MOLECULAR IDENTIFICATION OF CELLULASE PRODUCING STRAINS *BACILLUS SP.* HSTU-2 AND *BACILLUS SP.* HSTU-3 AND MAKING A BACTERIAL CONSORTIUM TO DECONSTRUCT THE RECALCITRANT STRUCTURE OF LIGNOCELLULOSE

A THESIS

BY

MD. ASHIKUJJAMAN ASHIK

Registration No.: 1705247

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MASTER OF SCIENCE (M.S.)
IN
BIOCHEMISTRY AND MOLECULAR BIOLOGY

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY
HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY
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DECEMBER, 2018

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Submitted to the Department of Biochemistry and Molecular Biology
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DECEMBER, 2018


Dedicated to
My
Parents

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ABSTRACT

The world is now facing one of the greatest difficulties of modern time. As she is running out of energy, scientists need to think of a new and alternative way to produce sustainable energy source. Luckily bio-ethanol production from lignocellulosic waste can solve this problem completely. However the structure of lignocellulose poses substantial recalcitrance to the path of ethanol production. In order to produce bioethanol two basic steps are involved; first one is the pretreatment of lignocellulosic waste such as rice straw, maize straw, corn stover, barley straw etc. and the hydrolysis step which can be achieved by enzymatic manipulation using different microbes. Many articles have already reviewed different pretreatment methods and hydrolysis mechanisms in order to conquer the recalcitrant structure of cellulose. However no significant advancement in this criteria has still been achieved as various obstacles such as cellulose crystallinity, degree of polymerization, lignin residues, and hemicellulose content keep getting in the way of our success. The use of bioconsortium in order to break the recalcitrant structure of cellulose has not been done very briefly and appears to promise more than the conventional biological treatments of lignocellulose. Bio-consortium can be the breakthrough that scientists are looking for. As one species of bacteria can't break through the recalcitrant cellulose, two or three different bacterial strains together in the right environment can produce significant results. In this article a total of two cellulase producing bacterial strains (HSTU-2 and HSTU-3) have been isolated from cow dung and identified at molecular level using 16s rRNA gene sequencing. Both the strains (HSTU-2 and HSTU-3) have been identified as *Bacillus sp.* and have been submitted to gene bank in National Centre for Biotechnology Information (NCBI). *Bacillus sp.* HSTU-2 (accession no. MK659878) and *Bacillus sp.* HSTU-3 (accession no. MG582599.1) have also been run through different bioinformatics software and online tools for their sequence similarity including AT-GC content determination and evolutionary analysis using 'MEGA X'. Phylogenetic tree has also been described for all the six strains with maximum likelihood method and proper node and branch lengths has been maintained to identify all of these strains. Four more bacterial strains have also been used (HSTU-6, HSTU-7, HSTU-9 and HSTU-10) in this study which have been studied previously in the Molecular biology lab of Hajee Mohammad Danesh Science and Technology University, Dinajpur as part of the bioethanol production research. HSTU-6 (MG582600.1) and HSTU-7 (MG582601.1) are pectinase producers isolated from cow rumen content while HSTU-9 (MG582602.1) and HSTU-10 (MG582603.1) are amylase producing strains which have been isolated from vermicompost. Phylogenetic trees have also been described for all the six strains (HSTU-2, HSTU-3, HSTU-6, HSTU-7, HSTU-9 and HSTU-10) with maximum likelihood method and proper node and branch lengths has been maintained to identify all of these strains. Various biochemical tests have been done using *Bacillus sp.* HSTU-2 and *Bacillus sp.* HSTU-3 to identify their metabolic habits. Both of them (*Bacillus sp.* HSTU-2 and *Bacillus sp.* HSTU-3) are gram positive bacteria as indicated by gram's staining test, citrate test and different selective media growth (Gelatin mannitol salt agar, MacConkey

agar, *Salmonella shigella* agar etc) analysis. Both the strains produced acetyl-methyl carbinol from pyruvate as indicated by Voges-Proskauer test. These strains are able to utilize complex sugars such as lactose, maltose and sucrose as depicted by the differential sugar fermentation tests. *Bacillus sp.* HSTU-2 has been found to be catalase positive while *Bacillus sp.* HSTU-3 has been detected to utilize cytochrome C. oxidase. *Bacillus sp.* HSTU-2 and *Bacillus sp.* HSTU-3 are efficient cellulose degrading bacteria since both strains performed great in the Congo-red agar media with holo zone diameter of 8 mm and 6 mm respectively. In order to prove the hypothesis that these strains can effectively make the perfect bio consortium that can reduce the lignin-hemicellulose content as well as the crystallinity and degree of polymerization, these six strains (HSTU-2, HSTU-3, HSTU-6, HSTU-7, HSTU-9 and HSTU-10) have been used to directly treat four types of fibers (cotton, areca, banana and coir fiber). The strains have been used in pair (amylase, pectinase and cellulase producing pairs) to make several bio consortiums by changing the concentration of bacteria and manipulating the temperature and time of the treatment. In order to determine the structural deformities of cellulose after treatment with the different bio consortiums, the fibers (cotton, areca, coir and banana) have been analyzed by FTIR analysis and found significant improvement of band transmittance at particular wavelengths of cellulose structure indicating band sharpening. The curves clearly indicate the removal of impurities such as lignin, hemicellulose, pectin, waxes etc. Also the XRD analysis have been performed for the treated cotton and areca fiber for determining the crystallinity index (CrI) of cellulose. Astoundingly, after treating the cotton and areca fiber with *Bacillus sp.* HSTU-2 and *Bacillus sp.* HSTU-3, the CrI dropped from 31.5% to a staggering 13.69%, which proves that these strains under optimum temperature can overcome the recalcitrance structure of cellulose. *Bacillus sp.* HSTU-2 and *Bacillus sp.* HSTU-3 have also been used to treat maize straw in order to see their combined effectiveness in reducing sugar yield as this is the primary concern of the experiment. Both of the strains (*Bacillus sp.* HSTU-2 and *Bacillus sp.* HSTU-3) performed splendidly as with a combined dose of HSTU-2 and HSTU-3 the reducing sugar yield have been found 27%, 35%, 43%, 60% and 63% in 20h, 30h, 40h, 50h and 72h timeframe respectively. All of these evidence points out to the fact that the use of these strains in making bio consortium will not only benefit in bioethanol production of lignocellulose but also will be able to overcome the cellulose recalcitrant challenge.

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LIST OF ABBREVIATIONS

g	:	Gram
ml	:	Millilitre
µl	:	Microlitre
g/L	:	Gram per litre
mg/ml	:	Milligram per millilitre
ng/µl	:	Nanogram per microlitre
et al	:	And others
n.d	:	No date
pH	:	Negative logarithm of Hydrogen ion concentration
rpm	:	Revolutions per minute
V	:	Volt
U/ml	:	Units per millilitre
U/mg	:	Units per milligram
RNA	:	Ribonucleic acid
DNA	:	Deoxyribonucleic acid
bp	:	Base pair
kb	:	Kilobase pair
NCBI	:	National Centre for Biotechnology Information
BLAST	:	Basic Local Alignment Search Tool
°C	:	Degree Celsius
$(NH_4)2SO_4$	:	Ammonium sulphate
$MgSO_4 \cdot 7H_2O$	:	Magnesium sulphate heptahydrate
KH_2PO_4	:	Monopotassium phosphate
$CaCl_2$.$2H_2O$	:	Calcium chloride dihydrate
NaH_2PO_4		Monosodium phosphate
Na_2HPO_4	:	Disodium phosphate
NaOH	:	Sodium hydroxide
HCl	:	Hydrochloric acid
NaCl	:	Sodium chloride
DNS	:	3,5- Dinitrosalicylic acid
nm	:	Nanometre

Alk. $CuSO_4$	:	Alkaline copper sulphate
Psi	:	Per square inch
dNTP	:	Deoxynucleotide triphosphate
M	:	Molar
mM	:	Millimolar
N	:	Normal
EDTA	:	Ethylenediaminetetraacetic acid
UV	:	Ultra violet
RFLP PFGE	:	Restriction Fragment Length Polymorphism Pulsed Field Gel Electrophoresis
ND	:	Nano Drop
PCR	:	Polymerase Chain Reaction
RFP	:	Restriction Fragment Length Polymorphism
SDSPAGE SP	:	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis Species
TAE	:	Tris Acetate EDTA
TBE	:	Tris Borate EDTA
KDa	:	Killo Daltone
NA	:	Nutrient Agar media
cm^{-1}		Per centimeter
FTIR		Fourier-transform infrared spectroscopy
XRD		X-ray powder diffraction
a.u.		arbitrary unit

CHAPTER- I

INTRODUCTION

One of the main problems that the world is facing right now is to find a source of sustainable energy which is renewable since energy needs have skyrocketed dramatically in the last two centuries, especially because of the transportation and industrial sectors. However, fossil fuels are polluting the environment and their reserves are limited (Erakhrumen, 2011; Noura EL-Ahmady, 2014; Ragauskas AJ & Al., 2006). Bangladesh is a developing country with a growing population and is also facing the same problem. In 2013 Bangladesh produced 4,500 bbl/d of oil against a demand of 119,000 bbl/d according to U.S. Energy Information Administration (EIA) which is quite scary. In the upcoming years energy crisis in Bangladesh will increase if not halts our economic development completely. To meet the energy demand for many years to come and also to decrease the emission of CO_2 which contributes to global warming Biofuel is now the most effective if not the only way to deal with this crisis (Erakhrumen, 2011; Mill & Mill, 2011). As we know biofuel are fuels produced from biomass, the two main biofuels are bioethanol and biodiesel (Noura EL-Ahmady, 2014). Bioethanol can be produced from lignocellulosic biomass which can be found vastly in our country as nothing but waste products. This process not only lowers the cost but also keeps the environment clean and is an efficient fuel alternative which is renewable (N. Nassi, 2011; Noura EL-Ahmady, 2014). Lignocellulosic biomass comprises of 40 or more cellulose chains making up the micro fibrils which are then cross-linked by hemicellulose and pectin matrixes to form macro fibrils giving structural integrity to the plant cell wall (Brown, 2003; Malherbe & Cloete, 2002; Noura EL-Ahmady, 2014). Hemicellulose and pectin matrixes make up a space which is occupied by lignin (Malherbe & Cloete, 2002). Lignin also binds with hemicellulose which provides mechanical strength to cell wall of plant (Noura EL-Ahmady, 2014). There are also many more components such as phenolics, fats, minerals, resins etc which are also present in lignocellulosic biomass. In addition cellulose have inter-intra molecular hydrogen bonding between sugar subunits that make some of the regions very ordered which are called crystalline region (Erakhrumen, 2011). Some regions are less ordered having accessible fibril surfaces that are in contact with water; they are called amorphous region (Erakhrumen, 2011). In order to produce bioethanol from lignocellulosic biomass the conventional processes that can be used are enzymatic hydrolysis and dilute acid hydrolysis (Noura EL-Ahmady, 2014). However the dilute acid hydrolysis has the problem of using different chemicals which are both costly and harmful for the environment (Brown, 2003). And so the bioconversion of lignocellulosic biomass using enzymatic hydrolysis is the best approach

for high efficiency and a clean environment (Tran, 1986). Many microbes have the capability to produce cellulase enzyme which in turn can break up cellulose chains liberating free sugar units (Puentes-téllez & Salles, 2018). These actions are basically done by three types of Cellulases: Endoglucanases or carboxy methyl cellulases which attacks the internal sites of the amorphous region of cellulose (de Souza, 2013; Rabinovich, M.L., 2002); exoglucanases or cellobiohydrlases which can degrade the crystalline structure of cellulose by attacking the reducing or non-reducing ends of polysaccharides chain and finally β-glucosidases which splits cellobiose into two glucose units (Noura EL-Ahmady, 2014; Wood, 1991). Bacteria and fungi are now widely accepted source of cellulase enzyme and this enzyme is now being produced industrially from these sources (de Souza, 2013; Puentes-téllez & Salles, 2018; Wood, 1991). Fungal source such as genera of *Trichoderma*, *Aspergillus* (de Souza, 2013; Wood, 1991) etc has already been established as cellulase producers and still an area under research. However despite the fact that bacterial production of cellulase has been scientifically proven for some of the genera such as *Clostridium*, *Cellulomonas*, *Thermomonospora* etc (Kuhad & Singh, 2011), many other bacterial strains are still to be tested and optimized to get better enzyme production. *Bacillus sp.* has also recently been reported in various articles to be an efficient cellulase producer and also some research have been carried out to clone the cellulase gene from *bacillus* (Pratama & Putra, 2014). So, this genera promises a great deal in terms of cellulase production. The deterioration of biomass into bioethanol by means of enzymatic hydrolysis requires several steps including pretreatment, cellulose hydrolysis, separation of sugar solution from lignin, microbial fermentation of sugar solution and distillation (Noura EL-Ahmady, 2014; Zhu & X.J. Pan, 2009). By following these steps 95% pure alcohol can be extracted. To further purify the alcohol dehydration can be used by molecular sieves to upscale the purity to 99.5% (Noura EL-Ahmady, 2014). The pretreatment process can be classified into physical pretreatment involving milling, irradiation etc, physico-chemical pretreatment involving steam explosion or auto-hydrolysis, chemical pretreatment and finally biological pretreatment involving microbes such as bacteria and fungi (Kuhad et al., 2011; Noura EL-Ahmady, 2014). The biological pretreatment using white-rot fungi or *Bacillus sp.* has significant results considering the fact that it requires low energy and mild environmental conditions (Sun, 2002). The main reason for this step is to break the protective coating that is made by lignin and hemicellulose network and also to lower the order of crystallinity in cellulose (Noura EL-Ahmady, 2014). The physical pretreatment

allows for easier binding of cellulase enzyme by increasing the surface area of biomass (Mais, 2002; Zeng & Huang, 2007). It also decreases the crystallinity and degree of polymerization of cellulose (Mais, 2002). Milling which is also a physical pretreatment is basically cutting the lignolocellulosic biomass into smaller pieces just like food chewing for size reduction (Mais, 2002; Palmowski, 1999; Sun, 2002). Irradiation such as gamma rays and microwaves can essentially increase the rate of enzyme hydrolysis and in combination with other treatments like dilute acid treatment hydrolysis will be more successful (Kumakura, 1983; Taherzadeh, 2007). In terms of chemical pretreatment dilute acid treatment is one of the most common method for the pretreatment of lignocellulosic materials (Noura EL-Ahmady, 2014; Taherzadeh, 2007). When treating with acid two types of condition can be maintained: High temperature and low acid concentration or low temperature and high acid concentration. This method can actually be used for fermenting the sugars also (Taherzadeh, 2007). Chemical pretreatment basically removes the amorphous lignin and hemicellulase to decrease the crystallinity (Gharpuray, M.M., 1983). Alkaline pretreatment is another example of chemical pretreatment which uses alkaline solutions like caustic soda (Noura EL-Ahmady, 2014) or lime (Yang, 2004) for withdrawing lignin part and breaking the intermolecular ester bonds between lignin, hemicellulose and cellulose (Fan, L.T., 1980; Tarkow, 1969). Treating with caustic soda increases the surface area as well as decreases the degree of polymerization of cellulose (Gaspar, M., 2007). However since caustic soda is expensive alternatives such as limes (calcium hydroxide) can be used for the disruption of lignin structure (Chang, V.S., 1997). Biomass can also be pretreated with ozone by maintaining optimum water content in biomass feed which is by standard 30% (Neely, 1984). This process is called ozonolysis and it depends on the sample content, particle size and ozone concentration in the gas flow (Neely, 1984). However this process itself is too expensive since a large amount of ozone is required (Sun, 2002). Pretreatment of the lignocellulosic biomass can be done using both physical and chemical means simultaneously. These type of pretreatments are called physico-chemical pretreatment (Chandra & A. Berlin, 2007). An example would be Ammonia fiber explosion (AFEX). Here the biomass sample is treated with ammonia in low pressure and high temperature to alleviate the lignin fractions in lignocellulose (Holtzapple, 1991). The AFEX process can be quite efficient in the lignin reduction but it cannot remove the lignin fractions and other cell wall components from the solution and therefore has to be washed with water to remove these inhibitory compounds (Holtzapple, 1991). Also AFEX is not very effective on

lignocellulosic samples that contains high lignin content and doesn't solubilize hemicellulose like other pretreatment methods (Noura EL-Ahmady, 2014). Almost all sorts of chemical pretreatment processes produce some kind of inhibitory compounds (Chandel, A.K., 2009; Chandel, A.K., 2010). These compounds have been classified as organic acids such as acetic, formic or levulinic acids, furan derivatives such as furfural and 5-hydroxy methyl furfural [5-HMF] and phenolic compounds (Chandel, A.K., 2009). These inhibitors have negative impact on production yields and thus can be dealt with in a number ways including the use of ion exchange resins, active charcoal, enzyme laccase, overliming (using CaOH) or using ethanolgenic microorganisms which have been reported to have the capacity to degrade these inhibitory compounds (Jurado, 2009; Taherzadeh, 2007). Many bio detoxification methods are now establishing using various microbes such as *Trichoderma* reesei, *Bacillus sp*ecies etc since bio detoxification provides less cost, minimum wastage of water, low energy costs etc (Fonseca, 2011; Yu, 2011). The only drawback is that it slows down the detoxification process (Noura EL-Ahmady, 2014). To degrade lignocellulosic biomass to fermentable sugars with the consumption of lower energy and creating less by-product enzymatic hydrolysis presents the most desirable effects (de Souza, 2013; Kumakura, 1983; Noura EL-Ahmady, 2014). Now with the recent advancements in environmental science and microbiological field various research articles suggests two systems of enzymatic hydrolysis including a complexed and a non-complexed systems (Lynd, 2002). A non-complexed system presents less challenge since the enzymes are secreted by the microbe and freely roam into the solution while a complexed system relies on the microbes being attached to the cell wall of lignocellulosic feedstock and then secreting the enzyme (Lynd, 2002). In order to hydrolyze the pretreated lignocellulosic biomass a process named 'separate enzymatic hydrolysis and fermentation (SHF) is followed where cellulose hydrolysis by cellulase and fermentation of sugars can be done simultaneously (Xianzhi & Chang, 2016). An optimum temperature has to be maintained to hydrolyze the cellulose. Microorganisms for harvesting ethanol basically needs 30-37c temperature (Olsson & H. Christensen, 2006; Saha & Iten, 2005). The drawback of this process is the depletion of cellulase activity by releasing sugar units such as cellobiose and glucose. After an initial good rate of cellulose hydrolysis, these sugars at a specific concentration (cellobiose $6g^{-l}$) can slow down the hydrolysis by cellulase to a significant amount (about 60%). Plus this process can take long time to be finished (Philippidis, 1995). Simultaneous saccharification and fermentation (SSF) is another process which can neutralize a big

fallback of SHF (Demain, A.L., 2005; Karimi, K., 2006; Sun, 2002). In this process two types of microorganisms are present in one container, one type hydrolyzes cellulose by releasing cellulase in the solution and other ferments the hexose sugar released as a result (Krishna, S.H., 2001). Of course the lignocellulose has to be pretreated first to break the lignin-hemicellulose network (Noura EL-Ahmady, 2014). The main drawback of this process is to find the optimum temperature and pH for both hydrolyzing and fermentation process since both processes need different temperatures to operate (Noura EL-Ahmady, 2014). Cellulase works best at about 40-45℃ whereas most ethanol producing bacteria don't function at that temperature (Demain, A.L., 2005; Kuhad et al., 2011; Philippidis, 1995). Also some articles reported that a certain amount of ethanol can also inhibits the cellulase activity and hampers the whole process (Ballesteros & M.J. Negro, 2004; Golias, 2002; Hong, J., 2007). In simultaneous saccharification and co-fermentation (SSCF) the mixture contain lignocellulosic biomass, cellulase enzyme secreting bacteria, pentose sugar fermenting bacteria and hexose sugar fermenting bacteria (Teixeira, L.C., 2000). So, it is basically one step ethanol production procedure. However some bacteria prefers both penotoses and hexoses resulting in a competition between organisms which gives lower yields (Noura EL-Ahmady, 2014). Among all the processes discussed for the hydrolysis and fermentation process, perhaps the most economically cheaper and moderate ethanol yielding process is 'consolidated bioprocessing (CBP)' where a single microbial consortium will have the ability to not only release cellulase enzyme but also the same group of bacteria will be able to produce ethanol from sugar units (Cardona, 2007; Lynd, 2002). While the solution seems simple it is not easy to make a consortium that will have this capacity (Noura EL-Ahmady, 2014). In fact, it is almost impossible to find bacterial species naturally that can both degrade cellulose and perform fermentation. The right combination of species may perform better and still an area under research (Hamelinck, C.N., 2005). One approach that has been already taken is to modify certain species of bacteria genetically so that they can perform various function at the same time. Modification of excellent ethanol producers so that they can hydrolyze cellulose also and modification of excellent cellulase producers so that they can produce ethanol (Den Haan & S.H. Rose, 2007). Now when we are talking about digesting cellulose several factors have to be considered including cellulose crystallinity, degree of polymerization, accessible surface area, particle size, lignin content, hemicellulose content, acetyl content etc (Noura EL-Ahmady, 2014). Cellulose digestion depends much on the crystalline structure of

cellulose (Noura EL-Ahmady, 2014). Different lignocellulosic material varies in crystallinity and their Particle size presents a subsequent challenge in the digestion since increase in particle size would eventually decrease the surface area and it would be harder for the enzyme to attack lignicellulose material (Eriksson, 1993). The more the lignin content of a lignocellulosic sample the more it is shielded from the cellulase since lignin has the tendency to attach with cellulase and masks the actual cellulose content (Noura EL-Ahmady, 2014). Hemicellulose contain different sugar units but easy to break down because of their low degree of polymerization and amorphous nature (Bai et al., 2012). Complex enzymes such as 1,4-β-D-xylaneses, exo-1,4-β-D-xylosidases, endo 1,4 β-D mannases, β-mannosiadse, α-glucornisidase, galactanses, endo arabinanses, α-L-arabinofuranosidases and α-galactosiadses can degrade hemicellulose (Jorgensen & J. Borjesson, 2003) One of the major hemicellulases are xylanases which yield xyloligomers by hydrolyzing β-1,4 bond (Noura EL-Ahmady, 2014). Accessory enzymes such as feuroyl esterase aids the release of hemicellulose content from lignin by hydrolyzing ester bonds. So, hemicellulose content does matter in digesting the lignocellulosic biomass (Prates & Fontes, 2001). Lignin and hemicellulose are covalently linked and after pretreatment phase less hemicellulose content aids in the digestion of cellulose. Also hemicellulose contains acetylated xylan contents which can intervene with the enzyme recognition and greatly decreases the cellulose digestibility. To promote biodegradation of lignocellulosic biomass, the use of microbial consortia is becoming increasingly popular among scientists since one species can't compensate for the diverse function that is needed for the multistep process of bioethanol production (Puentes-téllez & Salles, 2018). In nature microbial community survive by resource complementarity and this is the clue that we have to take and develop unique bacterial communities that provide less complex, cheaper and efficient way to produce bioethanol and remove the energy problem thats bothering us (Bai et al., 2012; Kuhad et al., 2011; Puentes-téllez & Salles, 2018). By trying to achieve this goal not only we are making progress on environmental science but also in microbiology as diversity and functionality in different species of microbes will give us new insight into their world. Textile industries in our country provide huge economic stability in Bangladesh and are considered as the biggest exporting sector (according to BGMEA). But this industry is also polluting the environment by using harsh chemicals for the processes such as bleaching, dyeing, washing, sizing and desizing etc (Kuhad et al., 2011; Shah, 2014; Tavčer, 2013). These processes can be done using enzymes from microbes such as in bacteria or Fungi (Kuhad

et al., 2011; Tavčer, 2013). For example the impurities that reside inside cellulose can hamper other textile processes such as desizing, scouring, polishing, washing, degumming, bleaching etc. These impurities are basically pectin substances, waxes etc and can be removed enzymatically by pectinase and amylase producers (Aly & Zahran, 2010).

