Isolation and Screening of Cellulolytic Fungi from Soil and Evaluation of its Potential to utilize different Cellulosic Material for the Production of Ethanol

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ABSTRACT

Cellulose is an abundant biopolymer on our earth; it is made of glucose polymer. Cellulose is not readily utilized by microorganisms, many fungi and bacteria utilize cellulose and obtain energy from it. Certain fungi like *Aspergillus niger*, *Rhizopus sp* and *Fusarium* sp are capable of producing cellulase and releasing fermentable sugar. In this study, *Fusarium sp* DBC1 was isolated and its enzyme activity was checked using cellulose as a substrate. Enzyme activity was recorded 1.29 ± 0.005 U/ml. This fungus generates fermentable sugar. When grown on pure cellulose, it generates 60 mg/ml of reducing sugar whereas as on filter paper it generates 40 mg/ml and on algae cellulose it releases only about 20 mg/ml. The sccharified cellulose, filter paper cellulose and algae cellulose when subjected to fermentation using *Saccharomyces cerevisiae* for up to one week. After incubation, ethanol was qualitatively checked using idoform test. The distillation of fermented medium was carried out and a pure distillate was obtained. The percentage of ethanol in distillate was checked using a specific gravity method. Pure cellulose recorded as 14% alcohol, filter paper recorded 9% whereas algae cellulose recorded only 3 % ethanol.

Keywords: Ethanol, saccharification, Fermentation, cellulosic, Fusarium sp

1. INTRODUCTION

Cellulose (C_6 H_{10} O_5) and hemicellulose are abundant biopolyers on our globe. It is synthesized by photosynthetic processes in green plants, algae and blue green algae (1). Cellulose is composed of about 1000 to 1200 residues of glucose. Glucose polymer are cellulose is liked by β 1-4 glycoside linkage, cellulose is a water soluble and biodegradable renewable biomass (2,3).

Some fungi like *Aspergillus niger, curvularia, Fusarium, T.viride* etc are able to produce cellulase enzymes. Cellulases are a group of hydrolytic enzymes and hydrolyze the β 1-4 glycosidic linkage in cellulose and generate cellobiose and finally generate free glucose molecule. Cellulase enzymes are endogluconase, exoglucanase and β glucosidase. These enzymes jointly act on cellulose and generate free fermentable sugar. (4). The world energy demand increases due to increasing population which ultimately impact on the existing fossil the existing fossil fuel will vanishing in the near future, to overcome this problem, an

alternative renewable energy source (cellulose, hemicellulose) could be a raw material for future biofuels. Cellulose from this biomass can be converted into bioethanol by a two-stage process. In the first stage, saccharification of cellulose with cellulase enzyme to generate sugar and in the second stage sugar subjected to fermentation using *Saccharomyces ceriviase* for the production of bioethanol. Commercial production of ethanol from cellulose through a single stage process is not reported. Presently, Brazil is the leading bioethanol producing country; the USA stands second. Bioethanol produced in these countries is from a first generation biomass like starch. Ethanol from cellulosic material has till to date not been commercialized. Many researchers have reported one stage of ethanol production [5]. In this study we used two stage processes for bioethanol production from cellulosic biomass [7.9]
In the present study, cellulase producing fungi was isolated and identified on a morphological and cultural basis. The pure cellulose, filter paper and algae were sccharified to generate fermentable sugar, which then fermented to ethanol by yeast *Saccharomyces cerivisae* this study reports, highest ethanol production from pure cellulose and least in algae and medium with filter paper.

2. MATERIAL AND METHODS

1. Collection of soil sample

The soil sample was collected from ACS college campus garden where the litter falls frequently. About 10 gm of soil was collected in a sterile container from a different place in the same garden.

