Isolation and characterization of cellulose degrading fungal strains

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Abstract

The study was done for the isolation, screening and characterization of cellulose degrading enzyme from sugarcane bagasse, vegetable and wood morphologically and through biochemical analysis. Cellulase enzyme is a major coast of the world enzyme market.

Trichoderma reesei is the chief fungus for the production of cellulase industrially. Its genome encodes 10 cellulases and 16 hemi-cellulases and also produces two exoglucanases, eight endoglucanases, and seven β-glucosidases. Cellulases are inducible enzymes; the regulation of the production of cellulase is controlled by activation and repression mechanism.

The production of cellulolytic enzymes such as cellulase is generally been promoted when the substrate that has been provided contains enough and good amount of either the cellulosic biomass or the lignocellulosic biomass. This happens with the cooperation of the very specific and significant transcription factors. The study discusses the molecular mechanism of cellulase production system in *T. reesei* and genetic engineering based approaches for refining the cellulases production in *T. reesei.*

Cellulose is a type of complex sugar which is been generally degraded or hydrolysed into the simpler sugars by action of the enzymes produced by the enzymes which have the efficient capacity to produce the cellulases or other such cellulolytic enzymes. The degradation of the complex sugars into simpler monosaccharides and disaccharides can later be broken down into the simpler ones by the process known as fermentation and later form products such as bio-ethanol, single cell proteins, etc. The cellulose can also be formed into other bi-products by the action of heat such as crystals of cellulose, butynol fuel etc.

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Introduction

One of the most abundantly available raw material present on the earth is the ligno-cellulosic biomass whether we talk about the commodity businesses and whether it is a matter of R&D development field [1, 2]. Lignocellulosic biomass consists of two main components such as lignin and cellulose. More than 60% of them is of cellulose, which can be converted into various secondary products. As cellulose is a stable component and is generally a complex sugar that is further degraded by the microorganisms like many bacteria and fungi. Despite of having many such degraders and reformers of the cellulose by help of either chemical, physiological or the microbial actions, cellulose is commonly degraded efficiently by an enzyme called cellulose [3]. Cellulose breaks down into the simpler sugars to form ethanol and other bi-products. For the ligno-cellulosic biomass alteration breakdown by the cellulase enzyme is the most promising technology. Being it the most cost efficient and the effective technology for the same, studies have been going on for developing identifying and characterising the most efficient strains for the production of cellulase [4, 5].

Out of many microorganisms that produces the cellulose degrading cellulase enzyme, the main cellulase producing microbes is fungi, though there are also other few bacterias that have been studied to yield the enzyme(s) for cellulase activity. *Aspergillus* and *Trichoderma* are well known and among the most

efficient producers of cellulases in the game [6]. There huge amounts of agro based biowastes that are produced and are rich in complex lignocellulosic substances and can be used as a low cost carbon and energy source for the growth of cellulolytic fungal species that can be degraded into simpler sugars.

Cellulases have great prospective in saccharification of lignocellulosic substances for fermentation of sugars which can used for production of bioethanol or biofuel that can be used for blending with petroleum or other domestic fuels, lactic acid, and single cell proteins. Cellulose degrading fungal strains that are from fruits and vegetables wastes can be used for production of cellulases [7-9].

Cellulose is a homopolymer of D-glucose units linked by β-1,4 bonds. Cellulase breaks down the cellulose molecules into monosaccharides and such as beta-glucose or shorter polysaccharides and oligosaccharides. Cellulose degrading includes the reaction of hydrolysis of hemicellulose , lichenin and cereal beta D-glucose. Cellulases do have attracted a large amount interest by the commercial and the industrial sector because of its various applications being it very efficient in cellulose conversion into simpler sugars and later on with certain post processing can be used in the industries of varying domains including some from the food processing , in the biofuel producing industries , in the industries innovating their skills in the detergents for effective and eco friendly cleaning , and many more. [10].

