

## Adsorption of Cellulose Enzymes on Lignocellulosic Materials and Influencing Factors: A Review

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### Abstract

Adsorption of enzymes onto lignocellulosic substrates and their integration is a major concern in the production of bioethanol. Information of the adsorption of enzymes, adsorption characteristics and practical viewpoints are necessary for understanding of an adsorption system. Adsorption of enzymes depends on so many factors, instead of examining the role of all these, this study deals with the factors affecting the key adsorption process such as accessibility of enzymes to lignocellulosic substrate or substrate components and adsorption isotherms.

Extensive analysis of the published literature on enzymatic adsorption onto lignocellulosic materials from 1994 to 2016 has consolidated in this review. The observations have enlightened some of the conflicting results in literature, especially increase or decrease to enzymatic adsorption under the influence of temperature, pH, presence or removal of lignin and hemicellulose for adsorption, enzyme to substrate ratio, inhibition by end-product and synergy of the components of cellulases mixture. It was concluded that an optimum removal of lignin is more important for creating accessibility of enzymes to substrates than non-removal. Effect of pH along with varying temperature on natural substrates has more room to work. End-product inhibition may be controlled by pretreatment and optimum enzyme to substrate ratio. The synergic actions of cellulases are obstructed by high enzyme concentration or high substrate concentration. This review will help in design of enzymatic adsorption step for bioethanol production from lignocellulosic materials.

**Keywords:** Adsorption; Cellulose; Lignin; Pretreatment; Inhibition; Enzyme/substrate; Synergism; Bioethanol

### Introduction

To start enzymatic hydrolysis of a lignocellulosic substrate, enzymes have to adsorb on the substrate and hydrolysis of substrate enzymes have to leave (desorb) the substrate because the attached site of substrate has been hydrolyzed to reducing sugar. Therefore, adsorption of enzymes is the basic step for conversion of cellulose to glucose for production of bioethanol from lignocellulosic biomass [1-3].

Understanding of mechanism of enzymatic adsorption and its controlling factors is important to improve production of sugars and hence enhance bioethanol production from lignocellulosic biomass. Adsorption is mainly a physical contact of its two components: substrate, and enzyme. The factors affecting both components and hence adsorption were studied [4-8]. The parameters studied were crystallinity index of substrate, degree of polymerization of substrate, accessibility of cellulases, and synergy of cellulases. For a substrate which is comprised of pure cellulose, mainly physical parameters such as pore structure of fibers, crystallinity and external surface area govern adsorption. For a natural lignocellulosic biomass, in addition to these physical parameters, the chemical composition also becomes important. Lignocellulosic biomass (i.e. wheat straw, corn stover etc.) are mainly composed of cellulose, lignin, hemicellulose which make the structure compact and the cellulose is protected by lignin. The parameters related to chemical properties are: i) lignin content, or the extent of lignin removed [9-12], ii) substrate particle size [13,14], and iii) substrate accessibility to cellulase [15]. According to some of the

researchers removal of lignin was important because it increased conversion of lignocellulosic biomass [16-18], while for others removal of both the lignin and xylan helped to increase the conversion. Some other researchers proved that the ease of accessibility of cellulases to cellulose was the main parameter for lignocellulosic conversion [19,20]. Particle size reduction was apparently energy consuming and hence cost increasing process [21], and for others fiber size does not matter [22]. A typical enzymatic lignocellulosic conversion process goes through four phases: i) rapid adsorption of cellulases on accessible substrate, ii) initial rate of adsorption/hydrolysis/desorption, iii) intermediate phase where 70% of substrate is hydrolyzed, iv) a very slow but steady decrease of adsorption due to inaccessible substrate. A long time of process for complete conversion lignocellulosic material adds operating costs to the process [23,24]. Enzymatic conversion of lignocellulosic materials is a heterogeneous biochemical reaction. It includes the three important practices: i) the physical contact of the reactants (adsorption), i.e., cellulose accessibility and adsorption of enzymes onto cellulose, ii) the reactivity of the substrate/s and cellulases, e.g., cellulase activity and cellulose structure, and (iii) the adsorption/hydrolysis conditions, such as temperature, pH, etc. There are controversial reports (as given above) about enzymatic lignocellulosic conversion process such as the effect of temperature, pH, lignin content and accessibility of enzymes. The accessibility of cellulases is considered as the most influencing factor for the adsorption of cellulases on the lignocellulosic substrates [25-27].

In lignocellulosic materials, the rate of enzymatic hydrolysis and its yield was observed to be directly related to the amount of the adsorbed enzymes [6,27-31] little information were reported on substrate-enzyme interactions, which include adsorption of enzymes and the