2. Isolation and screening of cellulosic fungi

Cellulose-producing fungi were isolated by inoculating 1 ml of serially diluted soil sample in PDA media containing 1% carboxy methyl cellulose and incubated at 28 ± 20 C for 72 hours for enrichment. After enrichment, a loop full culture was streaked on the PDA cellulose media and incubated, after that replica plate was made and incubated for 48 hours. .Fungal isolates were able to hydrolyse cellulose observed by flooding with 1% aqueous Congo red solution for 15 min. The Congo red dye was removed with 1N sodium chloride solution; the zone of hydrolysis around the fungal growth was measured in millimetres. This zone indicates fungal cellulase activity.

3. Morphological identification of cellulolytic fungi

Cellulolytic fungi were identified based on colony morphological and by microscopic observation with Lacto phenol cotton blue suggested by Navi et.al, Unchulcwa and N.Wakanma et.al.

4. Saccharification of cellulose

1 gm cellulose was added into the cellulase production medium, containing 3.0 g urea,1.4g (NH_4)2 So_4,2.0 g KH_2Po4 g. peptone 0.25 g ,yeast extract 0.005g, Feso4.7 H_2O, 0.0016g $MnSO_4$,7.H_2O, $ZnSO_4.7H_2O$, 0.002g, CoC_{l2}. 6 H_2O in 1000 ml distilled water. A set of three flasks containing basal medium with 1% cellulose, in the second flask basal medium with 1% filter paper and in the third flask basal medium with 1% algae powder. 5 ml sterile distilled water was inoculated with fungal mycelium. From this 1 ml of fungal culture was added to three separately prepared 50 ml basal media containing 1 gm cellulose, in the next flask containing basal media with 1 gm filter paper and in third flask containg basal media with algae powder and incubated these flasks at 28±2 0C for 48 hour. After incubation filtrate is obtained which on centrifuge to obtained supernatant that contains crude enzyme which was stored in a sterile bottle in a refrigerator for further analysis.

5. Enzyme assay

Cellulase activity was measured following the method of miller (18) briefly, a reaction mixture composed of 0.2 ml of crude enzyme solution, plus 1.8 ml of (0.5%) carboxy methyl cellulose in 50M (PH 7) was incubated at 30 ^{0}C in a shaking bath for 30 min, the reaction was terminated by adding 3 ml DNS reagent. The colour was taken and then developed by boiling the mixture for 5 min. The absorbance of the sample was measured at 575 against a blank, containing the entire reagent minus crude enzyme.

6. Bioethanol production

Isolated *Fusarium sp DB1* were grown in culture using a basal salt medium salt medium, separately in filter paper, pure cellulose, and algae power. All flasks were incubated for 6 days for saccharification, after saccharification filtrate was obtained and the total sugar was estimated by DNS method. The filtrate was sterilized and active yeast culture of about 10% volume was added and allowed to ferment for up to 8 days and the distillate was obtained

7. Estimation of ethanol

Idoform test

10 ml of distillate and 25 drops of iodine along with 10 drops of NaOH were added to the test tube, after a few minutes cloudy formation appears that confirms the presence of ethanol. It also gives a yellow precipitate and antiseptic smell.

8. Determination of alcohol by specific gravity method

Distilled bioethanol was obtained and was checked using specific gravity bottle, weigh a specific gravity bottle accurately. Fill the bottle with the alcohol distillate, insert the stopper, remove the spill- over solution with tissue paper and weigh. Transfer the solution back to the volumetric flask, clean and fill the bottle with distilled water and weigh as done for the sample. Calculate the specific gravity of the sample distillate and read the alcohol percentage from the table of specific gravity Vs alcohol % by volume.