In this article we have tried to isolate, characterize and create a new microbial consortia with the help of several bacterial strains and tried to understand their function in conjunction with the production of bioethanol. Also the effect of bacillus strains in the treatment of various cotton fiber to find the possibility of cotton scouring by assessing the bacteria treated sample in XRD and AFM analysis.

Objectives of the research work:

1. Isolation of bacterial strains from rumen fluid.
2. Screening for cellulase producer strains be means of cellulose plate assay
3. Biochemical characterization of the selected isolated cellulose degrading bacteria by means of biochemical tests analysis, morphological and microscopic characteristics.
4. Molecular identification using 16S rRNA gene sequencing and Bioinformatics analysis.
5. Fiber (cotton, banana, areca and coir) treatment using selected bacterial strains to distort cellulose structure.
6. FTIR and XRD analysis of the treated fibers to understand the structure distortions.
7. Determination of reducing sugar yielding capacity of cellulase utilizing strain HSTU-2 and HSTU-3 for maize straw.

CHAPTER-II

METHOD AND MATERIALS

This section shows all the utilized method for conducting this theses including isolation, biochemical and molecular characterization of cellulose degrading bacterial strains as well as the methods of semi-qualitative and quantitative analysis of the treated fibers (cotton, banana, areca and coir) with the selected bacterial strains and consortium using FTIR and XRD analysis.

2.1 Place of study

The experiment was designed and executed at Biochemistry and molecular biology research lab in Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur.

2.2 Handling of laboratory apparatus and glassware

All the laboratory equipment that were used in this study including conical flasks, glass pipettes, beakers, glass rods, petri dishes, micropipette tips, eppendorf tubes, falcon tubes, PCR tubes etc had been autoclaved at 121°c in 15 psi for 15 minutes before usage to make sure they were in sterile condition.

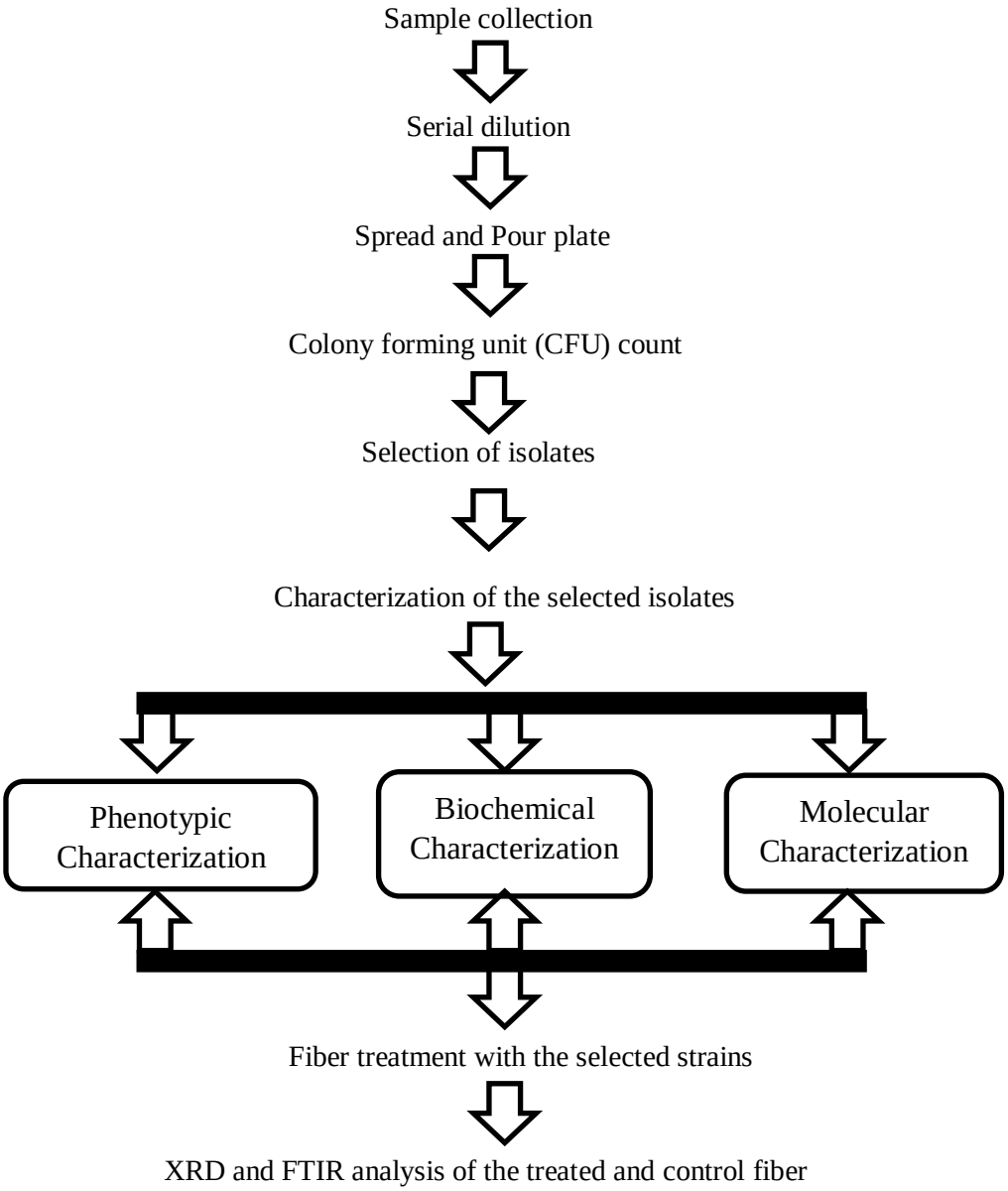
2.3 Solutions and reagents

Required solutions and reagents used in the study were freshly prepared before use. All chemicals needed were acquired from the laboratory. None of the solutions or reagents were further purified since they were of a reagent grade. The list of reagents is given in appendix A.

2.4 Media

In this study different types of media were used for selective growth, enrichment culture, and indication of specific properties. Media preparation and sterilization were done according to the protocol and standard recipe in appendix B.

2.5 Flow chart of the study



2.6 Materials and Methods

2.6.1 Chemicals and glassware

Chemicals and glassware used in this study are presented in Appendix A.

2.6.2 Sample collection

The cow dung sample was collected from the village kornai, near Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur, Bangladesh, in a sterile container and transported to the laboratory. After that the sample underwent serial

dilution and spread plate technique was used to transfer the bacteria to nutrient agar media. The plates showing distinct colonies of bacteria were chosen for further work.

2.6.3 Serial dilution

Test tubes containing 9 ml of physiological saline (0.9% NaCl) were autoclaved before use. Initially, 1 ml of fluid sample was mixed with 9 ml of saline water in a test tube in order to dilute 10^{-1} and mixed with 9 ml of saline by repeated pipetting in order to make tenfold dilution. Again, 1 ml from the 10^{-1} test tube was transferred to 10^{-2} labeled test tube and mixed with 9 ml saline solution in it by repeated pipetting. This action was repeated for the test tubes labeled as 10^{-3} to 10^{-6}.

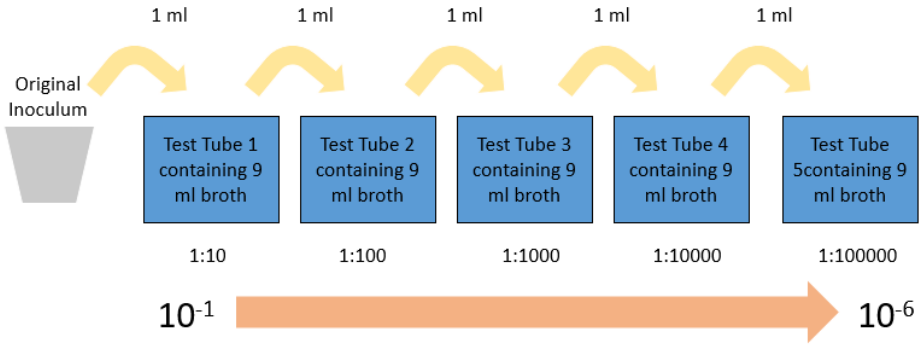


Fig 1: Serial dilution

2.6.4 Isolation of cellulase enzyme secreting bacteria

Cellulose degrading bacteria were isolated and identified from cow dung following sequential steps. Appropriate dilutions (10^{-4}) and (10^{-6}) of bacterial samples ware spread on agar plates containing 1% yeast extract, 1.5% Tryptic Soy Broth (TSB), 1.5% agar, pH 7.0 (Gerhardt, 1994). These plates were incubated for 24 h at 37° C. After that bacterial colonies were picked from each plate and streaked on agar plates for further purification. The purified colonies were subjected to congo red agar media (composition: KH_2PO_4 0.5 g, $MgSO_4$ 0.25 g, cellulose 2 g, agar 15 g, Congo-Red 0.2 g, and gelatin 2 g; distilled water 1L and at pH: 6.8–7.2) for the rapid and sensitive screening test of cellulase producers by observing clear zones after flooding with 1% congo red and 1M NaCl (Mishra & Dubey, 2015). The grown colonies that showed discoloration of Congo-Red plate were considered as cellulose degrading bacteria.

2.7 Phenotypic identification of bacterial strain

The potential cellulose degrading strains were observed under microscope in order to study their properties.

2.8 Morphological & Biochemical characterization

The selected bacterial strains were differentiated based on their metabolic pattern by using different biochemical tests (Williams, 1994) including catalase, oxidase, MR-VP, TSI, Citrate utilization, Indole, MIU and Individual sugar fermentation tests (dextrose, lactose, maltose and sucrose) (James Cappuccino, 1998). Morphological features were identified by growing the isolated cultures on the minimal nutrient media (composition: $(NH_4)_2SO_4$, 0.1%, KH_2PO_4, 0.05%; K_2HPO_4, 0.05%; $CaCl_2$, 0.01%; NaCl, 0.01%; $MgSO_4.7H_2O$, 0.02%; yeast extract, 0.1%, and cellulose, 0.2%) (Williams, 1994).

2.8.1 Gram staining

The bacterial samples were subjected to gram's staining by using a primary stain crystal violet and a counterstain safranine to distinguish between gram positive and gram negative strains (Colco, 2005). 95% ethyl alcohol was used as decolorizing agent. This method differentiates between gram positive and gram negative bacteria based on the presence or absence of peptidoglycan layer. Since gram positive bacteria has a thick peptidoglycan layer it retains the primary stain, however gram negative bacteria retains the counter stain safranine (Williams, 1994).

2.9 Biochemical characterization

2.9.1 Catalase test

The selected bacterial isolates were tested for the presence of catalase enzyme by adding 3% H_2O_2 into a small amount of bacterial colony on a clean glass slide. A positive catalase test provides rapid production of bubbles indicating catalase utilization by the isolate. A negative result indicates the absence of catalase enzyme. This test identifies members of Enterobacteriaceae family which are catalase positive (Williams, 1994).

2.9.2 Oxidase test

Oxidase test was done on the selected isolates to see if they can utilize cytochrome c oxidase which is basically an enzyme of the electron transport chain (Williams, 1994). A

clean filter paper was placed on a petri dish. A drop of oxidase reagent (1% tetramethyl-p-phenylenediaminedi hydrochloride) was added onto the filter paper. Using an inoculating loop, a small amount of bacterial pure culture of the selected isolates were streaked onto the oxidase droplet. Any formation of deep blue or dark purple color within 10-30 seconds of the reagent was observed. Oxidase positive bacteria are basically aerobic and can use oxygen as a terminal electron acceptor.

2.9.3 Methyl Red-Voges-Proskauer Test/ (MR-VP) Test

Methyl Red (MR) and Voges-Proskauer (VP) broth is utilized for identifying an organism's metabolic pathway. MR is performed to detect the ability of an organism to produce stable acids end products which is achieved if the organism uses mixed acid fermentation pathway. When an organism is MR positive, it creates an acidic environment that will overcome the buffers (Potassium phosphate that resist pH) in the medium. So, after adding methyl red, the broth will stay red. The organisms that gives positive VP result further metabolizes pyruvic acid to form acetyl-methyl carbinol (acetoin). This end product, in the presence of atmospheric oxygen and 40% potassium hydroxide is converted to diacetyl. Diacetyl, under the catalytic action of alpha-naphthol and creatine, is converted into a red complex. (Voges, 1898; werkman, 1930).

MR-VP broth was prepared with autoclaved distilled water and 3.5 ml broth was transferred to each test tubes. After that the selected isolates were inoculated into the each tubes and incubated at 37°C for 24 hours. After incubation, five drops of methyl red reagent was added for detecting MR positive isolates by searching for red colors in the test tubes. For conducting VP test after inoculation and incubation at 37°C for 24 hours 0.6 ml of 5% alpha-naphthol was added to individual test tubes. Following after 0.2 ml of 40% KOH was added and the test tubes were shaken gently in atmospheric oxygen. After that the tubes were allowed to remain for 10-15 minutes, they were observed for a pink-red color development which indicates a positive result. Both MR-VP tests will produce a yellow color on the surface to indicate a negative result.

2.9.4 Triple Sugar Iron (TSI) test

TSI media is a semi-solid media containing three sugar (Lactose, Sucrose, and Glucose) and also iron which is done to confirm the ability of the bacteria to utilize these sugars (Rutter, 1969). After preparing the media 7 ml of the broth was distributed to each test

tubes. The media was sterilized by autoclaving at 15 psi, 121°C for 15 min. After sterilization, the media was left to cool by keeping the test tubes in a slanted position in order to form a butt and a slant. The selected isolates were inoculated and the tubes were subjected to Incubation at 37°C for 24 hours. After incubation, the appearance of the media was observed by following below protocol (Table 1) (Rutter, 1969).

Table 1. TSI result evaluation parameters

Slant/Butt condition	**Slant color/Butt color**	**Decision taken**	**Gas formation**
Alkaline slant/Alkaline butt	Red/Red	glucose, lactose and sucrose non-fermenter	Depending on the visibility of bubbles or cracks in the test tubes
Alkaline slant/acidic butt	Red/Yellow	Glucose fermentation only	Depending on the visibility of bubbles or cracks in the test tubes
Acidic slant/acidic butt	Yellow/Yellow	glucose, lactose and/or sucrose fermenter	Depending on the visibility of bubbles or cracks in the test tubes
Note: Production of black color means the bacterial isolate can utilize iron and have produced H_2S.			

(Rutter, 1969)

2.9.5 Citrate Utilization Test (CIU)

The citrate test is utilized to identify the ability of the bacterial isolate to utilize citrate as a carbon and energy source. This test is basically necessitates in the identification of gram negative bacteria based on their metabolic product since gram negative isolates contain citrate-permease that uses citrate present in the medium as a carbon source of energy. This test is also used to distinguish between members of the Enterobacteriaceae family. A positive CIU test will rely on the generation of alkaline by-products of citrate metabolism which will subsequently increase the pH of the medium demonstrated by the color change of a pH indicator. Citrate enters into the cell and is cleaved by citrate lyase releasing oxaloacetate and acetate after which this oxaloacetate is metabolized to pyruvate and CO_2. After that CO_2 reacts with water and sodium carbonate to release an

alkaline by-product. The ammonium salts that are present in the media are also used by the microorganisms which ultimately produces another alkaline product ammonia and turning the media from deep green to intense Prussian blue (Coulter, 1931; Koser, 1923).

A sterile glass pipette was used to transfer 10 ml of the media to each of the test tube. After that, the media was left to cool until it had a semi solid consistency. The selected isolates were inoculated in individual test tubes and incubated at 37°C for 24 hours. The intense Prussian blue appearance of the media and the rise in pH above 7.6 (which is due to carbonates and bicarbonates produced as by-products of citrate catabolism) indicates a positive result. Also some bacterial growth will be visible on the surface of the test tube.

2.9.6 Indole test

The indole test screens for the ability of an organism to degrade the amino acid tryptophan by using the enzyme tryptophanase and produce indole. Indole can then react with (p)- dimethyl aminobenzaldehyde (Kovac's reagent) or (p)- dimethyl aminocinnamaldehyde produce a red colored complex (K. J., 1982; Turner, 1961). Peptone broth was formulated and the test tube containing the media were sterilized by autoclaving at 15 psi. pressure (121°C) for 15 minutes. The selected isolates were inoculated right after and incubated at 37°C for 24 to 48 hours. After incubation 5 drops of was added to each test tubes. The cultures producing the cherry red color ring were indicated as positive and the absence of red color was indicated as a negative result.

2.9.7 Motility Indole Urease (MIU) Test

The MIU test is done for multiple reasons including to detect if the selected isolate can utilize urea, if the organism is mobile or not and if the bacteria can produce indole (Williams, 1994). The media was prepared using MIU agar medium and 5 ml sterile 40% urea solution per 95 ml basal medium. After that, 6 ml solution was transferred to each sterilized test tube and autoclaved at 15 psi. pressure (121°C) for 15 minutes. The media was left to cool down completely until it had a semi solid consistency by gently placing the tubes in a horizontal manner. The selected isolates were inoculated and incubated at 37°C for 24 hours. The results were interpreted as per table 2.

Table 2. MIU result evaluation parameters

Conditions	Positive	negative
Urea	A color change from yellow to orange to pink-red	No color change
Motility	A cloudy mass of bacteria is seen	Hazy medium without any accumulation
Indole	pink-mauve color on the bottom of the tube	No pink-mauve color is formed

(Williams, 1994)

Note: Basically the results for motility and urease are observed first and then the medium is read for indole production. But to be absolutely sure Indole and urease test has been done separately to identify the ability of the selected isolates to use urea and tryptophan.

2.9.8 Urease test

Urease test utilizes the ammonia production from urea by microorganism using urease enzyme. Organisms containing urease hydrolyses urea into ammonia and CO_2. With formation of ammonia the media color changes from light orange to magenta pink (pH 8.1) since ammonia alkalines the medium (Christensen, 1946; Vuye & Pijck, 1973). If the organism utilizes urease, the medium will change color indicating positive result due to the presence of phenol red indicator in the urea agar media.

The media was formulated using urea agar base and a pH of 6.8 was maintained. After formulation, 6 ml solution was transferred to each sterilized test tube and autoclaved at 15 psi. pressure (121°C) for 15 minutes. The media was left to cool down completely and after reaching room temperature the selected isolates were inoculated and incubated at 37°C for 24 hours for observation of urease activity.

2.9.9 Fermentation of sugar: lactose, maltose, sucrose, dextrose

Specific sugar fermentation test was done using phenol red carbohydrate broth to observe if the selected isolates can utilize these sugars. Different sugars (Lactose, Maltose, Dextrose, and Sucrose) media were made according to protocol provided by the manufacturers and 6ml of the media were transferred into each test tubes. The final pH

of 7.4 ± 0.2 was maintained (Rutter, 1969). After that the selected isolates were inoculated and incubated at 37°C for 24 hours. The appearance and color of the media was observed after incubation. After incubation the tubes that turned yellow as a result of acid production by the bacteria were taken as a positive and the ones with no color change was indicated as negative.

2.10 Specific media growth

The isolated and selected strains were subjected to different media to observe their growth and metabolic pattern more clearly.

2.10.1 Gelatin mannitol salt agar

Gelatin mannitol salt agar is the media very much selective for *Staphylococcus aureus* but also facilitates growth for gram-positive, spore-forming, rod shaped, aerobic or anaerobic bacteria such as *Bacillus anthracis, B. cereus, B. subtilis, Clostridium perfringens and C. tetani*. The media contains casein enzymic hydrolysate and yeast extract as sources of carbon, nitrogen and other essential nutrients and growth factors including vitamins. It also contains D-mannitol and lactose as carbohydrate source. After bacterial growth ammonium sulphate is used to confirm if the isolate can liquefy gelatin or not (chapman, 1948).

The gelatin mannitol salt agar media was prepared accordingly and autoclaved at 15 psi. Pressure (121°C) for 15 minutes. After that the media was poured into specific petri dishes inside a laminar flow cabinet. Then the plates were solidified, specific strains were inoculated in the plates and incubated at 37°C for 24 hours. After incubation 3 drops of bromothymol blue was added onto the places of the plate where colonies had been removed for further confirmation.