accessibility of enzyme to lignin and cellulose in pretreated lignocellulosic materials [32]. The maximum capacity of substrates to adsorb enzymes were measured and reported in literature by conducting practical adsorption of enzymes (cellulases) onto lignocellulosic substrates. The techniques reported to measure the amount of adsorbed enzymes (cellulases) and hence, to evaluate accessibility of cellulases to substrates can be divided into two groups: indirect measurements, and direct measurements. Indirect measurements include determination of surface area by using: i) Water Retention Value (WRV) method [33], ii) Differential Scanning Calorimetry analysis, [34], NMR porosimetry [35,36], iii) Solute Exclusion Technique [37,38]. It has been established that, some of the techniques used to predict the available surface area of lignocellulosic materials are not mostly accurate (e.g., water retention value, mercury porosimetry etc.). The direct methods include i) Staining method which measures the adsorption of dyes [36,39], ii) cellulase adsorption methods that measure the amount of cellulase adsorbed [40-42]. Thygesen et al. [43] used polarized light microscopy along with confocal microscopy to investigate the degradation mechanism of filter paper and pretreated wheat straw cellulosic fibers by a mixture of Cellulast and Novozym 188. A fluorescently labeled cellulase (endoglucanase GH45) was used to study the changes appearing at adsorption locations present in the wheat straw fibers by noticing the adsorption of the labelled cellulases. It was observed that as adsorption progressed, the fibers remaining in the tested sample became shorter and the number of vacant locations was reduced [43]. Recently, Luterbacher et al. [44] also used confocal fluorescence microscopy, monitored the adsorption of fluorescently labelled Cel7A 6 CBHI (cellulase) and degradation of bacterial microcrystalline cellulose (BMCC) by adsorption/hydrolysis of cellulases. It was concluded that the accessibility of cellulases was a key factor for the degradation of BMCC [44]. In order to correlate accessibility of substrates to the amount of cellulases adsorbed, the maximum enzyme adsorbed to a substrate became a requirement for efficient adsorption. The correlation between enzymes adsorbed and accessibility is given by adsorption isotherms.

Adsorption isotherms is a curve relating the equilibrium concentration of adsorbate/s (enzyme/s) on the surface of an adsorbent, to the concentration of the enzymes in the solution with which it is in contact at fixed temperature at equilibrium [2,3,45]. The adsorption capacity of adsorbent may be determined by the use of an adsorption isotherm. Some reports are available on cellulases adsorption on various lignocellulosic substrates, such as microcrystalline cellulose [40,46], corn stover [47], steam-exploded Douglas fir [48], pretreated hardwood [49], isolated lignin from softwood [50] and lignin preparations from lodgepole pine [51]. The information about adsorption of cellulases onto lignin is very rare. Some of the researches have shown that adsorption of cellulases enzymes onto microcrystalline cellulose can be represented by Langmuir model [45,52]. Others have suggested that Freundlich isotherm for adsorption of cellulases [53,54]. Few interesting reports used both Langmuir and Freundlich isotherms to represent adsorption of cellulases on their substrates [55-57]. Therefore, a thorough study on the adsorption of cellulases on cellulose and lignin was required.

A literature survey was conducted on the reviews published from 1994 to 2016 on the enzymatic immobilization, adsorption and hydrolysis [4,6,30,58-68]. The gaps identified in literature were that the extensive data was not available for the parameters such as substrates types, enzyme preparation method, enzyme/substrate ratios, substrate adsorption capacity, and ease of adsorption due to accessibility. Instead

of examining all the parameters that affect adsorption of cellulases during hydrolysis of cellulose for production of ethanol from lignocellulosic materials, in this review a general approach based on recent literature data has been discussed. The objective of this investigation was to: i) consolidate some of the conflicting results on enzymatic adsorption of lignocelluloses and, ii) improve understanding of adsorption mechanism of cellulase enzymes onto lignocellulosic materials. Wherever it was found necessary and the examples from adsorption of enzymes on lignocellulosic materials were not available, the examples from adsorptions of atoms, ions and molecules were given to make a point. This review paper will also give insights to settle the conflicts about adsorption pattern on lignocellulosic materials and how to improve mechanism of adsorption onto substrate fibers by examining influencing factors. The influencing factors discussed are temperature, pH, product inhibition, E/S ratio, and synergy of action of cellulases onto substrates.

### Accessibility of Cellulases to Cellulose

The accessible surface area of the cellulose is one of the most important factors which shape the rate and extent of enzymatic adsorption of lignocellulosic substrates [1-3]. As the enzymatic adsorption of cellulose is a surface-subjugated phenomenon, and direct physical contact between the cellulase enzymes and substrate must occur. Converse et al. [69] studied adsorption of cellulases on cellulose obtained from a pretreated wood at 40°C and pH 5 and found that adsorption was dependent on surface area. It was also pointed out that the surface area provided for adsorption was not a function of substrate concentration rather it was a function of pretreatment conditions which means accessibility of cellulases to the substrate surface [69]. Some other researchers also suggested that the accessible surface area of the cellulose was one of the most important factors for adsorption of cellulases [25,68-71].

Cellulose accessibility was generally measured by the molecule adsorption methods, and a wide range of gross area values were reported in literatures. For example following Table 1 give surface area (accessibility) measurement of cellulose obtained from different type of cellulosic substrates while corresponding technique of measurement accessibility is also given.

Due to variations in experimental conditions (techniques used) such as adsorption time, vacuum time, vacuum pressure, sample preparation, varying quality of cellulose depending on batches and production location and sample origin [72,74], the values were found different. The surface area measured follow two techniques: i) external surface area, ii) external plus internal surface area. The external surface area of cellulose is related to the shape and particle size of cellulosic samples, therefore it was estimated by microscopy [4,72,75]. The internal surface area techniques falsely overestimate the accessible surface area [69]. To avoid the confusion in overestimation, labelled enzymes were used for assessing the cellulose accessibility to cellulases, it was found that the molecule used to probe into the substrate reached to the pores where average enzyme cannot reach. Enzymes were found adsorbed onto lignin as well [41,76]. The analysis of adsorption data reported lead to the conclusion that there was a linear dependency of the accessibility of crystalline and non-crystalline cellulose surface on the enzymes adsorbed [76] and the accuracy of measurement of the enzymes adsorbed depends on the technique used. The more surface was exposed the more adsorption there was. In fact, more surface exposure means more accessibility to enzymes.

