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Parameter Optimization of Enzyme Saccharification for low Lignin High Biomass Sorghum (CSV 15 X IS 21891)-1-1-1-1 and Estimation of Process Kinetics

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Abstract*: Optimization of Enzymatic saccharification conditions for acid treated bmr sorghum biomass was studied with the inhouse enzyme produced from Aspergillus sp.. Optimization of enzyme loading, substrate concentration, temperature and time yielded a maximum of 416.00 ± 0.55 mg/g sugars with an improved hydrolysis efficiency of 55.97 ± 0.45%. Protein adsorption onto lignocellulosic substance was investigated at pH 4.8 and at two different temperatures (4°C and 45°C) with the produced cellulase enzyme and compared with the commercial cellulase. The maximum adsorption capacities, the affinity constants and the binding strengths were more for sorghum biomass as compared to cellulose powder.*
Key words: *Enzyme Saccharification, Aspergillus sp., bmr Sorghum Biomass, Langmuir*

I. INTRODUCTION

The use of biofuel in replacement of gasoline due to consequences of rising oil prices, diminishing global oil reserves and concern about the fuel emissions and also the Kyoto protocol made by D3 countries to reduce fossil fuel usage & Bali action plan on the carbon emissions made to search for alternative to fossil fuels. However, the transition from petroleum to a biomass-based economy is not easily accomplished (Dahai Gaoa et al 2013). Biomass recalcitrance to biological conversion is one of the major hindrances to the production of cheap biofuel (Chundawat SPS et al 2011). Lignocellulose is the most abundant renewable material on earth (Perlack et al. 2005). Proper deconstruction or fractionation of cell wall components can facilitate the development of a variety of high value materials (Ingrid C. Hoeger et al 2013). Structural carbohydrates like Cellulose (a ß-1, 4-glucose polymer) are recalcitrant to enzymatic hydrolysis because of its highly self-associated (through hydrogen bonding and stacking forces) and microfibrillar nature. It is hydrolyzed by an array of cellulase enzymes that can be endo- (endoglucanase I or EG-I; also known as Cel7B) or exoactive (cellobiohydrolases I and II or CBH-I and CBH-II, respectively; also known as Cel7A and Cel6A, respectively) are the two major components of aerobic fungal secretomes (e.g., like *Trichoderma reesei*, *Aspergillus niger*) etc (Dahai Gaoa et al 2013s). However, the inherent disadvantages of processivity to polysaccharide hydrolysis and the high abundance of processive enzymes necessary for efficient lignocellulose hydrolysis suggest the need to better understand and eventually overcome the factors contributing to biomass recalcitrance. A pretreatment step such as dilute acid, ammonia, alkaline green, etc., is required to remove the natural resistance of lignocellulose cell wall to microbial deconstruction recalcitrance for efficient enzymatic saccharification (Ingrid C. Hoeger et al 2013). The purpose of the pretreatment is to remove lignin and hemicellulose, reduce cellulose crystallinity, and increase the porosity of the materials.

There are various factors, which affect the enzymatic hydrolysis rate such as substrate concentration, cellulase activity and reaction conditions (temperature, pH, as well as other parameters). The enzymatic hydrolysis rate can be improved by optimizing these conditions (Sun and Cheng, 2002). Our major focus of this study is to optimize the enzymatic saccharification conditions to enhance the enzymatic hydrolysis rate and sugar production from *bmr* sorghum biomass. The maximum amount of protein that can be adsorbed during enzymatic saccharification of cellulose to glucose is a controlling factor for hydrolysis rates and directly depends on enzyme accessibility to active sites on the solid substrate (Zhang YH et al 2006, Nidetzky B et al 1994, Lee Y-H 1982, Hongan CM et al 1990, Zhang YH et al 2005). Little information is available on substrate–enzyme interactions, including cellulase adsorption kinetics and the accessibility of enzyme to lignin and cellulose in pretreated biomass. Among the primary substrate features impacting enzymatic conversion of biomass, lignin is believed to impede enzyme access to glucan chains by its protective sheathing and also reduce cellulase effectiveness as a result of unproductive binding and steric hindrance (Chang and Holtzapple, 2000; Mansfield et al., 1999). In this study, cellulase adsorption capacities were determined at 4 and 50°C for pretreated *bmr* sorghum biomass and cellulose powder.