2.10.2 MacConkey Agar

This media is generally used to identify non-fastidious gram-negative rods, particularly members of the family Enterobacteriaceae and the genus Pseudomonas. These media can also be used to differentiate lactose fermenter and lactose non-fermenter gram negative bacteria. This media contains pancreatic digest of gelatin and peptones (meat and casein) that gives essential nutrients, vitamins and nitrogenous factors required for growth of microorganisms. This media also provides lactose monohydrate as a source of

carbohydrate and crystal violet and bile salts which gives the medium selectivity for gram negative bacteria (Allen, 2005).

The MacConkey agar was supplied by the manufacturer and prepared accordingly. Then the media was sterilized using autoclave. After that the media was poured into specific petri dishes inside a laminar flow cabinet. Then the plates were solidified, specific strains were inoculated in the plates and incubated at 37°C for 24 hours. After incubation the plates were observed for interpretation of results.

2.10.3 Salmonella Shigella (SS) Agar test

SS agar is a selective media for isolating and differentiating Salmonella spp. Strains. Bile Salts, Sodium Citrate and Brilliant Green are the selective ingredients in this media that inhibits gram-positive, coliform organisms and Proteus spp. The colonies that produce hydrogen sulfide grows as black centered colonies due to the presence of Thiosulfate and Ferric Citrate in the media. The source of nitrogen, carbon and vitamins for proper growth of microorganism is provided by beef extract, enzymatic Digest of Casein, and Enzymatic Digest of animal tissue in the media (James Cappuccino, 1998).

The SS agar was formulated and sterilized using autoclave. After that the media was poured into specific petri dishes inside a laminar flow cabinet. After the plates were solidified, specific strains were inoculated in the plates and incubated at 37°C for 24 hours. After incubation the plates were observed for interpretation of results (James Cappuccino, 1998; Williams, 1994).

2.11 Molecular Characterization

2.12 Genotypic identification of bacterial strain

The selected cellulose degrading isolates were characterized by 16s rRNA gene sequencing. The 16s rRNA is a ubiquitous housekeeping genetic marker that ribosomes can’t translate without. Furthermore the gene sequence itself is very much conserved with a bp of 1500 which makes it the perfect candidate for identifying new species of bacteria by performing phylogenic and taxonomic analysis (Janda & Abbott, 2007; Sacchi et al., 2002). The protocols used for this method has been described below:

2.13 DNA sequencing protocol

For the DNA sequencing of bacterial isolates fresh 24 hour TSB cultures had been prepared. Primers used for the partial sequencing included universal 16s primer. The procedures involving the sequencing of 16s rRNA gene sequencing include:

a) DNA isolation using 'Boiling method'
b) PCR and PCR product purification
c) Measurement of concentration of PCR purified DNA
d) Cycle sequencing and product purification
e) Data analysis

2.13.1 DNA isolation using Boiling method

To extract sufficient amount of DNA from bacterial isolates 300 µl deionized water had been taken in each of the 1.5 ml eppendorf tubes. For individual samples two or three single pure colonies of bacteria had been taken using sterilized inoculating loop and mixed with the deionized water (using hands). After that the tubes were subjected to heat shocks at 100° C for 10 minutes using 'Heat Block' machine. After 10 minutes the tubes were immediately put into ice (Icebox utilized) for 10 minutes. Then these tubes were centrifuged for 15 minutes at 10,000 rpm. The pellet was removed and the supernatant was transferred into new tubes for individual samples. For extraction confirmation, measurement was taken in nano-drop machine immediately after the procedures were finished (Queipo-Ortuño & Morata, 2008).

2.13.2 PCR and PCR product purification

For PCR amplification of the extracted DNA 16s universal primers were used as shown in table 3

Table 3. Universal Primer sequences

Primer	Sequence	Type
Forward: 27F	5'-AGA GTT TGA TCM TGG CTC AG- 3'	Universal
Reverse: 1492R	5'-GGT TAC CTT GTT ACG ACT T- 3'	Universal

For individual samples 12.5 µl master mix, 7.5 µl nuclease free water, 1 µl each for forward and reverse primer and 3 µl DNA sample were mixed into a PCR tube making a total volume of 25 µl and vortex properly eliminating any chance of bubble formation. After the procedures the PCR was run (Sander & Road, 1976) following the accurate PCR steps described in table 4.

Table 4. PCR parameters

PCR conditions	Temperature	Time (minutes)
Initial denaturation	94° C	2
Denaturation	94° C	30
Annealing	50° C	30
Extension	72° C	2
Final extension	72° C	5

After finishing the PCR samples were kept in -20° C for later use.

2.13.3 Agarose gel electrophoresis

The PCR products were subjected to agarose gel electrophoresis in which case 1% agarose gel (0.25 agar+ upto 25 ml TBE buffer) was used to stain the gel and the samples were loaded into the well with dye (3µl samples + 2µl dye). After running for 1 hour in the gel the samples were visualized using a gel documentation machine (Sambrook J, 2001).

2.13.4 DNA Purification after Gel Documentation

After completing PCR, DNA was purified from the amplicon using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The individual PCR tubes holding each sample were taken and transferred into a ‘spin column tube’. Binding buffer was added to each of them 4 times the amount of the sample and mixed using vortex machine. After mixing the tubes were centrifuged in 10,000 rpm for 1 minute. After centrifugation the unnecessary precipitate was discarded from the tubes and 650 µl wash buffer was added. The tubes were centrifuged again in 10,000 rpm for 1 minute and precipitate was discarded. The tubes were subjected to centrifuge for the third time in 12,000 rpm for 2 minutes to ensure the wash buffer is discarded. At the last step of purification 50 µl of elution buffer was added to each tube and allowed to stand for 1 minute. And lastly the

tubes were centrifuged again in 12,000 rpm for 2 minutes and stored at -20̊ C (Sander & Road, 1976).

2.13.5 Measurement of PCR purified DNA using Nano-drop

After the PCR products were purified, nano-drop was used to know the concentration of DNA present in each tube. The standard DNA concentration for the next step of the molecular characterization was considered to be 9-10 nano-gram per µl (ng/ µl). At first 2µl elution buffer was taken as blank and following after samples were loaded one after another to get the reading. The sample holder was sterilized each time after being used. The OD of 260/280 ratios were measured with the integrated software which indicated the purity of the sample (Sanger & Nicklen, 1977).

2.13.6 Cycle Sequencing Preparation

Before cycle sequencing, the sample DNA was diluted for it to be in the exact concentration as the working solution which was 5ng/µl. After diluting, reagents were prepared for cycle sequencing which is mentioned in table 5.

Table 5. Reagent preparation for Cycle Sequencing

Reagents	**Amount (µl)**
RRP (Ready reaction premix)	2.0
Buffer	2.0
Primer	0.32
DNA	7.0
Nano polar water or N. H2O	2.0
Up to	20.0 for each sample

Ready Reaction Premix contains $MgCl_2$, dNTPs, DNA polymerase and an addition of ddNTPS for chain termination. After reagent preparation for each sample, each tube was rotated to eliminate bubbles by using rotator and finally the samples were placed in the PCR machine for cycle sequencing. The process took up to two hours and 20 minutes (Sander & Road, 1976).

2.13.7 Purification after Cycle sequencing

After performing cycle sequencing the individual DNA samples were purified in a three step process. At first 2 µl 3M sodium acetate and 50 µl 100% Ethanol were added sequentially into each PCR tube holding individual cycle sequencing product and incubated at -20̊ C for 15 minute. After that the tubes were centrifuged at 13,000 rpm for 15 minute and the supernatant was discarded. Lastly, 200 µl of 70% Ethanol was added and centrifuged at 13,000 rpm for 15 minute. The supernatant was discarded and the tubes were dried overnight inside the PCR cabinet for the final step (Sanderv & Road, 1976).

2.13.8 Sequencing DNA sample using Genetic analyzer 3130

Before running the samples in the genetic analyzer, 10 µl highly de-ionized form amide (HIDI) was added to each tube containing individual samples. The samples were spun for the elimination of bubbles and incubated at 95°C for 5 minute inside a thermo cycler for denaturation of DNA. Immediately after incubation the samples were put into ice (using ice box) for making sure the DNA stayed denatured. Then, the samples were loaded into ABI 3130 Genetic Analyzer which uses 4 capillary systems. For each sample it took up to 4 hours to get a sequence (Sanger & Nicklen, 1977).

2.14 Bioinformatics analysis

2.14.1 Conversion

After getting the sequence output from the genetic analyzer as '.ab1' files, the sequences were converted into 'FASTA' format using DNA sequence assembler v4 (2013), Heracle BioSoft. After getting the FASTA sequences the files were kept for identification in a species level.

2.14.2 Identification

The FASTA format files of the sequences were checked for species level identification using National Center for Biotechnology Information or 'NCBI Blast' (nucleotide blast) tool for which 'megablast' optimization program was used.

2.14.3 Sequence similarity checking

The DNA sequences of each species was checked for similarity with other sequences of cellulase utilizing bacteria collected from NCBI using 'Clustal Omega' (Sievers et al., 2011).

2.14.4 Phylogenetic tree analysis

After sequence similarity analysis the evolutionary tree of the identified strains were formed in order to get a better understanding about their origin. With the help of maximum likelihood method the optimal tree was formed. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Since the analysis covers some of the bacterial strains previously identified (HSTU-6, HSTU-7, HSTU-8 HSTU-9 and HSTU-10) to make a bio consortium in the treatment of cotton fabric; a super phylogenetic tree has also been formed including all these strains to get a basic understanding about their evolution. All positions containing gaps and missing data were eliminated and all sites were used for making comparison. Evolutionary analyses were conducted in 'MEGA X' (F. J., 1985; Kumar S., 2018).

2.15 Fiber treatment with selected bacterial strains

For treating the fibers with the selected bacterial strains an optimal media was formed which included several reagents (0.1% $(NH_4)_2SO_4$, 0.05% K_2HP0_4, 0.05% KH_2PO_4, 0.02% $MgSO_4.7H_2O$, 0.01% $CaCl_2$, 0.01% NaCl, and 0.1% yeast extract, 1.8% agar) for bacterial growth. The media was autoclaved at 15 psi. Pressure (121°C) for 15 minutes and allowed to cool. After cooling the bacterial strains HSTU-2 and HSTU- 3 and previously identified and isolated 4 bacterial strain HSTU-6, HSTU-7, HSTU-9 and HSTU-10 were inoculated with 0.5% of the selected fibers (cotton, banana, areca and coir) which were already dissolved into the media (Haque et al., 2015; Haque et al., 2013). HSTU-6 and HSTU-7 seven are pectinase producers with NCBI accession number MG582600.1 and MG582601.1 respectively while HSTU-9 and HSTU-10 are amylase producers with NCBI accession number MG582602.1 and MG582603.1 respectively.

For treating these four selected fibers (cotton, banana, areca and coir) two types of consortium were made where HSTU-6, HSTU-7, HSTU-9 and HSTU-10 bacterial strains were used. After inoculation with the bacterial consortium each of the four fibers were incubated in different time periods, temperature and rpm as shown in table 6.

Table 6. Strains used in making bacterial consortium

Fiber used with labelling	Bacterial strains used	Incubation time	Rpm used
Cotton S	HSTU-9 and HSTU-10	4 days	130
Cotton S	HSTU-6 and HSTU-7	3 days	110
Banana 4	HSTU-9 and HSTU-10	4 days	130
Banana 4	HSTU-6 and HSTU-7	3 days	110
Areca 5	HSTU-9 and HSTU-10	4 days	130
Areca 5	HSTU-6 and HSTU-7	3 days	110
Coir 1(a)	HSTU-9 and HSTU-10	4 days	130
Coir 1(a)	HSTU-6 and HSTU-7	3 days	110

In addition cotton and areca fibers along with maize straw were treated individually with cellulose degrading bacterial consortium in which case strains HSTU-2 and HSTU-3 were used and incubated in different time periods, temperature and rpm shown in table 7.

Table 7. Individual treatment using cellulase producers (HSTU-2 and HSTU-3)

Fiber used	Bacterial strains used	Incubation time	Rpm used
Raw Cotton	HSTU-2,3	7 days	130
Areaca	HSTU-2,3	5 days	130
Maize straw	HSTU-2,3	7 days	140

After incubation these samples were sun dried and preserved for next phases of the experiment.

2.16 FTIR analysis

A Thermo Nicolet Nexus FTIR spectrophotometer was used to obtain the IR measurements connecting with liquid nitrogen cooled MCT-A detector. A MIRacle, single reflection horizontal ATR accessory (PIKE instruments) having a diamond ATR

crystal fixed at incident angle of 458 was used to collect the spectra. The each fiber samples (approximately 10 mm) were mounted on top of ATR crystal and pressed gently by a pre-mounted sample clamp. The IR spectra of each sample fibers were obtained in the range of 500-4000 cm^{-1} with an average of 4 cm^{-1} resolution. The Omnic software was used to correct the ATR effect and atmospheric contributions from CO_2 and H_2O vapor (Arman et al., 2012; Haque et al., 2015; Haque et al., 2013).

2.17 XRD analysis

To analayze the crystallinity of the treated fibers X-ray diffraction (XRD) was used. The XRD instrument that was used was a diffractometer with GADDS and uses powder as sample component. 40 kV and 40mA operating voltage and current was selected for the entire operation. 10° to 40° in steps of 0.02° was the size range of 2θ and time interval was 0.4 seconds (Arman et al., 2012; Haque et al., 2013). The crystallinity of cellulose was calculated according to method described by (Segal, L. & Conrad, 1959) using the following equation

$$\mathrm{CrI} = \frac{\mathrm{i}_{002} - \mathrm{i}_{\mathrm{am}}}{\mathrm{i}_{002}} \times 100$$

(Segal, L & Conrad, 1959)

Where i_{002} is the maximum intensity meaning all the peaks under designate baseline, i_{am} is the intensity of diffraction in the same units at 2θ = 18 meaning area of amorphous peaks. If this calculation is simplified It brings us to the following calculation

$$\text{Crystallinity Index}(\%) = \frac{\text{Area of crystalline peaks}}{\text{Area of all peaks}(\text{amorphous} + \text{crystalline})} \times 100$$

Area of crystalline peaks and all peaks were determined using 'OriginPro' software by using optimal baseline correction and the control sample percentage have been compared with the treated fiber to get the crystallinity data.

2.18 Enzymatic saccharification

Enzymatic saccharification was carried out using DNS method (Quinlan, 1982) and reducing sugar yield was confirmed by preparing a standard curve with absorbance at 540 nm on Y-axis and amount of reducing sugar on X-axis and flowing the below equation:

$$\text{Yield, Y (\%)} = \frac{\text{Weight of reducing sugars (mg)}}{\text{weight of substrate used for enzymatic hydrolysis (mg)}} \times 100$$

(Arman et al., 2012; Haque et al., 2013)

The reagents used for DNS method are shown in appendix A. To test for the presence of free carbonyl group or so called reducing sugars yield for the strains HSTU-2 and HSTU-3, a standard glucose solution was prepared and diluted into 200 µg/ml, 150 µg/ml, 100 µg/ml, 75 µg/ml, 50 µg/ml, 25 µg/ml, 10 µg/ml with our test solutions where HSTU-2 and HSTU-3 have been used for the enzymatic hydrolysis of maize straw.

HSTU-2 and HSTU-3 were applied for the saccharification process and a total of 3ml with selected strain dose was used. The dry powder concentration of the maize straw was 10 mg/ml for the two strains (HSTU-2and HSTU-3). The hydrolysis was performed in a hot water shaker bath at 50° C. The reaction was also monitored from time to time by withdrawing samples from the supernatant and measuring the release of soluble reducing sugars by a DNS assay using D-glucose as standard as said earlier. The total reducing sugars was measured using DNS method which were able to form some aldehyde and ketones (Arman et al., 2012; Haque et al., 2013).

CHAPTER III

RESULTS AND DISCUSSION

This section shows all the available results including isolation, biochemical and molecular characterization of cellulose degrading bacterial strains as well as the results of semi-qualitative and quantitative analysis of the treated fibers (cotton, banana, areca and coir) with the selected bacterial strains and consortium using FTIR and XRD analysis.

3.1 Isolation of cellulase enzyme secreting bacteria

Cellulose degrading bacteria were collected and screened from cow dung. The appropriate serial dilutions of samples were made. The bacteria acquired from 10^{-4} and 10^{-6} of cow dung (CD) dilutions were selected and inoculated on cellulose agar plates. Isolated bacterial colonies with cellulose degrading activity were further screened to obtain the pure culture. The colony number 2 from CD 10^{-4} and colony number 3 from CD 10^{-6} showed cellulase secreting activity (Fig 2). Later the colony number 2 from CD 10^{-4} and 3 from CD 10^{-6} were named as HSTU-2 and HSTU-3, respectively. The downstream experiments were conducted with the pure bacterial strains HSTU- 2 and HSTU-3. As the sole carbon source commercial grade cellulose was used because it induces the highest level of cellulase productions from the bacterial strains showing their cellulose degrading capacity.

The HSTU-2 and HSTU-3 produced clear zone of hydrolysis when it was flooded with 1% congo red and 1M NaCl respectively due to hydrolysis of cellulose. The congo red solution forms a dark-black complex with cellulose but not with hydrolyzed cellulose, giving a sharp and distinct clear zone around the cellulase producing bacterial colonies within 10 minutes. The zone diameter of the strain HSTU-2 and HSTU-3 were 8 and 6 mm respectively (Fig 2). The zone diameters of the strains are shown in the Table 8.

Table 8. The zones of clearance (mm) in cellulose plate are represented in details

Isolate number	Source	Zone diameter (mm)
HSTU-2	Cow Dung (CD) colony-1	8 mm
HSTU-3	Cow Dung (CD) colony-2	6 mm

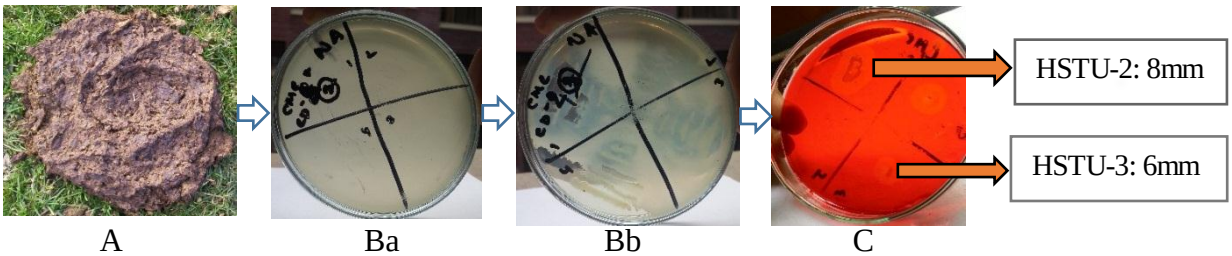


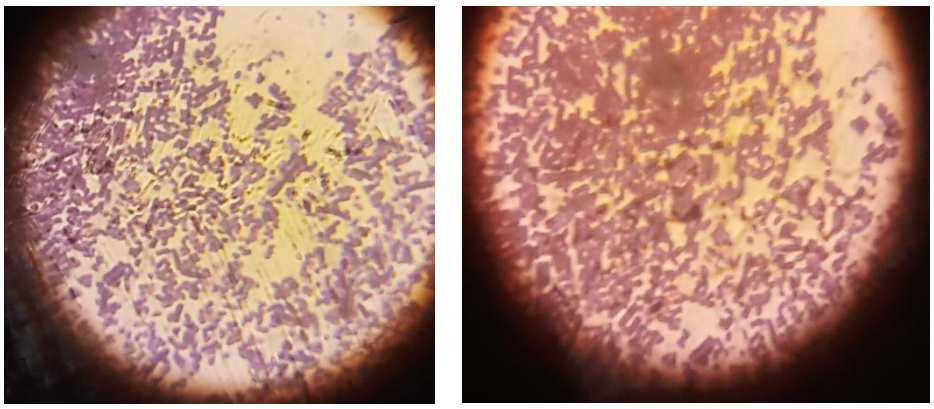
Fig 2: Screening of cellulase enzyme secreting bacteria from cow dung (CD) HSTU-2 and HSTU-3. Where (A) indicates cow dung sample source, (Ba and Bb) indicates culture media, (C) indicates zone diameter of the strain HSTU-2 and HSTU-3.

3.2 Identification of selected cellulase producing bacteria

After isolating the two cellulase producing bacterial strain HSTU-2 and HSTU-3, it was time to identify the bacterial strains based on morphology, metabolic pattern (Baron, 1996) and ultimately at a molecular level since unknown bacteria can be given a name by comparing it with a known bacterium and hence can be placed in a taxonomic class (Cowan, 1965).

3.2.1 Gram Staining

The strains HSTU-2 and HSTU-3 were identified morphologically through the gram staining procedure. Both the strains were stained with primary and secondary stain following accurate principle (Colco, 2005). HSTU-2 and HSTU-3 both were seen as rod shaped, purple colored symmetrically arranged single bacterial cells in chains under the light microscopic (Fig 3). So, both the strains were indicated as gram positive bacteria.



HSTU-2 HSTU-3

Fig 3: Strains HSTU-2 and HSTU-3 under Microscopic (1000x) showing purple color symmetrical arrangement and confirming booth the strains as gram positive bacteria.

3.3 Biochemical tests

Bacterial strains were collected from culture media according to their growth pattern, morphology, and appearance. The isolates were then sub-cultured and some specific biochemical tests were done for identification. The biochemical tests that were performed are described precisely in materials and method chapter 3.

The following biochemical tests were performed for isolate strain HSTU-2 and HSTU-3 and the results of the isolates are given below in table 9.