Since the very beginning and over many decades the certain development in the R&D has certainly lead for more discovery of such cellulose degrading fungi for various applications and uses. Cellulolytic enzymes are produced by a number of microorganisms being it the bacteria or the fungi. And as above said the fungi and bacteria are the major utilizers of the cellulose for degrading it into simpler sugars and other smaller bi-products [10]. Mesophilic bacteria whether being aerobic or anaerobic , filamentous fungi, bacteria heat stable or alkali stable, actinomycetes and certain protozoa are included in cellulose utilizing population. Among all, fungi such as *Aspergillus sp., Fusarium sp., Penicillium sp., Trichoderma sp., Chaetomium sp*., have been reported to be the most effective cellulase producing fungi [11].

But since the studies have been going on the fungi has proven to be the more reliable on the cellulosic biomass for its survival and converting them into simpler by action of enzymatic counter reactions done by usually cellulases. Cellulose is the primary structural component of plant cell wall helping it by providing definitive structure and rigidity. Cellulose is a linear polymer of D-glucose residue linked by β (1-4) glycosidic bonds. In plant cell wall, the cellulose fibres are embedded in and cross linked by a matrix of several polysaccharide that are composed of glucose as well as other monosaccharides [12-13] The peroxide and hydroxyl radicals that are produced by the peroxidases and on an addition the iron semi-oxidizes the cellulose, making it

much of an ease for the cellulases to degrade cellulose and complex sugars having glycosidic linkages, more effectively. The two major types of fungi responsible for the breakdown of the cellulose from the wooden biomasses. But among both of them the brown rot fungi has the capacity to use some varying set of the enzymes for their action on utilizing the cellulose are lacking the CBMs and the processive cellulases, as these have the potential to degrade the more stable crystalline cellulose. Both CBMs and processive cellulases are produced by aerobic microorganisms that use the free cellulase mechanism for degradation and also by anaerobic microorganisms that produce cellulosomes [14].

Microorganisms that secrete a series of enzymes collectively known as cellulase enzyme, are capable of hydrolyzing the β (1-4) linkages of cellulose. Cellulase has a variety of types and those all act in a chain of successive progress. The basic enzyme for the process of hydrolysing into simpler sugars of cellulose requires three types of enzymes: Endoglucanase (breaks down linkages of β (1-4) glucan chain of cellulose), Exoglucanase (removes complex cappings of major molecular compounds from the non- reducing end of cello-oligosaccharide) and beta-glucosidase (converts cellobiose to yield two shorter molecules of simpler sugars that later adds on to the final hydrolysis of cellulose). In industrial application cellulases have massive potential including

its beneficence in the commercial application for the production of biofuel, bio-ethanol, and other bi-products of the cellulose on further processing. [15-17].

Cellulase have been used from several years for different purposes such as food processing such as for the production of coffee by performing hydrolysis during the drying of the beans, feed preparation, waste water treatment by breakdown of celullosic biomass by improving the composting potential, in the textile industry and detergent formulation by mixture of various fungi, and in some other areas [18]. The additional applications include its application in the food processing industries such as for the production of wine, and other alcoholic fermented beverages. There are also some applications in the medicines e.g., fungal cellulases used for the treatment of phytobezoars [19].

There are a large number of microorganisms that are capable of degradation of cellulose, only a few of these microorganisms produce significant quantity of enzymes that are capable of completely degrading the complex sugars as cellulose. Aside from bacteria the Fungi are the most efficient producers of cellulolytic enzymes. For the given perspective of the study, two fungal species belonging to two genera i.e. *Trichoderma* and *Aspergillus* were isolated from different substrates of enough cellulose sources, screened and compared for their ability to degrade cellulose [20]. Although there are many microorganisms that decays the cellulosic biomasses but only a few aerobic fungi are there that degrades cellulose but not lignin, among which the most efficient is

Trichoderma reesei, being the most efficient converter of the cellulose uses the mechanisms including the free enzymes on the other hand the true brown rot fungi uses both cellulases and peroxidases for its enzymatic reactions [21].

Material Required

The substrates used for the experimentation were sugarcane baggase, broccoli and wood. Conical flask, petri plates, inoculum, Bunsen burner, 500 ml potato dextrose agar media, lactophenol cotton blue.

