Cellulases Production and Optimization in *Thermobifida fusca*

A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF

MASTER OF TECHNOLOGY

IN

INDUSTRIAL BIOTECHNOLOGY

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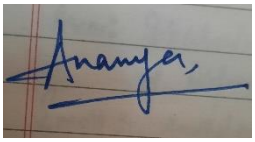
CANDIDATE'S DECLARATION

I Ananya Chouhan, Roll no: 2K21/IBT/02 student of M.Tech (Industrial Biotechnology), hereby declare that the project Dissertation titled "Cellulases Production and Optimization in *Thermobifida fusca*" which is submitted by me to the Department of Biotechnology, Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of Master of Technology, is original and not copied from any source without proper citation. This work has not previously formed the basis for the award of any Degree, Diploma Associateship, Fellowship, or other similar title or recognition.



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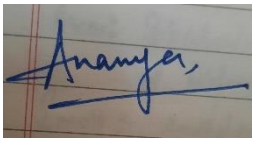
Ananya Chouhan

ACKNOWLEDGEMENT

Apart from the efforts of oneself, the success of my project depends largely on the encouragement and guidelines of many others. I am highly grateful for having a chance to meet so many wonderful people and professionals who led me through this Dissertation period. I am using this opportunity to express my deepest gratitude to **Dr. Shyam Kumar Masakapalli, School Biosciences and Bioengineering, IIT-Mandi, Himachal Pradesh,** whose continuous guidance keep me on a correct path to carry out my project correctly.

I also express my deepest thanks to **Ms. Jyotika Thakur** for taking part in the useful decisions and giving necessary advices and guidance and arranged all facilities during the project.

This project would not have been possible without the help and motivation of **Ms. Shagun**, **Mr. Ashutosh Sahoo, Arjita and Navya**. I am highly grateful for their help. At the end I would like to thank all the **MSBL (Metabolic System Biology Laboratory)** members for being so polite and generous during my entire dissertation tenure.



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CERTIFICATE

I hereby certify that the project Dissertation entitled "Cellulases Production and Optimization in *Thermobifida fusca*" which is submitted by Ananya Chouhan, Roll no 2K21/IBT/02 to the Department of Biotechnology, Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of Master of Technology, is a record of the project work carried out by the student under my supervision. To the best of my knowledge this work has not been submitted in part or full for any Degree or Diploma to this University or elsewhere.

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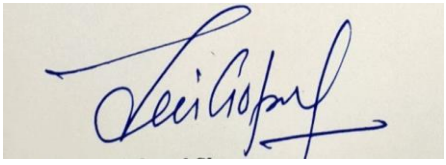
I hereby certify that the Dissertation titled **"Cellulases production and optimization in *Thermobifida fusca*"** which is submitted by **Ananya Chouhan, Roll No. 2K21/IBT/02** (Industrial Biotechnology), Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of Master of Technology (Industrial Biotechnology), is a record of the project work carried out by the student at IIT Mandi under the supervision of Dr. Shyam Kumar Masakapalli. To the best of my knowledge this work has not been submitted in part or full for any degree or diploma to this university or elsewhere.



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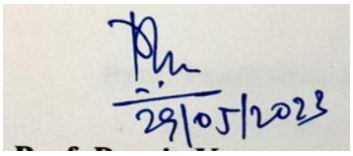

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ABSTRACT

Cellulases are the third largest Industrial Enzymes because of their importance in paper recycling, detergent enzymes and as animal feed additives. It is very likely that the cellulases will become the major Industrial enzymes, if bioethanol produced from biomass turns into a main transportation fuel. The recalcitrant structure of cellulose prevents the proper degradation of biomass into monomeric sugars. Cellulase enzymes are responsible for the bioconversion of lignocellulosic biomass into soluble sugars which can further be converted into ethanol. Among cellulolytic microbes, *Thermobifida fusca* possesses great physiological and cellulolytic characteristics i.e thermostability, high activity and tolerance to high pH range. Secreted cellulases are obtained from *Thermobifida fusca* under bioreactor condition and by giving different substrate concentrations of avicel (cellulose) i.e 0.5 %, 1 % and 2 % to *Thermobifida fusca* for cellulase production. After cellulase production the activity of crude and concentrated cellulases were checked and compared on different cellulosic substrate i.e avicel, carboxymethyl cellulose and cellobiose for determining exoglucanase, endoglucanase and β-glucosidase activity respectively. Enzyme activity assays were performed on both crude and concentrated cellulases. The cellulase activity of *Thermobifida fusca* was compared with the already available commercial cellulases of *Trichoderma reesei* and *Aspergillus niger*. Higher β-glucosidase activity of 1.57 IU/ml and endoglucanase activity of 1.43 IU/ml were obtained from *Thermobifida fusca* cellulases as compared to the activity of other two commercial cellulases which makes it a potent enzyme to be used as an important cellulose degradation material.

Keywords: *Thermobifida fusca*, *Aspergillus niger*, *Trichoderma reesei*, cellulases, avicel, carboxymethy cellulose, cellobiose.

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ABBREVIATIONS

1. *Tfu: Thermobifida fusca*
2. *Tre: Trichoderma reesei*
3. *Ang: Aspergillus niger*
4. kDa: Kilo Dalton
5. HM: Hagerdahl Medium

1. Introduction

Lignocellulosic biomass has the potential to get converted into fermentable sugars, which can be used for the production of fuels and chemical feedstocks (Binder et al., 2010). Lignocellulosic biomass contains cellulose whose hydrolysis is very difficult (Akhtar et al., 2016). Cellulose is a homopolysaacharide containing β (1 - 4) glycosidic linkages. Cellulose is tightly bonded to hemicelluloses which form a compact matrix with lignin forming a recalcitrant structure and preventing the efficient hydrolysis of cellulose into glucose monomers which can then be converted to ethanol (Woiciechowski et al., 2020).

Cellulases are the principle enzymes which are responsible for the hydrolysis of cellulose. Cellulases are the third largest industrial enzyme in the world for their immense use in paper industry (Singh et al., 2016). Cellulases do not work as a single enzyme rather it is a group of modular enzymes that work synergetically to carry out the degradation process (Escuder-Rodríguez et al., 2018). Cellulases are classified into different families based on the sequence similarity. They are referred as glycoside hydrolases enzymes.

Cellulase enzyme is made up to catalytic domain (CD) and carbohydrate binding module (CBM). The catalytic domain and carbohydrate binding module are connected to each other through a linker which is a short polypeptide sequence. The carbohydrate binding module recognizes the cellulose surfaces and gets bind to it whereas the catalytic domain carries out the degradation of the cellulose into the fermentable sugars (Moser et al., 2008). The degradation of cellulose to fermentable sugars mainly consists of four types of cellulases enzymes which are: endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.74), cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) (Barbosa et al., 2020).

Endoglucanase cleaves the internal glycosidic bonds in a glucose polymer. Exoglucanases acts on the terminal chains which are generated by the action of endo cellulases and releases cellobiose or glucose units. Cellobiohydrolases cleaves the internal glycosidic bonds and releases only cellobiose units. β-glucosidase catalyses the hydrolysis of oligosaccharides and converts cellobiose to monomeric glucose units (Adav et al., 2010). Among cellulolytic microbes, *Thermobifida fusca* possesses good physiological and cellulolytic characteristics i.e good thermostability, high activity, and tolerance to high pH range which makes it an interesting organism to be studied for cellulases production. *Thermobifida fusca (T.fusca)* is a filamentous

soil bacteria, moderately thermofilic and cellulolytic actinobacterium. *T.fusca* is a thermostable bacterium and shows best growth at 50 - 55 ˚C temperature. It also possesses activity against a wide pH range ranging from pH 4 to pH 10. It has a GC content of 67.5 % which is predictable for organism growing in extreme environment. *T.fusca* has endospores which is necessary to survive in extreme environmental stress.

T.fusca has the ability to efficiently degrade cellulose into the monomeric glucose units by the action of cellulase enzymes. *T.fusca* has high activity because it produces very competent multienzyme heterogeneous complex which leads to more synergism and high activity. Since *T.fusca* generate multienzyme complex, its cellulases have been classified into different families which are glycosil hydrolase (GH) family 5 which contains cel5B and cel5A. Glycosil hydrolase (GH) family 6 which contains cel6A and cel6B and glycosil hydrolase (GH) family 9 which contains cel9A and cel9B (Lykidis et al., 2007).

cel5A and cel5B are endoglucanases which cuts the cellulose chain in the middle and reduces the length of the polymer. It performs cellulose hydrolysis but shows little or no effect on other hemi cellulosic substrates. Cel6A is an endoglucanase which perform cellulose hydrolysis by single displacement mechanism. It is considered for carrying out site directed or site saturation mutagenesis studies. Cel6B is an exoglucanase which represents reducing end chain activity and processivity. Its active site is surrounded by two long loops, forming a tunnel, which permits processive movement on a cellulose chain. Cel9A is a processive endoglucanase which mean it performs multiple round of catalysis around a cellulose chain until that cellulose gets converted into monomeric sugars. There is another cellulase cel48A that belongs to glycosil hydrolase (GH) family 48.

cel48A is the exoglucanase and acts on the terminal region for the release of the successive glucose units. CelR is the main cellulase expression regulator in *T.fusca*. It belongs to lactose repressor family and is characterized as the regulator of gene expression in *T.fusca* (Wilson et al., 2004). CelR binds to cellobiose which is located at 14 base pair inverted repeats located upstream of many cellulase genes (Lykidis et al., 2007). The production of cellulases in *T.fusca* only takes place in the presence of the substrate cellulose. It means that only cellulose can induce the cellulase production otherwise the transcription will be off and there will be no production of the cellulase enzyme. *T.fusca* genome contains all the enzymes necessary to carry out glycolytic

degradation of monosaccharide. There is the presence of Entner-Douodoroff pathway in *T.fusca* which can be used for glucose utilization. All the necessary tricarboxylic acid cycle enzymes are also present in the genome of *T.fusca*. It also has glyoxylate cycle presence which improves its capability to grow on additional carbon sources such as citrate. There is the presence of pyruvate carboxylase, phosphoenolpyruvate, carboxy kinase and fructose-1, 6-bisphosphate which is a clear indication of the presence of gluconeogenesis pathway (Shi et al., 2016). It has machinery for carrying out fatty acid synthesis and can also perform de novo biosynthesis of all amino acids the exception is asparagine. It can hydrolyze lipids and can also utilize them as carbon source.