Table 9. List of performed biochemical tests

Performed Biochemical tests	
Catalase test	Motility Indole Urease (MIU) Test
Oxidase test	Fermentation of sugar: maltose, sucrose, dextrose and lactose
Methyl Red (MR) Test	Gelatin mannitol salt agar
Voges-Proskauer (VP) Test	MacConkey Agar
Triple Sugar Iron Agar (TSI) test	Salmonella Shigella Agar
Citrate utilization test	Indole test

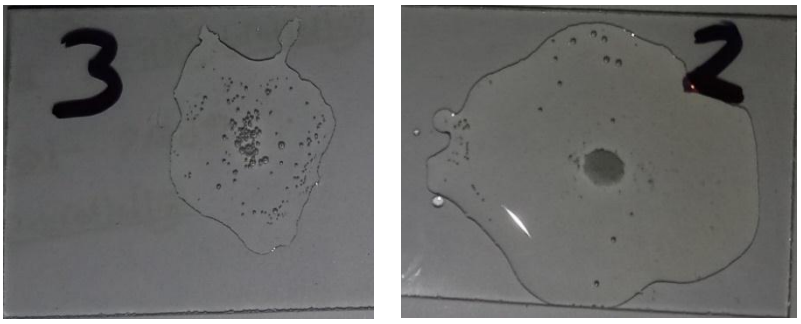
The results for the performed biochemical tests done on strain HSTU-2 and HSTU-3 are presented in Table 10 where a positive result is represented by + and a negative result is represented by - sign.

Table 10. Biochemical tests and results

Biochemical tests	**Results of HSTU-2**	**Results of HSTU-3**
Catalase test	+	+
Oxidase test	-	+
Methyl Red (MR) Test	-	-
Voges-Proskauer (VP) Test	+	+
Triple Sugar Iron Agar (TSI) test	-	+
Citrate utilization test	-	-
Indole test	+	-
Motility Indole Urease (MIU) Test	Non-motile and -	Non-motile and +
Urease test	+	+
Fermentation of maltose	+	+
Fermentation of sucrose	+	+
Fermentation of lactose	+	+
Fermentation of dextrose	+	+
Gelatin mannitol salt agar	-	-
MacConkey Agar	-	-
Salmonella Shigella Agar	-	-

3.3.1 Catalase test

Both the bacterial strains HSTU-2 and HSTU-3 showed strong catalase activity by producing bubbles in the presence of H2O2 as shown in figure 4.



HSTU-3 (catalase positive) HSTU-2 (catalase positive)

Fig 4: HSTU-2 and HSTU-3 both showing strong catalase activity

3.3.2 Oxidase Test

After adding a drop of oxidase reagent (1% tetramethyl-p-phenylenediaminedi hydrochloride) up onto the filter paper, bacterial strains HSTU-2 and HSTU-3 was smeared into the reagent. After exactly 12 seconds HSTU-3 showed dark purple color formation whereas HSTU- 2 showed no color formation even after 30 seconds. This indicates that HSTU- 2 doesn't utilize cytochrome c. oxidase but HSTU-3 does. The results are shown in figure 5.

HSTU- 2 (oxidase negative) HSTU-3 (oxidase positive)

Fig 5: HSTU-2 showing oxidase negative whereas HSTU-3 showing strong oxidase activity.

3.3.3 Methyl Red-Voges-Proskauer Test/ (MR-VP) Test

Following the principle of MR-VP test as stated by (Voges, 1898; WERKMAN, 1930) HSTU-2 and HSTU-3 both showed negative MR results as in both cases the MR-VP

broth changed colors from red to yellow indicating no acidic environment was established. The MR results are shown in figure 6

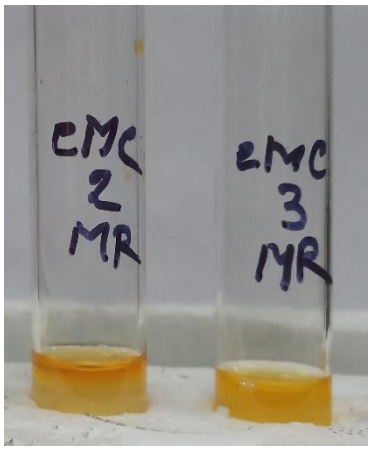


Fig 6: Strain HSTU-2 and HSTU-3 showed negative results after 24 hours at 37°C of incubation.

Both the strains HSTU-2 and HSTU-3 showed positive VP results since in the presence of alpha naphthol, 40% KOH and atmospheric oxygen the MR-VP broth changed into red complex as shown in figure 7

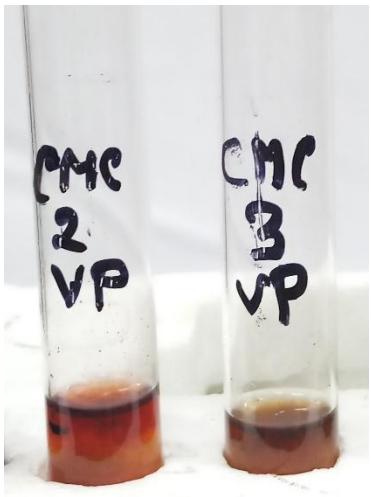


Fig 7: Strain HSTU-2 and HSTU-3 showed positive result by forming red complex with alpha naphthol and KOH results after 24 hours at 37°C of incubation.

3.3.4 Triple Sugar Iron (TSI) test

HSTU-2 showed alkaline slant and alkaline butt as indicated by the color of the media after incubation at 37° C for 15 minutes. So, it is evident that this strain is incapable of fermenting glucose, lactose and sucrose. There is also no bubbles in the tube and no black color formation indicating no gas or H_2S production.

As for strain HSTU-3 the yellow slant and butt indicate that the strain can ferment glucose, lactose and sucrose. Also there is black color formation inside the test tube indicating H_2S production. Evident bubble production is seen and gas production. The results are shown in figure 8

Fig 8: Strain HSTU-2 showed negative result while HSTU-3 showed positive result after 24 hours at 37° C of incubation. H_2S and gas. Production is also prominent in HSTU-3.

3.3.5 Citrate Utilization Test (CIU)

The selected bacterial strains HSTU-2 and HSTU-3 both showed negative results as after proper incubation time the medium color inside the tubes remain unchanged (fig 9) indicating the isolates didn't use citrate as carbon source of energy thereby not producing any alkaline by-products that can subsequently raise the pH of the media (Koser, 1923).

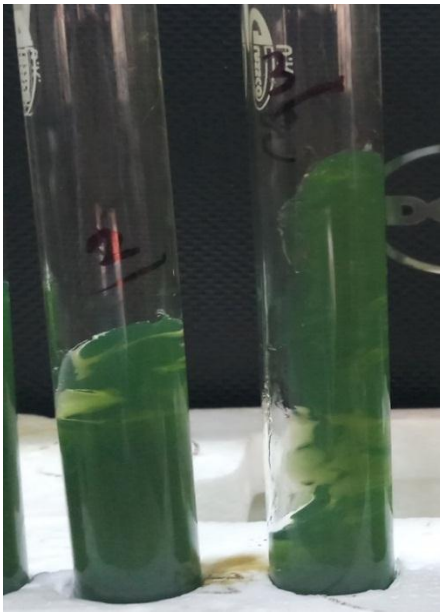


Fig 9: Strain HSTU-2 and HSTU-3 both showing negative result since after incubation at 37° C for 24 hours the color of the tubes remain unchanged.

3.3.6 Indole test

HSTU-2 was able to produce indole (Fig 10) thereby producing a reddish complex within 30 seconds after adding kovac's reagent (K. J., 1982) while HSTU-3 remain unchanged (fig 10) due to the inability to produce indole.

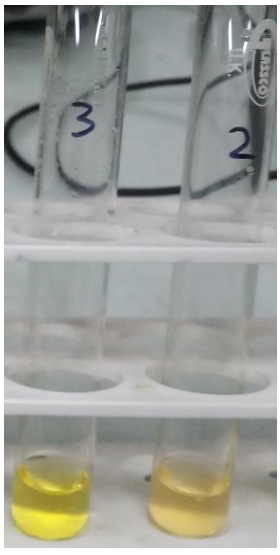


Fig 10: Indole test of HSTU-2 (positive) and HSTU-3 (negative)

3.3.7 Motility indole urease (MIU) test

The selected isolates HSTU-2 and HSTU-3 has been tested for their motility, production of urease and indole. The results are shown in table 11

Table 11. MIU test result

Isolates	Motility	Urea hydrolysis/urease	Indole production
HSTU-2	negative	positive	positive
HSTU-3	negative	negative	negative

HSTU-2 and HSTU-3 clearly show no sign of motility since there is no growth extension from the inoculating line. Moreover HSTU-3 is both urease and indole negative indicated by the fig 11. However, HSTU-2 is both urease and indole positive which is corroborated by the next urease test and the previous indole test results.

Fig 11: HSTU-3 showing indole and urease negative result whereas HSTU-2 is indole and urease positive. However both the strains are motility negative since their growth is limited to inoculating line.

3.3.8 Urease test

After 24 hours of incubation HSTU-2 and HSTU-3 was able to produce pink color (fig 12) in the medium indicating strong urease activity. Since the medium changed color within 24 hours of incubation extended incubation time period was out of question.

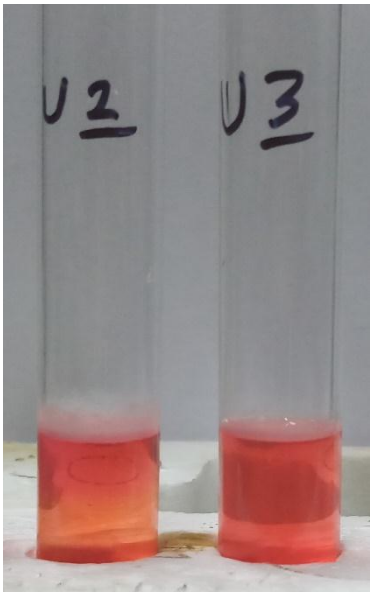
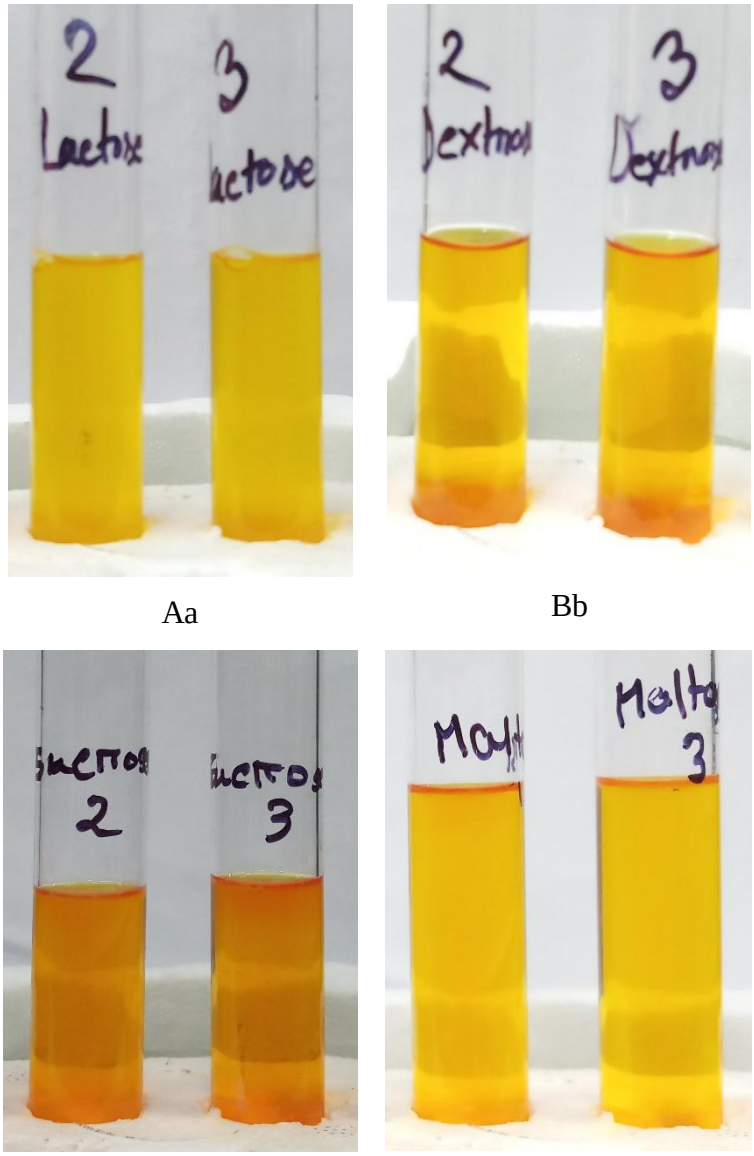


Fig 12: HSTU -2 and HSTU-3 both showing urease activity after 24 hours indicated by the color change from light orange (initial color) to pinkish red.

3.3.9 Fermentation of sugar: lactose, maltose, sucrose, dextrose

In sugar fermentation test both HSTU-2 and HSTU -3 could utilize lactose, dextrose, sucrose and maltose as seen fig 13 (sub figures Aa, Ab, Ac, Ad) respectively as in all cases the media turned to yellow due to the acidic environment that was created with fermentation of sugars and phenol red indicator present in the medium.



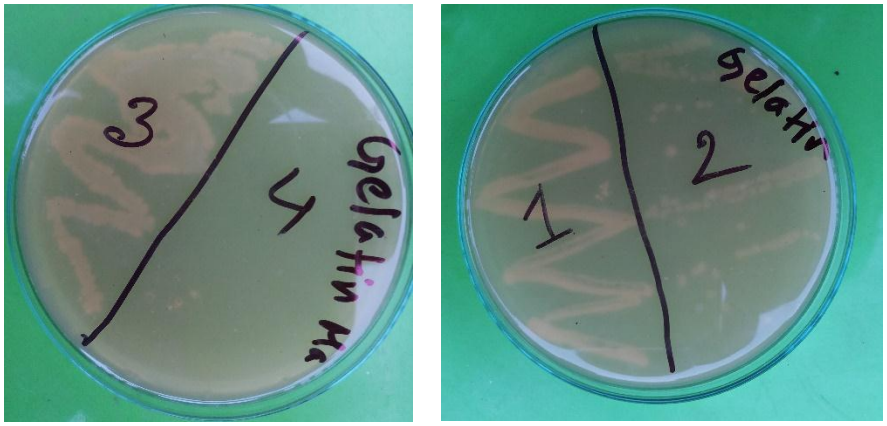
Aa Bb

Ac Ad

Fig 13: Fermentation of lactose, maltose, dextrose and sucrose by HSTU-2 and HSTU-3 as both strain showed positive result in all cases (sub figures Aa, Ab, Ac, Ad).

3.4.1 Gelatin mannitol salt agar

Both HSTU-2 and HSTU-3 didn't show any gelatin liquefaction (fig 14) when applying ammonium sulfate after incubation. The two isolates did grow on the medium suggesting that these isolates are halophiles and they can utilize casein as an energy source of carbon.



Aa Bb

Fig 14: HSTU-2 and HSTU-3 both growing on the medium proving they are halophiles and can utilize casein as carbon source. However both the strains failed at gelatin liquefaction test confirming the strains are not *staphylococcus aureus*.

3.4.2 MacConkey Agar

After proper culturing techniques using macConkey agar neither of the isolates (HSTU-2 and HSTU-3) grew on agar plates indicating clearly that the isolates are gram-positive organism (fig 15)

Fig 15: Both HSTU-2 and HSTU-3 showed no growth what so ever in the mcConkey agar indicating they are gram positive.

3.4.3 Salmonella Shigella (SS) Agar test

Both HSTU-2 and HSTU-3 failed to grow in the SS agar after proper incubation period since SS agar is a selective media only for gram-negative bacteria. Fig 16 shows the plate condition after 24 hours incubation time

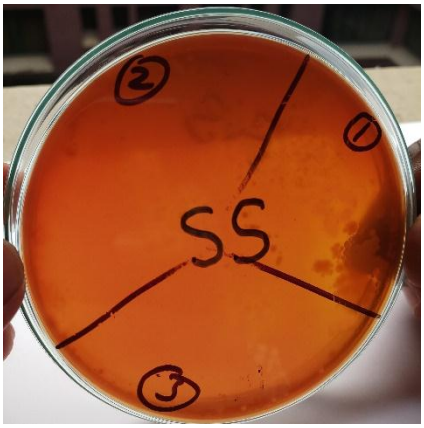


Fig 16: Both HSTU-2 and HSTU-3 showed no growth what so ever in the SS agar indicating they are gram positive isolates.

3.5 Molecular Characterization

The selected cellulose degrading isolates HSTU-2 and HSTU-3 were characterized by 16s rRNA gene sequencing. This section describes the results of DNA sequencing analysis using 16s primer mentioned in chapter 2 as well as the bioinformatics analysis.

3.6 Measurement of DNA concentration after boiling method

After extracting DNA with the help of boiling method, measurements were taken using 'nano-drop' to ensure DNA extraction was completed with sufficient quantity. Table 12 shows the results

Table 12. 'Nano-drop' measurement after DNA extraction

Sample	Conc.	OD (260/280)	OD (260/230)
2	191.3	2.09	0.71
3	190.1	1.69	0.67

3.7 Agarose gel electrophoresis

Following after amplification of the DNA sample with the help of universal 16s primer, the samples were run in the agarose gel electrophoresis which gave specified band data within 1500 bp confirming a successful PCR. The results are shown in fig 17

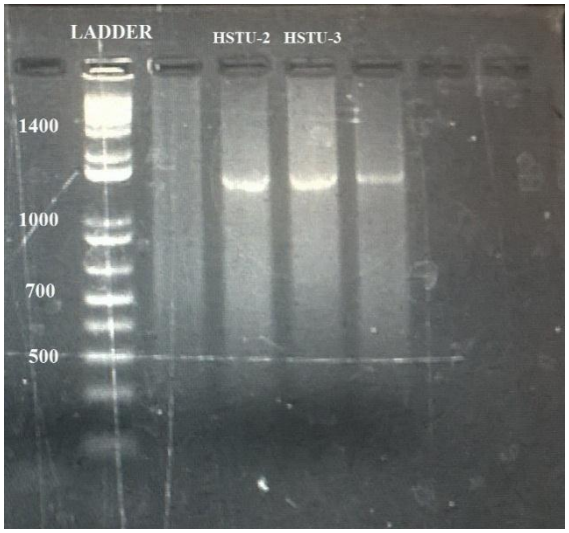


Fig 17: Gel documentation image showing HSTU-2 and HSTU-3 band size.

3.8 DNA concentration measurement after PCR

After confirmation of PCR bands, the PCR products of HSTU-2 and HSTU-3 were purified and the PCR purified products were measured using 'nano-drop' to know the concentration and purity of the DNA sample. Table 13 shows the concentration and OD of the samples HSTU-2 and HSTU-3 with a baseline correction of 340 nm.

Table 13. DNA concentration of the PCR purified products

Sample	Conc.	OD (260/280)	OD (260/230)
2	19.5	2.56	1.91
3	7.6	2.08	1.46

3.9 Sequencing analysis

After measurements using 'nano-drop', the PCR products were subjected to cycle sequencing and purified afterwards. At the final stage the samples were run through the genetic analyzer 3130 and after 8 hours the sequences of HSTU-2 and HSTU-3 were obtained. Both identified sequences of HSTU-2 and HSTU-3 are given in table 14 and table 15 respectively.

Table 14. Identified nucleotide sequences of Bacterial Isolates HSTU-2

Sequence Name	16S rRNA Gene sequence
HSTU-2 NCBI Accession number (MK659878)	GCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGG GTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTC CGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATG GTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGAC CCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGC AACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGG ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGA ATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTG AGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAG AACAAGTACCGTTCGAATAGGGCGGTACCTTGGACGGTACCTAA CCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATA CGTAGGTGGCAAAGCGTTGTCCGGAATTATTGGGCGTAAAGGG CTCGCAGGCGGTTTCTTTAAGTCTGATGTGAAAGCCCCCGGCTC AACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAA GAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGA TGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAAC TGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGAT ACCCTGGTAGTCCACGCCGTAAAACGATGAGTGCTAAGTGTTAG GGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTC CGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTG ACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAA GCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATC CTAGAGATAGGACGTCCCTTTCGGGGGGCAGAGTGACAGGTGG TGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT CCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGT TGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGT GGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACA CACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAG GTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTC TGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGAT CAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGC CCTTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTA ACCTTTAGGA
Total bases	1403 bp

The HSTU-2 strain has been submitted to National Center for Biotechnology Information (NCBI) as part of the partial sequencing experiment data.

Table 15. Identified nucleotide sequences of Bacterial Isolates HSTU-3

Sequence Name	16S rRNA Gene sequence
HSTU-3 NCBI Accession number (MG582599.1)	GGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGG GCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAGACATAAA AGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTA GCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAG CCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGG CCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAAT GGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGT TTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGT TCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCAC GGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGGCA AGCGTTGTCCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGT TTCTTAAGTCTGATGTGAAAGCCCCCGGGCTCAACCGGGGAGGG TCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGA ATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACA CCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGA GCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTC CACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGGTTTCCGCC CCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGGAG TACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCG CACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAG AACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGG ACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCG TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG CAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAG GTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCA AATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAAT GGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCC CACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTG CGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCG GTGAATACGTTCCCGGGCCTTGTACACACCACCCGTCACACCAC GAGAGTTTGT
Total bases	1315 bp

Strain HSTU-3 has been submitted and accepted in NCBI database as partial sequence of the *Bacillus sp*. The NCBI accession number of HSTU-3 is MG582599.1.

3.10 Bioinformatics analysis

3.10.1 Conversion

Both strains HSTU-2 and HSTU-3 came out of the genetic analyzer as .ab1 files which had to be converted to 'FASTA' format which is a universal readable text format. For

this conversion DNA sequence assembler v4 (2013), Heracle BioSoft was used. Also the images that came out of the analyzer has been given in appendix F.