The medium used for the isolation contained the given composition as- 30 gms sucrose, 0.2% of sodium phosphate, 0.05% K_2HPO_4, 1% agar, 0.03% Magnesium sulphate ($MgSO_4.7H_2O$), 0.25% Ammonium sulphate $(NH_4)_2SO_4$ and 0.2 % gelatin , were incubated for a period of 48 hours at room temperature (30 ºC) at a maintained pH of 7.0. Fungal colonies were purified by subculturing by process of multiple repeated streaking. The purified colonies were taken and preserved at 4°C for further identification and screening for cellulase production and characterizing on the basis of their cellulase degrading capacity.

Methodology

Sample preparation: Sugarcane bagasse, broccoli and wood were taken and soaked in water. The samples were kept in air tight container separately in a place without sunlight until fungus was grown on it making sure that no other type of fungus rather than cellulose degrading (contamination) is observed. After some days fungus was observed on each sample which was later used as the first or primary inoculum for growing the fungus.

Media preparation: 500 ml Potato dextrose agar media was prepared and autoclaved in conical flasks of 250ml in two different flasks in order to avoid the spilling. After autoclaving the media was taken to the LAF and cooled down at room temperature after which the pouring of the media was done on petri plates.

After the plate preparation inoculation of the fungi from substrates were done using inoculum loop sub –culturing of each sample was done separately by streaking the culture on the petri plates. The petri plates were sealed using paraffin film and labelled accordingly. The sub–cultured plates were then incubated at 37 °C for 5 -7 days. Pure cultures were observed after 2-3 times sub-culturing. The resulting strains that have been significantly isolated were carefully identified on the basis of their morphological characteristics that included colour of the colony and growth pattern, with the help of lactophenol

cotton blue their vegetative and reproductive structures were observed under the microscope [22].

Inoculation of the fungal strain was then done on the media. The plates were carefully incubated at 37 ºC for 5 days. After the continuous incubation of 5 days, the plates were taken from 37 ºC and incubated again for 18 hr at 50 ºC. After the incubation the plates were removed from 50 ºC and were taken for staining with 1% Congo red staining solution and were shaken at 50 RPM for 15 min. The plates were then destained with 1M NaCl solution and were again shaken at 50 RPM for 20 min. The Diameter of zone of decolourization was measured around each colony. The fungal colony showing largest zone of decolourization was selected for cellulase production which will certainly be depicting the maximum utilization of the cellulose [23].

Cellulose content estimation from substrates:

Inoculum preparation: The selected culture *was* maintained as stock culture on Potato dextrose agar slants. Then the given culture was grown at 30°C for 5 days and then stored at 4°C for regular sub culturing. Another 50 ml of inoculum was prepared using potato dextrose broth in 250 ml conical flask and autoclaved for sterilization.

Cellulose activity on Carboxy methyl agar was recorded as the Index of Relative Enzyme Activity (ICMC) was calculated as follows:

Clear zone ratios = Clear zone diameter / Colony diameter

The net growth simulated per the amount of the growth inhibited was being indexed and later the colonal region on the basis of diameter was computer according to the amount of agar utilized [24].

For Morphological charaterization:

Gram staining is done for the studying of the classification on the morphological basis. For performing the same a bacterial smear from a pure culture was prepared using an inoculation loop and fixed on a clean glass slide. After forming the streak and heat fixing it for few seconds, the slide was rinsed with crystal violet solution of 4-5 drops and waited for 1 minute, and later being rinsed with running tap water to avoid the over staining and non differentiating visuals. The slide was then rinsed with Gram's iodine with approx. 2-3 drops for 1 minute, and again rinsed with running tap water. This was followed by rinsing with 95% ethanol and rinsing with tap water again. The slide was counter stained with Safranin for 1 minute, rinsed with running tap water and allowed to air dry. The resultant slide was observed under microscope with different magnifications [25].

Filter Paper Assay:

As per to the standard method for calculating the total enzymatic activity for degradation in the culture filtrate was being determined. An Aliquot with 0.5 ml

of cell suspension was taken and later the supernatant was transferred into a clean and dry silicone tube. For the performing of assay 1 ml of sodium citrate buffer (pH 4.8) was added and Whatmann filter paper strip (6 x 1 cm) was immersed in the 1 ml of 0.5 M Sodium citrate buffer of pH 5.0. After incubating the sample at 50 C for 1 hr, the reducing sugar released was estimated by DNS method. 10 ml of distilled water was added to each tube, and the tubes were mixed and absorbance were noted at a standard wavelength of 540nm in colorimeter for checking the optical density of the resultant [26].