Taking all these points into consideration it is very evident that *Thermobifida fusca* has a great potential for cellulases production. This project is mainly focused on the production of cellulases from *Thermobifida fusca* and subsequently checking its enzymatic activity against different substrate for making it a potential commercial cellulase enzyme that can be used as an industrial enzyme for the conversion of lignocellulosic biomass into fermentable sugars that can subsequently be converted to ethanol (Gomez del Pulgar et al., 2014).

2. Literature review

India has an urgent need for renewable transportation fuels and bio-ethanol is considered as one of the most favorable options. India has mandated the use of 5% ethanol to be mixed in motor gasoline in various states. Sources of ethanol production are mainly molasses feedstock. But this is not sufficient to meet the present demand. Lignocellulosic biomasses can be taken as a source for bio ethanol production.

But there are various drawbacks in this fields which include poorly documentation of resource, no advanced technology used and the technology used for ethanol production from lignocellulosic biomasses are under preliminary stages of development. With the increasing energy demand and fast depletion of petroleum resources, country is shifting towards alternative fuel resources to meet the demand of the growing population (Sukumaran et al., 2010).

Bioethanol production from lignocellulosic biomass is considered as one of the most favorable options because of its higher octane rating than motor gasoline (Bergthorson et al., 2015). One of the major drawbacks associated with lignocellulosic ethanol production is weak commercialization and costly enzyme production technologies (Sukumaran et al., 2010). India is a country giving positive outlook to the renewable energy technologies and is committed towards the use of renewable sources to meet its energy requirements. India is among few countries having ministry for renewable energy, addressing the development of biofuels along with other renewable resources (Planning commission, 2003).

Bioethanol and Biodiesel soon to be identified as the major biofuel to be developed for the nation. Lignocellulosic Biomass production and its conversion to bioethanol comprise of various pretreatments and enzyme mediated hydrolysis processes. Due to the presence of cellulose in lignocellulosic biomass, its conversion to fermentable sugar has become a major challenge worldwide in the process to be economically competitive. The current technologies used for biomass conversion came out to be very costly, so there is a high need for the development of efficient systems for cellulose degradation.

There are various cellulolytic microorganisms like *Aspergillus niger*, *Trichoderma reesei* etc, which produces cellulases that are used for cellulose degradation to fermentable sugars. Among cellulolytic microbes, *Thermobifida fusca* possesses great cellulolytic characteristics like high

thermostability (50 - 55 ˚C), High activity (Produces heterogeneous enzyme complex) and tolerance to high pH range (4 - 10) which makes it an interesting organism to be studied for cellulases production for lignocellulosic biomass degradation (Gomez del Pulgar et al., 2014). In recent research it has been reported that the thermophilic *Thermobifida fusca* strain (UPMC 901) is showing great potential in producing thermostable cellulases. It has shown very good endoglucanase activity at only 24 hrs of incubation using carboxymethyl cellulose (CMC) as substrate and filter paper. Further studies suggested that endoglucanase activity was most stable at 70 ˚C for 24 hrs. This endoglucanase activity was maintained till 144 hrs at 50 ˚C and 60 ˚C temperatures which makes it most stable than other endoglucanases reported in various other literature. The endoglucanases stability of cellulases produced from *Thermobifida fusca* at different temperature for prolonged period of time makes it an appropriate candidate for biorefinary application (Zainudin et al., 2019).

Various extracellular cellulases and intracellular β-glucosidases have been purified and characterized from *Thermobifida fusca* for cellulose degradation. Structural genes encoding these enzymes have been cloned and sequenced to study the three dimensional structures of cellulase catalytic domains. Some studies have reported that there are two types of exoglucanases being produced by *Thermobifida fusca* from which one exoglucanse attack the non-reducing end and the other one attacks the reducing end of the cellulose chain (Wilson et al., 2004). Plant biomass on an average consists of 40 % cellulose, 33 % hemicelluloses and 23 % Lignin (Ahmed et al., 2009).

Cellulose is the most abundant carbohydrate and is one of the most important structural materials of plant cell walls (Dodd et al., 2009). Cellulose is polymer made up of glucose units connected by β-1, 4 linkages. Cellulose is converted to glucose by cellulase producing microorganisms. Cellulase is the enzyme mostly used for cellulose degradation and it is able to hydrolyze β-1, 4-glycosidic bonds of cellulose to release glucose units (Tsuji et al., 2012). Cellulases have been widely used in industries such as brewery, wine, laundry, paper, agriculture and pulp industries (Kuhad et al.,2011). They are also used in bioprocessing of lignocellulosic biomass containing cellulose into fermentable sugars and ethanol production (Joshi et al., 2023).

There are many organisms that have the capability to produce cellulase for cellulose degradation. But only a few of them produces significant quantity of cellulases which are thermostable and

possesses high activity and are compatible enough to convert or degrade crystalline cellulose (Teeri et al., 1997). Bacteria and fungi are two of the most common microorganisms that produce cellulases (Immanuel et al., 2006). Although some of the bacteria were reportedly yield cellulase activity. Fungi are considered as the major organism for cellulases production but they can produce cellulases only in the presence of cellulose (Suto et al., 2001). On the other bacterial cellulases are produced constitutively (Suto et al., 2001). *Thermobifida fusca* is one of the most prominent producers of cellulases. It is aerobic, moderately thermophilic, and filamentous soil bacterium which degrades the cell walls of plants containing cellulose (Deng et al., 2010). The strain of *Thermobifida fusca* belongs to actinobacteri phylum which can grow on most simple sugars and carboxylic acids and is capable of degrading all major plant cell wall polymers except for lignin and pectin (Lykidis et al., 2007).

The cellulolytic fungus *Trichoderma reesei* is one of the most widely studied microorganisms for producing cellulases (Gupta et al., 2016). Approximate production of extracellular cellulase from *Trichoderma reesei* was reported to be 100 g/L (Cherry et al., 2003; Yan et al., 2021). The secreted cellulases consists of two major cellobiohydrolases CBHI/CEL7A and CBHII/CEL6A, endoglucanases EGI/CEL7B AND EGII/CEL5A, and β-glucosidases BGLI/CEL3A accounting for 90% of the extracellular protein (Paula et al., 2018). Besides the cellulases there are some other proteins present in *Trichoderma reesei* which participate in efficient degradation of cellulose. These proteins are lytic polysaccharide monooxygenase and cellulose induced protein (Gupta et al., 2016). Such an amazing cellulase producing ability is attracting various industries to use this as an alternative for the depleting fossil fuels to meet the demand for sustainable development (Kubicek et al., 2013).

Traditionally the bioconversion of biomass requires large amount of cellulases which are costly. So it is important to reduce the cost of cellulases produced which further reduces the cost of biodegradation of cellulose by cellulases. Although *Trichoderma reesei* possesses good cellulase production capacity but it contains only few number of cellulases encoding genes other than fungi (Martinez et al., 2008). The specific regulator and exceptional cellulase response mechanism gives *Trichoderma reesei* superb cellulase production ability and greater potency in a saprotrophic habitat (Paloheimo et al., 2016).

Other than *Trichoderma reesei* cellulases, cellulases from *Aspergillus niger* have also been reported. Large number of fungi has been reported in municipal solid waste (Gautam et al., 2011). The *Aspergillus niger* was predominantly high among the other fungal species. Most of the work of the fungal cellulases is mainly focused on saccharification of cellulose by *Aspergillus* (Kang et al., 2013). Also cellulase production on different carbon sources by *Aspergillus* and other fungi have also been reported (Gautam et al., 2010). Cellulose degrading fungi have been used to degrade cellulosic containing materials to fermentable sugars that have various biotechnological and industrial applications. Most of the commercial cellulases available in the market is of fungal origin i.e they are produced by *Trichoderma reesei* and *Aspergillus sp*. Considering the importance of cellulases in industries, this study aims to provide good understanding of conditions required for the production and activity of cellulase by *Thermobifida fusca* along with other commercial cellulases from *Trichoderma reesei* and *Aspergillus sp.*

In the present study, cellulase production from *Thermobifida fusca* is performed and its activity is checked on various carbon sources: on avicel to check the exoglucanase activity, on carboxy methyl cellulose to check the endoglucanase activity and on cellobiose to check the β-glucosidase activity. Cellulase activity of available commercial cellulases from *Aspergillus sp.* and *Trichoderma reesei* have also been performed on the above mentioned three substrates to compare the efficiency of cellulases produced from *Thermobifida fusca* with the commercial cellulases. Furthermore the effect of buffer on enzyme activity has also been studied and compared.

3. Materials and Methods

3.1. Microbial Strain and Media Components

Microbial strain of *Thermobifida fusca (T.fusca)* ATCC 27730 obtained from Microbial Type Culture Collection and Gene Bank (MTCC) was used.