3.10.2 Identification

Identification was done for both these strains (*Bacillus sp.* HSTU-2 and *Bacillus sp.* HSTU-3) using 'NCBI megablast'

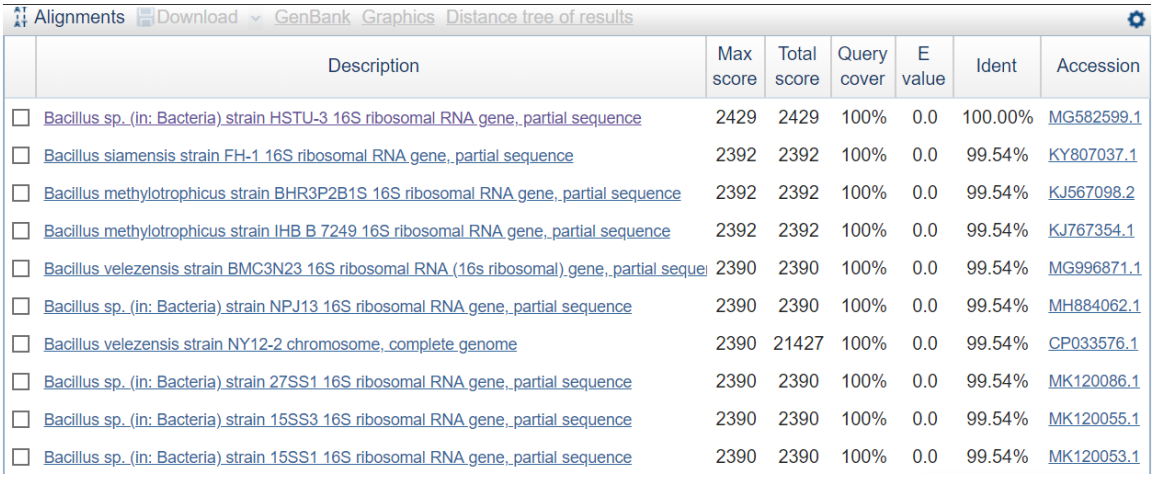
3.10.3 NCBI BLAST analysis

The nucleotide sequences of HSTU-2 and HSTU-3 were identified using NCBI nucleotide 'BLAST' (basic local alignment search tool) analysis. For analysis using 'BLAST', nucleotide collection database of NCBI has been used. The default optimization tool 'Megablast' which is an intra-species comparison tool for identifying unknown nucleotide sequences by aligning and comparing with a known similar sequence has been used for this method. After analysis both the strain HSTU-2 and HSTU-3 have been identified as *Bacillus sp.* (fig 18 and fig 19). HSTU-2 strain is 99.5% identical with various submitted sequences of *Bacillus tequilensis* and *Bacillus subtilis* strains while HSTU-3 is 99.5 % identical with various submitted sequences of *Bacillus siamensis* and *Bacillus methylotrophicus* strains as shown in fig 18 and fig 19

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus tequilensis strain EPP47 16S ribosomal RNA gene, partial sequence	2549	2549	100%	0.0	99.50%	MH085040.1
Bacillus subtilis strain I5-4 16S ribosomal RNA gene, partial sequence	2549	2549	100%	0.0	99.50%	KU229980.1
Bacillus subtilis strain B30 16S ribosomal RNA gene, partial sequence	2549	2549	100%	0.0	99.50%	KC686715.1
Bacillus subtilis strain PK5-70 16S ribosomal RNA gene, partial sequence	2547	2547	100%	0.0	99.50%	MG988256.1
Bacillus subtilis strain PK5-6 16S ribosomal RNA gene, partial sequence	2547	2547	100%	0.0	99.50%	MG988239.1
Bacillus sp. (in: Bacteria) strain tequilensis 16S ribosomal RNA gene, partial sequence	2547	2547	100%	0.0	99.50%	MG818958.1
Bacillus subtilis subsp. stercoris strain WTB53 16S ribosomal RNA gene, partial sequence	2547	2547	100%	0.0	99.50%	MK241851.1
Bacillus subtilis subsp. stercoris strain WTB23 16S ribosomal RNA gene, partial sequence	2547	2547	100%	0.0	99.50%	MK240444.1
Bacillus subtilis strain SA 197 16S ribosomal RNA gene, partial sequence	2547	2547	100%	0.0	99.50%	KY194763.1
Bacillus subtilis strain SA 122 16S ribosomal RNA gene, partial sequence	2547	2547	100%	0.0	99.50%	KY194735.1

Fig 18: Nucleotide BLAST analysis using 'Megablast' in NCBI for HSTU-2.



Alignments | Download | GenBank | Graphics | Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus sp. (in: Bacteria) strain HSTU-3 16S ribosomal RNA gene, partial sequence	2429	2429	100%	0.0	100.00%	MG582599.1
Bacillus siamensis strain FH-1 16S ribosomal RNA gene, partial sequence	2392	2392	100%	0.0	99.54%	KY807037.1
Bacillus methylotrophicus strain BHR3P2B1S 16S ribosomal RNA gene, partial sequence	2392	2392	100%	0.0	99.54%	KJ567098.2
Bacillus methylotrophicus strain IHB B 7249 16S ribosomal RNA gene, partial sequence	2392	2392	100%	0.0	99.54%	KJ767354.1
Bacillus velezensis strain BMC3N23 16S ribosomal RNA (16s ribosomal) gene, partial seque	2390	2390	100%	0.0	99.54%	MG996871.1
Bacillus sp. (in: Bacteria) strain NPJ13 16S ribosomal RNA gene, partial sequence	2390	2390	100%	0.0	99.54%	MH884062.1
Bacillus velezensis strain NY12-2 chromosome, complete genome	2390	21427	100%	0.0	99.54%	CP033576.1
Bacillus sp. (in: Bacteria) strain 27SS1 16S ribosomal RNA gene, partial sequence	2390	2390	100%	0.0	99.54%	MK120086.1
Bacillus sp. (in: Bacteria) strain 15SS3 16S ribosomal RNA gene, partial sequence	2390	2390	100%	0.0	99.54%	MK120055.1
Bacillus sp. (in: Bacteria) strain 15SS1 16S ribosomal RNA gene, partial sequence	2390	2390	100%	0.0	99.54%	MK120053.1

Fig 19: Nucleotide BLAST analysis using 'Megablast' in NCBI for HSTU-3.

3.10.4 Sequence similarity checking

Since *Bacillus sp.* HSTU-2 and *Bacillus sp.* HSTU-3 both had been identified as *Bacillus sp*ecies, a comparison analysis between these two strains had been done as shown in fig 20. The nucleotide composition and comparison between these two strain HSTU-2 and HSTU-3 has also been shown table 16

Table 16. Comparison and Similarities Analysis of *Bacillus sp.* HSTU-2 and *Bacillus sp*. HSTU-3

Orgnisms	T(U)%	C%	A%	G%	Total%
Bacillus sp. HSTU-2	20.2	23.4	25.1	31.3	1403
Bacillus sp. HSTU-3	19.9	23.8	25.0	31.3	1315
Avg.	20.1	23.6	25.1	31.3	906

As we can see from table the two sequence has considerable differences in Thymine content while having slight differences in cytosine and adenine content. The guanine percentage however is same for both species.

3.10.5 Multiple sequence alignment result

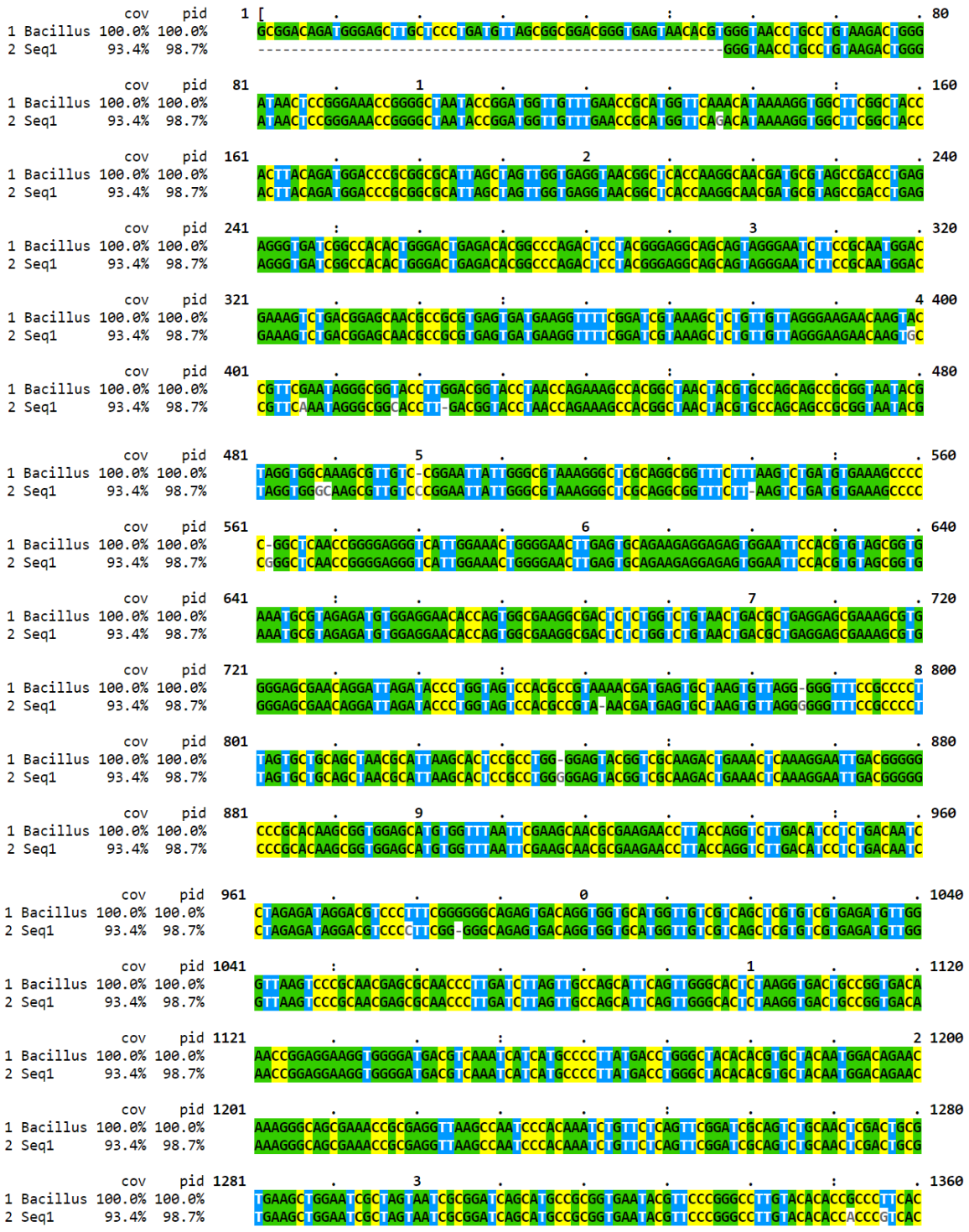


Fig 20: Sequence alignment using clustal omega to determine similarities and dissimilarities between the two identified strains.

The multiple sequence alignment of *Bacillus sp.* HSTU-2 and *Bacillus sp.* HSTU-3 has been done to oversee the differences between these two identified sequences. For sequences alignment the online standalone tool 'clustal omega'(Sievers et al., 2011) and

windows software ‘jalview’ (Troshin PV, 2011; Waterhouse AM & Clamp M, 2009) has been used. The score of the alignment between *Bacillus sp.* HSTU-2 and *Bacillus sp.* HSTU-3 is 128540.0 with the alignment having a length of 1320. Two sequences are 98.71% identical if the gap regions are excluded.

3.10.6 Phylogenetic analysis of (*Bacillus sp.* HSTU-2 and *Bacillus sp.* HSTU-3)

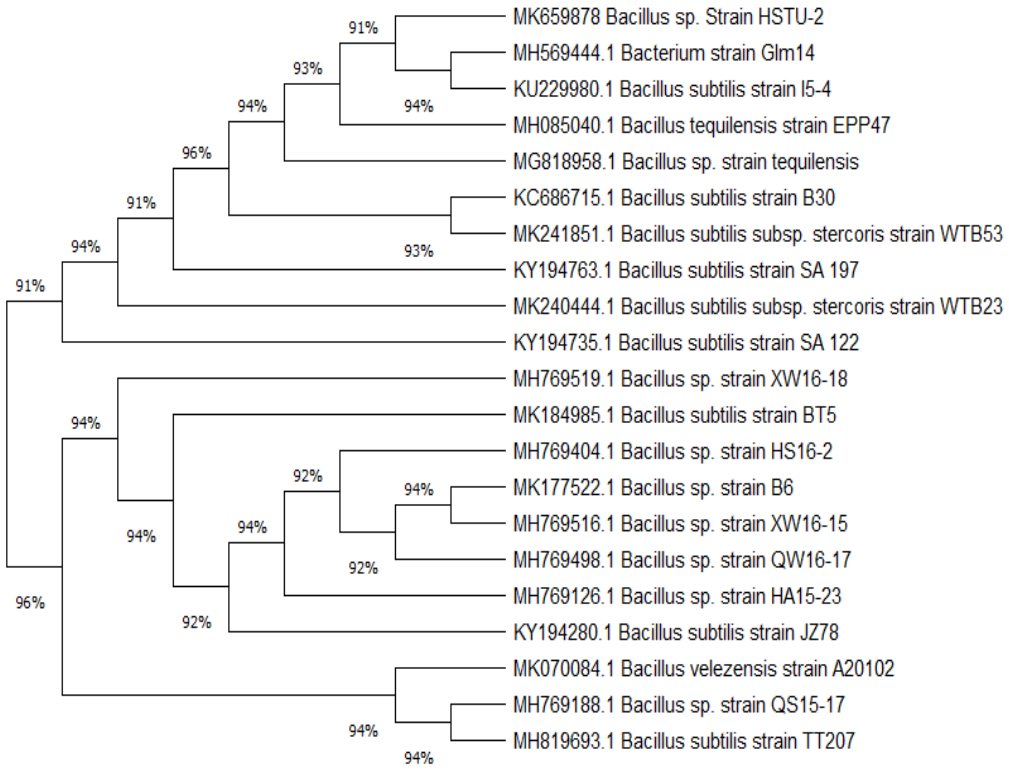


Fig 21: Phylogenetic tree of HSTU-2

The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model (C.R., 1969) (fig 21). The bootstrap consensus tree inferred from 500 replicates (F. J., 1985) is taken to represent the evolutionary history of the taxa analyzed (F. J., 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (F. J., 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 22

nucleotide sequences. There were a total of 1541 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar & Tamura, 2018).

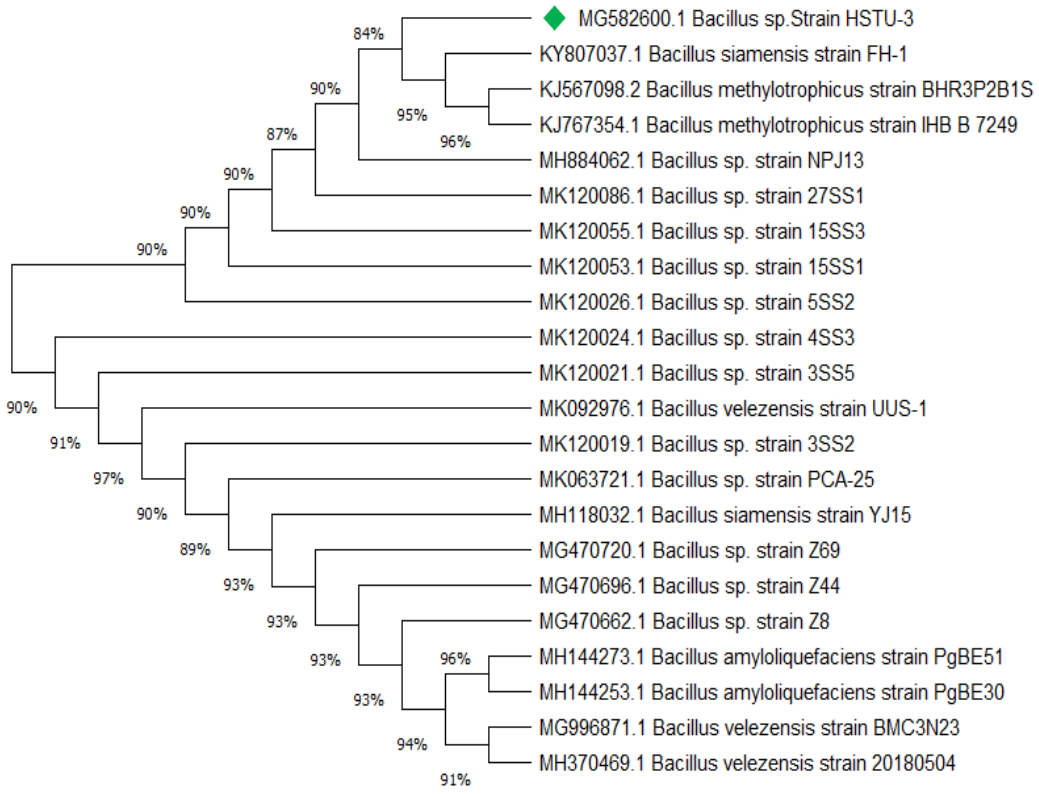


Fig 22: Phylogenetic tree of HSTU-3

The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model (C.R., 1969) (fig 22). The bootstrap consensus tree inferred from 500 replicates (F. J., 1985) is taken to represent the evolutionary history of the taxa analyzed (F. J., 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (F. J., 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 21 nucleotide sequences. There were a total of 1563 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

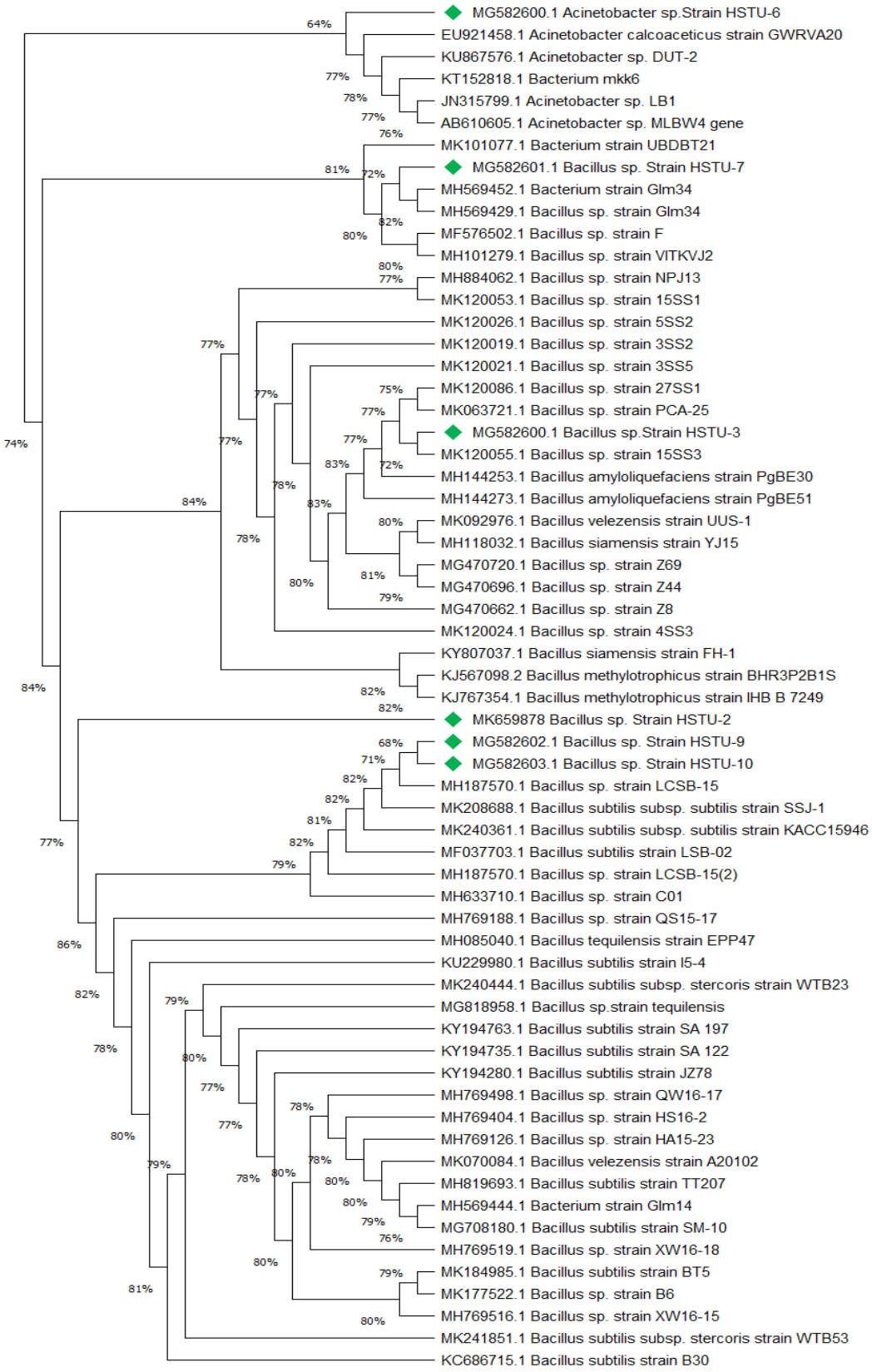
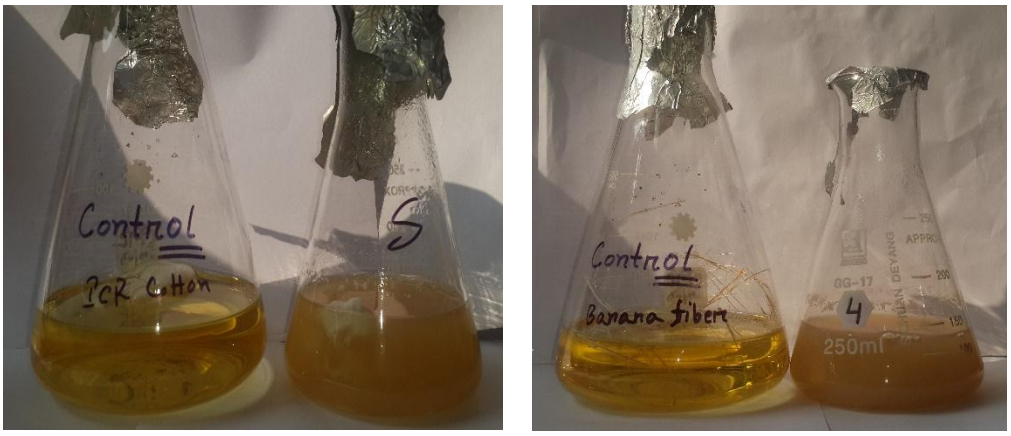


Fig 23: Phylogenetic tree of HSTU-2, HSTU-3, HSTU-6, HSTU-7, HSTU-9 and HSTU-10.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model (C.R., 1969) (fig 23). The tree with the highest log likelihood (-3872.68) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 66 nucleotide sequences. There were a total of 1819 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

3.11 Fiber treatment with selected bacterial strains

After treating the fibers (cotton, areca, banana, coir) with selected bacterial strains and consortium the results were promising as compared with the control almost all the fibers were utilized by bacteria as seen in naked eye. Fig 24 shows the results after the treatment period of fibers and Fig 25 shows areca fiber, cotton fiber, banana fiber and coir fiber treated with (*Bacillus sp.* HSTU-2 and *Bacillus sp.* HSTU-3) respectively along with their control



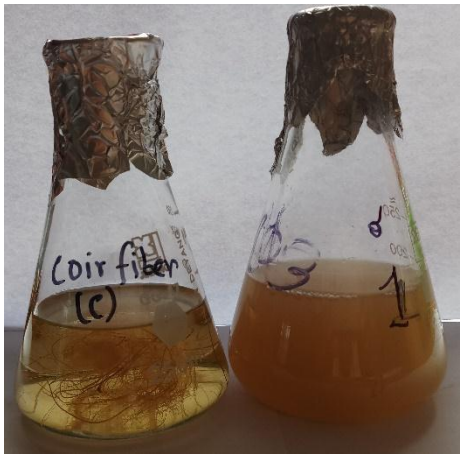
Aa Ab

Fig 24: Sub fig (Aa and Ab) showing ICR cotton and banana fiber treated with consortium (HSTU-2, 3, 6, 7, 9 and 10) respectively along with their control. It is evident that compared to the control the treated fiber is showing a dense solution slightly proving that the bacteria have successfully removed some impurity in them.