Cellulase activity assay:

The cellulase activity of each culture can be measured by determination of the amount of the total reducing sugars consumed while the action of enzymes, using DNS method. One unit of FP–ase , CMC–ase was defined as the amount of enzyme, which released µmole of reducing sugar measured as glucose per min under the assay condition [27].

Endoglucanase assay:

The endoglucanase activity in the filtrates of the cultures has to be quantified by using the carboxy-methyl cellulase (CMCase) method. The amalgam of the reaction mixture was prepared with 0.5 mL of crude enzyme, 1.0 mL 0.05 M sodium citrate buffer with pH 5.3 and 0.5 mL substrate. The CMC prepared in the same buffer was to be incubated in the water bath at 50°C for 30 minutes. The released reducing sugar has to be determined according to Miller's method.

FPase assay:

The net total cellulase activity has to be measured by FPase assay. The reaction mixture with composition of 0.5 mL of crude enzyme, 1.5 mL 0.05 M sodium citrate buffer pH 5.3 and another strip of Wattman filter paper (50 mg, 1 X 6 cm) to be incubated in the water bath at 50 C for 1 hour. The reducing sugar was determined after being released according to Miller's method of DNSA. Appropriate control without enzyme was simultaneously run [27-29].

Influence of various agro-wastes as a carbon source on cellulase production:

For performing the above test, the respective four lignocellulosic agro-wastes were being selected on the basis of their cellulose content and were used to check their influence on the enzyme production. Fermentation was carried out on the selected samples for 6 days at a temperature of 30 ℃ and the total cellulase production was measured [30].

Influence of various alkali pre-treated substrates on cellulase production:

Out of all the given substrates, the substrates which give the maximum production during the study based on their influence on the enzyme production and based upon their enzyme activity they were selected for the study. Different alkali pre-treated substrates with different compositions i.e. 5%, 10%, 15% and 20% NaOH, were used for the production and the optimization for proofing the

effect of the alkali pre-treated substrates on the enzyme production and their specific activity.

Influence of incubation period on enzyme production:

For effective fermentation and cost efficient enzyme activity over the substrates the incubation period for which they have been allowed to ferment and utilize the necessary components for the breakdown into finer simple sugars, that provided incubation period is a major parameter for checking and regulating the cellulolytic enzymatic reactions. Here, the experiment for the degradation of complex sugar was carried out for a time period of 7 days and the observations were taken continuously every day in the working hours of the laboratory in difference of every 17-24 hr [31-32].

Influence of pH on enzyme production: Different values of pH are taken that is 4.0, 4.8, 5.0, 5.3, 6.0, and 6.5 for the very different substrates that have been used for the study. The CMCase and FPase activity is measured at 50ºC. The different values of pH was adjusted by adding Mandel s medium having various pH mentioned above and their optimization have to be done if the pH are not as mentioned.

Influence of temperature on enzyme production: To check the effect of temperature on the enzyme production by different substrates and the fungus present on them leading to particular enzyme activity, fermentation was carried

out at two different temperature 28 °C (RT), 30 °C and 50 ºC and the enzyme activity was determined based upon the given temperature.

Influence of spent wash as moistening agent on cellulase production: For checking the effective enzymatic activity and the net enzyme production, effect of distiller waste spent wash is to be checked on the enzyme production by using it with various concentrations and ratios of MW media like 3mL MW media, 2:1 (MW media: spent wash), 1:2 (MW media: spent wash) and 3mL spent wash [33-34].

Partial purification:

After calculating and indentifying the sample with maximum enzymatic response for the cellulase purification or outcome, all procedures of the cellulase purification were carried out at cooler temperature 4°C. The most effective and efficient step for the same can be done by culture supernatant being separated by centrifugation process and pellet down, by using in buffer like buffer A, 50mM citrate buffer (pH 5.3), and later followed by fractional ammonium sulphate precipitation by adding solid ammonium sulphate to the culture filtrate composition ranging from 60% to 80% saturation[36-38]. After resting for a period of 48 h a centrifugation set at 10,000× *g* for 30 min was done and the precipitate was being collected and later dissolved in the given buffer A and dialysed overnight against three changes of the same buffer. After

which the resulting insoluble material was to be removed by centrifugation at 10,000× *g* for 10 min. After the removal of the pellets of the insoluble waste molecules, the clear supernatant can be filter sterilized using a micro filter injection and then to be stored at 4 C [38-42].