Two types of Microbial culture media were used in this study:

- Tryptone-Yeast extract (2TY) Liquid media (Enrich media)
- Hagerdahl medium (Minimal media)

Detailed composition of each medium is given further in section 3.2. All the prepared media were autoclaved at 120 ˚C for 20 minutes and thermo-degradable components (Biotin and thiamine) were filter sterilized through 0.22 - micron filters.

3.2.Medium Composition

1. Enrich media composition (Tryptone-Yeast extract (2TY) (Liquid media)
    - Tryptone → 3.0g
    - Yeast extract → 3.0g
    - Glucose → 3.0g
    - K_2HPO_4 → 1.0g
    - Agar → 20.0g
    - Distilled water → 1L
    - Adjust pH to 7

2. Minimal Media Composition (Hagerdahl Medium)

Minimal media prepared for T.fusca growth contains macro salts which are Nacl, $(NH_4)_2SO_4$, Na_2HPO_4, and KH_2PO_4. Micro salts include EDTA, $MgSO_4.7H_2O$, $ZnSO_4.7H_2O$, $FeSO_4.7H_2O$, $MnSO_4.H_2O$, $CaCl_2.2H_2O$. 1X Hagerdahl medium was prepared using macro and micro nutrients. Other additions were Biotin and Thiamine which were added in autoclaved media. Glucose was added as a carbon source.

3.3. Culturing, Maintenance and Inoculum preparation

T.fusca cells preserved in a glycerol stock were inoculated in 50 ml falcon tubes containing prepared HM media along with 2 % glucose at 55 ˚C in an incubator shaker with 180 rpm shaking for 3 days. The growth of bacteria was monitored after every 12 hrs. Two falcon tubes were also kept as blank to check the contamination. After 48 hrs the bacteria was fully grown and it was then centrifuged at 9000 rpm for 10 minutes and the supernatent was discarded. The resulting pellet was washed with HM media without any carbon source by resuspending the cells in 10 ml media and centrifuging once again at 5000 g for 5 minutes. This cell pellet was subsequently used as inoculum for experiments.

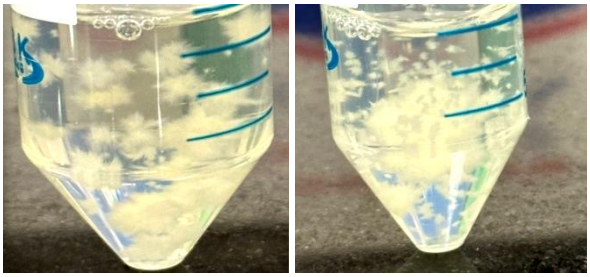


Figure 1: Liquid culture of *T.fusca* in HM media

3.4. Bioprocessing of cellulose with optimized parameters

Experiment 1: Set up 3 bioreactors of volume 250 ml, 250 ml, and 1000 ml for Cellulase production:

- For 1000 ml Bioreactor: 2 % Avicel (Cellulose) = 2 g / 100 ml = 20 g / 1000 ml
- For 250 ml Bioreactor: 0.5 % Avicel (Cellulose) = 0.5 g / 100 ml = 0.75 g / 250 ml

Experiment 2: Set up Batch experiment in falcon tubes for cellulase production

- Four 50 ml falcon tubes containing : 0.5 % Avicel (Cellulose)
- Four 50 ml falcon tubes containing : 1 % Avicel (Cellulose)
- Four 50 ml falcon tubes containing : 2 % Avicel (Cellulose)

Optimum Parameters for Bioreactor and Batch Experiment:

- PH = 7
- Temperature = 55 ˚C
- Dissolved oxygen set to 100 at time zero

3.5. Methodology

3.5.1. Bioreactor experiment

Three Bioreactors were disinfected with ethanol in laminar air flow hood. Previously prepared 1X HM media was poured into the bioreactor with carbon source. Optimum parameters like pH, temperature and Dissolved oxygen for *T.fusca* were maintained as described in section 3.5. Bioreactors were inoculated with *T.fusca* inoculum and the sample was taken at 0 hr. For 1000 ml bioreactor 20 ml sample was taken and for 250 ml Bioreactor 2 ml sample was taken. Subsequently samples were taken at 24, 48, 72 96 and 120 hr respectively. Samples obtained were centrifuged at 7000 rpm for 5 minutes. Supernatent and pellets were kept in separate falcon tubes and were stored in 4 ˚C refrigerator. DC assay and DNS assay were performed on all the previously collected samples for determining extracellular protein and sugars respectively. Bioreactors were dismantled after 120 hr and the ammonium sulphate precipitation is performed on the medium harvested from the bioreactor to precipitate out the protein from the medium.

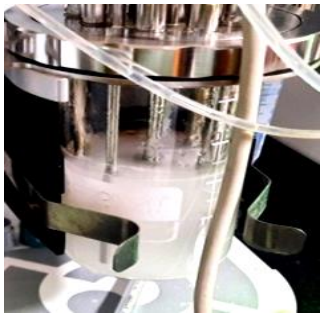
A)   B)

Figure 2: A) Bioreactor setup for cellulase production at 0 hr. Substrate given was avicel (Cellulose) and media used was 1X HM. B) Bioreactor setup for cellulase production at 96 hr, bacterial growth was visible in the form of lumps at 96 hr.

3.5.1.1. Protein Content

DC assay Kit based on Lowry method was used to estimate protein content. BSA standard of different concentrations were prepared i.e 0.0, 0.2, 0.4, 0.8 and 1.6 (mg/ml) using BSA standard (30 mg/ml) for getting calibration curve. 5 µl of each supernatent obtained at different intervals from the bioreactors were taken in which 25 µl of Reagent A (Folins reagent) and 200 µl of Reagent B (Alkaline copper tartarate solution) is added. DC assay of BSA standards at different concentrations were also performed in order to get the Standard BSA curve for calculating our extracellular protein concentration. All these samples were poured in multiwell plate and the plate is kept in dark for 15 minutes for the reaction to occur. After 15 minutes OD was taken at 750 nm and quantification calculations were performed.

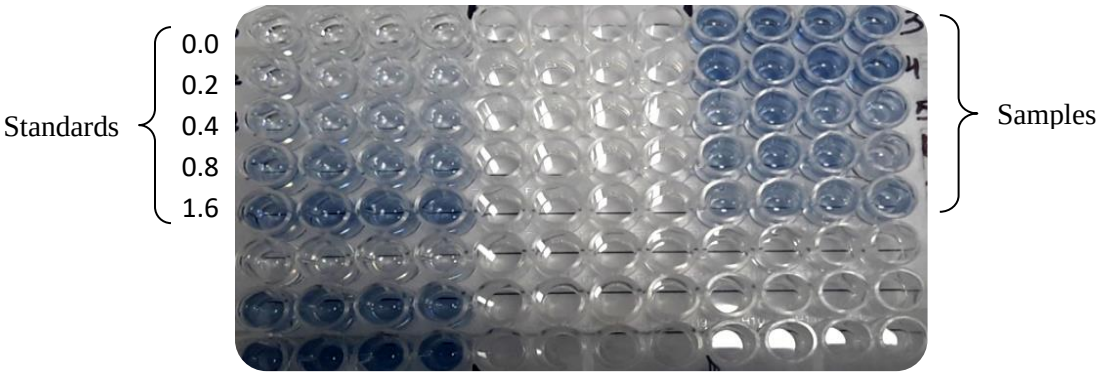


Figure 3: DC assay for BSA (protein standard) and secreted protein samples from *T.fusca.*

3.5.1.2. Total Residual sugar in culture filtrate

DNS (3, 5-dinitrosalicyclic) assay was performed for determining total reducing sugar content. Glucose standards of different concentrations were prepared i.e 0.2, 0.4, 0.8, 1.6 (mg/ml) using glucose standards (20 mg/ml) for getting calibration curve. 100 µl of supernatent obtained at different time intervals from the bioreactor were taken in which 200 µl of prepared DNS solution is added. All the samples were heated at 90 ˚C using heating block for 10 minutes and after 10 minutes all the samples were immediately plunge into ice for 5 minutes to stop the reaction. 200 µl of each sample were poured into multiwell plate and absorbance is recorded at 540 nm.

Fig 4: DNS assay on 96 well microtitre plate. A) Represents the crude enzyme samples taken at different time intervals i.e 24, 48, 72, 96 hrs. B) Represents the glucose standards of different concentrations i.e 0.2, 0.4, 0.6, 0.8, 1.6 mg/ml prepared for making calibration curve.

3.5.1.3. Ammonium Sulphate Precipitation

200 g of Ammonium sulphate is weighed and kept for drying overnight to remove the moisture in 60 ˚C oven. Dried Ammonium sulphate salt is then grinded into fine powder and stored in 500 ml dried bottle. Dried Salt is then added into the supernatent harvested from 3 bioreactors. Salt is added according to 0 %, 20 %, 40 %, 60 %, 80 % & 90 % saturation using the saturation chart. After addition of Salt into the supernatent, the bottle is kept into magnetic stirrer for 12 hrs at

600 rpm. After incubation the supernatent is centrifuged at 8500 g at 4 ˚C for 25 minutes. Pellets were collected and resuspended in 2 ml of Tris HCL buffer of PH 7.

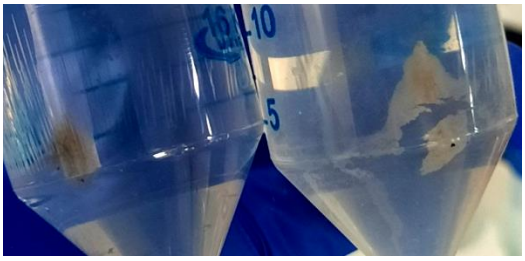


Figure 5: Ammonium sulphate precipitation pellets were observed at 40 % saturation after incubation period of 17 hours.