Cellulose	Accessibility, m <sup>2</sup> /g	Technique	Ads max, mg/g	Reference
Filter paper	9.76	Nonhydrolytic TGC Protein Adsorption	86.2	Hong et al. [40]
CF	0.158	Microscopy	-	Weimer et al. [72]
Sigmacell 20	0.301	Microscopy, ESA	-	Weimer et al. [72]
Avicel PH 105	2.38	Nonhydrolytic TGC Protein Adsorption	21	Hong et al. [40]
Avicel PH 101	-	Adsorption	84	Kumar and Wyman [31]
Avicel PH 101	-		34.9	Zheng et al. [73]
AC	0.606	Microscopy, ESA	-	Weimer et al. [72]
Avicel 101	20	BET, N <sub>2</sub> adsorption	-	Marshall and Sixsmith [74]
BMCC	33.5	Nonhydrolytic TGC Protein Adsorption	295	Hong et al. [40]
RAC	41.9	Nonhydrolytic TGC Protein Adsorption	369	Hong et al. [40]

**Table 1:** The maximum cellulases adsorbed (Adsmax) and their accessibility on various cellulosic substrates.

### Accessibility of Cellulases to Lignin

Lignin is a natural protective barrier that constraints water and enzyme accessibility to cellulose. The lignin samples used in all adsorption reported studies were prepared from pretreated from agricultural and forestry materials by extensive enzymatic hydrolysis or by a pretreatment technology. This materials were then exposed to enzymes for adsorption and the equilibrium behavior was determined. Table 2 showed maximum amount of cellulases adsorbed on lignin prepared from different techniques and different substrate. The reported adsorption capacity values of lignins varied from 1.91 to 133.6 mg enzymes/g lignin and increased with respect to the pretreatment types using ammonia fiber expansion (AFEX), dilute acid, steam explosion and lime or the case of CEL-SELP and CEL-EPLP. Zheng et al. [73] adsorbed cellulases on two substrates corn stover lignin and Rice straw lignin. While corn stover was pretreated by two different techniques (Dilute acid and Steam explosion). Steam exploded corn stover and rice straw had almost similar maximum cellulases adsorbed [73-80]. Kumar and Wyman [18] compared cellulases adsorbed on corn stover lignin prepared from five different techniques [31]. There is no match in the amount of cellulases adsorbed on dilute acid pretreated corn stover by the two authors (e.g., 2.34 27 mg/g and 90.7 mg/g).

Gharpuray et al. [80] worked on wheat straw, decreased its lignin contents from 11.53% to 1.33%, its surface area increased from 0.64 m<sup>2</sup>/g to 1.8 m<sup>2</sup>/g. In the next step they adsorbed cellulases QM 9414 on the delignified wheat starch and found that the relative rate of hydrolysis increased more than 10 times of the original wheat straw [80]. Similarly, Kong et al. [81] delignified Aspen Wood from 21.3% to

4.3% lignin contents by alkaline pretreatment and adsorbed Cellulase Cytolase TM 123 on Aspen wood and found that the adsorption of proteins increased which in turn increased sugar yield from 50.7% to 70.1% [81].

Lignin	Enzyme type	Ads max, mg/g	Reference
APCL	Accellerase 1000	2.34	Zheng et al. [73]
SPCL	Accellerase 1000	1.91	Zheng et al. [73]
SRPL	Accellerase 1000	2.3	Zheng et al. [73]
CEL-SELP	Celluclast 1.5	10.2	Tu et al. [54]
CEL-SELP	MSUBC	4.87	Tu et al. [54]
EL-SEHW	Cellulase GC 123	12.3	Nonaka et al. [78]
CEL-EPLP	Celluclast 1.5	2.73	Tu et al. [54]
LHW-Wood10.44	Cellic Ctec 2	37	Ko et al. [77]
LHW-Wood11.39	Cellic Ctec 2	44.8	Ko et al. [77]
LHW-Wood11.56	Cellic Ctec 2	44.5	Ko et al. [77]
CS-AFEX	Spezyme cellulase CP	99.7	Kumar and Wyman [31]
CS-ARP	Spezyme cellulase CP	113.8	Kumar and Wyman [31]
CS-Controlled pH	Spezyme cellulase CP	101.7	Kumar and Wyman [31]
CS-DA	Spezyme cellulase CP	90.7	Kumar and Wyman [31]
CS-Lime	Spezyme cellulase CP	133.6	Kumar and Wyman [31]
CS-SO <sub>2</sub>	Spezyme cellulase CP	124.8	Kumar and Wyman [31]
Eucalyptus-L	Cellulase ATCC2692	60	Ooshima et al. [79]
Eucalyptus-SEL	Cellulase ATCC2692	80	Nonaka et al. [78]

**Table 2:** The maximum cellulases adsorbed (Ads max) on lignin substrates. APCL: Dilute Acid Pretreated Corn Stover Lignin; SPCL: Steam Explosion Pretreated Corn Stover; SPRL: Steam Explosion Pretreated Rice Straw; CEL: Cellulolytic Enzyme Lignin; SEHW: Steam Exploded Hard Wood (Birch 90% + Maple 10%); Accellerase 1000, Celluclast, GC123: Commercial Cellulase Preparation from *T. reesei* produced by Du pont, and Gencore respectively; CS: Corn Stover; LHW-Wood: Lignin prepared by Liquid Hot Water (LHW) Pretreatment.