3. RESULT

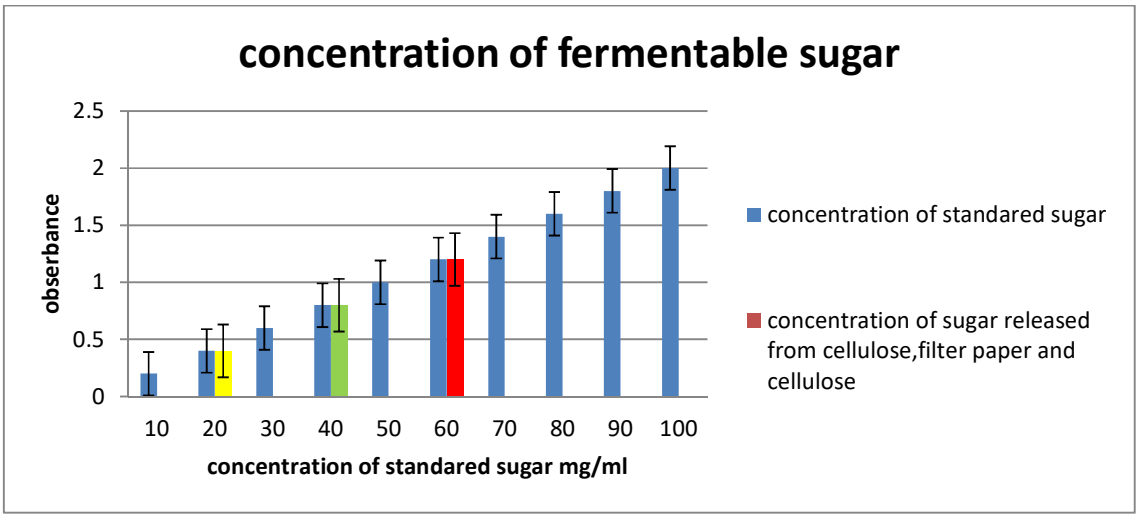
Fossil fuel is the source of the present energy demand, this existing fuel will exhaust shortly. Hence sustainable energy for the future is now being proposed, in this study, the renewable energy source is being tested for its potential for bioethanol production.

1. Isolation and screening of cellulolytic fungi

Cellulase producing fungi was isolated and after studying its morphological and biochemical properties. Isolate was identified as *Fusarium sp DB1* (Figure 1). The ability of cellulase production by isolated is shown in figure 2.

2. Saccharification of cellulosic biomass

Saccharification of pure cellulose, filter paper and algae cellulose was done and the amount of reducing sugar was estimated. The value of sugar obtained from standard graph. (Graph 1) pure cellulose (red bar) released about 60mg/ml, filter paper 40mg/ml (green bar) and algae released 20 mg/ml. (yellow bar). Algae cellulose very poorly utilized by Isolate.



Graph 1. Estimation of fermentable sugar

Fig. 1. A, Growth of isolate on Basal media. B. microscopic image of isolate

Figure 2. Cellulase activity of isolate

figure 3. Distillate

Enzyme assay

Isolated Fusarium spp DBC1 produced enzymes and the enzymes production was recorded as 0.19I /U ml, in pure cellulose, in filter paper it was found 0.14U/ml and in algae powder it was recorded as 0.75U/ml.

Bioethanol production

The ethanol production was carried out by simultaneous saccharification and fermentation using mixed culture of isolated Fusarium sp DBC1 and *Saccharomyces ceriviase* during this process pure cellulose generate about 13% ethanol and filter paper was produced 9% ethanol algae biomass release small amount of sugar and produce very little amount of the ethanol, i.e about 3%.

DISCUSSION

Today, commercially bioethanol is produced from starchy material, these material are being consider as the essential food staff, and can be used to reduced the hunger index of the growing population, this study was aimed to use third generation biomass for the production of bioethanol (12) Venkatraman et.al has utilized *Fusarium* species to degrade cotton cellulose, isolated fungi was stained with lacto phenol cotton blue and observed under microscope and identified as Fusarium spp . Olumuyiwa Adeyemo et.al identified the selected fungi using colony and cellular characteristics complied and referred Navi. et.al.