3.5.1.4. Enzymatic assays

After determining the secreted protein and sugar content, enzymatic assays were performed using avicel as substrate for determining the activity of the enzyme obtained after bioprocessing. The Enzymatic assay was performed for the sample giving highest secreted protein concentration from DC assay. For this assay 1ml of the enzyme is taken in which 0.6 mg of avicel is added and the reaction is kept at micro centrifuge shaker for 2 hrs at 55 ˚C at 900 rpm. After 2 hrs DNS assay is performed on the reaction mixture and total sugar units released were determined and enzyme activity was calculated (µmol/min). Buffer used was Tris HCL having pH 7.

Table 1: Enzymatic assay reaction setup at different enzyme and buffer dilutions. Three reactions were made as shown in the table in which reaction 1 is containing only enzyme i.e 1:0 dilution, reaction 2 is containing equal concentration of enzyme and buffer i.e 1:1 dilution, and reaction 3 is containing buffer concentration three times higher than the enzyme concentration i.e 1:3 dilution.

S.No	Substrate Concentration (mg)	Enzyme (ml)	Buffer (ml)
1	0.6	1	0
2	0.6	0.5	0.5
3	0.6	0.25	0.75

3.5.2. Cellulase production at 0.5 %, 1 % and 2 % cellulose concentration

Batch experiment was set up for comparing *T.fusca* cellulase activity grown under Batch and Bioreactor conditions. For Batch experiment 10 % *T.fusca* inoculum was used. The growth of bacterium was monitored on three different cellulose compositions which were 0.5 %, 1 % and 2 %. All the three experiments were set up along with their replicates. The working volume of falcon tubes was kept as 15 ml. The detailed composition of the Batch experiment was given as follow:

Table 2: List of replicates and their composition. 1a, 1b, 1c, 1d are the biological replicates having avicel concentration of 0.5%, 2a, 2b, 2c, 2d are the biological replicates having avicel concentration of 1% and 31, 3b, 3c, 3d are the biological replicates having avicel concentration of 2%.

S.No	Avicel Concentration(mg)	Working volume (ml)	PH	Temperature (˚C)
1a	75	15	7	55
1b	75	15	7	55
1c	75	15	7	55
1d	75	15	7	55
2a	150	15	7	55
2b	150	15	7	55
2c	150	15	7	55
2d	150	15	7	55
3a	300	15	7	55
3b	300	15	7	55
3c	300	15	7	55
3d	300	15	7	55

The above mentioned experiments were monitored for 5 days and the samples were taken after every 24 hrs for 5 days. Samples collected were centrifuged from which supernatent was kept for further studies. DC and DNS assays were performed for determining the total secreted protein and total reducing sugar content of the samples collected at different time intervals respectively. One replicate each of different substrate concentration were harvested after 72 hrs.

Fig 6: Experiment set up for cellulases production at different concentrations. Experiments were set up at 3 different concentrations of avicel i.e 0.5 %, 1 % and 2 %. Each concentration was set up with four biological replicates.

3.5.2.1. Protein concentration using Cutoff (kDa) filters

The samples harvested after 72 hrs were concentrated using cutoff filters. The samples with volume of 10 ml from which 2 ml was stored as crude sample were poured into the filter which was then centrifuged at 4000 rpm at 4 ˚C for 25 minutes. The samples were concentrated 15X times then their original concentration. Each samples of 72 hrs were concentrated and their crude replicate was stored respectively.

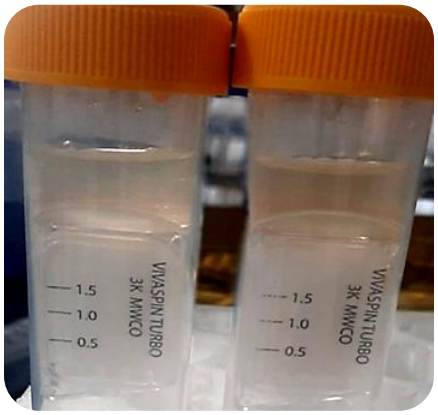


Figure 7: Protein Concentration using Cutoff filters. The filters used has 3kDa cutoff value. These filters will fiter out all the water content and other impurites present in the crude sample and concentrate only the protein content of the sample having molecular weight greater than 3kDa.

3.5.2.2. DC assay for crude and concentrated samples

DC assay for crude and concentrated samples were performed in order to see the increase in the total secreted content of concentrated sample as compared to the crude.

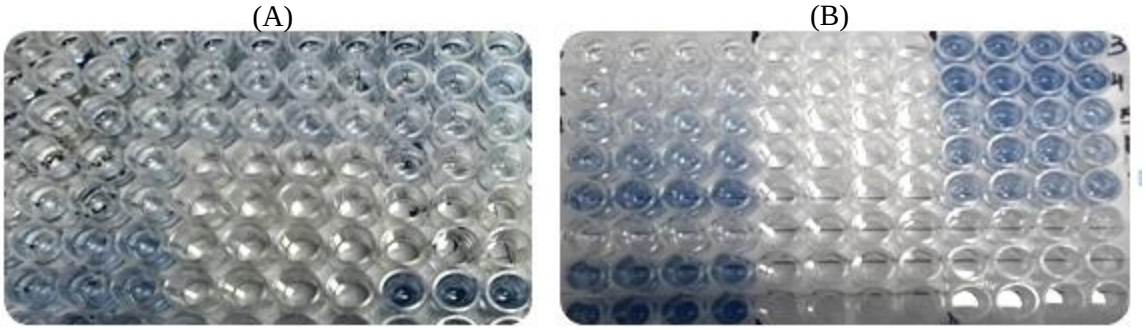


Figure 8: DC assay for crude and concentrated samples. A) represents the DC assay plate for the crude enzymes and B) represents the DC assay plates of the enzymes concentrated using kDA filters.

3.5.2.3. pH optimization experiment

Concentrated replicates of 72 hrs each of different concentrations were pooled for check the effect of PH on total sugar release. The Enzymatic assay for total reducing sugar release were performed at Acidic (4.28), Neutral (7) and Basic (10) PH. These assays were performed along with the positive and Negative controls. *T.reesei* and *Aspergillus niger* cellulases were used as positive control. For the following experiment 2% avicel is taken as the substrate as follow:

Table 3: PH optimization reaction set up. Three reactions of dilution 1:1, 1:0 and 1:3 were set up for all the three acidic (4.28), Basic (10) and Neutral (7) pH

S.No	Substrate concentration (mg)	Enzyme (ml)	Buffer (ml)
1	6.25	0.5	0
2	6.25	0.25	0.25
3	6.25	0.125	0.375

The above mentioned enzyme mixture were kept at 55˚C for 2hrs on micro centrifuge shaker and then the DNS assay was performed and the total reducing sugar content was calculated. The total reducing sugar content the crude samples were also calculated to get the exact number of sugars released from the sample.

3.5.2.4. Enzymatic assays

From the above experiment we get to know the optimum pH required for the doing enzymatic assays. Enzymatic assays were performed to check the exoglucanase, endoglucanase and β-glucosidase activity of the cellulases produced using Avicel, Carboxymethyl cellulose (CMC) and cellobiose respectively. For enzymatic assays following methodology was adopted:

Table 4: Enzymatic assays reaction setup. Dilution used was 1:1 i.e equal concentration of enzyme and buffer. pH kept was 7 and the buffer used was Tris-HCL

S.No	Substrate	Substrate concentration (mg)	Buffer (ml)	Enzyme (ml)	Temperature	Incubation period (min)
1	Avicel	1.9	0.15	0.15	55˚C	120
2	CMC	3	0.15	0.15	55˚C	120
3	Cellobiose	0.77	0.15	0.15	55˚C	120

Avicel is used for determining the exoglucanase activity, during the incubation period the enzyme mixtures were kept on a microcentrifuge shaker for 2 hrs at 55 ˚C, the sample vials were directly plunge into ice to stop the reaction and the DNS was added for determining the sugar content in the sample. After DNS addition the sample vials were kept on a heating block at 90 ˚C for 10 minutes in order to initiate the reaction of the DNS with the enzyme mixture. After 10 minutes the sample vials were again plunge into the ice for 5 minutes to stop the reaction and the absorbance was recorded using microtitre plate reader at 540 nm and sugar units were calculated. Glucose standards of different concentrations were used for making the calibration curve.

3.5.2.1. ^{1}HNMR for detecting product glucose release

^{1}HNMR was performed to obtain a metabolite concentration. The intensity of the NMR (Nuclear Magnetic Resonance) signal is proportional to the Boltzmann equilibrium magnetization and ultimately depicts the absolute metabolic concentration. Hydrogen is used in NMR because it has an odd mass number which means that it has spin and is affected by external magnetic fields. The number of signal molecules produce in ^{1}HNMR spectrum can be determined by counting the number of distinct hydrogens on one side of the plane of symmetry. In this experiment ^{1}HNMR was performed to confirm the presence of glucose in the samples which further conveys the good activity of cellulases being produced.

4. Results and Discussion

4.1. Bioreactor Experiment

4.1.1. Total Secreted Protein analysis

After *T,fusca* inoculation, samples were taken after every 24 hr for 5 days and total secreted protein was calculated at each time interval using DC assay kit (section 3.5.1.1.). From the below mentioned graph (Figure 9) we obtained a maximum protein concentration of 0.105 mg/ml which was observed after a time interval of 72 hr. This extracted protein was further used for carrying out enzymatic analysis. The X – axis of the graphs represents the time interval at which different samples were taken and Y – axis represents the concentration obtained at different time intervals.