II. MATERIALS AND METHODS

2.1 Pretreatment Of The Sorghum Biomass

Lignocellulosic feedstock used in the study was sorghum biomass and pretreated by dilute sulphuric acid (**Table 1**). Sorghum biomass was subjected to acid hydrolysis with 0.45% (v/v) sulfuric acid at 120°C for 20 min. The contents were filtered and the residue was washed with the water till it reaches pH-7 and stored at 4°C and used for enzyme hydrolysis experiments. The chemical composition of the pretreated materials was determined according to a standard method (T222 om-88; Technical Association of the Pulp and Paper Industry). All experiments in the study were performed in triplicate and mean of the experimental values were taken.

2.2 Source Of Cellulase Enzyme For Enzymatic Saccharification

The growth of *Aspergillus sp.* and enzyme production under submerged fermentation conditions by using sorghum biomass as carbon source was carried out at 30 °C. The extracellular enzyme used was extracted by centrifuging the fermented broth on 3rd day of cultivation. Cellulase activity (FPase) was analyzed on filter paper, according to Ghose (1987). One unit of enzyme corresponds to the amount of enzyme necessary to form 1 µmol of glucose per ml per minute. The reducing sugars were measured by the dinitrosalicylic acid (DNS) method according to Miller (1959). The maximum of 5 IU/ml Fpase activity and 1.2 mg/ml of protein was observed in crude enzyme under the optimum conditions. The saccharification of the inhouse enzyme was compared with the commercial Dyadic cellulase 1.5L (14,000 IU/gm; protein content 400 mg / g of cellulase powder); Protein concentrations were determined using Lowry method.

2.3 Enzymatic Hydrolysis

The enzymatic hydrolysis experiments were carried out in 100 ml flasks with a working volume of 20 ml. Acid treated sorghum biomass and cellulose powder derived from cotton were hydrolyzed with 20 FPU/g of crude cellulase in 0.5 M citrate buffer (pH 4.8) at a substrate concentration of 12% (w/v). The substrates were soaked in buffer for 2 h before adding the enzyme. Sodium-azide (0.05%) was added to the reaction mixture to prevent microbial or fungal contamination. The flasks were incubated at 50 °C on an orbital shaker at 150 rpm for 96 h. Sample aliquots of 1 ml were taken at different times, centrifuged and the supernatants were analyzed for reducing sugars to determine the percentage of hydrolysis (% saccharification).

$$\% \text{ Saccharification} = \frac{Reducing\ sugars\ in\ enzyme\ hydrolyzate}{total\ holocellulose\ in\ pretreated\ sample}$$

2.4 Parametric Optimization of Enzyme Sacchrification Conditions on Biomass

Various parameters such as hydrolysis time, temperature, enzyme loading and substrate concentrations were studied to find out the best hydrolyzing conditions for sorghum biomass using cellulase enzyme produced by *Aspergillus sp.*. The optimum condition obtained from each experiment was used in the next optimization study unless otherwise stated. The optimum saccharification time was determined by withdrawing the samples at 8, 16, 24, 32, 36, 48, 72, and 96 h of enzymatic hydrolysis and maximum hydrolysis (42.5%) was achieved at 48 h. Further optimization studies were carried out for 48h of saccharification time. Five different solid loadings of 8, 10, 12, 14, and 20% were investigated in the batch enzymatic hydrolysis to know the effect of biomass loading on enzyme hydrolysis. Various cellulase concentrations of 5, 15, 20, 25, 30 FPU/g of pretreated biomass was tested to know the optimum enzyme concentration of saccharification. To determine the optimum temperature of saccharification, the reaction mixture was incubated at different temperatures ranging from 25°C to 65°C. The enzymatic hydrolysis studies of pretreated sorghum biomass vice versa cellulose powder derived from cotton was carried out at optimum conditions and the same was compared with the commercial enzyme.

2.5 Studies of Enzyme Adsorption on Biomass

The adsorption of the cellulase protein onto the acid treated sorghum biomass was studied and compared against cellulose powder. The adsorption studies were performed in polypropylene tubes (2 mL) using 12% (w/v) sorghum biomass and 20 FPU/gm of biomass in 0.5 M citrate buffer (pH 4.8). In order to study the effect of temperature on the enzyme adsorption, incubations of reaction mixtures were carried out for 48 h at both 4 °C and 50°C in a rotary shaker. Aliquots withdrawn at various time intervals were centrifuged (10,000g, 10 min) and the liquid containing unbound enzymes was collected (adsorption supernatant) for further analysis. The adsorbed cellulase protein concentration was calculated as the difference between initial (blanks) and unbound protein concentration.