Similarly, Chang and Holtzapple [82] delignified Switch grass (original 22.3% lignin), Poplar wood (original 26.1% lignin) and Bagasse (original 24.2% lignin) and delignified lignocellulosic materials to varying lignin concentration by applying lime pretreatment [82]. On the basis of their results plotted they predicted that if lignin contents could be reduced to 15% of the original lignin concentration (i.e., 3.34%, 3.91% and 3.63%) in the tested substrates and an adsorption mixture of CBH I and  $\beta$ -glucosidase can produce

100% sugar conversion in 72 hours of hydrolysis. Exam reported that the ethanol yields increased with increased delignification [83]. It also stated that with decrease of the lignin contents of furfural residues from 43.46% to 8.7% that ethanol yield increased up to 75.6% of theoretical yield [84]. Therefore, removal of lignin to an optimum level increased the adsorption of cellulases onto the substrate because lignin adsorb cellulases. Very rare studies were found to compare cellulases (enzymes) adsorbed onto same type of substrates and under same experimental conditions.

### Accessibility of Cellulases to Lignocellulosic Substrates

Pretreatment of lignocellulosic materials modify the surface properties of a substrates and hence alters adsorption capacities of the substrate during hydrolysis. Cellulases showed a quick initial adsorption followed by increasing adsorption on the pretreated Douglas-fir (softwood) [48,85]. The adsorption capacity of the total proteins on the three cellulosic substrates was studied: i) These included Avicel PH101 (a microcrystalline cellulose), ii) SO<sub>2</sub> impregnated, steam-exploded Douglas-fir (DF), and iii) the water-insoluble fraction of the DF substrate obtained from hot alkali peroxide (DFP). The DF and DFP substrates demonstrated similar amount of cellulases adsorbed but they differed from that of Avicel (95 mg/g) and the maximum protein adsorbed was much higher (171.3 mg/g, 162.4 mg/g, respectively). While the initial adsorption/hydrolysis rate for the DFP was almost 3 times of the DP. On the contrary, Mooney et al. [86] found that a delignified refiner mechanical pulp had almost the same adsorption capacity as a regular refiner mechanical pulp [86]. On the other hand, during the study of pretreated lodgepole pine, it was observed that the adsorption remain at a constant level [87]. Cellulase adsorption onto ethanol pretreated lignocellulosic substrate (EPLP) was 87.69 mg/g and on steam exploded lodgepole pine (SELP) substrate, it was 101.05 mg/g with the affinities to substrates were 3.48 mL/mg and 1.48 mL/mg respectively. The cellulase adsorption onto corresponding isolated lignin showed a much lower adsorption capacity (2.73–10.20 mg/g) and higher affinity (3.21–6.44 mL/mg). It provided explanation of phenomena why lignin in the lignocellulosic substrates made a negative effect on the adsorption/hydrolysis because lignin has higher affinity for cellulose [54]. It was never tried to determine what features could give similar or different behavior of adsorption onto substrates prepared from different pretreatment technologies.

Some researchers claim that deacetylation had a higher impact on cellulose accessibility than delignification [19,88-93]. In order to evaluate impact of acetylation Kumar and Wyman [31] decaylated corn stover and CBH I cellulases were adsorbed on it and found that initial glucose and xylose release was increased by 65% and 74% respectively with respect to the non-deacetylated corn stover. While a mixture of CBH I and  $\beta$ -glucosidase was adsorbed on delignified corn stover an initial increase of 198% and 780% in glucose and xylose release was observed. Therefore, saying same thing in another way, it still means that it is the lignin whose removal increased access to cellulose. Chang and Holtzapple [16] concluded that extensive delignification was sufficient to obtain high conversion of substrates regardless of acetyl content and crystallinity. Complete lignin reduction invites an extra cost; therefore, it is not justified. These results indicate that a delignification treatment process should remove optimum level of lignin. The accessibility of substrate to adsorption/hydrolysis of proteins were studied [28,41,94] but not in combination, with the surface properties of pretreated lignocelluloses. The

experiments reported in order to measure adsorption experiments were rarely extended to measure surface area. A correlation between enzyme adsorption characteristics of the natural existing substrate, pretreated substrate and the substrate surface area may be developed. In order to compare adsorption of cellulases to the pretreated substrates under same experimental conditions, no data is available in the literature.

### Adsorption Isotherms

Adsorption is the ability of cellulases to stay on a substrate. The adsorption studies also provide insight about the structure of adsorbed layers, the type of interaction of cellulases with the substrates and, hence about desorption because desorption is dependent on surface coverage. 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 [95]. The adsorption isotherms have history for being used in representing adsorption of gases on liquids [96,97] and liquids on solid surfaces [98,99]. Klyozov [100] worked with cellulase preparations, among these 10 were highly purified cellulases suggested that there exist a strong co-relation between hydrolysis rate and the adsorption equilibrium constant of enzymes adsorbed [100]. Recently, some reports are available on cellulases adsorption on various lignocellulosic substrates, such as microcrystalline cellulose [40,46], corn stover [47], steam-exploded Douglas fir [48], pretreated hardwood [49], isolated lignin from softwood [50] and lignin preparations from lodgepole pine [51]. Peitersen et al. [45] determined that adsorption of cellulases onto microcrystalline cellulose can be represented by Langmuir model [45,101,102]. Some of the researchers have suggested Freundlich isotherm for adsorption of cellulases [51,53]. Few reports used both Langmuir and Freundlich isotherms to represent adsorption of cellulases [55-57]. Adsorption of cellulases on cellulose and lignin under similar experimental conditions was never studied although both will be present on lignocellulosic substrate at the same time. The information about adsorption of cellulases onto lignin is very rare. A dedicated study on the adsorption of cellulases on cellulose and lignin under similar conditions was required.

