	73.67	74.67	0.90
K_a				
1.50	1.51	1.48	1.5	0.016
lnK_a				
0.41	0.41	0.39	1.21	

Step 2, Variance

$$\sigma lnK_a = \sqrt{\left(\frac{\sigma P_a}{P_a}\right)^2 + \left(\frac{\sigma P_f}{P_f}\right)^2 +} \quad \ldots \quad \text{A-16}$$

$$= \sqrt{0.00064}$$

$$= 0.84$$

Therefore,

$$\ln K_a = 1.21 \pm 0.84$$

$$K_d = \frac{P_d}{P_a} \quad \ldots \quad \text{A-17}$$

For desorption, the same steps as lnKa were followed, except that variance was calculated as following formula

$$\sigma lnK_d = \sqrt{\left(\frac{\sigma P_a}{P_a}\right)^2 + \left(\frac{\sigma P_d}{P_d}\right)^2} \quad \ldots \quad \text{A-18}$$

Calculations for ΔH, ΔS and ΔG

$$lnK = -\frac{\Delta H}{R}\frac{1}{T} + \frac{\Delta S}{R} \quad \ldots \quad \text{A-19}$$

Plot ln K_a vs 1/T for all temperatures, 25ºC, 40 ºC, 50 ºC, 70 ºC. Lower plot in Figure 25 is for Avicel PH 101 to get ΔH_a, ΔS_a and ΔG_a as

Slope = -ΔH / R

ΔH = - Slope x R … A-20

= - (1962) x 8.314

= -16.32

Similarly

ΔS = Intercept x R … A-21

= (6.0957) x 8.314

= 50.69

Sample calculation for activity of cellulases from supernatants

Activity of unknown enzymes from the supernatant of adsorption which contain non-adsorbed cellulases [P_{fa}] and similarly from the supernatant of desorption which contain desorbed enzymes [P_{fd}] can also be found from by the Equation A-22.

$$\frac{FPU}{ml} = \frac{A_{540}}{0.206}\ x\ \frac{0.5\ mL}{0.180\ \mu mol}\ x\ 60\ min \qquad \ldots \qquad \text{A-22}$$

Absorbance for true sample A_{540} for [P_{fa}] was calculated by subtracting A540 from the A540 of substrate control, using stand calibration curve given in Figure 10.

For a sample which gave [Glucose] =0.524 showed A_{540} = 0.108 at the standard calibration curve in Figure 10.

The same curve showed A_{540} for Avicel = 0

Therefore, true A_{540} for the sample = 0.108-0.000 = 0.108

Putting this A540 = 0.108 in Equation A-22, we get activity

$$\frac{FPU}{mL} = \frac{0.108}{0.206}\ x\ \frac{0.5}{0.180}\ x\ 60$$

$$Activity = 87.37\ \frac{FPU}{mL}$$

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