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RESEARCH ARTICLE

Characterisation of β glucosidase from wood rotting fungi to facilitate efficient plant biomass biodegradation for biofuel production.

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Abstract

β-glucosidases catalyzes hydrolysis of cellobiose and cellodextrins releasing glucose as main product which is used in food, pharmaceutical and biofuel industries, it is an important component of the cellulase complex, it not only hydrolyzes cellobiose and short-chain cello oligosaccharides to glucose, also removes inhibitory effect of cellobiose on β-1, 4-endoglucanase and exoglucanase, thereby increasing overall rate of cellulose biodegradation.
Rotten wood samples were collected for isolation and identification of different fungi. 5 fungal isolates of the 20, showed higher cellulose degrading ability which were identified as *Pisolithus*spp., *Phanerochaete*spp., *Syncephalastrum*spp., *Aspergillus*spp. and *Nigrospora*spp. *Phanerochaete* spp. showed highest ability to degrade cellulose with least cellulose concentration of 22µg/ml and 10µg/ml, in biomass production. Glucose urea media supported more fungal growth than Potato dextrose broth. *Nigrospora*spp. showed 560µg/ml of protein content. The β-glucosidase activity (pNPG and salicin as substrates) results indicated that *Pisolithus* spp. exhibited highest enzyme activity i.e., 237 and 300 µg/ml/min respectively and showed highest alcohol production of 290µl/gm of fungal mat. Therefore, if the fungi are appropriately screened and purified for β-glucosidase, they can be potentially improved for bio-conversion of cellulose to glucose and facilitate efficient plant biomass biodegradation for biofuel production.

Introduction:-

The ever-increasing energy consumption and the depletion of fossil resources have laid the foundation for a shift towards sustainable production of biofuels from renewable sources. In 2008, Ministry of New and Renewable Energy established a National Policy on Biofuels to limit the country's future carbon footprint and dependence on foreign crude, Government had proposed a target of 20% ethanol blending by 2017 and laid down a roadmap for phased implementation. The pronounced scarcity of fossil fuels related to environmental problems resulting from their processing and consumption has prompted the search for alternative sources of biofuels and renewable energy. This in turn, has generated significant interest in use of cellulases and other enzymes to convert vegetable biomass into fermentable sugars (Singhania*et al.*, 2010).

Bioethanol production had started long ago, the first attempt at commercialization of a process for ethanol production from wood was done in Germany in 1898 (Saeman, 1945). Currently, ethanol is being produced from corn in the US and from sugarcane in Brazil as well as India. Ethanol production from these substrates does not appear to be advantageous so use of lignocellulosic biomass is the best way out to reduce energy burden on a developing country like India as it is cheap and easily available which consists mainly of cellulose (33-40%), hemicellulose (20-25%) and lignin (15-20%) (Volynets and Dahman,2011).

Ethanol can be produced by variety of microorganisms; cellulose-to-ethanol bioconversion can be conducted by various anaerobic thermophilic bacteria as well as by some filamentous fungi including *Aspergillusoryzae*(Ito *et al.*, 1990) and *Trichodermaviride*(Gervais and Sarrette, 1990).

Fungi plays role in degradation of plant biomass producing an extensive array of carbohydrate active enzymes responsible for biomass degradation, several fungal beta-glucosidases have furthermore been shown to produce glucose from larger cellodextrins, thus having the potential to increase the reaction rate and extent of cellulose hydrolysis (Lynd *et al.*, 2002 and Zhang and Lynd, 2004).β-glucosidases catalyze the selective cleavage of glucosidic linkages and are an important class of enzymes having significant prospects in industrial biotechnology. These are classified in family 1 and family 3 of glycosyl hydrolase family. β-glucosidases, particularly from the fungus Trichoderma, are widely recognized and used for the saccharification of cellulosic biomass for biofuel production. With the rising trends in energy crisis and depletion of fossil fuels, alternative strategies for renewable energy sources need to be developed. However, the major limitation accounts for low production of β-glucosidases by the hyper secretory strains of Trichoderma.