2.6 Langmuir adsorption studies

The adsorption of cellulase enzyme on both the substrates were studied by using the Langmuir adsorption isotherm at various concentrations (10–100 mg/ml) of protein with the 1.2 gm of acid pretreated

biomass suspended in 20 ml of 0.5 M citrate buffer (pH 4.8). The crude enzyme produced from *Aspergillus sp.* was concentrated for the Langmuir adsorption studies by freeze drying. The adsorption parameters of maximum adsorption capacity (P_{max}) and the equilibrium constant (K_d) were determined by non-linear regression using MATLAB software of the adsorption data using below equation Eq.

$$P = \frac{P_{max} . E}{K_d + E}$$

Where, P denotes the amount of adsorbed protein (mg of protein/g cellulose); P_{max}, the maximum protein adsorption at equilibrium (mg of protein/g cellulose); E, the free cellulase concentration (mg of protein/gm of cellulose); and K_d, the dissociation constant (mg/ml).

III. RESULTS AND DISCUSSION

3.1 Pretreatment of sorghum biomass

The composition of sorghum biomass was found to contain (36.08 ± 0.78% cellulose, 30.01 ± 0.64% of hemicellulose, 12.01 ± 0.9% of lignin on dry solid (DS) basis. The presence of lignin in cellulosic substrates hinders the saccharification of them into its constituent monomeric sugars (Vimala Rodhe et al 2011). Therefore, to overcome the lignin barrier lignocelluloses are usually perpetrated to remove lignin and hemicellulose by dilute acid pretrement for easy access of the cellulase enzyme. The chemical composition of raw and acid treated sorghum biomass was shown in **Table 1**. As shown in table cellulose a polymer of glucose was the major component followed by hemicellulose. Acid pretreatment of sorghum biomass removed most of hemicelluloses portion leaving cellulose and some amount of lignin in the residual biomass (**Table 1**).

3.2 Effect of Saccharifiation time on sugar production

For the improvement of enzymatic hydrolysis rate, it is necessary to optimize the critical process parameters such as optimum cellulase loading, temperature, saccharification time and substrate to liquid ratio etc. Enzymatic saccharification of the acid pretreated sorghum biomass into glucose was optimized using the enzyme supernatant of *Aspergillus sp.* These process parameters play a crucial role in enzymatic hydrolysis of lignocelluloses to get satisfactory yield of monomeric sugars (Vimala Rhode et al 2011). Optimization of time of enzymatic hydrolysis results showed that maximum sugars were released after 48 h showing the hydrolytic efficiency of 52.8% with 386.5 ± 0.5 mg/g sugars at 50°C (**Figure 1**). The enzymatic hydrolysis of acid treated sorghum biomass at biomass concentration of 12% w/v initially proceeded very rapidly up to 46 % till 48 hours. Afterwards there was no significant increase in saccharification approaching 53% when reaction was carried out at 50 °C for 72 hours as reported in **Figure 1**.

3.3 Effect of hydrolysis time and solid loading on the enzymatic hydrolysis of pretreated sorghum biomass

It can be seen in **Figure 2** that percent saccharification of sorghum biomass obtained was 65.7, 54.3, 53.6, 50.02 and 46.5%, when 8,10,12,14 and 16% of acid treated sorghum biomass was used at 48 hours of incubation period. It was observed that the amount of reducing sugars formed increased with increasing the substrate concentration (M.S.Akhtar et al, 2001). It was found that the amount of reducing sugars released increased with increasing substrate concentration while the percent conversion was reduced (Alrumman et al, 2016).

3.4 Effect of cellulase concentration on the enzymatic hydrolysis of pretreated sorghum biomass

It was found that the increase in enzyme concentration from 10-15 IU in the reaction mixture containing sorghum biomass as a carbon source resulted in 2 fold increase in percent saccharification (**Figure 3**). Further 2.7 fold increase was observed by further increasing the amount of enzyme, i.e., 15 to 20 IU. one fold increase in saccharification of 1.2g pretreated biomass occurred, respectively with increasing the amount of enzyme from 20 to 25U in 20 ml reaction mixture after 48 hours of incubation period.