### Conditions of Adsorption

#### Effect of temperature

Temperature is one of the two principal adsorption environment parameters. Some results of influence of temperature on adsorption are presented in this section. Ooshima et al. [103] adsorbed cellulases on Avicel at 5°C and 50°C [103]. The amount of cellulases adsorbed was decreased with increase in temperature. Kim et al. [104] also adsorbed purified components (CBHs and EGs) of cellulases on Avicel PH 101 and CMC for the temperature range of 25°C to 45°C [104]. The amount of cellulase components adsorbed decreased with increasing temperature. Kyriacou et al. [105] studied adsorption of EG I, EG II, EG III and CBH I (components of cellulases) on Solka floc (purified wood cellulose) at temperature 5°C, 30°C and 50°C [105]. It was observed that increasing the temperature from 5°C to 50°C had a negligible effect on adsorption of CBH I. For EG I, increase in adsorption by 3 times of that of CBH I between temperature 30°C to 50°C. For EG II and EG III, the effect temperature was almost the same but less than other two components. Over all there was an increase in cellulases adsorbed with increase in temperature. Kim et al. [102]

adsorbed enzymes obtained from *T. viride* on to Sigmacell 20 and Sigmacell 50 and observed that with the increase in temperature, the adsorption capacity of Sigmacell (cellulose) was decreased [52]. As it was expected for Langmuir adsorption isotherms. The van der Waals forces between the proteins and cellulose were considered responsible for the adsorption, and leading towards an exothermic and enthalpy-controlled adsorption process. The effect of temperature on adsorption to lignin was inverse. It was an endothermic process which could be result of hydrophobic interactions between lignin and enzymes involved in the adsorption process [54,106,107]. The findings agreed with that of other researchers in stating that there was an entropy-dominant adsorption process [108,109]. Therefore, hydrophobic interaction between enzymes and lignin and the nature of the structure of lignin have impact on the adsorption capacities of lignin. Increasing temperature would also effect on protein structures and the hydrophobic regions of proteins would be more exposed to surroundings [110], and as a result, the adsorption capacity of lignin increased. Andreaus et al. [111] reported that hours thermal pretreatment of proteins (without a substrate) could only reduce activity of proteins slightly [111]. On the contrary, Baker et al. [112] investigated the effect of temperature on all the components of an enzyme-mixture and reported that activity of the components reached to a maximum with increase in temperature from 55°C to 60°C [112]. Tomme et al. [113] adsorbed CBHI and CBHII from *T. reesei* on Avicel from changing temperature from 5°C to 50°C and the difference in the amount adsorbed in both the extreme temperature was less than 10% [113]. Lee [99] adsorbed cellulases on Solka floc SW-40 and the profile of the adsorbed cellulase at 4°C were closely parallels to that at 50°C. Some reported only a small effect on the adsorption by changing the temperature from 4 to 50°C [73]. There were conflicting reports on the effect of temperature on the adsorption of cellulases some group of researchers said it increased with increasing temperature and other said it decreased with it and there are some who claim that temperature has insignificant effect on adsorption. Therefore, a detailed study to determine the effect of temperature on enzymatic adsorption onto cellulose, lignin and lignocellulosic materials should be conducted.

### Effect of pH

pH may cause surface charge leading to altered surface hydrophobicity. The change in surface charge may affect electrostatic interactions and hence, adsorption between components of a substrate and cellulases. The cellulases QM 9414 were adsorbed on Avicel PH 102, Solka Floc SW 20 (hammer milled spruce pulp-40 mesh), Sweco 270 (Ballmilled spruce pulp-270 mesh) at pH 3.8, pH 4.3, pH 4.8 and pH 5.0 at 30°C and observed that the adsorption of enzyme was largely independent of pH [45]. It was noted that the studies used a narrow range of pH. It adsorbed cellulases (CTec2) on four (4) different lignin residues of acid pretreated Lodgepole pine. It was observed that as the pH increased from 4.8 to 5.5, the adsorption of cellulase to lignin was decreased. The decreased adsorption of cellulase to lignin was attributed to enhanced electrostatic interactions at raised pH through the increased negative charges of cellulase enzymes. This study contradicts the well-established concept that the optimal pH is 4.8–8.5 for enzymatic hydrolysis using *Trichoderma reesei* cellulose [113]. Kyriacou et al. [105] adsorbed fractionated *T. reesei* cellulases (EG I, EG II, EG III, CBH I) onto Solka floc BW-40 and observed that the maximum adsorption at 5°C and 50°C was similar even though the pH increased from pH 3 to pH 7 with the exception of EG I at 50°C. Changes in adsorption were attributed to structural reformation of

enzymes due to charge distribution. The sharp increase in maximum cellulases (EG I) adsorbed at 50°C between pH 5.0 and 7.0 remained impenetrable [105]. Other groups of researches have reported a decrease in the degree of adsorption with increasing pH [114-116]. Tolan and Foody [116] studied adsorption of cellulases on cellulosic substrate and concluded that the curves of activity of enzymes vs pH were bell shaped and maximum was between pH 1 to pH 3. Moloney et al. [117] examined the effects of pH on the adsorption of *Talaromyces* cellulase by using Avicel and filter paper as the substrates. The maximum adsorption (around 50%) of *Talaromyces* cellulases on Avicel occurred at pH 4.2 While 35% of adsorption was observed at the filter-paper. Therefore, adsorption characteristics of cellulases were influenced by the nature of the substrate [117]. Criquet [118] investigated effect of pH on cellulases activity using two types of buffer currently used in studies of cellulolytic enzymes [118]. The use of Na-acetate buffer showed a single and maximum peak at pH 6.0. The cellulase activity of the enzyme solution showed two optimum values at pH 4.5 and 6.0. From literature survey, it was observed that cellulase activity measured using the citrate buffer was lower than with the acetate buffer [119-121]. Vasquez et al. [122] prepared substrate Cellulignin by acid treatment of sugarcane bagasse and used the substrate to evaluate effect of some factors including pH on adsorption. The pH values used were 5, 5.5, 6 and it was found that the effect of pH was negligible [122]. A big spectrum of pH range was rarely used and the effect of pH along with temperature needs to be addressed. We are suggesting that the activity of cellulases should be measured on a global buffer say acetate buffer or citrate buffer which may reflects the activities of various cellulase on one scale. It would not be surprising to find contradictory results on adsorption of cellulases in the literature, given that the experimental conditions, substrates, type of buffer used were not similar.