Among the cellulolytic enzyme complex, β -glucosidases play a key role for the final conversion of cellobiose into glucose. β-glucosidases are prominent class of enzymes and catalyze cellulose degradation acting synergistically with cellobio hydrolase and endoglucanase, respectively (Henrissat and Driguez, 1985). The specificity of β-glucosidases is variable towards different substrates depending on the enzyme source. The enzyme is ubiquitously present in nature and found in fungi (Deshpande *et al.*, 1978). Activity of β-glucosidase was also detected in pure cultures of litter decomposing basidiomycetes by Steffen *et al.*, 2007 and Valakova *et al.*, 2007 and in ectomycorrhizal species by Mucha *et al.*, 2006 and β-Glucosidases isolated so far exhibit high structural variability, partly reflecting the intracellular/extracellular localization of the enzyme. Therefore, β-glucosidases are prospective toolbox in bioethanol production and in the near future it might be successful in meeting the requirements of alternative renewable sources of energy, this study provides an overview of fungal beta-glucosidases in relation to use of lignocellulosic materials.

Materials and methods:-

Collection and Isolation:-

Fifty six samples of wood rot fungi were collected from decayed wood and living trees of *Tectonagrandis, Tarmarindusindica, Borasus flabelliform* of Western Ghats Area of Tamilnadu and Karnataka, South India. The fruit bodies were collected along with supporting wood. The samples were marked with information such as collection number with names, procurement location and date of collection. Fruit bodies were wrapped in paper bags and brought to the laboratory. The collection sight is situated in the latitude of 11.58°S and longitude of 76.93° East at 420 ± 50 M MSL. It receives rainfall of about 300mm per year with high humidity and even temperature. The fungal mass was sterilized with 1% mercuric chloride solution, repeatedly washed with sterile distilled water and inoculated on Potato Dextrose agar (PDA) medium in petriplates and incubated for 7days at room temperature. The fungal growth which occurred on the plates were sub-cultured and maintained on PDA slants.

Isolation of discrete colonies:-The isolation of discrete colonies from a mixed culture was done by the selection of individual cells that were picked up with the sterile needle and transferred to separate Potato dextrose agar slants/plates to obtain pure slant/plate culture of single fungal species (Cappuccino and Sherman, 2004).

Study of colony characteristics: The morphological characteristics of the purified isolated fungal colonies were studied according to Barnett and Hunter, 1984.

Identification of isolated pure fungi:-The isolated samples were identified based on Bakshi, (1971)

Comparison between Potato dextrose broth and Glucose urea broth: For comparative analysis of biomass production isolated fungal samples were grown in 50 ml of PDB and Glucose urea broth at 27^0C for 7 days in a rotatory shaker cum incubator.

Estimation of cellulose: (Updegraff, 1969):-

10ml of microbial culture is homogenized in a 15ml centrifuge tube. It is centrifuged for 5min at 2000 to 3000 rpm, supernatant is discarded. 3.0ml of acetic nitrate is added. The mixture is refluxed for 30min in a boiling water bath.10ml of distilled water is added and centrifuged. To the pellet, 10ml of 67% H2SO4(v/v) is added and allowed to stand for 1hr.1ml of this mixture is diluted to 100ml with distilled water.10ml of cold anthrone reagent was

layered on this with a pipette. The mixture was vortexed well and placed in a boiling water bath for 16min.It is allowed to cool and read at 620nm against a reagent blank.

Estimation of protein:-Protein content was estimated by Bradford, (1976) method using BSA as a standard.

Preparation of fungal extract:-

The fungi was cultured in glucose urea medium and filtered. The fungal mat obtained was homogenized in phosphate buffer and the homogenate was centrifuged at 3000 rpm for 15 min. The supernatant obtained was used for the estimation of protein.

Preparation of standard graph:-

Different aliquots of the standard ranging from 0.2 – 1ml were pipetted out in test tubes and the volume in each case was made upto 1ml with distilled water. 5ml of Bradford reagent was added and the tubes were incubated at room temperature for 20 minutes and read at 595nm.