Effect of pH on the enzyme activity: The optimal pH for the main enzyme was to be determined for obtaining the effective enzymatic reaction at an optimized pH which can be done by incubating crude enzyme with substrate (1% CMC) which has to be prepared in appropriate buffers with standard compositions as- 0.05 M citrate buffer (with pH lying between 3.0 to 8.0). Crude enzyme mixed in those buffers was later to be incubated for 30 min at 50°C. Cellulase activity from the resulting outcomes can later be assayed by DNS method [43].

Effect of temperature on the enzyme activity:

Temperature being an important key for the whole study has to be characterize by checking the effect of temperature on activity of cellulase and its enzymatic activity, was determined for knowing the most efficient temperature range of the enzymatic degradation and that can be done by incubating crude enzyme with 1 % CMC in 0.05 M citrate buffer (pH 3.0 to 8.0) at temperatures ranges lying between 30 ºC and 80 °C. Enzyme activity was being assayed by DNS method at different temperatures as described above [44].

Effect of various metal ions on the enzyme activity: Various divalent metal ions including Ca^{2+}, K^{+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+} and Cu^{2+} were applied to check the optimum activity of enzyme to check that which metal ion is being consumed most by the microorganisms for showing the maximum degradation of enzymatic responses. Each variant for experimentation was done by taking the metal ions used at specific concentrations of 5mM [45].

Enzymatic hydrolysis:

Alkali pre-treated and untreated brocolli and sugarcane baggase substrates were taken, and carried in different 250mL of Erlenmeyer flask, mixed with extracted enzyme having 1.2 U/mL activity making an amalgam and then taking purified enzyme having 1.2 U/mL activity in each defined and labelled flask . The total volume of solution is adjusted to 50mL followed by addition of 50mM sodium citrate buffer optimised at a given pH of 5.3. This Enzymatic solubilisation was performed in a revolving lab shaker at 50°C, 150 rpm for 24 h. The content of reducing sugar solubilised into hydrolysate was later to be determined by DNSA method at every 2 hours[46-47].

Results & Observations

Initial results obtained-

After performing the required methodologies and experimentations the given results were taken and the major conclusions were made out of them. The fungi was isolated and sub cultured from the respective substrates and screened accordingly based upon their morphological properties and their functioning based on the enzymatic responses on the cellulose rich media provided to them in different conditions varying on the basis of temperature, pH, concentrations of the different nutritive components, and other such factors.