Ba

Bb

Bc



Bd

Fig 25: Sub fig (Ba, Bb, Bc and Bd) showing areca fiber, cotton fiber, banana fiber and coir fiber treated with (*Bacillus sp.* HSTU-2 and *Bacillus sp.* HSTU-3) respectively along with their control. It is evident that compared to the control the treated fiber is showing denser solutions slightly proving that the bacteria have successfully removed some impurity in them.

After the treatment phase the fibers were removed from the solution sundried and kept at room temperature for the next phase of the experiment.

3.12 FTIR analysis of the fiber (coir, banana, areca and cotton) samples

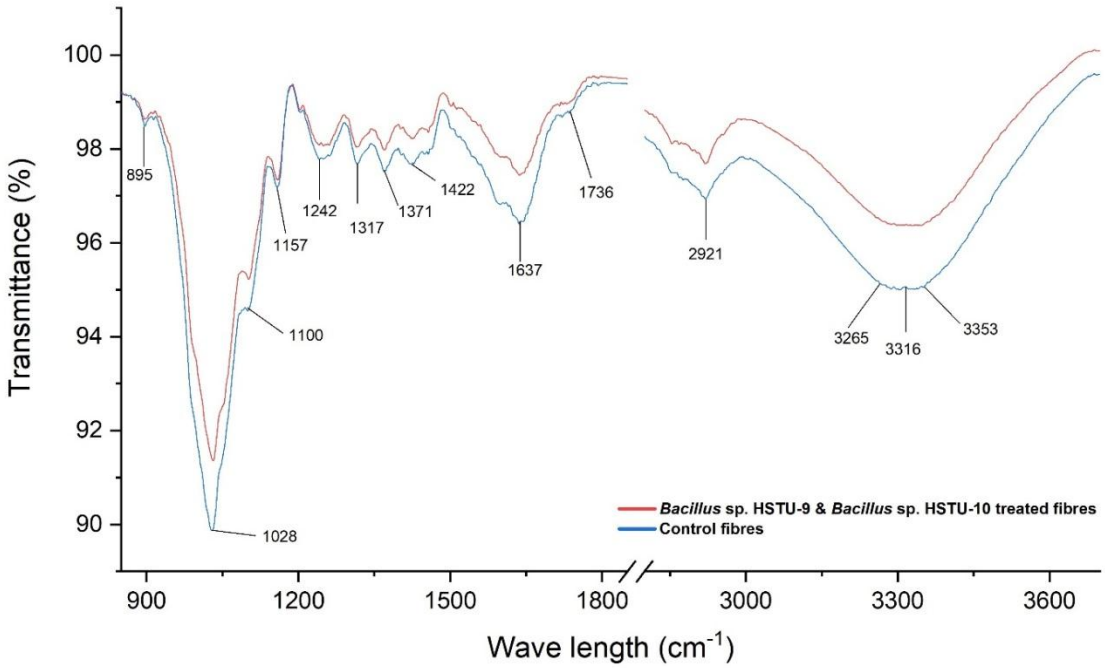


Fig 26: FTIR Curve of HSTU-9 (amylase producer) and HSTU-10 (amylase producer) treated areca fiber.

As seen in the fig 26, HSTU-9 and HSTU-10 treated areca fibers changed significantly compared to the untreated areca. The broad band from 3265-3353cm^{-1} which shows the OH-stretching vibration have become much sharper and more intense after treatment with HSTU-9 and HSTU-10, amylase producers. The peak also shifted to a higher wavenumber value indicating removal of impurities in the fibers such as waxes and pectin etc. The band shown at 895 cm^{-1} corresponding to C-O-C stretching at β-(1, 4)-glycosidic linkages didn't change indicating these two strains can't break these covalent linkages. Moreover, the C-H stretch of cellulose at 2921cm^{-1} changed slightly with more intense and sharper band indicating removal of impurities as well as scissoring of intra and inter molecular hydrogen bonds. The bands corresponding to 1637 cm^{-1} indicates benzene ring vibrations or in this case C-C aromatic structure of lignin have also sharpened considerably with more intensity. Also the band at 1736 cm^{-1} indicates C=O stretching of unconjugated ketone and carboxyl groups. The C-O-C antisymmetric portion of the cellulose indicated at 1157 cm^{-1} also sharpened. The band at 1422 cm^{-1} corresponding C-H bending vibration, 1371 cm^{-1} corresponding C-H rocking vibration, 1100 cm^{-1}, 1242 cm^{-1} and 1317 cm^{-1} corresponding C-O stretching and finally 1028 cm^{-1}

corresponding to =C-H bend all showing much sharper band with high intensities compared to the untreated cotton confirming that the impurities of the treated fiber have been removed effciently strains HSTU-9 and HSTU-10.

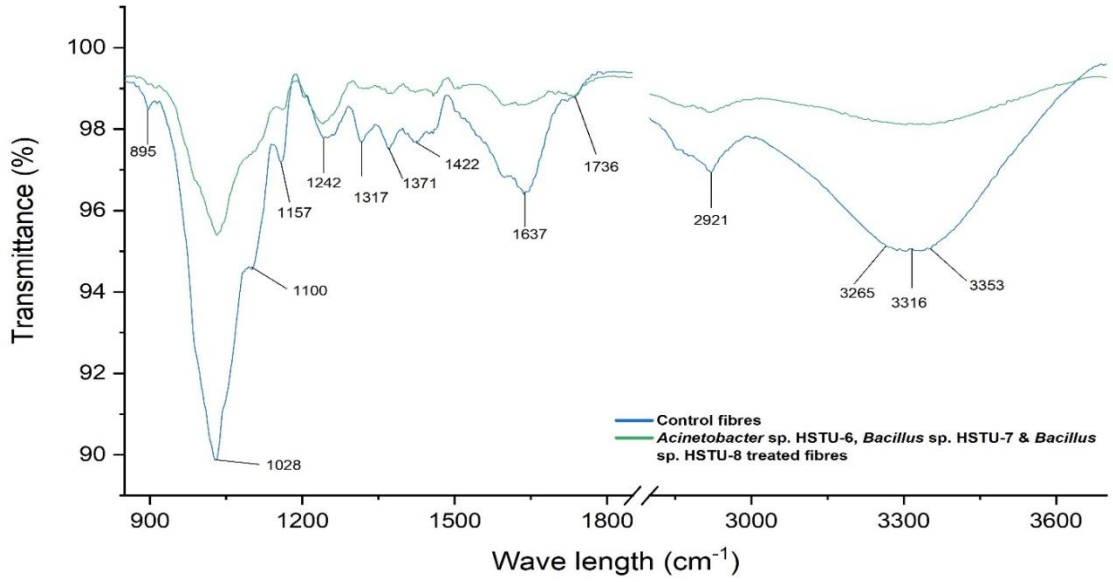


Fig 27: FTIR Curve of HSTU-6 (pectinase producer) and HSTU-7 (pectinase producer) treated areca fiber.

As seen in the fig 27, HSTU-6 and HSTU-7 treated areca fibers changed drastically compared to the untreated areca. The broad band from 3265-3353cm^{-1} which shows the OH-stretching vibration have become Incredibly sharp and more intense after treatment with HSTU-6 and HSTU-7, pectinase producers. The peak itself has changed significantly confirming the ability of these two strains to not only remove impurities from the cellulose structure but also scissoring of intra and inter molecular hydrogen bonds. Also the C-H stretch at 2921cm^{-1} have become sharper and more intense with slight change in the structure indicating removal of impurities as well as distorting the cellulose structure as well. The bands at 1637 cm^{-1} corresponding benzene ring has become sharper with high intensity, however the band at 1736 cm^{-1} also corresponding C-C in ring stretch vibration has not changed at all. The band at 1422 cm^{-1} corresponding C-H bending vibration, at 1371 cm^{-1} corresponding C-H rocking vibration, at 1100 cm^{-1}, 1157 cm^{-1}, 1242 cm^{-1} and 1317 cm^{-1} corresponding C-O stretching and finally at 1028 cm^{-1} corresponding to =C-H bend all showing much sharper band with high intensities compared to the untreated cotton confirming the pectinase activity of the strains HSTU-6 and HSTU-7. Most importantly the band at 899 cm^{-1} corresponding to C-O-C stretching

at β-(1, 4)-glycosidic linkages has almost vanished proving that HSTU-6, HSTU-7 and HSTU-8 have the ability to break β-(1, 4)-glycosidic linkages within cellulose structure.

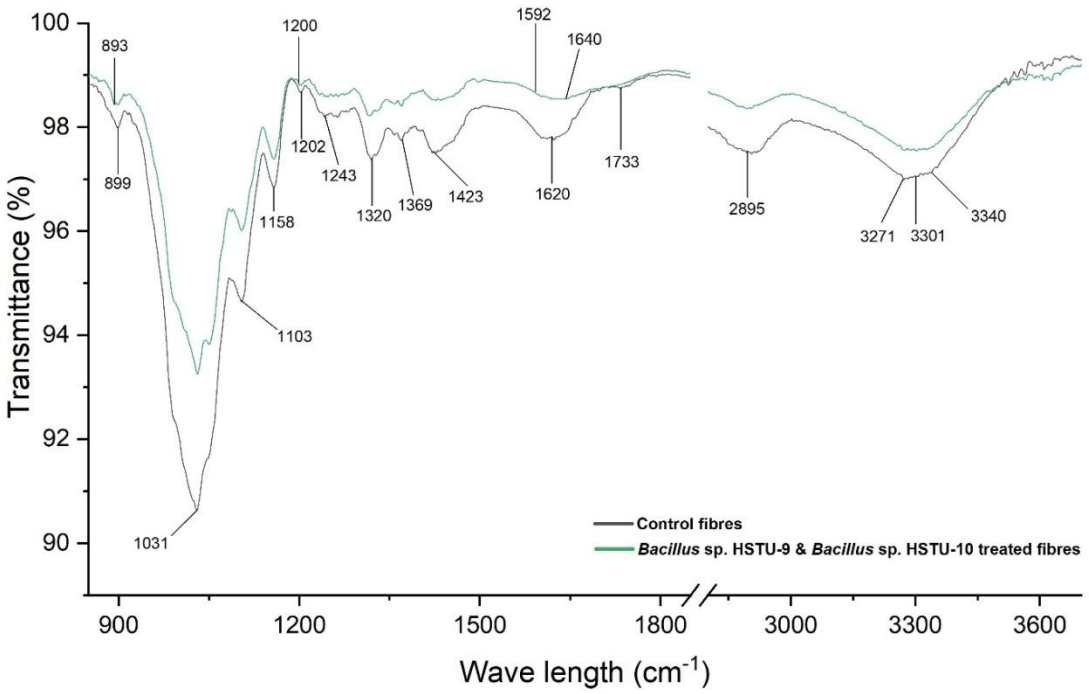


Fig 28: FTIR Curve of HSTU-9 (amylase producer) and HSTU-10 (amylase producer) treated banana fiber.

Compared to the change seen in banana fiber treated by HSTU-6, HSTU-7 and HSTU-8, fig 28 showing HSTU-9 and HSTU-10 treated banana fibers didn't change that much but the results are significant.

The band at 3271-3340 cm^{-1}, the OH-stretching vibration and the band at 2895 cm^{-1}, showing C-H stretch sharpened with more intensity. Also the band at 1733 cm^{-1}, corresponding to C-C in ring stretch, the band at 1423 cm^{-1} corresponding C-H bending vibration, at 1369 cm^{-1} corresponding C-H rocking vibration, at 1103 cm^{-1}, 1158 cm^{-1}, 1243 cm^{-1} and 1320 cm^{-1} corresponding C-O stretching and finally 1031 cm^{-1} corresponding to =C-H bend, all showing more intense sharpening band confirming the removal of certain impurities. Also the band at 1620 cm^{-1} corresponding C-C in ring stretch shifted to 1592 cm^{-1}, the band at 899 cm^{-1} corresponding to C-O-C stretching at β-(1, 4)-glycosidic linkages shifted to 893 cm^{-1} confirming the some distortion of the cellulose structure as well. The band at 1202 cm^{-1} corresponding C-O stretching also

shifted to 1200 cm^{-1}. The strains HSTU-9 and HSTU-10 changed properties of the fiber significantly with proper treatment.

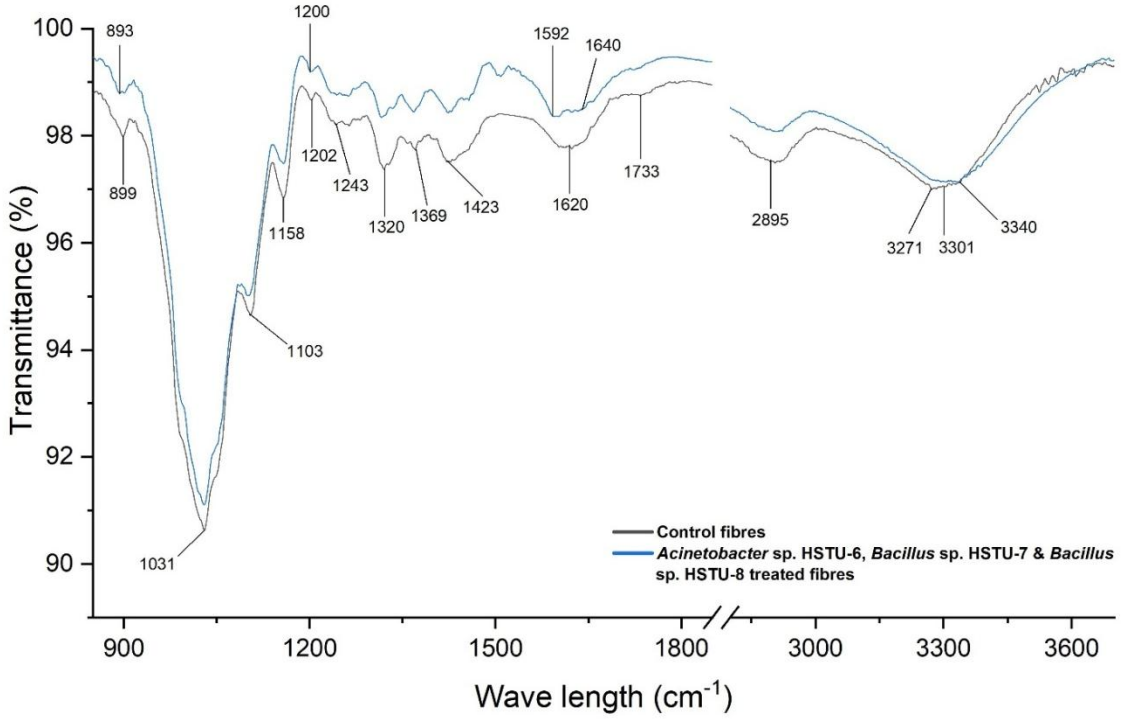


Fig 29: FTIR Curve of HSTU-6 (pectinase producer) and HSTU-7 (pectinase producer) treated banana fiber.

In this case (fig 29) the band at 3271-3340 cm^{-1}, the OH-stretching vibration is slightly sharpened with no change in the peak but the band at 2895 cm^{-1}, showing C-H stretch sharpened with tremendous intensity. Also the band at 1733 cm^{-1}, corresponding to C-C in ring stretch, the band at 1423 cm^{-1} corresponding C-H bending vibration, at 1369 cm^{-1} corresponding C-H rocking vibration, at 1103 cm^{-1}, 1158 cm^{-1}, 1243 cm^{-1} and 1320 cm^{-1} corresponding C-O stretching and finally 1031 cm^{-1} corresponding to =C-H bend, all showing more intense sharpening band than the banana fiber treated previously with amylase producers (HSTU-9 and HSTU-10) confirming that the impurities removal capacity of HSTU-6 and HSTU-7 is much higher. Also the band at 1620 cm^{-1} corresponding C-C in ring stretch shifted to 1592 cm^{-1}, the band at 899 cm-1 corresponding to C-O-C stretching at β-(1, 4)-glycosidic linkages shifted to 893 cm^{-1} confirming some distortion of the cellulose structure as well. The band at 1202 cm^{-1} corresponding C-O stretching also shifted to 1200 cm^{-1}. So, finally it can be said that the

strains HSTU-6, HSTU-7 and HSTU-8 changed properties of the fiber significantly with proper treatment.

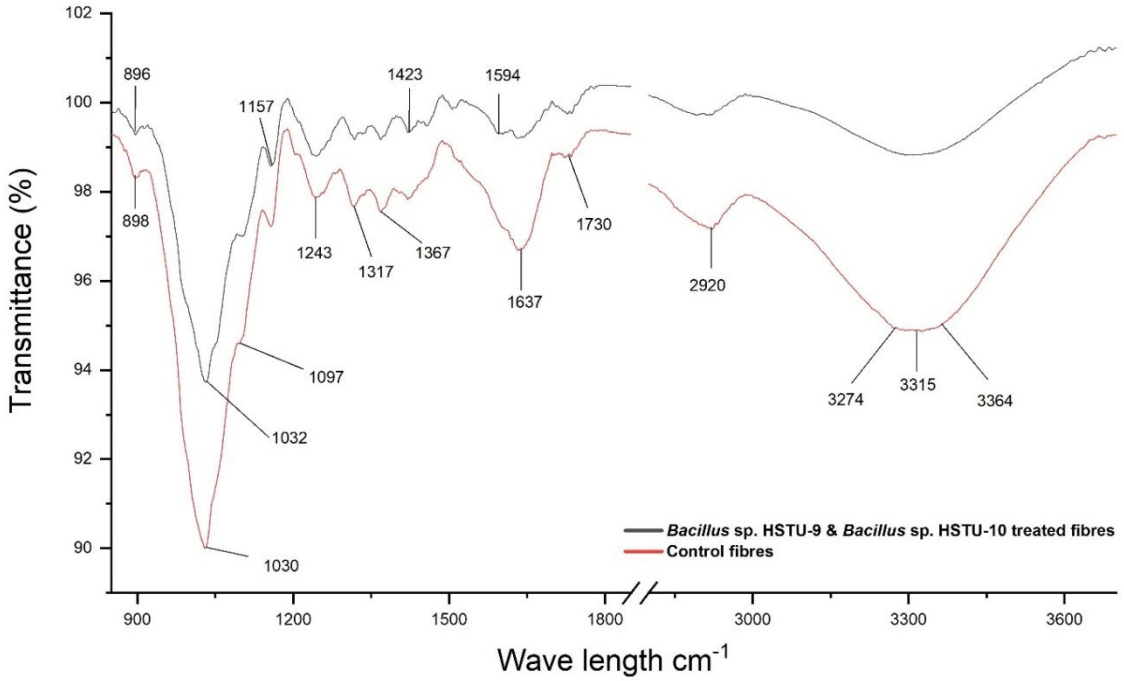


Fig 30: FTIR Curve of HSTU-9 (amylase producer) and HSTU-10 (amylase producer) treated coir fiber.

The OH- stretching vibration here from 3274 cm^{-1} to 3364 cm^{-1} is massively sharper (fig 30) and intense just like the curve shown in fig 27 for areca fiber treated by pectinase producers (HSTU-6 and HSTU-7). Almost all the band showing much sharper and more intense band including the C-H stretch at 2920 cm^{-1}, C-C in ring stretch at 1637 cm^{-1} which has shifted towards 1594 cm^{-1} confirming that the strains are degrading the polymer structure of cellulose as well. Other band positions inside 1500 cm^{-1} also showing huge sharpening of band and intensities while the peak is not changing confirms that the impurities that reside inside these fibers are clearly getting removed. Also in this fig 30 the band at 898 cm^{-1} is also shifted to 896 cm^{-1} just like in the case of other fibers and confirms that strains are hydrolyzing β 1, 4 glycosodic linkages within the structure and decreasing the degree of polymerization of cellulose.

Fig 31 showing the same intense sharp band at specified positions along with prove that Pectinase producer (HSTU-6 and HSTU-7) and amylase producer (HSTU-9 and HSTU-10) is able to remove impurities and slightly change the degree of polymerization of cellulose by keeping the main structure in an intact state.