Estimation of protein:-

1ml of fungal extract was pipetted out into a test tube and 5ml of Bradford reagent was added. The tube was incubated at room temperature for 20 min. The absorbance was read at 595nm. From the standard graph, concentration of protein in the sample was estimated.

β - Glycosidase enzyme assay:-

The enzyme activities were assayed using the method of Hongzhi Bai *et al.,* (2013) by p-NPG and salicin as substrates.

1. p-NPG method:-

150µL of p-NPG was dissolved in 0.1M sodium acetate buffer and 10µL of enzyme extract was added to it. The mix was incubated at different time intervals. Final volume was made up to 1ml with 100mM NaOH. 0.5 ml DNS reagent was added. Absorbance was read at 540nm.

2. Salicin method:-

1ml of respective enzyme dilutions were pipetted out into a series of numbered test tubes. A blank with 1ml distilled water was taken. Tubes were incubated at 37^0C for 6-8 minutes to achieve temperature equilibrium. At timed intervals, 4ml of salicin solution was added and mixed well. Each sample was incubated at different time intervals. 0.5ml DNS reagent was added. Reaction was stopped by placing each tube in a boiling water bath for atleast 15 minutes. Tubes were cooled in ice bath. Absorbance was read at 540nm.

Estimation of alcohol:-

Alcohol was estimated by potassium dichromate methodDifferent aliquots of absolute alcohol was pipetted out into a series of numbered test tubes. The solution was made upto 5ml with distilled water. 5ml of potassium di chromate solution was added into all the test tubes. Tubes were incubated at 60^0C for 15 minutes. Absorbance was read at 540nm.

Results and discussion

Isolation: About 20 different fungal isolates were obtained from rotten wood samples and distinctly grown colonies were selected for colony characteristics, similarly Goodell*et al.,* (2014) isolated *Gloeophyllumtrabeum*from wood rotting sample and identified its role in biodegradation.

Table 1: Fungal colony characteristics

Serial number	Dilution	Colour		Shape	Margin	Elevation	Configuration
		Upside	Downside				
1	WrS3 10^{-5}	Grey white	Brown white	Irregular	Lobate	Raised	Powdery
2	WrS3 10^{-6}	White	Cream	Irregular	Filamentous	Raised	Cottony
3	WrS3 10^{-6}	White	Cream	Irregular	Filamentous	Raised	Cottony
4	WrS3 10^{-6}	white	Brown	Irregular	Filamentous	Raised	Cottony
5	WrS2 10^{-3}	Greyish white	Cream	Irregular	Filamentous	Raised	Cottony
6	WrS4 10^{-5}	White	Cream	circular	Lobate	Raised	Cottony
7	WrS3 10^{-6}	White	Cream	Irregular	Filamentous	Raised	Powdery
8	WrS1 10^{-4}	Cream	Brown	Irregular	Filamentous	Raised	Cottony
9	WrS3 10^{-6}	White	Cream	Irregular	Filamentous	Raised	Cottony
10	WrS2 10^{-5}	White	Cream	Irregular	Filamentous	Raised	Powdery
11	WrS3 10^{-6}	Cream	Brown	Irregular	Filamentous	Raised	Cottony
12	WrS3 10^{-5}	Grey	Brown	Irregular	Filamentous	Raised	Cottony
13	WrS3 10^{-6}	Cream	Brown	Irregular	Filamentous	Raised	Cottony
14	WrS3 10^{-6}	Grey	Black	Irregular	Filamentous	Raised	Powdery
15	WrS3 10^{-6}	White	Cream	Irregular	Filamentous	Raised	Powdery
16	WrS5 10^{-5}	Greyish green	Cream	Irregular	Filamentous	Raised	Powdery
17	WrS3 10^{-5}	Grey	Brown	Irregular	Filamentous	Raised	Cottony
18	WrS3 10^{-6}	Grey	Brown	Irregular	Filamentous	Raised	Cottony
19	WrS5 10^{-5}	White	Cream	Irregular	Filamentous	Raised	Cottony
20	WrS3 10^{-6}	White	Cream	Irregular	Filamentous	Raised	Cottony