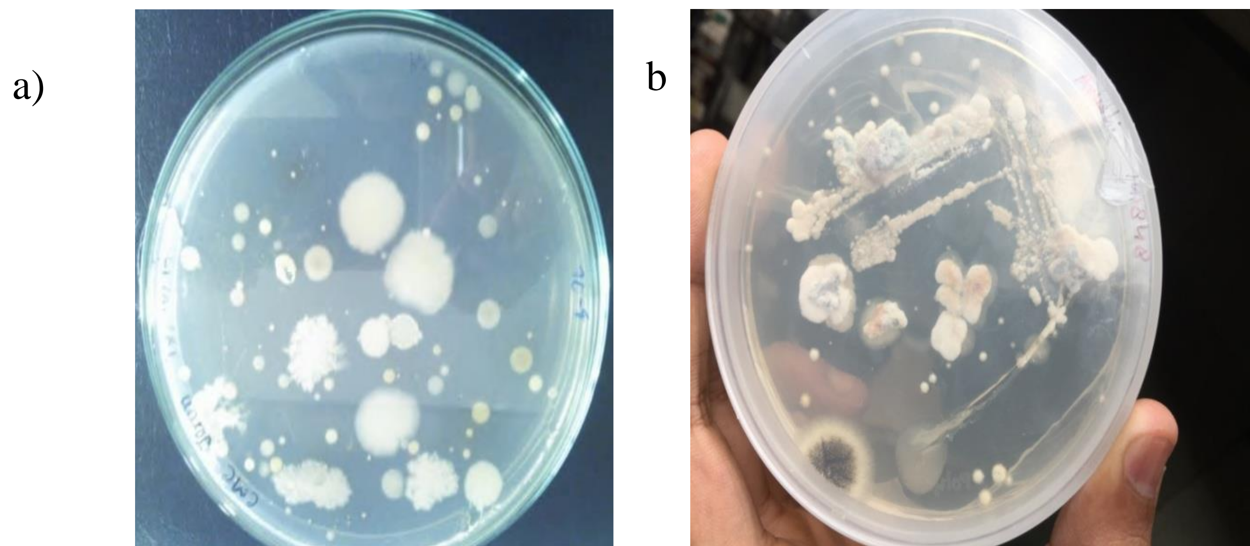

Figure 1. Fungal growth on Plate obtained from a) broccoli used as substrate b) sugarcane bagasse as substrate