3.5 Effect of Temperature on enzymatic hydrolysis

Figure 4 shows the effect of temperature on saccharification. Maximum hydrolysis of acid treated substrates occurred at 50°C that correspond to degree of saccharification of 55.9% shown in **Figure 4**. At 30, 40 & 60°C the degree of hydrolysis of sorghum biomass were 29%, 43.7%, and 45.05% respectively, which were considerably lower than that what occurred at 50^{o}C.

3.6 Enzymatic hydrolysis at optimum saccharification conditions

Enzymatic hydrolysis of acid pretreated sorghum biomass to reducing sugar was carried for the initial kinetics and cellulose digestibility. **Figure 5** shows total reducing sugar production for pretreated sorghum biomass at enzyme loading of 20 FPU/gm of biomass in comparison with cellulose powder. Taking into account the hydrolysis reaction stoichiometry, 1 g of cellulose upon complete hydrolysis produces 1.11 g of glucose (Dadi et al., 2006). Digestibility of dilute acid pretreated sorghum biomass reached 55.97 ± 0.45% (416.00 ± 0.55 mg of sugar/gm of biomass) where as the cellulose powder has 59.5 ± 0.50% (442.23 ± 0.50% mg of sugar / gm of cellulose powder) saccharification efficiency. The initial rates of hydrolysis to soluble reducing sugar are shown in **Table 2**. The saccharification rate of pretreated sorghum biomass was almost similar when compared to cellulose powder in presence of in-house enzyme. Cellulase produced on sorghum biomass was used for the hydrolysis of acid pretreated sorghum and has given good saccharification efficiency in comparison with commercial cellulase enzyme. The results of the hydrolysis were compared with the commercial dyadic enzyme (**Figure 5**.). Hydrolysis conversions obtained after 48hr of hydrolysis are presented in **Figure 5**. It could be seen that the enzyme produced on the same substrate could degrade the pretreated sorghum biomass more than the commercial enzymes.

3.7 Langmuir adsorption isotherms

Enzyme adsorption kinetics was studied using acid pretreated sorghum and cellulose derived from cotton at 4 and 50°C. The adsorption profiles of cellulase produced from *Aspergillus sp.* was determined during a period of 72 h (**Figure 6**). The adsorption parameters maximum adsorption capacity [P_{max}] and equilibrium constant [K_d] were determined by non-linear regression of the adsorption data to the Langmuir expression (Kumar and Wyman 2009) as Eq. 1, using MATLAB software (ver 10.0, SPSS Inc., Chicago) (**Table 2**). Time course studies of enzyme adsorption were conducted to investigate the time when equilibrium was reached. Equilibrium for adsorption was reached after 2 h, hence this time period was chosen for further adsorption studies (**Figure 6**). The time period required to attain equilibrium during adsorption varies according to the biomass, the protein, and the incubation conditions (Nidhi Pareek et al 2013). It is apparent that the acid treated substrate had the highest adsorption of cellulases than cellulose powder (**Table 2**). This was in agreement with the enzymatic digestibility in Fig. 5. The observation suggested that more protein adsorbed on substrate do not always mean a faster and better cellulose hydrolysis (Zhang et al 2013). If most of the enzymes were adsorbed nonproductively on lignin not productively on cellulose, the adsorption did not benefit the cellulose hydrolysis. The difference in substrates adsorbing enzymes was probably attributed to the affinity of lignin to cellulases. The effect of temperature on enzyme adsorption was evaluated by performing the adsorption experiments at 4 °C and 45 ° C. Similar adsorption profiles were observed at both temperatures, but generally the fraction of protein that adsorbed to the substances was higher at 45 °C than at 4 ° C (**Figure 7**). The increase in adsorption with corresponding increase in temperature might be due to increased hydrophobic interactions, which are believed to play an important role in protein adsorption onto substrate (Nidhi Pareek et al 2013).

The adsorption parameters were determined using Langmuir adsorption isotherm equation; the plot of P/Pad vs. P (free protein concentration) gave a fairly straight line which indicates that the adsorption of cellulase enzyme on pretreated sorghum and cellulose powder followed the Langmuir adsorption isotherm (**Figure 8**). The equilibrium constant (K_d) and maximum adsorption capacities were shown in **Table 2**. The higher value of equilibrium constant is indicative of higher binding affinity of the enzyme for the specific substrate and the value of P_{max} obtained is 86.24 and mg/ml for sorghum biomass at 50°C , the binding capacity provide information concerning the maximum number of sites that are available for adsorption (Xiaoran Shi et al 2014).