Fungal colonies:-

Among 20 isolates (Fig 1), five isolates which brings highest cellulose degradation were identified based on their morphology and staining with Lacto phenol cotton blue, they were identified as *Pisolithus*spp, *Phanerochaete*spp, *Syncephalastrum*spp, *Aspergillus*spp and *Nigrospora*spp (Fig 2). Selvam*et al.*, (2012) identified 129 isolates from rotten wood of Western Ghats area among them *Phanerochaete*sp. showed highest degradation activity.

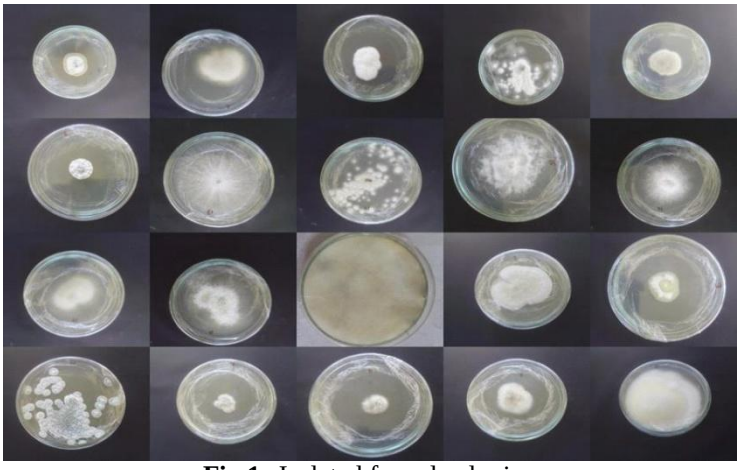

Fig 1:-Isolated fungal colonies

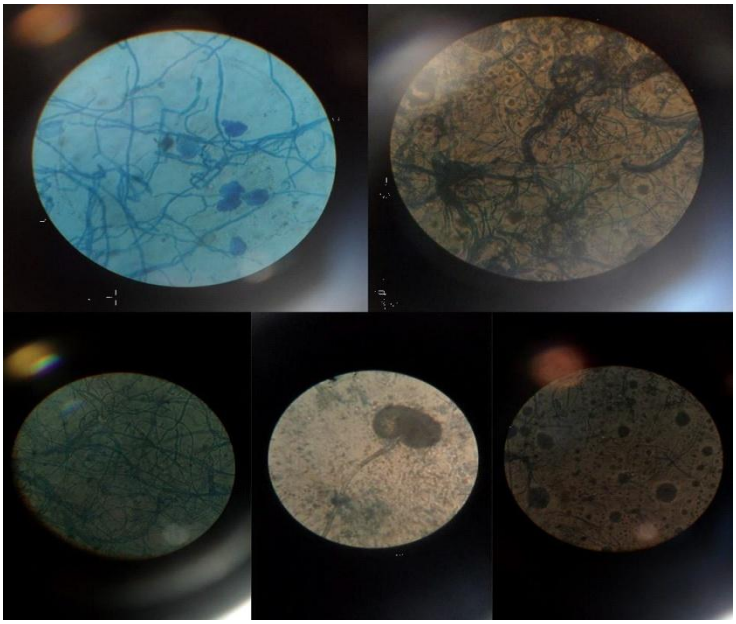

Fig 2:-Microscopic view of screened 5 fungal samples

Fungal growth on Potato dextrose broth and Glucose urea media:-

The 20 isolated fungi grown on Potato Dextrose Broth and Glucose urea media at 27°C for 7 days produced biomass which was weighed to obtain wet and dry weight in grams. A Comparative analysis showed relatively more fungal biomass of all the isolates in Glucose urea media (GU) than Potato Dextrose Broth (PDB) (Fig 3).Isolate Wr-9 had the highest biomass of 7.8g/50ml in GU media compared to 3.4g/50ml in PDB. Isolate Wr-6 had the highest biomass of 4.5g/50ml in PDB. JatinderKaur (2007) used glucose urea media for growing *Melanocarpus* spp.