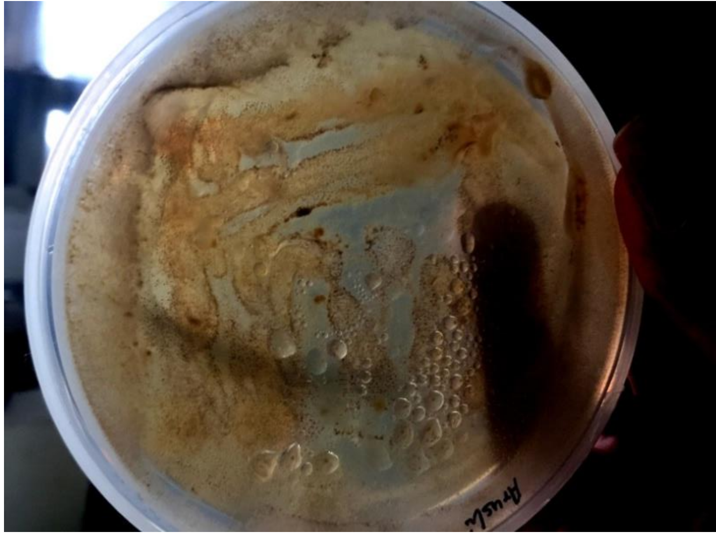

Figure 2. Fungal growth on Plate obtained from wood

Figure 3. Final Subculturing from the sugarcane baggase substrate

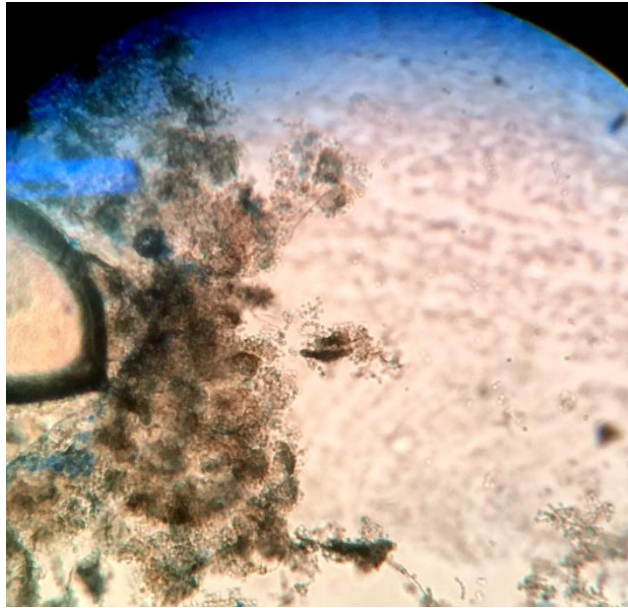

Figure 4. Microscopic view of the fungal growth

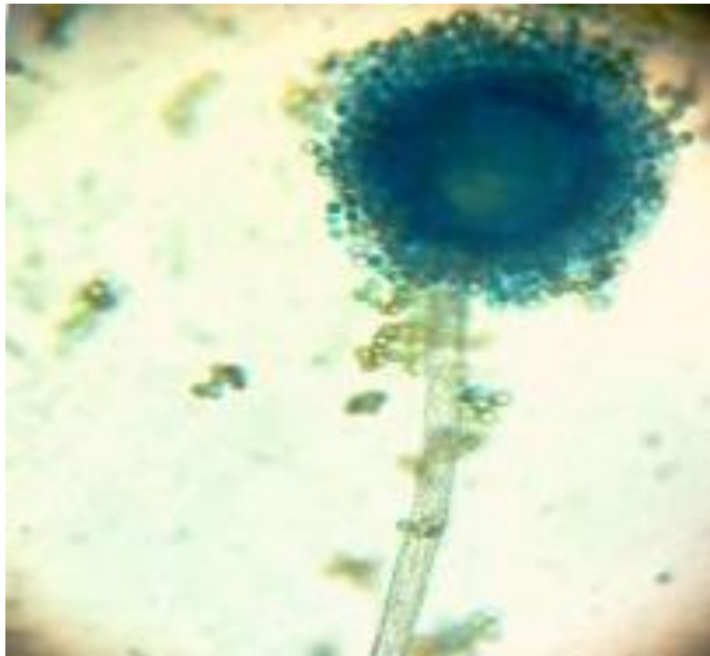

Figure 5. Microscopic view of Hyphae

Conclusions & Discussion

From the results as been taken after the given set of experiments we can conclude that all the fungi that has been isolated from the respective samples is able to degrade the cellulose into simpler sugars from the complex ones and they all have shown to produce cellulase enzyme activity for producing the bioethanol and degradation of the cellulose by freeing its complex carbon chains and glucose linkages.

Further fermentation of the provided samples under various conditions shows the optimizes and the most suitable conditions on the basis of factors such as the temperature, pH, concentration of the CMC, concentration of the ammonium sulphate, concentration of the sodium sulphate and the compositions of the respective chemical nutrients for the effective enzymatic reactivity toward the cellulose for breaking it down into simpler ones. The largest hydrolytic zone obtained after the studying was of around 15mm. As we have also look that whether the pre-treatment of the substrates with NaOH increases the respective enzymatic efficiency for the degradation of the cellulose. So after the observations it was made a clear conclusion that yes it increases the net cellulase production by the pre treatment in a basic medium and certainly leading to better degrading efficiency. For the fermentation efficiency from the agro based wastes it has been concluded that the sugarcane bagasse is the most efficient in the growth of the cellulolytic fungi and hence helping in the

destruction of the complex sugar molecules into the simpler ones. The microbes such as - *Trichoderma, Aspergillus, Penicillium, Botrytis, Neurospora etc*., are the most common ones which are grown in the suagrcane baggase based agro wastes. Talking about the incubation period for the fermentation that can be the most suitable one at the higher scales for the cellulose degradation so the incubation period varying in the time duration of 140-150 hours has turned out to be the most effective one with the maximum amount of cellulytic reaction by the help of the cellulase production in a very cost effective and efficient manner. The optimum pH should be used preferably for the better cellulase production by the fungi and later for the effective degrading and formation of the simpler sugars and later fermentation leading to the production of the bio-fuels and other bi-products. The temperature efficiency for the enzymatic production mainly depends upon the species of the microorganism which is producing the cellulase , which usually lies between the temperature range of 28-30 ^{o}C , but in case of the *Aspergillus niger,* it lies at a slight higher temperature of 40 ^{o}C. Presence of good mixture and the nutrients like zinc, magnesium, cobalt plays a beneficiary role in the addition of the net enzymatic activity of the given cellulolytic enzyme.

Based upon the above conclusions we can see that there is a very bright and fruitful economical benefit from these cellulose degrading microbes that can be very useful in the commercial and as well as the research and development

sector. As the temperature of the globe in increasing day by day and the major pollutant is the fuel combustion by the vehicles. So with studies like these and with their advancements the net production of the biofuels can be increased and these biofuels can act as a replacement for the usual fuels like petroleum which are having high carbon emissions and causing grater threats to the world economically and environmentally. As for the advancements of the same many countries are trying to also blend the petroleum with the biofuels in composition ratios of 8:2 which is really an effective step towards the collective good of all. But , for that step to be taken we are in the urgent need of the development of the sector in R&D for the identification , research and commercialization of such microbes that are contributing for the conversion of the cellulosic biomass(the most abundant raw material on our planet) into biofuels that too in an effective and efficient way.

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