### Effect of product inhibition

A lignocellulosic substrate is inhibited by nonspecific (nonproductive) adsorption of cellulase tonon-cellulosic components (e.g. lignin, hemicellulose) [123-125] and also by the end product formed which reduces effectiveness of enzymes (cellulases). Additives were applied to address inhibition adsorbed lignin [126-130]. The use of additives at the suggested dosage was found expensive and may cause potential problems. Holdzapple adsorbed cellulases on a pretreated poplar wood and found that sugars (glucose,  $\delta$ -gluconolactone, and cellobiose) and solvents (ethanol, butanol, and acetone) produced during hydrolysis decrease adsorption and hence hydrolysis [131]. Zhang et al. [132] pretreated swollen cellulose and it was adsorbed/hydrolyzed by two *Thermomonospora fusca* endoglucanase, EG II and EG V at (3- to 4-fold) slower rate than untreated swollen cellulose [132]. Xiao et al. [133] attempted to determine the inhibition effect of end products (glucose) on two (2) types of substrates (Avicel and Acetic acid pretreated soft wood) during ethanol production by using a mixture of cellulases and  $\beta$ -glucosidase [133]. The increased glucose resulted in a dramatic decrease in the adsorption/hydrolysis which was reflect by a decrease in activities of both enzymes. Bezerra and Dias (2005) adsorbed crude cellulase and purified exoglucanase Cel7A on Avicel and carboxymethyl cellulose and let the hydrolysis complete. End products found were cellobiose and ethanol. Integrated Michaelis-Menten equations were used to determine inhibition. The calculated inhibition constants showed that cellobiose inhibited much more than ethanol using exoglucanase (1.6  $\times$  10<sup>15</sup> mM, 0.035 mM) and with the crude enzyme (151.9 mM, 0.05 mM) as well. Among the cellulose degrading enzymes, beta-glucosidases are essential for efficient

hydrolysis of cellulosic biomass as they relieve the inhibition of the cellobiohydrolases and endoglucanases by reducing cellobiose accumulation [134]. Zhao et al. [135] through their adsorption/hydrolysis studies suggested the use of glucose tolerant non-cellulolytic  $\beta$ -glucosidase enzyme which will decrease inhibition caused by cellobiose and tolerate glucose [135]. Qing et al. [136] studied adsorption/hydrolysis of cellulases on Avicel PH 101 and that xylooligomers were more powerful inhibitors than glucose and cellobiose [136]. Cellulose accessibility and degree of adsorption of cellulases onto cellulosic substrates are controlling factors for conversion of cellulose and yields of production of glucose and bioethanol [19,137]. Based on the above, the use of a pretreated substrate,  $\beta$ -glucosidase should be used to impair substrate accessibility by inhibition.

### Effect of E/S

In adsorption studies a very crucial parameter is an enzyme/substrate (E/S) ratio. According to survey there exist a linear correlation between adsorption/hydrolysis rate and enzyme to substrate ratio. Bernardez et al. [49] adsorbed cellulases (obtained from *Clostridium thermocellum* strain ATCC 27405) on Avicel 105 and on pretreated mixed wood (90% Birch wood, 10% Maple) and studied the effect of enzyme to substrate ratio on adsorption. At low E/S ratios, the results of experiments showed a linear trend of adsorbed enzyme with respect to the initial enzyme concentration. At higher E/S ratios the adsorbed enzyme leveled off due to the saturation of the cellulosic substrate with enzyme. For PTW 220, as a lignin substrate the cellulases adsorbed in relation to increasing concentrations of pretreated mixed wood of 0 to 0.5 g/L and an enzyme activity of 95 to 100 U/L. Saturation was achieved around 0.1 g/L of cellulases concentration. Comparison of the results indicated that, 30-fold fewer enzymes were required to saturate Avicel than for PTW220 [49]. This comparison was indicative of the profoundly greater capacity of lignin to adsorb cellulase. Sattler et al. [138] studied the effect of enzyme (cellulases) concentration on adsorption/hydrolysis of Sigmacell 50 and pretreated poplar. The substrate concentration was 20 g/L while enzyme concentration varied from 5 FPU to 100 FPU/g [138]. It was observed that increase in cellulase loading increased adsorption for all enzyme concentrations. Moldes et al. [139] used delignified Eucalyptus globulus wood chips to study effect of enzyme to substrate ratio on adsorption and found that for low concentration 13 FPU/g 39% adsorption/hydrolysis was achieved when it was increased to 28 FPU/g 53.5% of adsorption/hydrolysis was observed [139]. The subjected acid treated spruce wood chips were to adsorption by the amounts of Celluclast 2 L added were 4%, 8%, 16%, and 24% w/w DM, corresponding to cellulase activities of 5, 10, 21, and 32 FPU/g cellulose, respectively. The whole slurry from the pretreatment stage, diluted to different dry weights of solid material (2%,16%,5%,7.5%,and 10%), was used as substrate. It was observed that maximum conversion of substrate was achieved with cellulase activities from 4 to 7 g/L for % substrate concentration. For 5% substrate concentration, the time to reach maximum adsorption/hydrolysis increased and this concentration was found optimal. A low concentration of substrate formed byproducts and high concentration was not suitable for downstream process (i.e. fermentation). At 7.5% substrate, the inhibition reduced the productivity whereas the final ethanol yield varied randomly. And the results of 10% substrate showed no fermentation at all. This is contrary to observations in previous separate hydrolysis and fermentation experiments at concentrations comparable to the SSF with 10% dry material [140]. The inhibitors