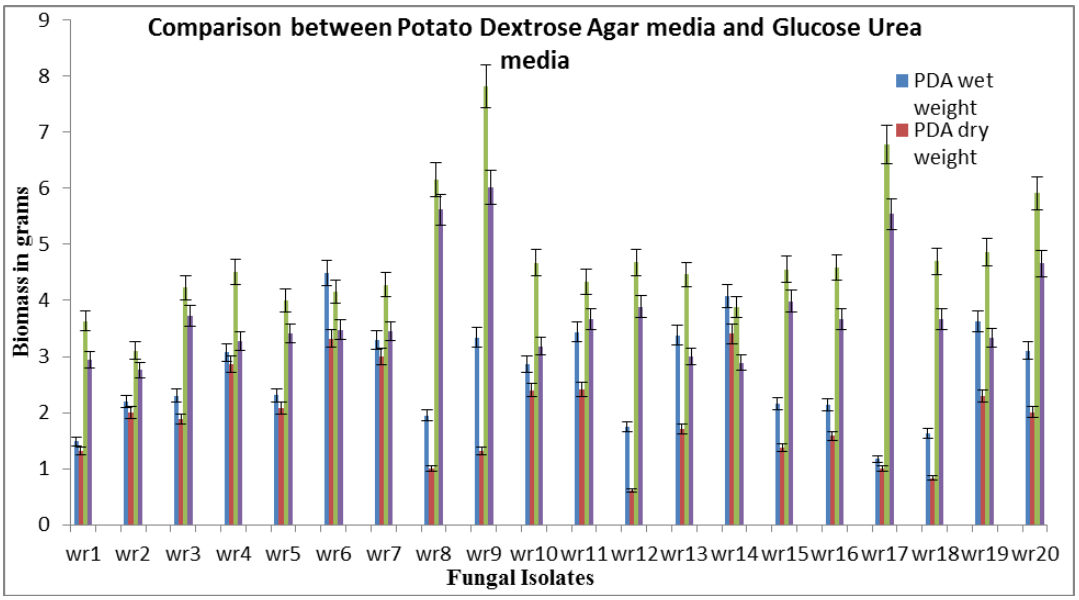

Fig 3:-Wet and dry weight of fungal biomass grown on Potato dextrose broth (PDA) and Glucose urea media (GU)

Cellulose: On 6th day *Phanerochaete* sp. showed highest ability to degrade cellulose with least cellulose concentration of 22µg/ml and 10µg/ml followed by *Pisolithus* sp. 26µg/ml and 9µg/ml, *Syncephalastrum* sp. 35µg/ml and 12µg/ml, *Aspergillus* sp. 34µg/ml and 15µg/ml and *Nigrospora* sp. 47µg/ml and 26µg/ml respectively (Fig 4). Hence, the above 5 isolates were selected for further studies. The general biochemistry of cellulosic enzymatic hydrolysis has been reviewed by (Wang *et al.*, 2013), cellobio hydrolases and endoglucanases are often

inhibited by cellobiose (Murphy et *al.*, 2013) making beta-glucosidases important for avoiding product inhibition through conversion of cellobiose to glucose and thereby avoiding decreased hydrolysis rates of cellulose over time. However, β-glucosidases are often themselves inhibited by their product glucose (Xiao *et al.*, 2004) making β-glucosidase the rate-limiting enzyme. Maintaining a high hydrolysis rate of cellulose ultimately requires highly efficient β-glucosidases that tolerate glucose at high levels. It has further been recognized that enzyme performance is reduced by interaction with lignin or lignin carbohydrate complex .However, of cellulase and xylanase enzymes tested β-glucosidase was least affected by lignin (Berlin *et al.*, 2006).