IV. CONCLUSION

The present study was aimed at enzymatic hydrolysis of *bmr* sorghum biomass using crude cellulase produced by *Aspergillus sp.* When acid treated sorghum biomass was subjected to enzymatic hydrolysis with cellulase enzyme, 416.00 ± 0.55 mg/g of sugars were recovered after optimization of enzyme loading, temperature, time and substrate to liquid ratio. As compared to the commercial enzyme, the crude cellulase enzyme has maximum saccharification rate (55.97 ± 0.45%) and high sugars were yielded as compared to cellulose powder. The Langmuir adsorption kinetic studies showed that the sorghum biomass has maximum adsorption capacity (94.13 mg/g of biomass) compared to cellulose powder (64.69 mg/g of biomass) at 50°C with cellulase enzyme produced from the *Aspergillus sp.* The result clearly indicates that the sorghum biomass is a suitable material with the maximum sugar which can be used for bioethanol production.

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Table1: Composition of Sorghum biomass

% Component (w/w)	Raw Biomass	Pretreated biomass
Hemi Cellulose	30.01 ± 0.64	7.8
Cellulose	36.08 ± 0.78	54.9
Lignin	12.01 ± 0.9 %	10.2

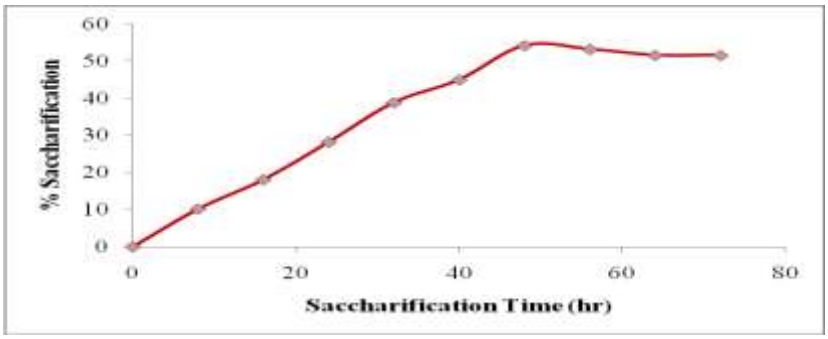


Figure 1: Saccharification time optimization for acid treated sorghum biomass

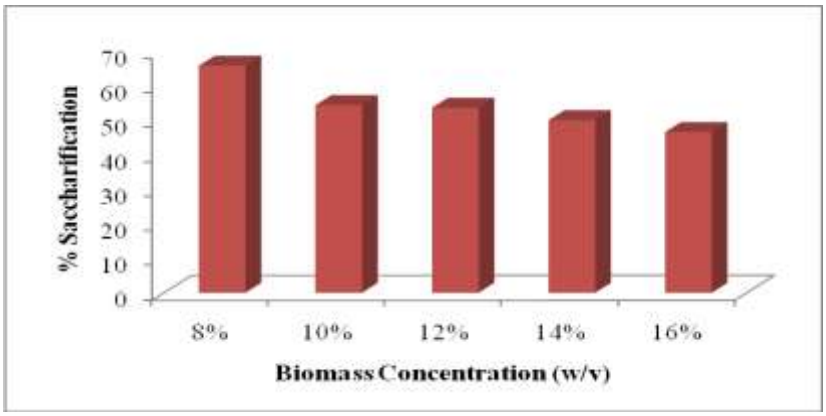


Figure 2: Optimization of solid to liquid ratio for acid treated sorghum biomass

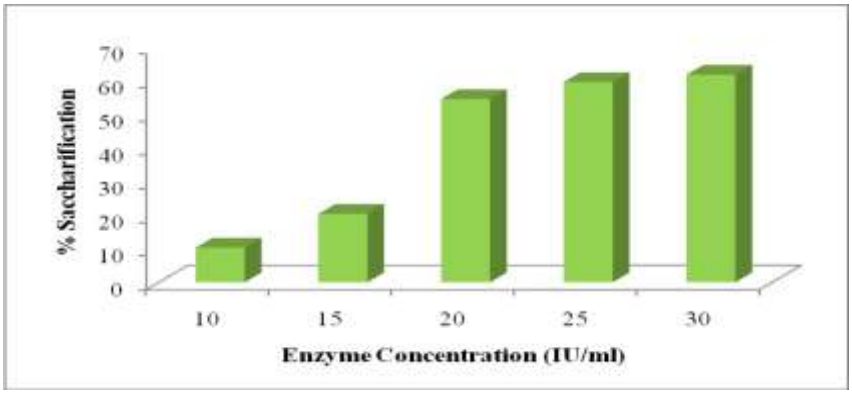


Figure 3: Optimization of enzyme concentration for acid treated sorghum biomass

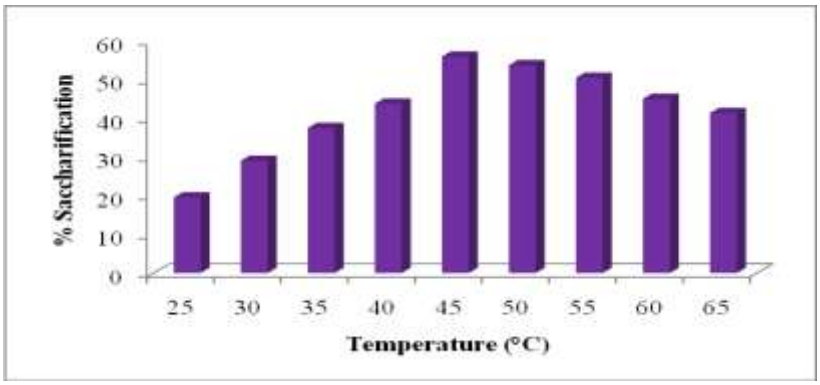


Figure 4: Optimization of Saccahrification temperature for acid treated sorghum biomass