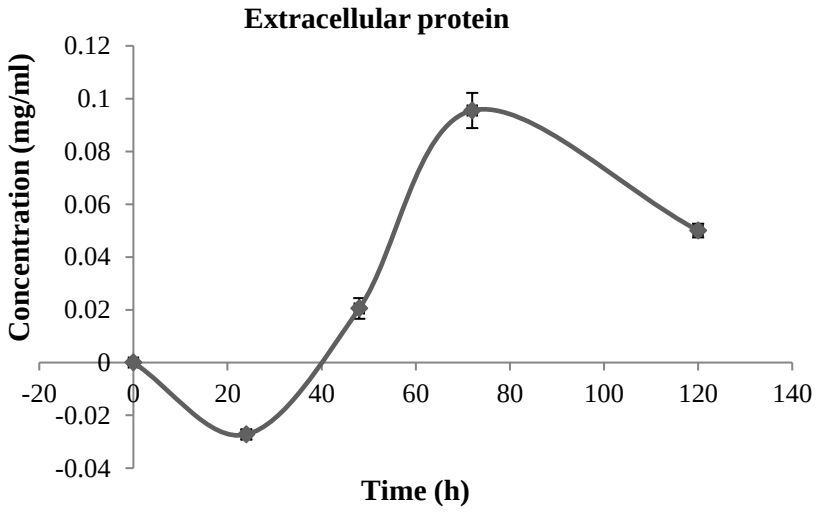


Figure 9: Graphical representation of extracted protein concentrations at different time intervals from 5 days bioreactor run.

Table 5: Extracellular Protein Concentration obtained at different time from Bioreactor using BSA calibration curve from.

S.No	Time(hr)	Concentration (mg/ml)
1.	0	0
2.	24	-0.0027
3.	48	0.021
4.	72	0.105
5.	120	0.05

4.1.2. Total Residual Sugar analysis

After *T.fusca* inoculation, samples were taken after every 24 hr for 5 days and total residual sugar content was calculated at each time interval using DNS assay kit (section 3.5.1.2.). From the graph given below (Figure 10) maximum residual sugar content of 0.0037 mg/ml was observed after a time interval of 96 hr. The sugar content was same throughout 5 days bioreactor run. The X – axis of the graphs represents the time interval at which different samples were taken and Y – axis represents the concentration obtained at different time intervals.

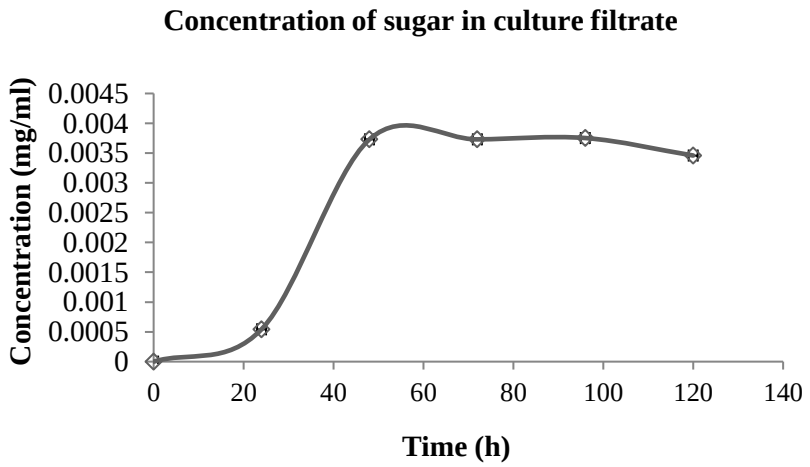


Figure 10: Graphical representation of residual sugar content obtained at different time intervals from 5 days bioreactor run

Table 6: Residual Sugar Concentration obtained from bioreactor at different time intervals using glucose calibration curve

S.No	Time (hr)	Concentration (mg/ml)
1.	0	0
2.	24	0.0005
3.	48	0.004
4.	72	0.0037
5.	96	0.0037
6.	120	0.0034

From the above mentioned table we can conclude that sugar content at time 72 hr and 96 hr were same and maximum. From determining sugar content we saw the growth of the bacterium as how our bacterium is taking cellulose and subsequently converting it into glucose by releasing cellulases.

4.1.3 Enzymatic assays

Sample (72 hr) showing the maximum secreted protein concentration (0.105 mg/ml) was taken along with 120 hr sample to perform total cellulase activity using avicel (cellulose) as substrate. Enzymatic activity assay methodology has already been described in section 3.5.1. Different dilutions of buffer and enzymes were made to study the effect of buffer on enzyme activity. Following table will represents the results obtained from enzymatic activity assay at different dilutions.

Table 7: Enzymatic activity calculated at different dilutions of buffer and enzyme using Avicel as substrate.

Sampling time (h)	Avicel (mg)	Buffer pH 7 (ml)	Enzyme (ml)	OD_{540}	Enzyme activity (µmol/min)
72	0.6	0	1	0.1428	0.0012
72	0.6	0.5	0.5	0.1419	0.0048
72	0.6	0.75	0.25	0.001	0.00005
120	6	0	10	0.11	0.001
120	6	5	5	0.13	0.003
120	6	7.5	2.5	0.01	0.002

- From the above results it was clear that:
  - Maximum enzyme activity of 0.0048 and 0.003 from 96 and 120 hr samples respectively was observed at 1:1 dilution.
  - Confirming that Equal concentration of enzyme and buffer is appropriate for carrying out enzymatic analysis.

4.2. Cellulases production at 5, 10 and 20 g/l of avicel as substrate concentration

4.2.1. Total Secreted Protein analysis

From the graph given below (Figure 11) it is very evident that the maximum protein concentration was observed at the 5th day i.e after 120 hr. Also replicates containing 10 g/l of avicel had shown low protein concentration which makes this concentration inappropriate for carrying out enzymatic analysis experiments. Although 20 g/l replicates had shown maximum results but the deviation within the replicates was higher which needs to be taken care for carrying out enzymatic analysis experiments further. 5 g/l replicates had shown good results with less error bars making this as an appropriate amount of substrate for carrying out enzymatic analysis.

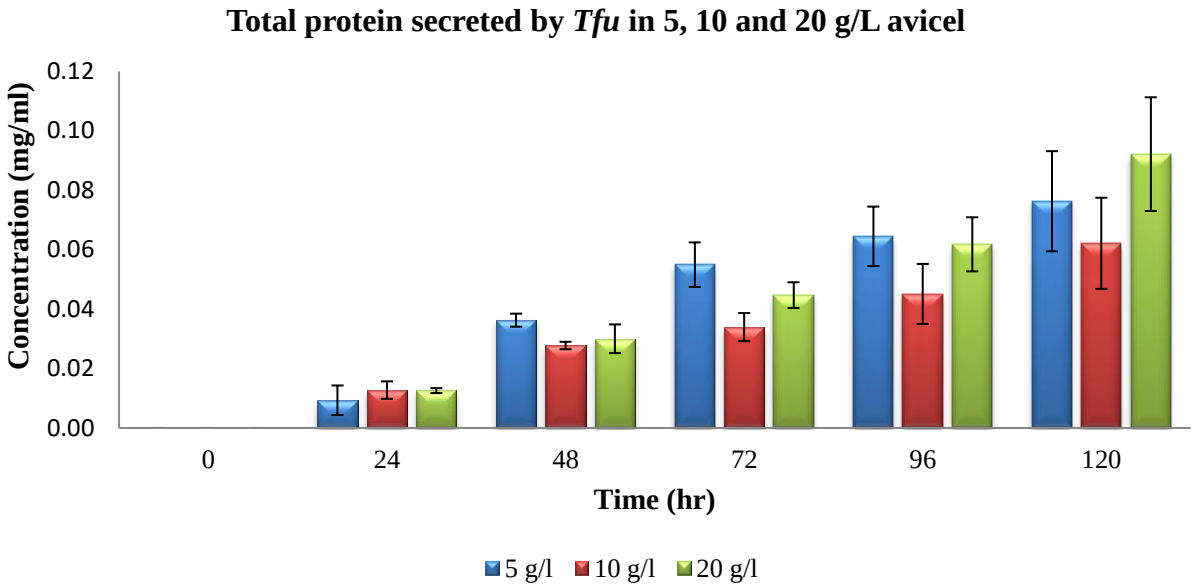


Figure 11: Graphical representation of total secreted protein secreted by *T.fusca* in 5, 10 and 20 g/l avicel. The X – axis represents the different time intervals at which the samples were taken and the Y – axis represents the concentration of the protein obtained from the samples taken at different time intervals.

Table 8: Concentration obtained at different time using BSA calibration curve.

Time (hr)	Concentration (5g/l)	Concentration (10g/l)	Concentration (20g/l)
0	0.00	0.00	0.00
24	0.01	0.01	0.01
48	0.04	0.03	0.03
72	0.05	0.03	0.04
96	0.06	0.05	0.06
120	0.08	0.06	0.09

4.2.2. Total Residual Sugar content analysis

After *T.fusca* inoculation, samples were taken after every 24 hr for 5 days and total residual sugar content was calculated at each time interval using DNS assay kit (section 3.5.1.2.). From the graph (Figure 12) it is very evident that maximum residual sugar content of 0.42 mg/ml was obtained at 4th day i.e after 72 hr in 20 g/l replicates. Since substrate concentration in 20 g/l replicates were higher means in some cases bacteria is feeding on the substrate and consequently releasing cellulases, in those cases the sugar content was higher and in other cases bacteria is feeding on the glucose content present in the media due to cellulose degradation and releasing less glucose into the media.