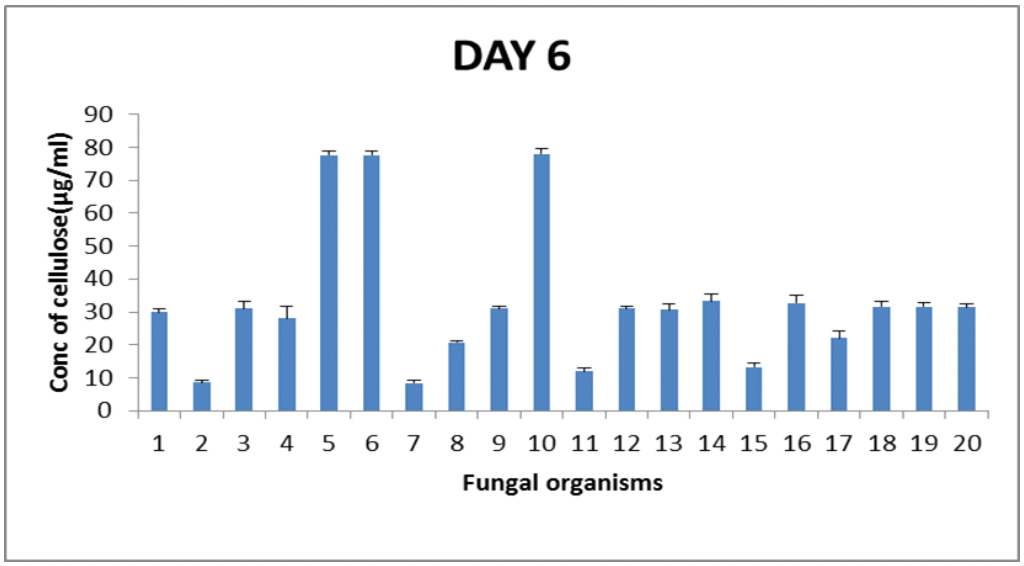

Fig 4: Cellulose degradation

P**rotein:** *Nigrospora*sp. showed highest protein content of 560µg/ml among all the 5 fungal isolates followed by *Syncephalastrum*sp. 542, *Pisolithus*sp. 520, *Aspergillus*sp. 500 and *Phanerochaete*sp. 470(µg/ml) ,Table 2 and Fig 5,when the concentration was checked against standard graph using BSA as standard. Proteins have flat substrate-binding surfaces and are capable of cleaving polysaccharide chains by oxidative reactions to disrupt polymer packing, thereby increasing its accessibility (Quinlan *et al.*, 2011), JatinderKaur*et al.*, (2007) identified protein in *Melanocarpus*sp. by Bradford method Km and Vmax for the hydrolysis of pNPG by β-glucosidase was calculated as 3.3 mM and 43.68 µmolmin-1mgprotein-1 respectively.

Table 2: Protein content in fungal isolates

ORGANISM	PROTEIN CONTENT (µg/ml)
*Phanerochaete*spp	470
*Pisolithus*spp	520
*Syncephalastrum*spp	542
*Aspergillus*spp	500
*Nigrospora*spp	560