Chetan Gupta and other also identified the cellulolytic fungi using lacto phenol cotton blue. In our study we used the Navi et.al literature and microscopic observation of isolate using lacto phenol cotton blue and was identified as Fusarium sp and it has been given the name *Fusarium sp DBC1.*

Cellulase production potential of *Fusarium species* was reported by G .Ramanath et.al, when grown on PDA medium containing cellulose, Fusarium sp use cellulose as carbon source and the after incubation growth observed for cellulase production , the cellulase production ability was checked using the Congo red solution. Zone of clearance around the colony is indicative of cellulase production. We have used the similar methodology to find the cellulase producing fungal colony shown in figure 2. Our results showed Similarities with the reported literature which supports our finding. [10]

Cellulase production from fungal species were studies by Lakshmi C.M. and reported that cellulase from Fusarium sp showed cellulase activity 1.90 U/ml, Prahes Khatiwada et.al obtained crude cellulase from Fusarium sp using substrate straw waste and enzyme activity was recorded as 2.0 U/ml. Soniya sethi et.al also studied the bacterial cellulase activity and reported about 1.92U/ml using filter paper as a substrate, in the light of the above data, we reported cellulase production from Fusarium sp DBC1 using pure cellulose, filter paper and algae cellulose with basal cellulase media and our fining showed that production of cellulase from pure cellulose yields 1.90 U/ml, filter paper showed 0.98U/ml and algae substrate produced 0.80U/ml. our result are in accordance with the pervious reported cellulase activity

but the substrate, we used in this study was first time reported among the Fusarium species.[11,12,17]

Dinitrosalicyclic acid (DNS) method is the most widely used method for the estimation of reducing sugar, G. Ramanathan et.al treated the agriculture cellulosic waste using fungi and recorded 2.38 mg/ml of reducing sugar, Lakshmi C.M used banana farm soil Fusarium sp and treated pure cellulose and recorded about 40 mg/ml reducing sugar. In our study we used pure cellulose and obtained 60mg/ml reducing sugar, filter paper generated 40mg/ml of reducing sugar, algal cellulose after saccharification generate 20 mg/ml of reducing sugar, our result are in close proximity with the above reported finding and slight better than G Ramanath et.al.[1]

Algal biomass is now being considered as third generation biomass and it is not compete with our foods, hence biofuels form algae cellulose can be used along with existing petrol as additives. From the extraneously literature survey on bioethanol production from algae biomass, C. N. Khobrgade et.al repotted about 20mg/ml of reducing sugar from Scenedesmus sp biomass and sccharified with Aspergillus niger sp. In our study we used spirogyra biomass and *Fusarium spDBC1* was used for saccharification and obtained 20mg/l of reducing sugar our result matches with the reported study.[11]

Studies on bioethanol production from cellulosic biomass is widely studied , Ajay Kumar et.al reported that bioethanol production from hemicellulose material using saccharification and fermentation, Sancher and Cardona also reported bioethanol production from cellulosic biomass, Lakshmi C.M used the same methodology and reported 32% ethanol. George Prasoul et.al reported bioethanol production form wood cellulose using mixed culture of yeast and Fusarium using simultaneous saccharification and fermentation, in this study, we used the same methodology with different substrates. When we used the pure cellulose it yields 13% ethanol with filtrate paper 9% ethanol and with algal cellulosed it yield very least bioethanol (3%). Partimas Gupta reported et.al reported about 2% bioethanol from cellulosic material, Dhanpal Chavan also reported about 3% bioethanol from algal biomass, our finds of bioethanol production from algae biomass when compared with the reported studies, it finds somehow similar with the reported data.[16.17.18]

Bioethanol from crude cellulosic material is still a challenging issue and the cellulose hydrolysis with biological catalyst with modified techniques is essential.

CONCLUSION

Cellulase producing fungi Fusarium sp DBC1 was isolated from garden soil of college campus and its cellulase producing ability was recorded as 1.90 U/ml with pure cellulose and with filter paper 0.98 U/ml and alga about 0.80 U/l ml.

Simultaneous saccharification and fermentation of substrate generate reducing sugar and bioethanol. Pure cellulose generated about 60 mg/ml of reducing sugar and 13% ethanol , filter paper yields 40 mg/ml of reducing sugar and 9% ethanol where as algal cellulose generate 25 mg/ml reducing sugar and 3% bioethanol.

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CONFLICT OF INTEREST

The author declare no conflict of interest

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