formed in the pretreatment of substrate were likely to limit the substrate concentration unless substrate were washed. Vasquez et al. [122] prepared substrate Cellulignin G by acid treatment of sugarcane bagasse and used this substrate to evaluate enzyme/substrate ratio for enzyme loading of 5.0, 17.5 and 30.0 FPU/g Cellulignin as substrate with concentration of 2.0%, 6.0% and 10.0%. The conditions of hydrolysis that yielded the highest glucose concentration, 58.40 g/L, were: temperature 47°C, enzyme loading 25.6 FPU/g Cellulignin G, and 10.0% substrate. Enzymes accessed to Cellulignin G and converted it to glucose, the highest value, 67.0%, was obtained when enzyme loading 24.4 FPU/g Cellulignin G, and solid percentage 2.0% [122]. Medve et al. [46] adsorbed CBH I and CBH II from *T. reesei* onto Avicel at various E/S ratios. It was observed that at E/S > 0.2, the adsorption of CBH I was decreased by up to 23% in the presence of equimolar amount of CBH II. The adsorption of CBH II was decreased by up to 45 % (at E/S = 2.56  $\mu$ mol/g) by competition from CBH I. At low saturation (E/S < 0.2  $\mu$ mol/g) the results indicated that the presence of the other enzyme has very little effect in this concentration range [46]. Van conducted adsorption/hydrolysis of ball milled Spruce wood pulps with varying concentrations of cellulases from (*T. viride*) and found that rate of adsorption/hydrolysis increased with initial substrate concentration until an optimum is reached whereupon it decreased. Overall rate of adsorption hydrolysis was dependent on concentration of endoglucanase at optimum substrate concentration. The decrease in hydrolysis was due to the inaccessibility of enzymes to adsorb onto the substrates [62]. Howell and Struck [141] studied adsorption/hydrolysis of cellulases QM9123 from *T. viride* on Solka Flocc (BW200) and reported a decrease of adsorption/hydrolysis rate at high substrate concentration [141]. For a fixed enzyme concentration when substrate concentration were increased the accessibility of enzymes 30 to adsorb decreased and hence the hydrolysis [142,143].

Wang et al. [144] investigated the adsorption of enzymes (cellulases from *T. reesei*), affectivity of the enzymes and concentration of substrate (Avicel PH 101). The enzyme concentration was increased equivalently with the substrate concentration and it was observed that the glucose yield decreased with the increased substrate concentration. The mechanism causing the glucose yield decreased at high substrate loading in enzymatic adsorption/hydrolysis of cellulose is yet to explore [144]. Similar results were reported by Triwahyuni et al. [145] and they also achieved maximum ethanol yield by adding substrate slowly and gradually (instead of filling all substrate in the stat). The efficiency of enzymatic digestibility and fermentation is significantly reduced at high concentrations of pretreated biomass, as mixing becomes difficult with increased viscosity [145]. For adsorption reactions, the rate of adsorption increased with initial substrate concentration until an optimum is reached whereupon it decreased. The increase in enzyme (cellulase) loading increased adsorption for all enzyme concentrations till the optimum value. For a fixed enzyme concentration when substrate concentration were increased the accessibility of enzymes to adsorb decreased and hence the adsorption. For a substrate to use an optimum E/S ratio should be determined under the given conditions and such a data bank is not available.

### Effect of enzymes synergy

Synergism of cellulase systems is a phenomenon when collective activity of the components of system exhibit higher activity than the sum of the activities of individual cellulase components. There are four (4) reported forms of synergism in literature: i) between endoglucanases and exoglucanases (endo-exo), ii) between exoglucanases processing cellulose from the reducing andnon-