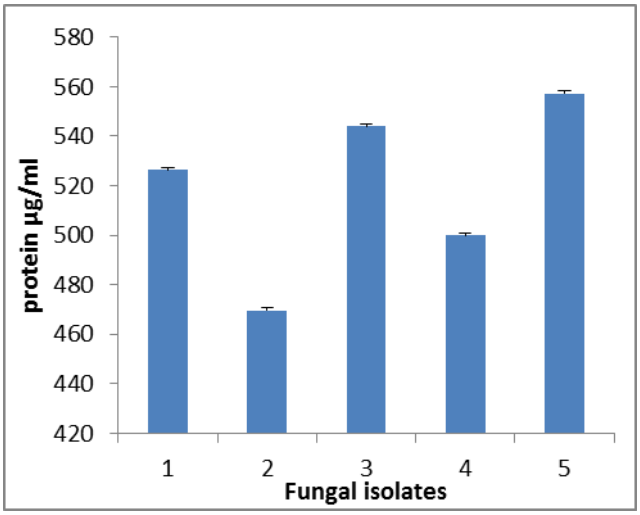

Fig 5:-Protein content in fungal isolates

β- glucosidase enzyme assay

β -glucosidase activity (Salicin method): Substrate specificity of β-glucosidase was determined by using salicin as substrate. Using DNS reagent reducing sugar was estimated. *Pisolithus* sp. showed highest enzyme activity of 237µg/ml/min and the enzyme activity of other isolates are *Phanerochaete*spp.187.5, *Aspergillus*spp.150, *Syncephalastrum*spp. 125, *Nigrospora*spp.100 (µg/ml/min),Table 3 and Fig 6. HongzhiBai*et al*.,(2013) assayed β-glucosidase from culture supernatant of a fungus *Penicillium simplicissimum* purified to homogeneity by using ammonium sulfate fraction, Sephadex G-100 chromatography and its properties were studied, the molecular mass of enzyme was about 126.0 kDa, as identified by 12% SDS-PAGE.

Table 3:-Enzyme activity of salicin on fungal extract

ORGANISM	ENZYME ACTIVITY (µg/ml/min)
*Phanerochaete*spp.	187.5
*Pisolithus*spp.	237
*Syncephalastrum*spp.	125
*Aspergillus*spp.	150
Nigrospora spp.	100

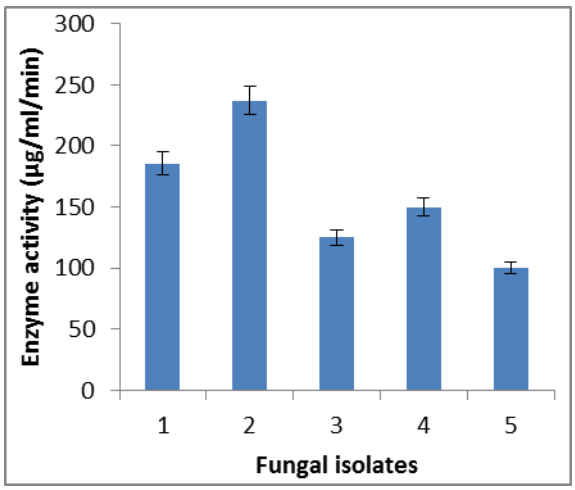

Fig 6: Enzyme activity of salicin on fungal extract

β-glucosidase activity (p-NPG method): Substrate specificity of β-glucosidase was determined by using p-NPG as substrate. The activities on p-NPG were estimated by assaying the amount of glucose released by using DNS reagent. *Pisolithus* spp. showed highest enzyme activity of 300µg/ml/min followed by *Aspergillus*spp. 250, *Phanerochaete*spp. 210, *Syncephalastrum*spp. 150 and *Nigrospora*spp. 129 (µg/ml/min). Ali M. Elshafei*et al*.,(2011) assayed β - glucosidase activity an intracellular β-glucosidase (EC 3.2.1.21) from *Aspergillu sterreus* NRRL 265 grown on whey permeate was purified to homogeneity as indicated by disc acrylamide gel electrophoresis with an apparent molecular mass of about 116 kDa. Optimal activity was observed at pH 5.0 and 60°C. The β-glucosidase had Km values of 2.5, 3.7 and 5.5 mM for p-nitrophenyl-β-D-glucopyranoside (p-NPG), cellobiose and salicin respectively. Based on substrate specificity, β-glucosidases have traditionally been divided into cellobiases (high specificity towards cellobiose), aryl-β-glucosidases (high specificity towards substrates such as p-nitrophenyl-β-D-glucopyranoside (pNPG) or broad specificity β-glucosidases (Eyzaguirre *et al*., 2005) most β-glucosidases are placed in the last category.