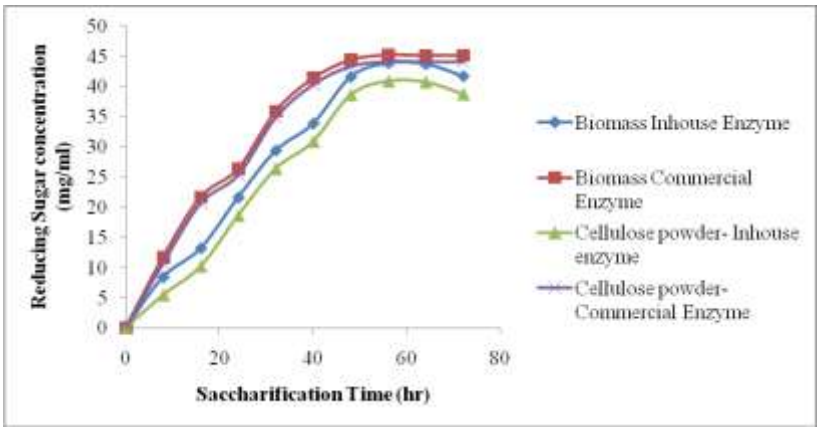


Figure 5: Optimization of acid treated sorghum biomass under optimized ondtions

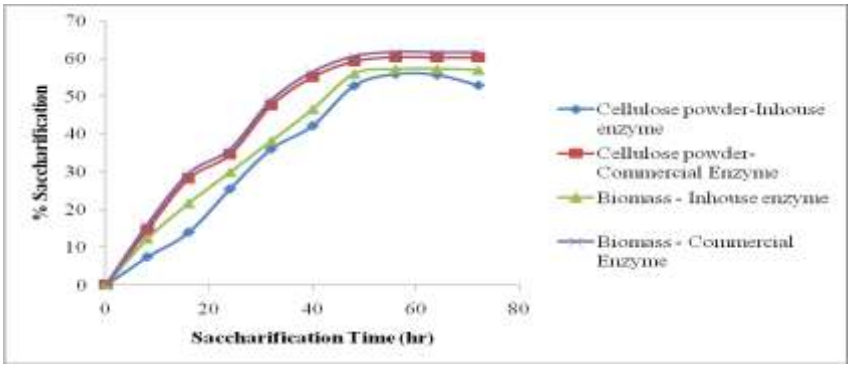


Figure 5: Optimization of acid treated sorghum biomass under optimized condtions

Adsorption Kinetics

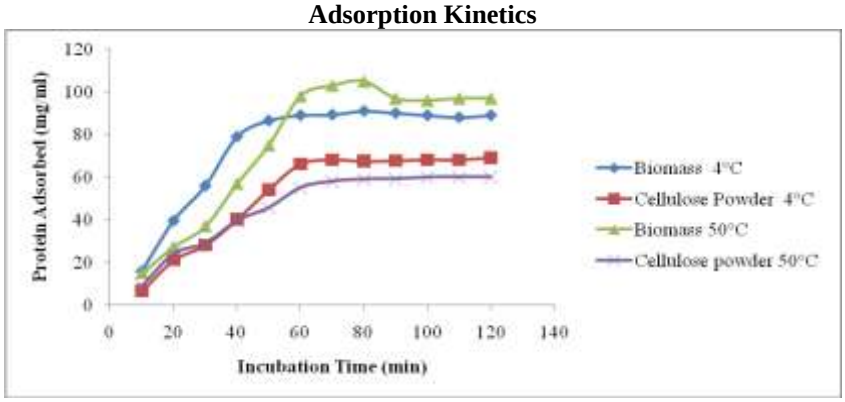
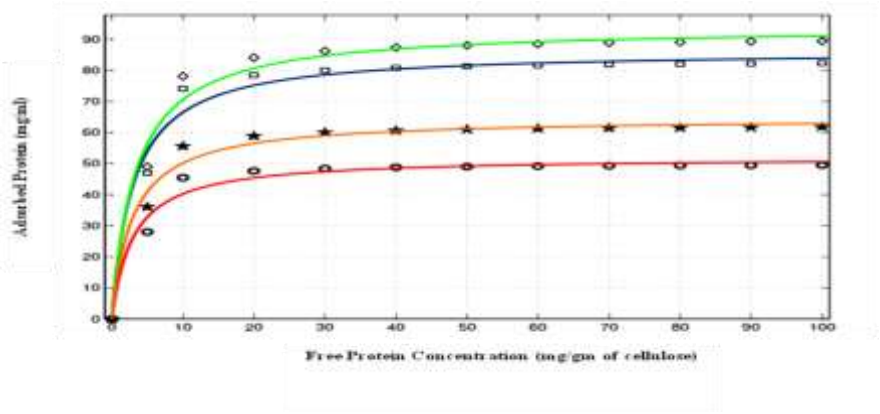


Figure 6 : Effect of incuabtion time on protein adsorption at different temperatures



○- Cellulose powder (4°C), □-Acid Treated Biomass (4°C), ★- Cellulose powder (50°C),◇-Acid Treated Sorghum Biomass (50°C)