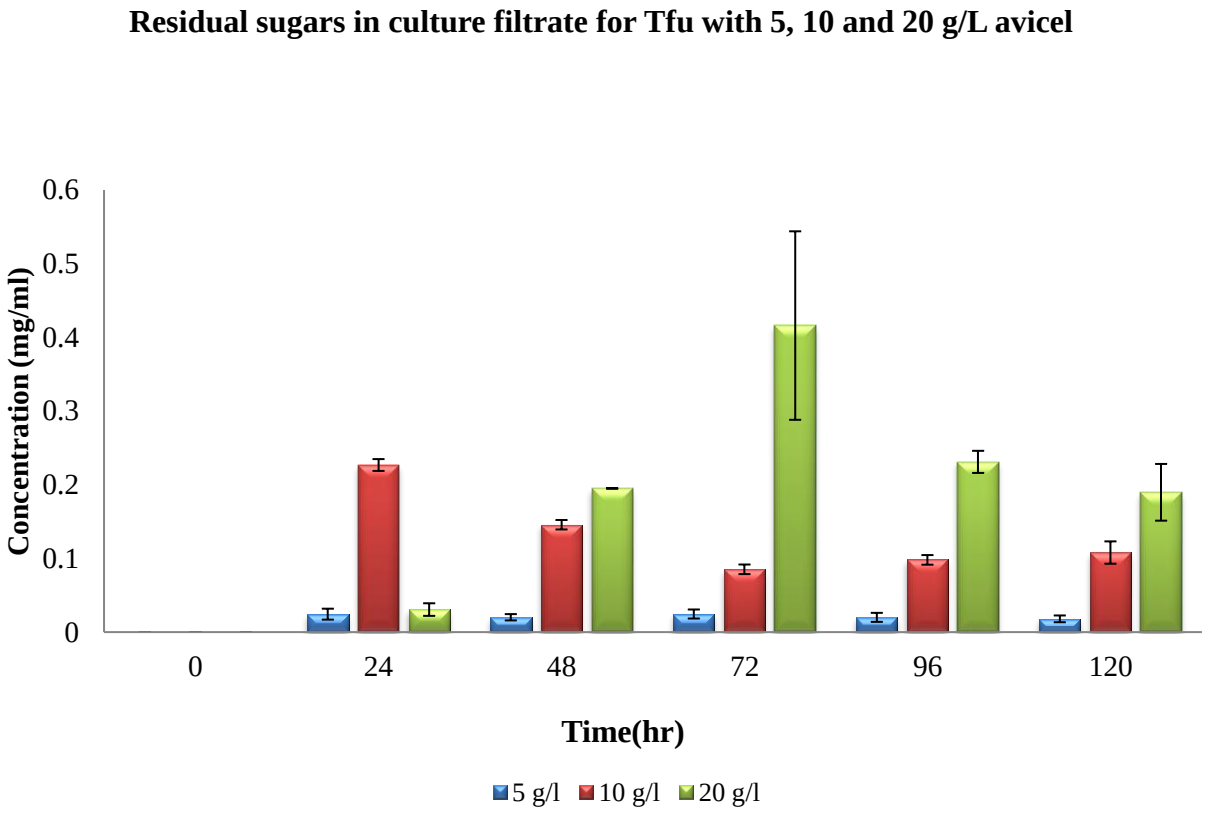


Figure 12: Graphical representation of residual sugar content released by *T.fusca* in 5, 10 and 20 g/l avicel. The X – axis represents the different time intervals at which the samples were taken and the Y – axis represents the concentration of the residual sugar content obtained from the samples taken at different time intervals.

Table 9: Concentrations obtained at different time intervals using Glucose calibration curve

Time (hr)	Concentration (5g/l)	Concentration (10g/l)	Concentration (20g/l)
0	0	0	0
24	0.02	0.23	0.03
48	0.02	0.15	0.20
72	0.02	0.09	0.42
96	0.02	0.10	0.23
120	0.02	0.11	0.19

4.2.3. Secreted protein concentration using KDa/cutoff filters

Protein samples showing the maximum protein concentration were concentrated using cutoff filters having cutoff of 3kDa from which all the water content is removed and only the protein content stays in the filter. Protein content of the concentrated samples was calculated using DC assay where BSA is taken as the calibration curve. The samples showing the maximum protein concentration were further selected for carrying out enzymatic assays on different substrates: Following table represents the amount the maximum protein concentration obtained at different concentrations and at different time intervals.

Table 10: Maximum protein concentration obtained from concentrated fractions obtained from KDa filters.

Substrate Concentration (g/l)	Secreted protein content in concentrated fraction (mg/ml)	Time (hr)
5	0.8	120
10	1.6	120
20	0.8	120

4.2.3.1. Comparison of Total Secreted Protein content of the crude and concentrated samples

Table 11: Comparison of Total secreted protein content of crude and concentrated samples

Maximum Secreted protein content of Crude sample (20g/l)	Maximum Secreted protein content of concentrated samples (5g/l)	Maximum Secreted protein content of concentrated samples (10g/l)	Maximum Secreted protein content of concentrated samples (20g/l)
0.05	0.8	1.6	0.8

From the above table it is very evident that the total protein content of concentrated samples was higher as compared to the total secreted protein content of the crude samples. This shows that the use of cutoff filters worked well for concentrating protein from crude sample.

4.3. pH optimization experiment

pH optimization experiment was performed to find the appropriate Ph for *T.fusca* cellulases before performing enzymatic activity assays on different substrates. Along with *T.fusca*, two other commercial cellulases from *Tre* and *Ang* were also taken and heir optimum pH was also determined. Since these experiments (Figure 13) were not carried out in replicates so the consistency of the results is not significant. We need to carry out this experiment again in replicates for getting significant results.

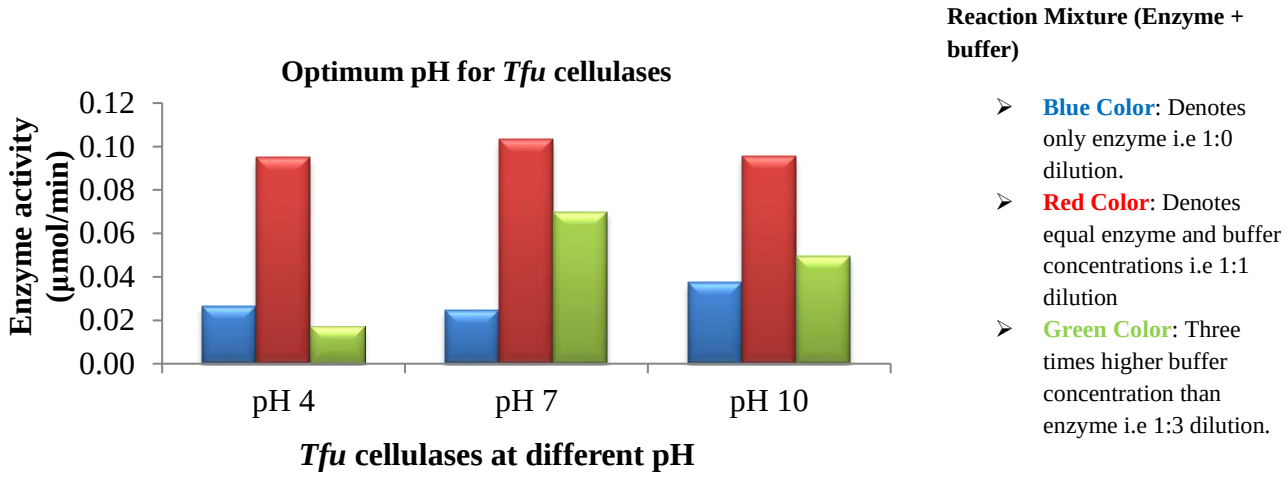


Figure 13: Determination of optimum pH for *T.fusca. T.fusca* had shown good activity on all the three determined pH depicting its activity on wide pH range. The X – axis represents the pH used at different dilutions and the Y- axis represents the enzyme activity obtained at different pH dilutions.

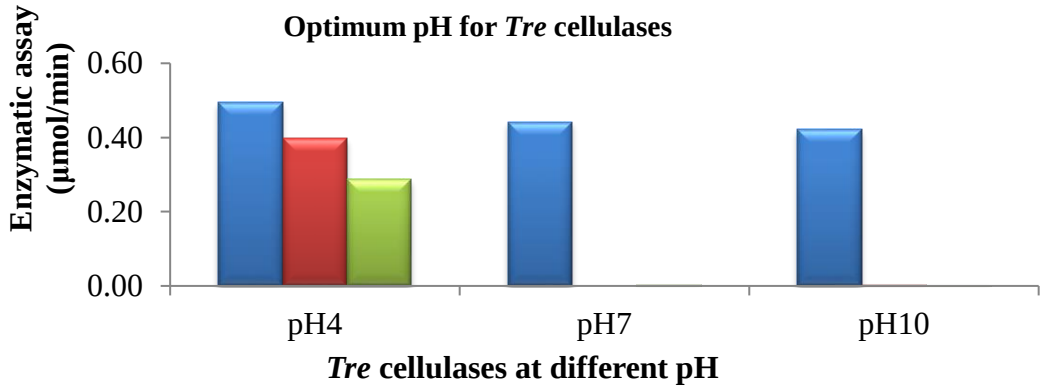


Figure 14: Determination of optimum pH for *Tre* cellulases. *Tre* cellulases have shown good activity at pH 4. The X – axis represents the pH used at different dilutions and the Y- axis represents the enzyme activity obtained at different pH dilutions.