Table 4:-Enzyme activity of PNPG on fungal extracts

ORGANISM	ENZYME ACTIVITY (µg/ml/min)
*Phanerochaete*spp.	210
*Pisolithus*spp.	300
*Syncephalastrum*spp.	150
*Aspergillus*spp.	250
*Nigrospora*spp.	120

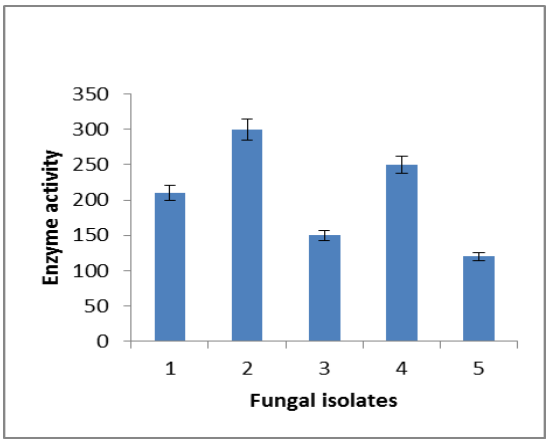

Fig 7:-Enzyme activity of PNPG on fungal extracts

Alcohol:-

Alcohol content was estimated by potassium dichromate method using 100% ethanol as standard. *Pisolithus*spp. showed highest alcohol production of 290 µl/gm of fungal mat among all 5 fungal species followed by Nigrospora spp. 272 µl/gm, *Phanerochaete*spp. 250 µl/gm, *Aspergillus*spp. 219 µl/gm and *Syncephalastrum*spp. 210 µl/gm. Nakade Dhanraj, (2014) estimated alcohol in sugar beet by modified potassium dichromate method, results indicated that this method is 99 % effective in estimation of ethanol and can be used as one of the best method for ethanol production.

Table 5:-Amount of alcohol in fungal isolates

ORGANISM	Amount of alcohol(µl/gm of fungal mat)
*Phanerochaete*spp	250
*Pisolithus*spp	290
*Syncephalastrum*spp	210
*Aspergillus*spp	219
*Nigrospora*spp	272

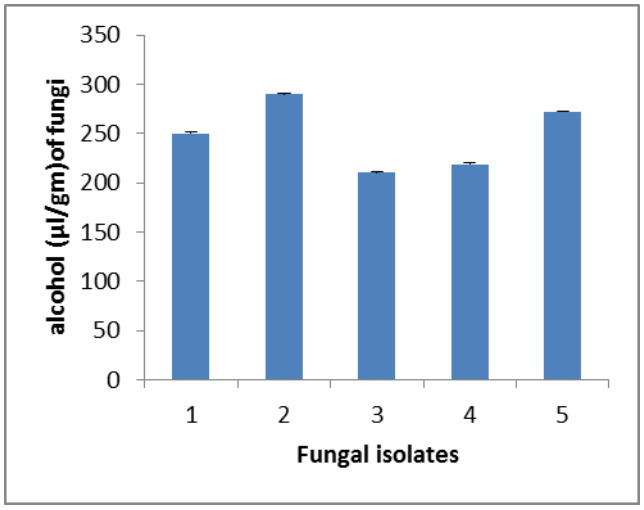

Fig 8:-Amount of alcohol in fungal isolates

Conclusion:-

Profitable biomass conversion processes are highly dependent on the use of efficient enzymes for lignocellulose degradation. Among the cellulose degrading enzymes, beta-glucosidases are essential for efficient hydrolysis of cellulosic biomass as they relieve the inhibition of the cellobio hydrolases and endoglucanases by reducing cellobiose accumulation.

Present work provides basic data to ascertain β-glucosidase producing fungi as potential microbes for degradation of lignocellulosic waste and bioethanol production, future research on optimization of their growth, scale-up and bulk production of the enzyme for increasing bioethanol production holds promise.
Most of the bioconversion processes used today does not allow complete saccharification of biomass. Hydrolysis of biomass can be enhanced by several approaches, one of which is by supplementation of cellulase complex with accessory enzymes. β-glucosidase characteristics associated with its ability to hydrolyze cellobiose, underscore the utility of this enzyme in diverse industrial processes.

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