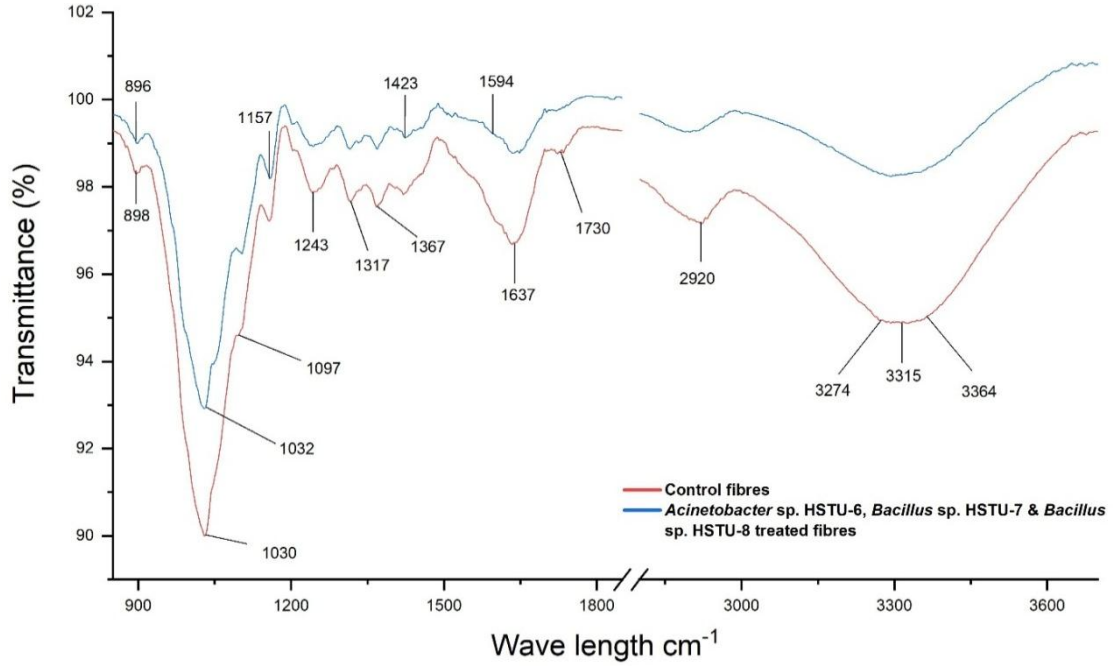


Fig 31: FTIR Curve of HSTU-6 (pectinase producer) and HSTU-7 (pectinase producer) treated coir fiber.

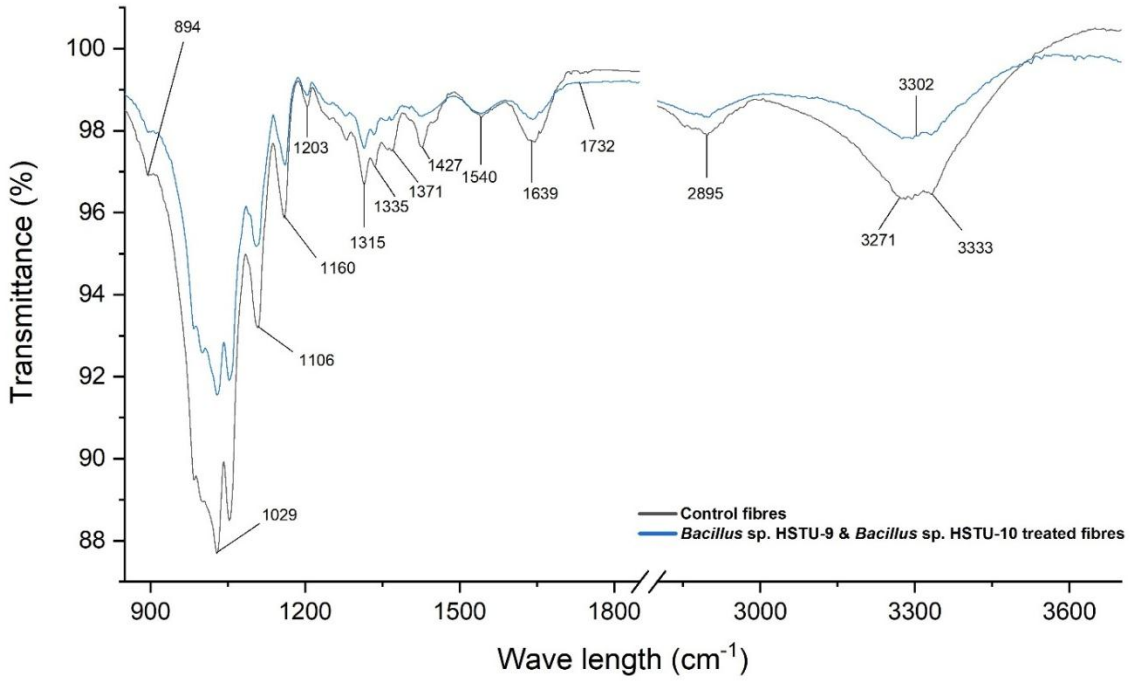


Fig 32: FTIR Curve of HSTU-9 (amylase producer) and HSTU-10 (amylase producer) treated cotton fiber.

As for cotton fibers the bands inside 1500 cm^{-1} corresponding to C-H rocking, bending stretching, =C-H bending, C-O stretching are slightly sharpened with one exception at 1029 cm^{-1} where the =C-H bending band is really sharpened (fig 32). HSTU-9 and HSTU-10 didn't really change the band at 894 cm^{-1} corresponding to the C-O-C stretching since these are amylase producer strains. However the OH stretch from 3271 other than this, the whole structure is completely intact with entire curve sharpening and with more cm^{-1} to 3333 cm^{-1} shifted towards 3302 cm^{-1}, which is very sharp with greater intensities indicating breaking of inter and intra molecular hydrogen bonds. Other than that the entire curve showing intense bands at exact position indicating removal of impurity without distortion of cellulose structure.

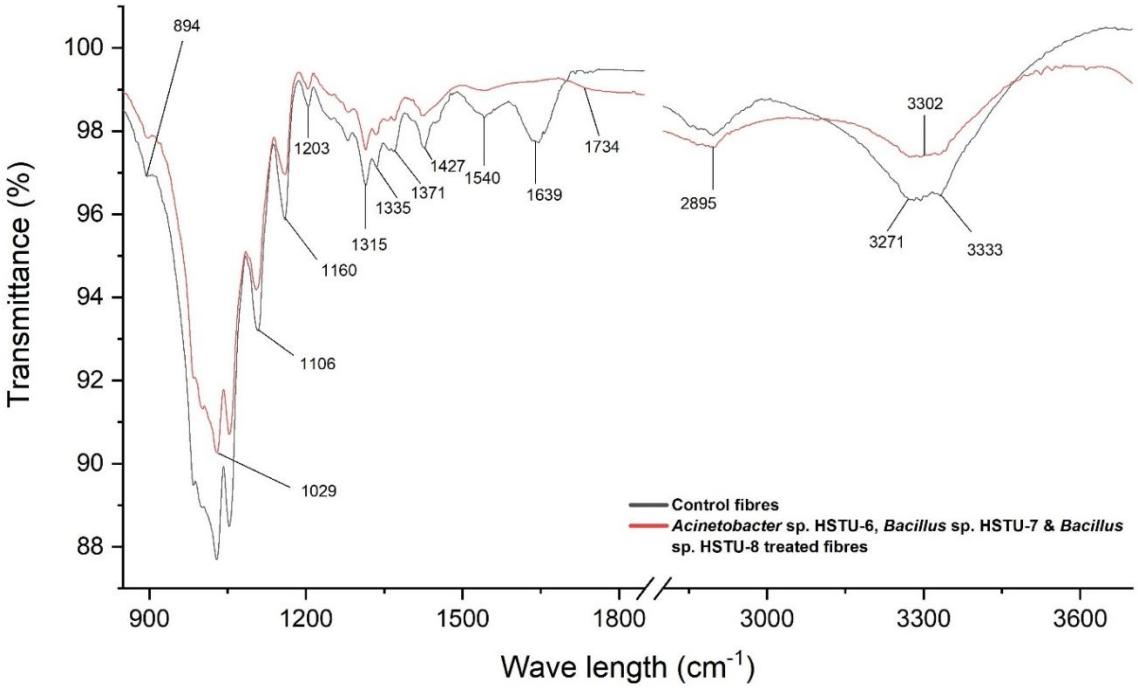


Fig 33: FTIR Curve of HSTU-6 (pectinase producer) and HSTU-7 (pectinase producer) treated cotton fiber.

Fig 33 showing the same intense sharp band at specified positions along with prove that Pectinase producer (HSTU-6 and HSTU-7) (fig 33) and amylase producer (HSTU-9 and HSTU-10) (fig 32) is able to remove impurities and slightly change the degree of polymerization of cellulose by keeping the main structure in an intact state. However in this case where the cotton fiber is treated with pectinase producers (HSTU-6 and HSTU-7) the bands are a little less sharp than when the same fiber is treated with amylase producers as shown in fig 33.

3.12.1 Structural change and scouring of cotton fabrics

In FTIR analysis the pectinase producing strains (HSTU-6 and HSTU-7) and the amylase producing strains (HSTU-9 and HSTU-10) have been used for four different fibers (cotton, areca, banana and coir). As all of the fibers contain cellulose as their base structure and the curves didn't change much but in almost all positions such as in –OH stretch, C-O-C stretch, carbon ring structure vibration band, the peaks were sharper with high intensity. Since the cellulose structure contain impurities such as pectin, waxes etc, it is clear that after treating the fibers with the designated strains (HSTU-6, HSTU-7, HSTU-9 and HSTU-10) the impurities were removed, thus the intensity was increased. These pectinase and amylase producer strains clearly can be used for the reduction of impurities of cotton fabrics. Table indicates the wave numbers and their correlation with bands of cellulose structure.

Table 17. Designated bands at particulate wave number for FTIR

Wave number (cm^{-1})	Band assessment
898	C-O-C stretching for β-1,4 glycosidic linkages
3265-3353	OH- stretching vibrations
1157	C-O-C antisymmetric
1242	Aromatic C-O stretching out of lignin
1371	CH_2 vibration of cellulose
1422	CH_2 vibration of cellulose
1637	C-C aromatic ring structure of lignin
1640	Absorbed residual water bending
1736	C=O stretching of unconjugated ketone and carboxyl groups
2921	C-H stretch of cellulose

(Haque et al., 2015, 2013)

3.13 XRD Analysis

XRD analysis had been performed for more clear justification that the cellulose degrading strains (*Bacillus sp.* HSTU-2 and *Bacillus sp.* HSTU-3) is clearly able to distort the cellulose structure of different (coir, areca, cotton and banana) fibers and also remove the impurities that lies within them.

XRD analysis generally shows the crytallinity index of the cellulose structure. It determines both the amorphous and crystalline region of the cellulosic samples. Amorphous cellulose is the portion of the cellulose structure containing hemicellulose and lignin. With proper chemical treatment these portions can be degraded in which case the XRD curve will give high intensities because the lignin part will be removed by chemical treatment and crystallinity will increase. However, in our experiment the cotton fibers were not subjected to chemical analysis meaning no lignin part was removed. The cellulase utilizing strains (*Bacillus sp.* HSTU-2 and *Bacillus sp.* HSTU-3) were able to distort the ordered regions of the cellulose by breaking the intra and intermolecular hydrogen bonds. So, the intensity of the bands decreased in the treated fiber indicating the reduction of crystallinity. Fig 34-41 shows the XRD curve of untreated and treated FIBERS (cotton, areca, coir and banana) respectively.

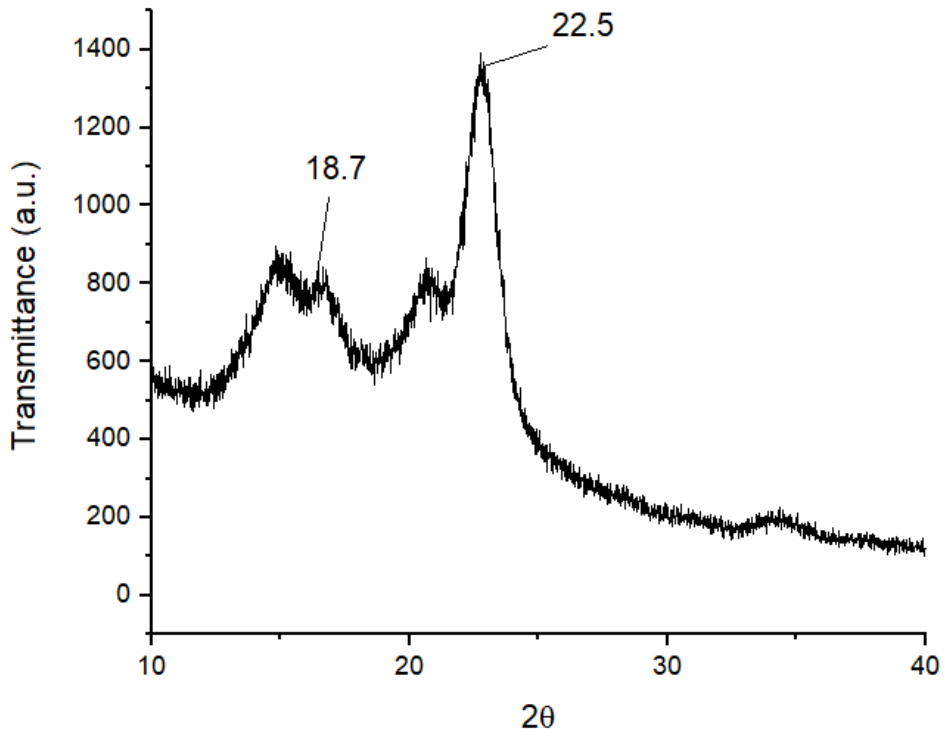


Fig 34: XRD curve of untreated cotton fiber.

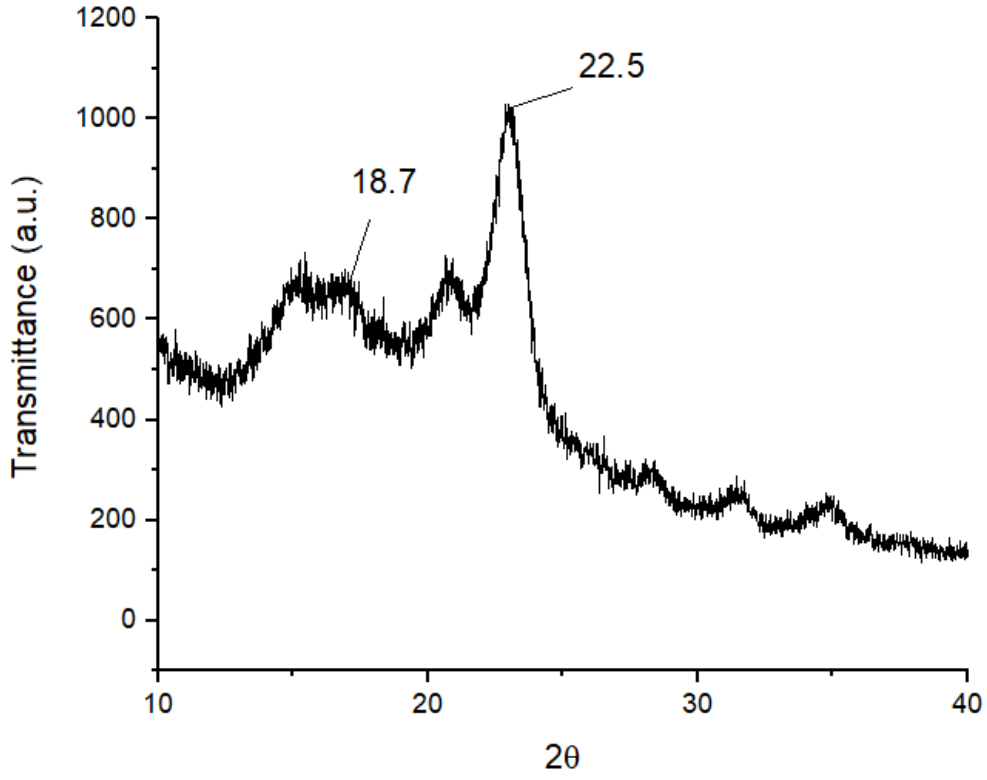


Fig 35: XRD curve of HSTU-2 and HSTU-3 treated cotton fibers.

From these curves the crystallinity index of untreated control fibers (cotton, coir, areca and banana) and treated fibers (cotton, coir, areca and banana) have been determined using the equation described by (Segal, L. & Conrad, 1959). The peak positions were determined in the section of 10° to 40° using 'OriginPro'. The untreated cotton, areca, coir and banana fibers showed crystallinity index of 31.5%, 21.71%, 35% and 29.26% respectively. After the treatment the treated cotton and areca fiber showed an astonishing crystallinity index of 13.69%, 18.27%, 17.72% and 25.20% respectively. The transmittance of the treated fibers has also been lowered compared to the untreated (fig 34-41). So, it is evident that these cellulose degrading strains (HSTU-2 and HSTU-3) can distort cellulose structure to a great extent which can be utilized in bioethanol production as well as in the scouring of cotton fabrics. It is clear that the crystalline cellulose structure of cellulose has been distorted by cellulase enzyme through breaking of inter and intra molecular hydrogen bonding with great efficiency (Arman et al., 2012; Haque et al., 2015, 2013).

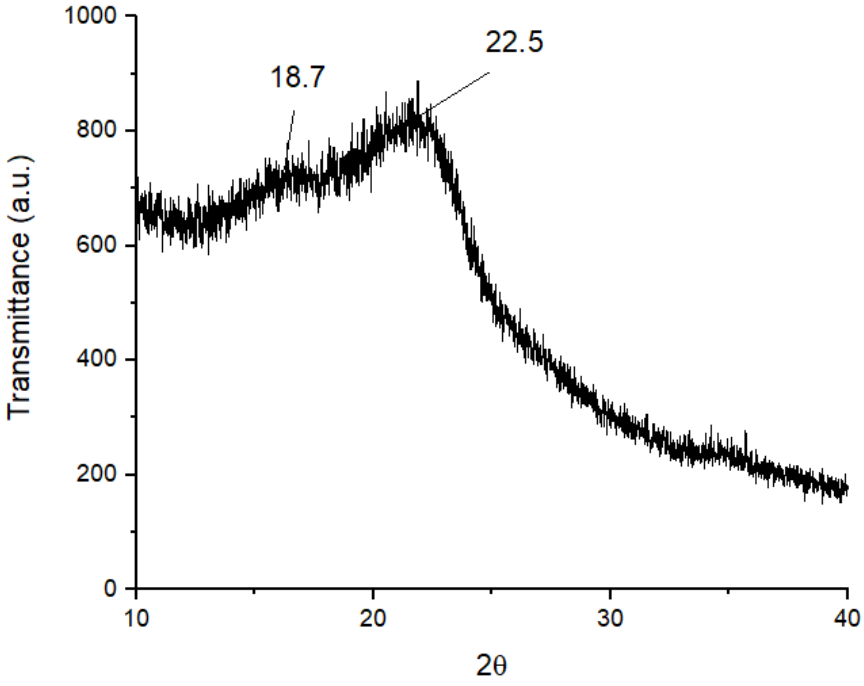


Fig 36: XRD curve of untreated areca fibers.

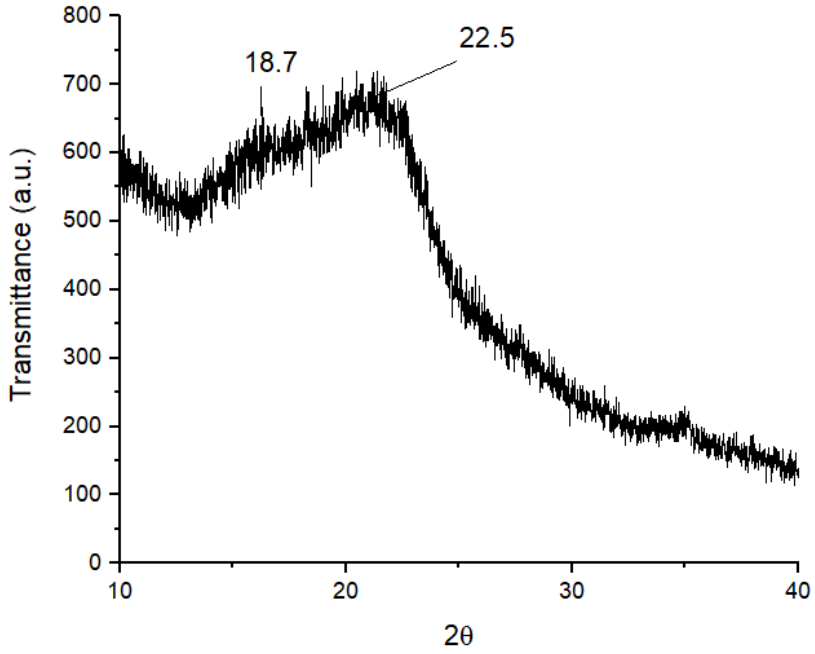


Fig 37: XRD curve of HSTU-2 and HSTU-3 treated areca fibers.

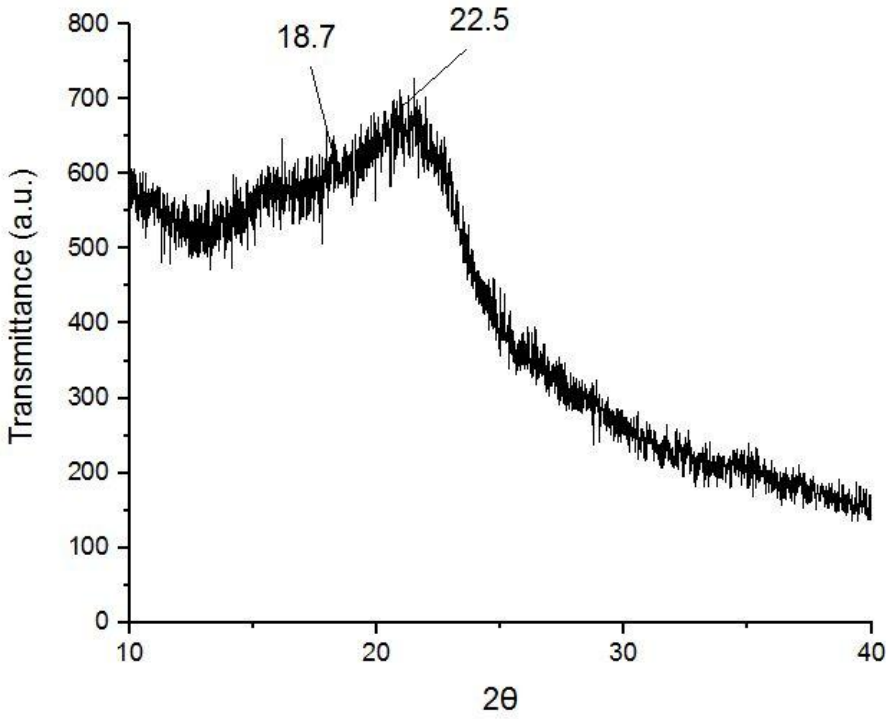


Fig 38: XRD curve of untreated coir fibers.