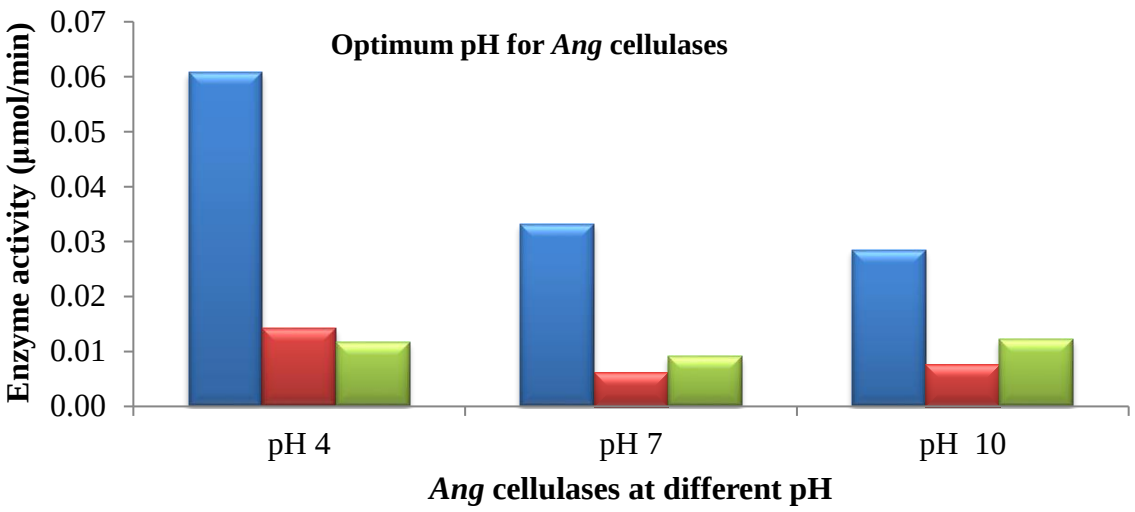


Figure 15: Determination of optimum ph for *Ang* cellulases. *Ang* cellulases have shown moderated activity on all the 3 pH. The X – axis represents the pH used at different dilutions and the Y- axis represents the enzyme activity obtained at different pH dilutions.

- *Tfu: Thermobifida fusca*
- *Ang: Aspergillus niger*
- *Tre: Trichoderma reesei*

} Commercial cellulases

4.3.1. pH optima for *Tfu* cellulases

T.fusca cellulases has shown maximum activity at pH 7 and at a dilution of 1:1 (equal buffer and enzyme concentration) which is shown in the below given graph (Figure 16).

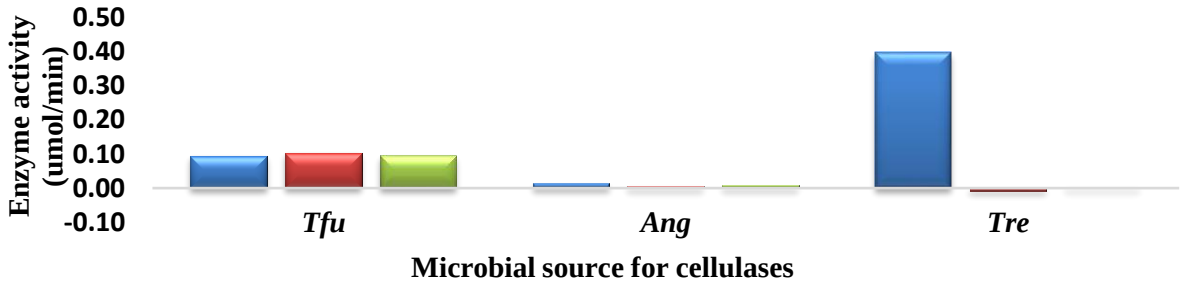


Figure 16: Cellulase activity of *T.fusca, Ang and Tre* at pH 7.

T.fusca cellulase has shown maximum activity at pH 7 and at a dilution of 1:1. All the enzymatic activity assays were performed at pH 7 for getting the exoglucanase, endoglucanase and β-glucosidase activity of *T.fusca* cellulases. Ang cellulases had shown no activity at pH 7 while Tre cellulases had shown good activity at a dilution of 1:0 i.e only enzyme and no buffer at pH 7.

4.3.2. Calculation and Comparison of Exo, Endo and β-glucosidase activity of *T.fusca* cellulases

Exoglucanase, endoglucanase and β-glucosidase activity of *T.fusca* were calculated using avicel, carboxy methyl cellulose and cellobiose as substrate respectively. The methodology for cellulase activity calculations were already described in section 3. Enzymatic activity assays had shown the following results.

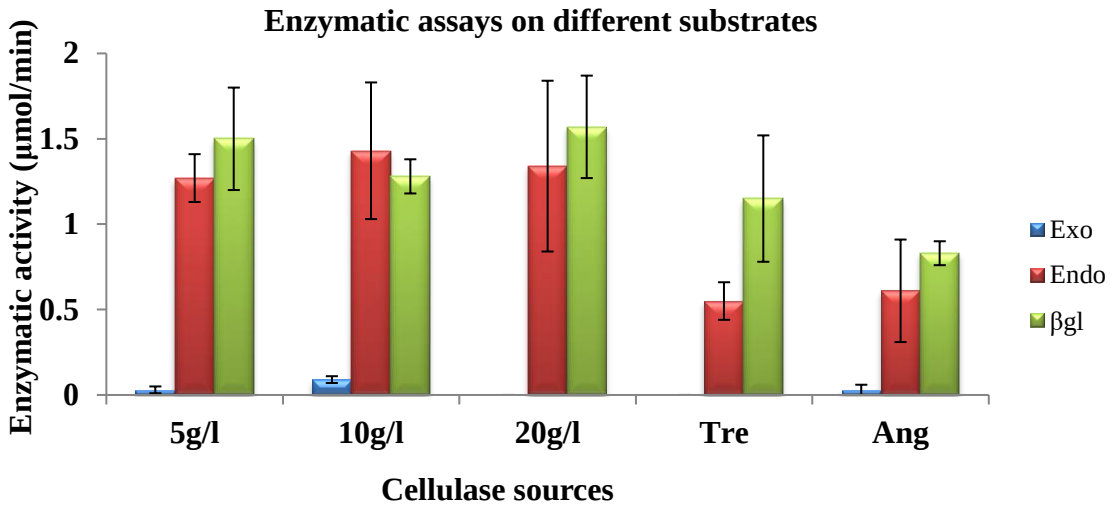


Figure 17: Enzymatic assays of *T.fusca* cellulases at different substrate

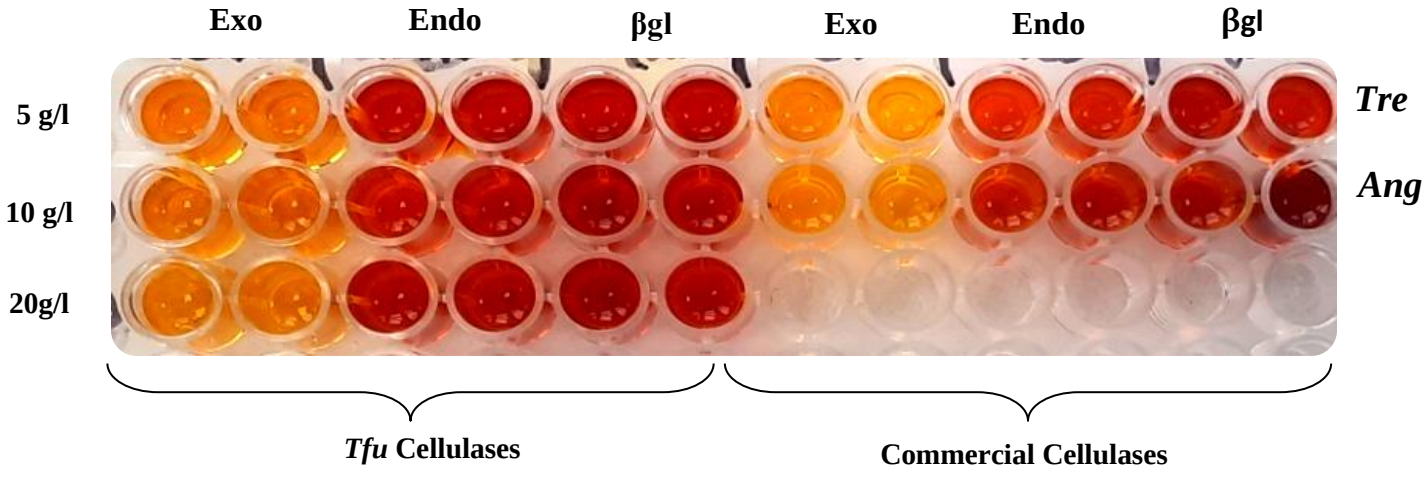


Figure 18: Visual representation of Exoglucanase, Endoglucanase and β-glucosidase activity from DNS activity assay for total sugar presence concentration. Brown color represents the sugar presence, darker the brown color more will be the sugar content. From the color it is very clear

that *T.fusca* cellulases have good endo and β-glucosidase activity but less or no Exoglucanase activity. Similar results were shown by *Tre* and *Ang* commercial cellulases.

Table 12: Comparative specific activity analysis of *T.fusca* cellulase at different concentrations (5g/l, 10g/l, 20g/l) with *Tre* and *Ang* commercial cellulases.

Cellulase sources (15X concentrated)	**Exoglucanase (IU/ml)**	**Endoglucanase (IU/ml)**	**β-glucosidase (IU/ml)**
5g/l	0.03	1.27	1.5
10g/l	0.09	1.43	1.28
20g/l	0	1.34	1.57
Tre (1X)	0	0.55	1.15
Ang (2X)	0.03	0.61	0.83

Following are the important points obtained from the above results:

- Higher β-glucosidase activity of **1.57 IU/ml** was observed as compared to endo and exo glucanase activity at 20g/l concentration.
- *T.fusca* cellulase activity was observed to be higher as compared to the activity of commercial cellulases.
- No Exoglucanase activity was observed in both *T.fusca* and commercial cellulases
- Zainudin et al., 2019 had also calculated endoglucanase activity of *T.fusca* which came out to be 0.9 IU/ml at pH 5 and 60 ˚C temperature. However in this experiment the maximum endoglucanase activity came out to be 1.43 IU/ml at Ph 7 and 55 ˚C temperature which is higher as compared to the other cellulase activity research papers.