reducing ends (exo-exo), iii) between exoglucanases and  $\beta$ -glucosidases that remove cellobiose (and cellobioses), and iv) between catalytic domains and carbohydrate-binding modules. Henrissat et al. (1985) studied the action of cellobiohydrolases (CBH I and CBH II) and endoglucanases (EG I and EG II) on various substrates and found that synergy between endoglucanases and CBH II follow the pattern of endo-exo cooperation [34]. The endoglucanase (Endo I, 11,111, IV, V, and VI) were adsorbed on Avicel along with CBH II by Beldman et al. [146]. The results showed that initial endoglucanase to exoglucanases ratio were dependent upon the adsorption of these enzymes to conduct hydrolysis of Avicel. Endo I, Endo III, and Endo V, adsorbed strongly on Avicel, proved the maximal synergistic effect with Exo III (adsorbed strongly on Avicel as well). The initial endoglucanase-to-exoglucanase ratio was much lower than these were used in incubations where Endo II, Endo IV, and Endo VI were used. The endo-exo synergy examined by a number of researchers and it was observed that: i) The degree of synergy (DS) was increases significantly with increasing degree of polymerization of substrate [147], as accessibility of enzymes increased [34,147], and as enzymes loading increased [147-149]. It is an endo-exo mechanism degrades the amorphous region of cellulose [150]. Tomme et al. [113] found that CBH I and CBH II adsorbed strongly on Avicel and equilibrium reached very slow (more than 40 min) and the synergistic effects were dependent on the ratio of the enzymes [113]. In order to obtain maximally synergistic effects, both enzymes were suggested to be added simultaneously and in non-saturating concentrations. Ikeo et al. [151] investigated the mechanism of the exo-exo synergism between CBH I and CBH II by comparing the characteristics of enzyme adsorption and desorption to the cellulose under agitated and static conditions [151]. Under both conditions CBH I and CBH II adsorbed 100% and 80% respectively. In adsorption study of cellulases to cellulose between different cellulase fractions, more adsorption was observed when both CBHs were present. Under agitation CBH I and CBH II adsorbed quickly and get deactivated which indicated that CBHs was deactivated by shear stress from agitation. Under static conditions at the end of hydrolysis 10-20% of CBH I and CBH II were found inactive. CBHs from family can occasionally transform to have some endo-character (never demonstrated for family CBHs), for the altered character a mechanism for endo-exo synergism was proposed also to be responsible for synergism between different CBHs [152-154]. For hydrolysis of lignocellulosic substrate the problem is enzyme inhibiting end-product (cellobiose). To overcome end-product inhibition the addition of  $\beta$ -glucosidase is needed and  $\beta$ -glucosidases work efficiently on cellulolytic residues in a synergistic manner and produce glucose. Activity of  $\beta$ -glucosidase can be increased by using a thermo-tolerant CBH I [155]. Anderson et al. [156] reported synergistic effect by a mixture of Cel 45A, Cel 6A (an endoglucanase and an exoglucanase respectively from *Humicola insolens*) and  $\beta$ -glucosidase from *Penicillium brasilianum* on amorphous cellulose [156]. Some reports has shown a competition in adsorption to cellulose between different cellulase fractions. On the crystalline region of cellulose, these enzymes inhibit each other due to the competition between them for binding sites on cellulose. The investigated the adsorption characteristics of families Cel 7A and Cel 6A, and attempted to elucidate the mode of action of between catalytic domains and carbohydrate-binding modules in synergy [157]. The adsorption affinity of the cellulase binding domain (CBD) for Cel 7A are larger than of Cel 6A, hence, CBD of Cel7A binds more rapidly and tightly to Avicel than that of Cel6A. Warner et al. [158] studied substrate binding of a cellulose binding module (CBM) and catalyticdomain (CD) from *Ruminococcus flavefaciens* (a glycoside

hydrolase family 44 xyloglucanase/endoglucanase), both were quantified when binding various  $\beta$ -1,4-linked glucans and xylans when separated and attached. It was found that the CBM was adsorbed on cello-oligosaccharides (xylo-tetraose, xylo-pentaose, or xylo-hexaose). The CD appeared to adsorb carboxymethylcellulose (CMC) and onto xylan very weakly, while the CBM and the CD/CBM adsorbed much more strongly. A synergistic effect was observed for the adsorption of cello-pentaose and cello-hexaose with the CD/CBM as compared to the separated CD and CBM alone [158]. Synergism is hindered at high enzyme concentrations due to saturation of the adsorption sites, therefore, an increase in synergistic action with increased enzyme/substrate ratio was not found. The mechanistic cause of the observed synergism is still unknown. The exo-exo, endo-exo, endo- $\beta$  glucosidase and intra molecular synergies were found, however, synergism between endoglucanases has not been clearly demonstrated till now. A reason could be that natural lignocellulosic substrates are not used commonly due to their heterogeneous nature and the performance of individual endoglucanases were not observed on natural substrates.

## Conclusion

A number of studies have emphasized on the importance of cellulose accessibility during adsorption and most of the studies have used a small number of samples or pure cellulosic substrates, which do not represent the natural heterogeneous, lignocellulosic materials.

External surface area plays small role in adsorption and similarly, not all internal area is good for adsorption. The only area is accessible which is wider than the size of an enzyme. The accessibility (available surface area) is determined by practical adsorption of enzymes (cellulases) on the substrates. The maximum adsorbed amounts on the substrates were used to determine accessibility of cellulases with the help of adsorption isotherm. The relationship between the accessibility and cellulose conversion is sometimes measured yet it was contradictory and in many cases inadequate.

In order to achieve efficient adsorption onto lignocellulosic substrates an optimum level of lignin removal is required. It appeared that accessible surface area of cellulose in pretreated substrate require lower enzyme levels to attain high conversions. The amount of enzymes required for a known level of lignin removed from lignocellulose substrates under given conditions need to be worked on.

There are conflicting reports about the effect of temperature therefore, for a test substrate the optimum temperature under the given condition is recommended to determine. Similarly, a narrow slit of the effect of pH was studied, and the effect of pH along with the temperature was rarely studied.

Regardless of significant differences in the origin, structure and chemical composition of the substrates and the pretreatment process used, the optimum E/S is required for efficient adsorption of pretreated lignocellulosic substrates. A data bank should be developed for E/S ratios for components of lignocellulosic materials and lignocellulosic materials as well.

The end-product inhibition can be controlled by using modified glucose tolerant  $\beta$ -glucosidase in an optimum amount. The acceptable glucose (end product) level for each lignocellulosic material (which can be used for bioethanol production) should be determined.

Enzymes perform their action in synergy and synergism is obstructed at high enzyme concentrations due to saturation of the adsorption sites. An optimum concentration of enzyme components to

convert a corresponding lignocellulosic material to reducing sugars should be determined. Working at low E/S is suggested to have more synergy among enzymes. Such strategic information is difficult to find in published literature, therefore, a data bank should be developed.

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