Figure 7: Figure showing the amount of cellulase protein adsorbed on sorghum biomass and cellulose powder at different temperatures

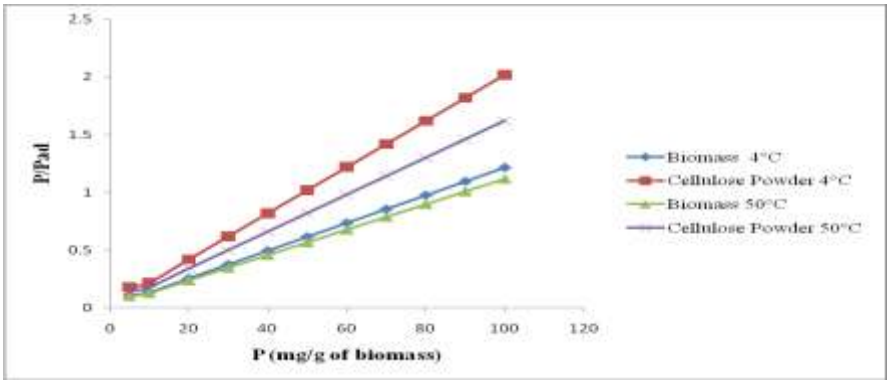


Figure 8: Graph of free protein concentration versus $P/P_{adsorbed}$

Table 2: Table showing the P_{max} and K_d values for sorghum biomass and cellulose powder at 4°C and 50°C

	Temperature (°C)	Maximum Adsorption Capacity (P_{max} mg/g of biomass)	Affinity of Adsorption (K_d)	R-square
Biomass	4	86.42	3.4	0.9806
Cellulose powder		52.08	2.9	0.9752
Biomass	50	94.13	3.34	0.9828
Cellulose powder		64.69	2.94	0.9808