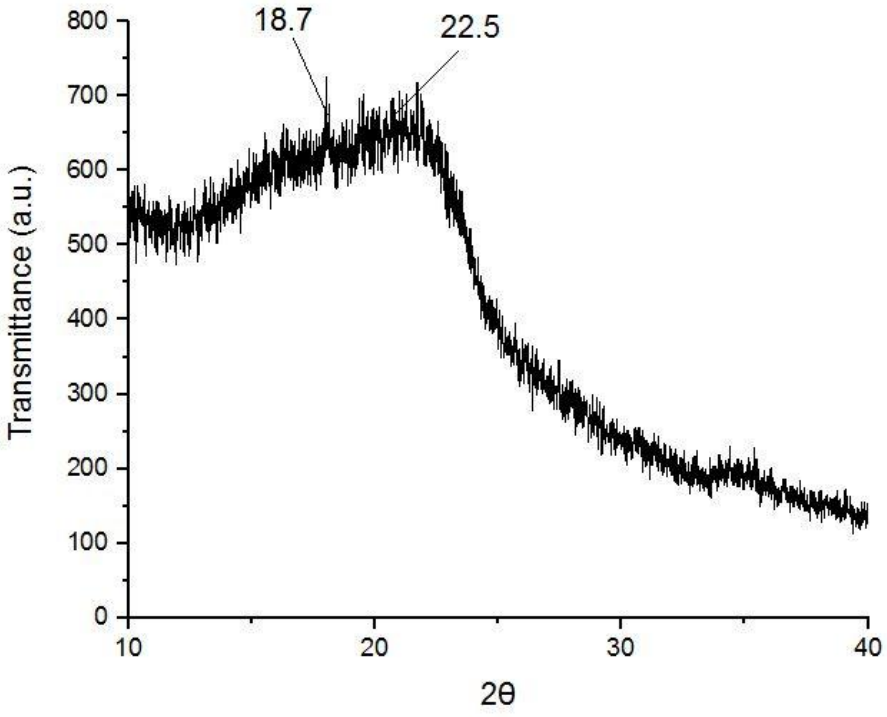


Fig 39: XRD curve of HSTU-2 and HSTU-3 treated coir fibers.

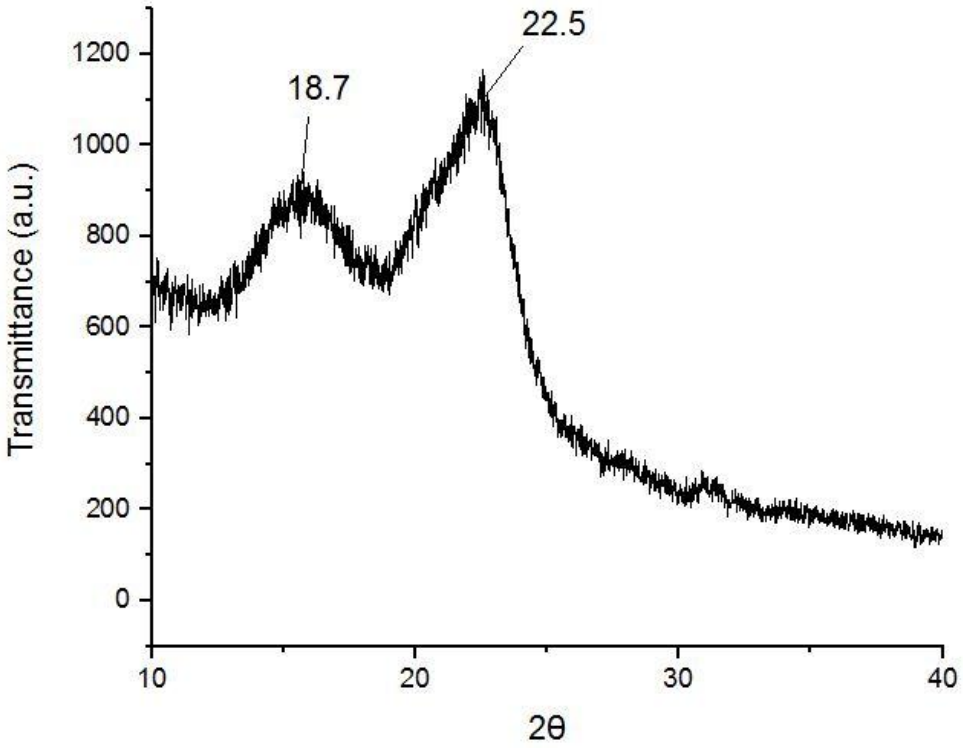


Fig 40: XRD curve of untreated banana fibers.

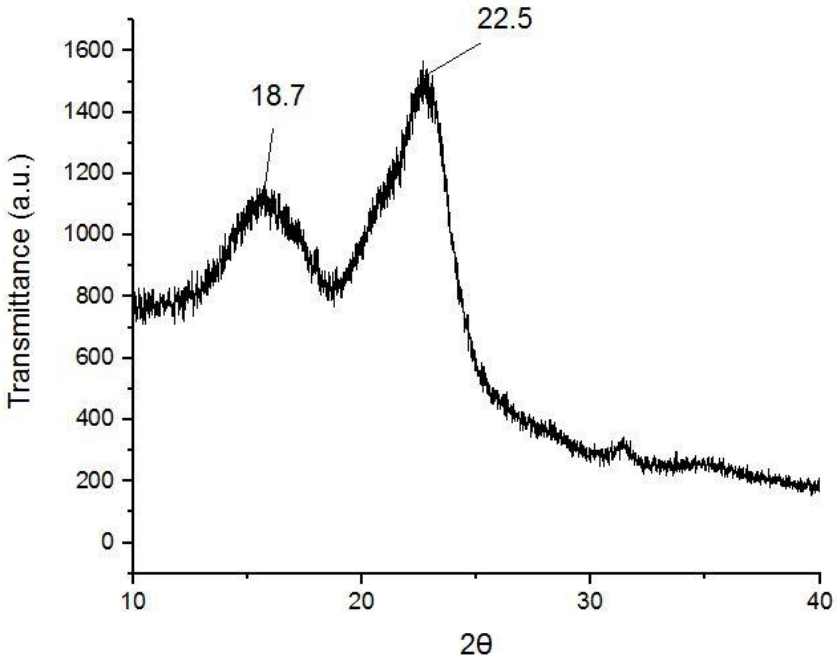


Fig 41: XRD curve of HSTU-2 and HSTU-3 treated banana fibers.

3.14 Enzymatic saccharification

Enzymatic saccharification using the selected bacterial strains (HSTU-2, 3, 6, 7, 9 and 10) on maize straw is one of the most important step in this experiment as it proved the reducing sugar yielding capacity of our selected bacterial isolates in question. At first standard curve was prepared for D-glucose using DNS method at 530 nm as shown in fig 38.

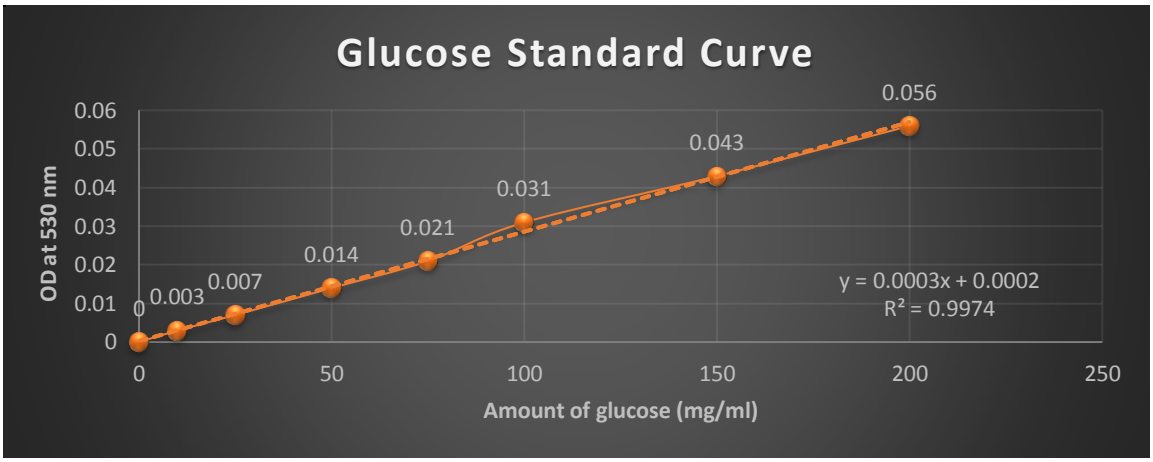


Fig 42: Standard curve for D-glucose using DNS method where Y-axis shows the OD at 530 nm against the amount of glucose in X-axis (200 µg/ml, 150 µg/ml, 100 µg/ml, 75 µg/ml, 50 µg/ml, 25 µg/ml, 10 µg/ml).

Reducing sugar yield was determined by comparing with the standard curve of glucose. Fig 39 shows the reducing sugar yield from maize straw after treating them with strain HSTU-2 and HSTU-3 with the help of DNS method (Quinlan, 1982).

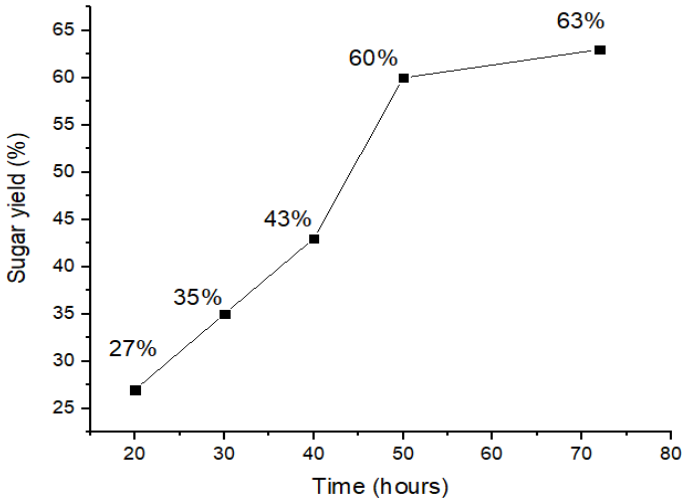


Fig 43: Showing the reducing sugar yield of HSTU-2 and HSTU-3 for maize straw.

After determining the reducing sugar yield it was clear that the lignocellulose pretreatment condition which was maintained greatly affected the hydrolysis of enzymes as in different conditions and in different time frames cellulase producer strains (*Bacillus sp.* HSTU-2 and *Bacillus sp.* HSTU-3) produced enzymes at different rates. As shown in fig 37, for treatment period of 20, 30, 40, 50 and 72 hours the strains HSTU-2 and HSTU-3 produced 27%, 35%, 43%, 60% and 63% reducing sugar respectively. It proves that the strains are able to produce enzymes at a very high rate and has great significance in bioethanol production.

Although the enzymes were not purified from the following strains due to instrumental and time related factors, this experiment does prove the capability of these cellulase producers (*Bacillus sp.* HSTU-2 and *Bacillus sp.* HSTU-3) in bioethanol production.

CHAPTER IV

SUMMARY AND CONCLUSION

The article provides information on bioethanol production and scouring of cotton fabrics by using newly isolated bacterial strains (HSTU-2, HSTU-3, HSTU-6, HSTU-7, HSTU-9 and HSTU-10) from cow dung. These bacterial strains are identified by using biochemical methods as well as using 16s rRNA gene sequencing. *Bacillus sp.* HSTU-2 and *Bacillus sp.* HSTU-3 are efficient cellulase producers which have been subjected to several bioinformatics analysis to ensure their identification. Both *Bacillus sp.* HSTU-2 and *Bacillus sp.* HSTU-3 have been checked using similarity analysis for identifying their differences. Also evolutionary tree has been formed for these two strains along with similar bacterial strains for contemplating their closest neighbours. In addition four more strains, *acinetobacter sp.* HSTU-6 (pectinase producer strain), *Bacillus sp.* HSTU-7 (pectinase producer strain), *Bacillus sp.* HSTU-9 (amylase producer strain) and *Bacillus sp.* HSTU-10 (amylase producer strain) which was previously identified by molecular biology lab of HSTU have been used to make bio consortium in order to treat different fibers (areca, banana, coir and cotton fiber). The two cellulase producing strains *Bacillus sp.* HSTU-2 and *Bacillus sp.* HSTU-3 have shown significant result in XRD analysis and have been proved as efficient cellulase producers. The two strains have also performed really well in the enzymatic saccharification test as the reducing sugar yield went up to 63% within 72 hours which is very progressive. So, it is evident that these two strains (HSTU-2 and HSTU-3) have the ability to produce large amounts of enzymes very rapidly and degrade lignocellulose. On the other hand *Acinetobacter sp.* HSTU-6 and *Bacillus sp* HSTU-7 along with *Bacillus sp.* HSTU-9 and *Bacillus sp.* HSTU-10 can remove the impurities from different fibers including cotton fibers which can greatly benefit the scouring of cotton fabrics. These strains (HSTU-6, HSTU-7, HSTU-9 and HSTU-10) have given significant results in the FTIR analysis, showing intense bands and much sharper image indicating the removal of pectin and other impurities lying within the cotton fabrics along with areca, coir and banana fibers. Finally, it can be concluded that these strains (HSTU-2, HSTU-3. HSTU-6, HSTU-7, HSTU-9 and HSTU-10) have great potential not only for the production of bioethanol but also in the scouring of cotton fabrics. Since bacterial enzyme production is cheap and eco-friendly, it is now a matter of time when the world will be changing its focus from conventional process to bio-ethanol production. By doing so, we can have an unlimited supply of natural bio-fuel that can solve the world's energy crisis.

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APPENDICES

APPENDIX A

CHEMICALS

Table A.1. Chemicals Used in Experiments

Sr.	Chemical Name	Code
1	Agar-Agar Merck	Merck 1.01613
2	D-Glucose	AppliChem A3666
3	Yeast Extract	Merck 1.03753
4	Glycerol	AppliChem A2926
5	NaCl	Biochem/ and NIB
6	K_2HPO_4	AppliChem A2942
7	$MgSO_4.7H_2O$	Merck 1.05886
8	Polygalacturonic acid	Fluka 81325
9	H_2SO_4	Merck 1.0735
10	Carboxymethyl cellulose sodium salt	Fluka Biochemical 21902 14 Soluble Starch Merck 1.01252.0250
11	Ammonium sulfate	AppliChem A3485
12	Potassium Di-hydrogen Phosphate	Merck 1.04871
13	Immersion oil	AppliChem A0699
14	Calcium chloride	AppliChem A3652
15	Crystal violet	Sigma C3886
16	Safranin	Merck 1.15948
17	Nutrient broth	Merck1.05443
18	Congo Red	Sigma C6767
19	Potassium Iodide	Sigma P8256
20	DNA isolation kit	AppliChem A3421
21	N, N, N`, N`-Tetra methyl-p-phenyl	enedione Sigma T3134
22	Tris Base	Sigma T6066
23	EDTA	AppliChem A2937
24	Isopropanol	AppliChem A3928
25	Proteinase	AppliChem A3830

26	Ethidium bromide	AppliChem A1151
27	Ethanol	AppliChem A3678
28	Taq DNA polymerase MBI,	Ferments EP0401
29	dNTP set MBI,	Fermatas R0181
30	Taq I	Fermatas ER0671
31	Primers	Ege 1, Ege 2, L1 Promega
32	Chloroform	AppliChem A3633
33	Standard agarose	AppliChem A2114

APPENDIX B

MEDIA

B.1 Media Used for Isolation (*Bacillus subtilis* Medium) (Broth)

Solution A

Yeast extract	1 g
$(NH_4)_2SO_4$	0.2 g
$MgSO_4 \times 7H_2O$	0.5 g
$CaCl_2 \times 2H_2O$	0.25 g
KH_2PO_4	0.6 g
Distilled water	500 ml
Adjust pH to	3.7 with 1 M H_2SO_4

B.2 Media Used for Extracellular Enzyme Screening

B.2.1 Media used for cellulase Screening

Medium I	**g/l**
Ammonium sulfate	1
Na_2HPO_4	6
KH_2PO_4	3
Agar	20

Ingredients (except agar) were dissolved in distilled water. The pH of the medium was adjusted to 4.0 with 1M H_2SO_4. Agar was added. Medium was sterilized by autoclaving at 121 ºC for 15 min.

Medium II	**g/l**
Yeast extract	1
Ammonium sulfate	2
Na_2HPO_4	6
KH_2PO_4	3
Commercial grade cellulose	5
Agar	20

Ingredients (except agar) were dissolved in distilled water. The pH was adjusted to 4.0 with 1M H_2SO_4. Agar was added and the medium was sterilized by autoclaving at 121º C for 15 min.

Medium III	**g/l**
Nutrient Broth	13
$CaCl_2$ x $2H_2O$	0.1

Ingredients were dissolved in deionized water. The pH of the medium was adjusted to 4.0 with 1 M H_2SO_4. Agar was added and the Medium was sterilized by autoclaving at 121º C for 15 min. Tween 20 was autoclaved separately and added to the medium.

APPENDIX C

PCR-RFLP RECIPIES

C.1 PCR Mixture

PCR reagents	Quantity
Mg free Taq DNA Polymerase buffer	5µl
MgCl2 (25 mM)	3µl
Sterile deionized water	35µl
Oligo forward 10 picomole/l	3µl
dNTP (2mMeach)	10X 5µl Taq
DNA Polymerase	0.25l (1.25 U) C.2 6X
Gel Loading Buffer	(20ml 10x TBE) 2ml
Glycerol	6ml
Deionized water	12ml

C.3 dNTP (10X)

100mM dATP, dCTP, dGTP and dTTP in separate vials were taken. They were mixed in 0.2 ml PCR tubes and sterile deionized water was added. They were mixed gently and 2mM concentrations of each was obtained and stored at -20º C.

C.4 Restriction Enzyme Mixture

Restriction enzyme buffer	5l
Sterile deionized water	35
DNA	10l
Restriction Enzyme	0.5 l (5U)

APPENDIX D

STAINS AND INDICATORS

D.1 Solutions for Gram Staining

D.1.1 Crystal Violet Staining Reagent

Solution A

Crystal violet	2g
Ethanol (95%)	20ml

Solution B

Ammonium oxalate	0.8g
Distilled water	80ml

Solution A and B were mixed to obtain crystal violet staining reagent.

D.1.2 Iodine Solution

Iodine	1g
Potassium iodide	2g
Distilled water	300ml

Iodine and potassium iodide were grinded. Water was added slowly and the solution was stirred until the iodine was dissolved. The solution was stored in amber bottle.

D.1.3 Safranin Solution

Safranin (2,5% in 95% alcohol) 10ml / Distilled water 100ml

D.1.3 Other Indicators

- Methyl red
- Kovac's regent
- KOH
- α-naphthiol
- Bromothymol blue
- Phenol red

APPENDIX E

BUFFERS AND STOCK SOLUTIONS

E.1 50X TAE -- Two hundred and forty-two grams Tris base was dissolved in deionized water. 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) were added. Volume was adjusted to 1000 ml with deionized water.

E.2 1X TAE -- Twenty ml of 50X TAE buffer was taken and the volume was adjusted to 1 liter with deionized water to obtain 1-liter 1X TAE buffer.

E.3 10X TBE -- One hundred and eight grams Tris Base and 55 g boric acid were weighed. They were dissolved in nearly 800 ml of deionized water and 40 ml 0.5 M EDTA (pH 8.0) was added. The volume was brought to 1 L with deionized water.

E.4 1M Tris-HCI (pH 8)

-- Tris base (121.1 g) was dissolved in 800 ml of deionized water. pH was adjusted to 8 with concentrated HCI. Volume was brought to 1L with deionized water.

E.5 1X TBE

-- Hundred milliliters 10X TBE was taken and the volume was brought to 1 liter with deionized water to obtain 1-liter 1X TBE buffer.

E.6 0.5 M EDTA pH 8.0

-- EDTA (186.12g) was dissolved in 800 ml of deionized water and pH was adjusted to 8.0 with 10 N NaOH. Volume was brought to 1000 ml with deionized water.

E.7 1X TE

-- 10mM Tris, pH 8, 1mM EDTA

E.8 Ethidium Bromide Stock Solution (10 mg/ml)

-- Ethidium bromide (0.5g) was dissolved in 50 ml of deionized water.

E.9 Chloroform-Isoamyl Alcohol Solution

-- Forty-eight milliliters of chloroform was mixed with 2 ml of isoamyl alcohol.

E.10 Phenol

Liquified phenol was taken from the freezer and it was melted at 68ºC in a water bath. Then equal volume of buffer (0.5 M Tris. Cl, pH 8 at room temperature) was added to the phenol. The mixture was stirred for 15 minutes. After the two phases have separated, the aqueous (upper) phase was removed by using a separation funnel. Equal volume of 0.1 M Tris. Cl (pH 8) was added to the phenol. The mixture was again stirred for 15 minutes. The aqueous phase was again removed. The extractions were repeated until the pH of the phenolic phase was >HSTU-2. HSTU-3. The phenol solution can be stored in this form at 4ºC.

E.11 CTAB/NaCl Solution

NaCl (4.1g) was dissolved in 80 ml water.10g CTAB was added slowly while heating and stirring. If necessary the solution was heated to 65 ºC to dissolve. The final volume was then adjusted to 100 ml.