4.3.3. 1H- NMR spectra for sugars released confirmation

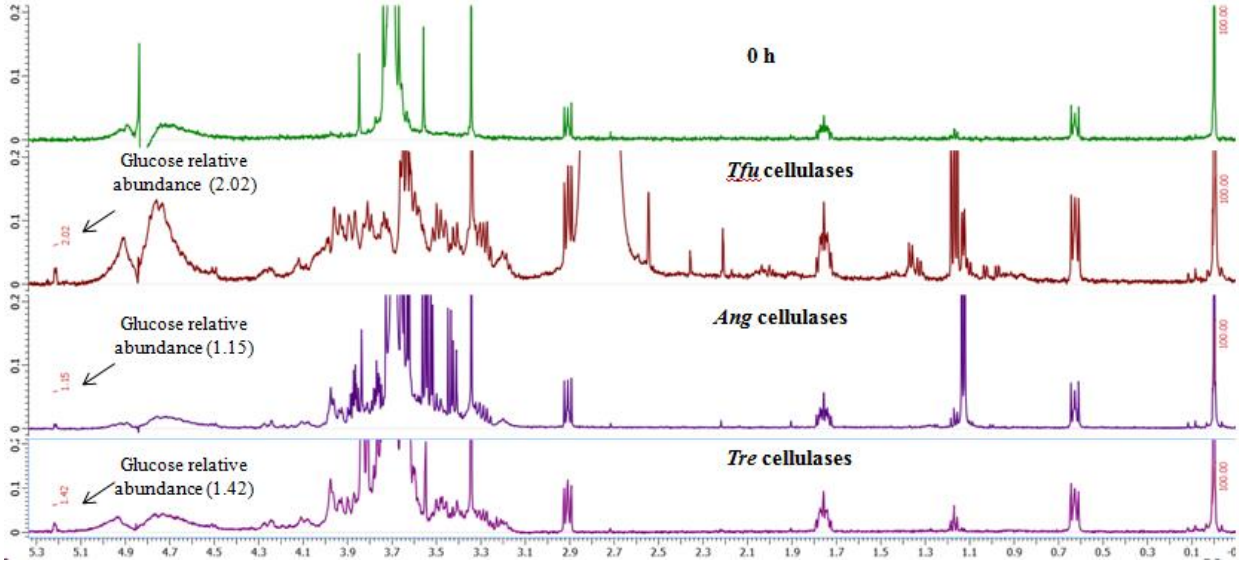


Figure 19: Representative ^{1}H- NMR spectra for sugars released after endoglucanases activity from *Tfu, Ang, Tre* cellulases on CMC.

Highest Glucose relative abundance of 2.02 was obtained in *Tfu* cellulases as compared to the other commercial cellulases whose relative abundances were 1.15 for *Ang* and 1.42 for *Tre* cellulases.

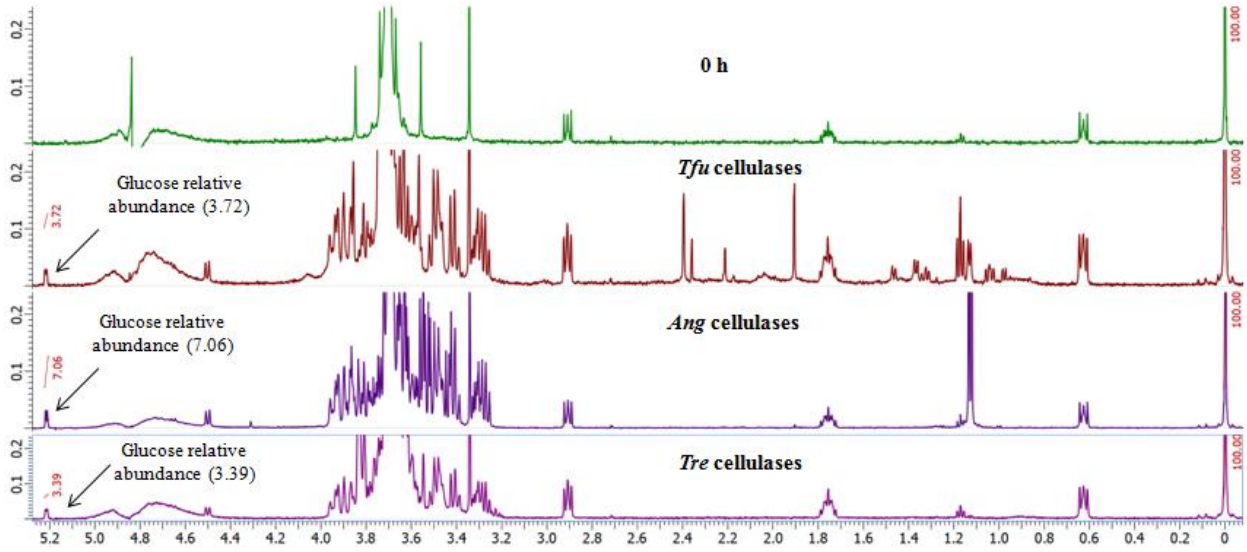


Figure 20: Representative ^{1}H- NMR spectra for sugars released after β-glucosidases activity from *Tfu, Ang, Tre* cellulases on Cellobiose.

Highest glucose relative abundance of 7.06 was obtained in *Ang* cellulases while *Tfu* and *Tre* cellulases have shown a relative abundance of 3.72 and 3.39 respectively.

4.3.4. Comparative NMR based analysis of different cellulases

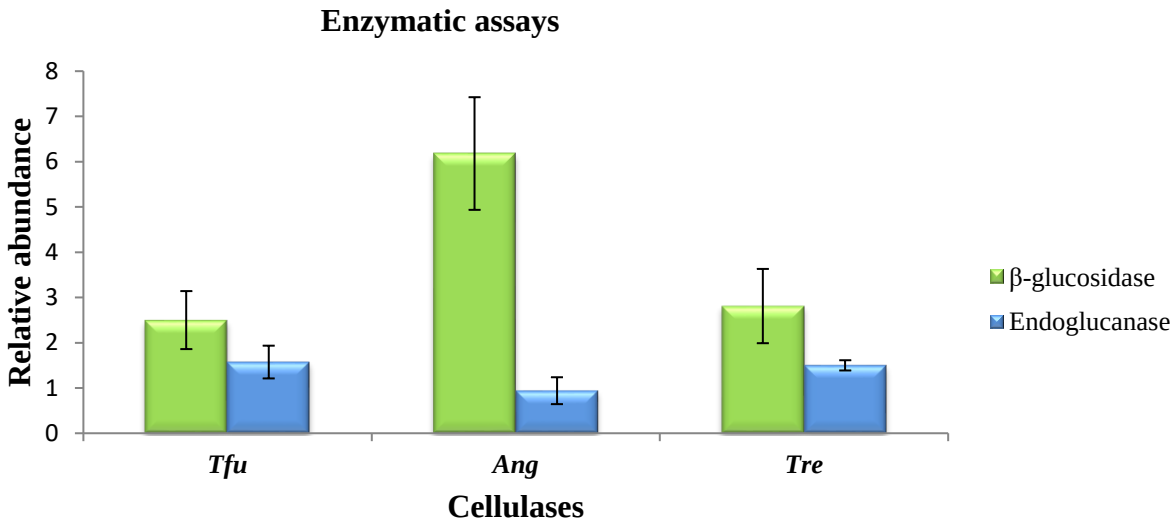


Figure 21: NMR based comparative analysis of glucose abundance present in *Tfu, Ang and Tre*

- ✓ Highest β-glucosidase activity of **7.06** was observed in ***Ang*** cellulases although *Tfu* and *Tre* cellulases also showed β-glucosidase activity.
- ✓ Highest Endoglucanase activity of **2.02** was observed in ***Tfu*** cellulases.
- ✓ 0 (0hr sample) was taken as the sample control for comparing after and before enzymatic activity of cellulases.

Conclusion

T.fusca cellulase has high endoglucanase and β-glucosidase activity but no exoglucanase activity. Endoglucanase activity of *Tfu* was higher as compared to the commercial cellulase activity. Biological replicates of 5g/l had shown maximum protein concentration within 3 days but other replicates (10g/l and 20g/l) had shown maximum protein concentration after 5 days. Although the maximum enzymatic activity had been seen at 20g/l but the deviation within the replicates was high which was not so in case of 5g/l replicates, which makes this concentration suitable for carrying out further enzymatic analysis. No exoglucanase activity was observed in NMR analysis because the concentration of glucose in that case was below the detection limit. All these biochemical assays and analytical techniques performed for sugar release had shown good glucose release content which clearly states that the obtained cellulase has good cellulose degradation capacity and has the potential to be used as a major industrial application tool for lignocellulosic biomass conversion to fermentable sugars.

Future Prospect

T.fusca cellulases obtained from the above experiment has shown good e ndoglucanase and β-glucosidase activity which shows that this secreted cellulase have great biotechnological promise for utilization in the degradation of the agricultural products and wastes to produce sugars that can subsequently be converted to ethanol. In future it is very evident that the cellulases will soon become the principal industrial enzyme for converting lignocellulosic biomasses into fermentable sugars.

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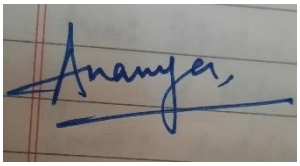

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