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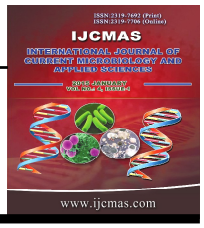

Original Research Article

Production, Optimization and Characterization of Fungal Cellulase for Enzymatic Saccharification of Lignocellosic Agro-waste

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K e y w o r d s

Fungal cellulase, Lignocellulolytic agrowaste, Enzymatic saccharification

A B S T R A C T

Cellulases are a group of hydrolytic enzymes and are capable of degrading lignocellulosic materials. Cellulases have wide range of applications. This work focuses on factors relevant for improvement of enzymatic hydrolysis of rice straw by using isolated fungal strain from compost pit. Different cultural conditions were examined to assess their effect in optimizing enzyme production as well as for characterization of enzyme after partial purification. In comparison with WR (White rot fungi), our isolated strain 2b reports higher production of cellulases.

Introduction

All over the world, there is a growing concern about the over dependence on fossil fuels as well as their possible roles in global warming. Because of this, there is a tremendous search for a biofuel to use as an alternative source of energy, utilizing the existing lignocellulosic biomass. In this endeavor, ethanol produced from foodstuffs such as wheat, maize, soybean, etc., is under scrutinized experimentation, while victimizing the food security of the world (Somerville, 2006).

The use of cellulase is the most promising technology in the conversion of lignocellulosic biomass (Demain *et al.*, 2005). Cellulosic ethanol is a biofuel produced from wood, grasses, and the nonedible parts of plants (Ingram *et al.*, 1999). Lignocellulosic biomass is an important resource for the production of biofuels because of its abundance in nature, it is inexpensive, and production of such resources is ecofriendly. Agricultural residues are the major source of lignocellulosic biomass, which is renewable and inexpensive. Most of these agricultural residues are nonedible lignocellulose which is used for ethanol production (Brustarr and Bakenhus, 2008). Biochemical conversion of biomass advantageously preserves the original carbohydrate structure in the form of monomeric sugars and enzyme technology generally considered the most sustainable technology for saccharification (Xiros and Christakopoulos, 2009).

Most lignocellulosic material contains three main components: lignin, hemicelluloses, and cellulose. Former is hydrophobic and

latter two components are hydrophilic. Lignin forms a three-dimentional network inside the cell wall. Cellulose and hemicellulose are densly packed by layers of lignin.

Thus, these components are sparingly insoluble in water and partially soluble in organic solvents because of adhesion of lignin to the polysaccharides. Such complex structure makes lignocellulosic biomass hard to be used by microorganisms and protect it against enzymatic hydrolysis. So, the enzymatic hydrolysis involves biomass pre-treatment which decreases crystallinity of cellulose, increase biomass surface area, remove hemicellulose, and break the lignin barrier. Pre-treatment makes cellulose more accessible to hydrolytic enzymes to facilitate the conversion of carbohydrate polymers into fermentable sugars in a rapid way with the concomitant yield (Khushal Brijwani *et al.,* 2011).

Pre-treatments include physical, chemical, and thermal methods, and their combinations. Chemical pre-treatment includes alkali, acid, lime and ammonia treatments while physical pre-treatments include steam explosion and microwave treatment (Table 2). Among all these, alkali pre-treatment has been proven to be a promising one. During the process of alkaline treatment, degradability remains mainly in the solid phase and the subsequent solid separation is easy. The most commonly used alkali is sodium hydroxide.

Cellulose is commonly degraded by an enzyme called cellulase. Enzyme is produced by several microorganisms, mainly by bacteria & fungi. Some of these produce significant quantities of extracellular enzymes capable of completely hydrolyzing crystalline cellulose *in vitro.* Fungi are the main cellulase producing microorganisms. Cellulase is an enzyme which is capable of breaking down a highly ordered cellulose polymer into sufficiently smaller sugars which are able to pass through the microbial cell wall (Eriksson, 1969). The enzymatic degradation of cellulsose is a complex process that requires the participation of at least three types of the enzymes:

ENZYME	SUBSTRATE
Endoglucanases (C_x) (1,4-β –D- glucan 4-glucanohydrolase) (E.C.3.2.1.4)	- CMC , HEC - Amorphous cellulose - cellodextrins
Exoglucanase (C_1) (1,4-β –D- glucan cellobiohydrolase) (E.C.3.2.1.91)	- Amorphous cellulose - Cellodextrins - crystalline cellulose.
β – glucosidase (E.C.3.2.2.21)	- cellodextrin - cellobiose

These enzymes act sequentially in the synergistic system and subsequently convert cellulose into an utilizable energy source – glucose and hence cellulases provide a key role in biomass utilization. The specificities of these enzymes have been studied by using a number of substrates. In 1950 Reese et al demonstrated their C_x-C_1 hypothesis outlined in following figure.

Aspergillus (Gokhale *et al.,* 1984) & *Trichoderma* (Benkun Qi *et al.,* 2007) are well known efficient producers of cellulases.

Cellulase production is the most important step in the saccharification of renewable cellulosic materials. The production of cellulase generally depends on variety of growth parameters which includes inoculum size, pH value, temperature , presence of inducers, medium additives, aeration, growth and time (Immanuel *et al.,* 2009) and

also the cellulase activity is appear to be depend on the presence of various metal ions as activators and inhibitors (Muhammad *et al.,* 2012). Cellulases have numerous applications in the area of industry and pharmaceuticals. The major industrial applications of cellulases are in textile industry for 'biopolishing' of fabrics and producing stonewashed look of denims, in household laundry detergents for improving fabric softness and brightness (Cavaco-Paulo, 1998), in food, leather, paper/pulp industries. Besides, cellulases are also used in ruminant nutrition for improving digestibility, in fruit juices processing and another emerging application is de-inking of paper (Sakthivel *et al.*, 2010). Nevertheless, all these uses are of rather small magnitude compared with cellulase requirements for bioconversion of lignocelluloses to fuel ethanol.

Recently Solid State Fermentation (SSF) gained fresh attention due to some major advantages it posses, especially in the utilization of agro-industrial residues and pollution prevention (Pandey *et al.,* 2011). In present study also cellulase production was carried out by SSF.

In view of biotechnological importance of cellulases, the present study emphasizes on screening of potential fungal cultures for cellulases production, optimization of various parameters that affects the enzyme production, partial purification of enzyme and utilization of partially purified enzyme for the enzymatic hydrolysis of various treated and untreated lignocellulosic agro-wastes.

Materials and Methods

Selection of agricultural wastes: Rice straw, Banana stem, banana leaves and Sugarcane bagasse were selected for the cellulase production and these wastes collected from the different fields of the Padara village.

Isolation and screening of cellulolytic fungi: For isolation and screening of cellulolytic fungi sample was collected from local compost pits of Bakrol village, coordinates: 22°33'4"N 72°55'3"E, Anand, Gujarat, India. Isolation was done by serial dilution method using Potato Dextrose Agar as growth medium. The isolated strains were carefully identified by morphological characteristics include color of the colony and growth pattern studies, as well as their vegetative and reproductive structures observed under the microscope.

Cellulase producing fungi were primarily screened on selective Basal Salt Media containing 1% carboxy methyl cellulose-Na Salt (g%): $NaNO_3$, 2.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; Carboxymethyl Cellulose-Na salt, 1.0; Peptone, 0.2; Agar, 3.0. Well of 6 mm size was made in the middle of the plate and inoculated with 0.1mL of spore suspension. Then the plates were incubated at 28ºC for 5 days.

After 5 days, these plates were again incubated for 18hr at 50 ºC. After incubation the plates were flooded with 1% Congo red staining solution, shaken at 50 rev/min for 15 min. Then plates were destained with 1M NaCl solution and shaken at 50 rev/min for 15–20min. Diameter of zone of decolorization was measured around each colony. The fungal colony showing largest zone of decolorization was selected for cellulase production.

Secondary screening was carried out by growing all the isolated strains in submerged fermentation by using Mandel and Weber's media. Macro nutrients (g/L): $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2.0; Urea, 0.3; $CaCl_2$, 0.3; $MgSO_4.7H_2O$, 0.3; Peptone, 1.0 and micronutrients (mg/L): $FeSO_4.7H_2O$, 5.0;

$MnSO_4.7H_2O$, 1.6; $ZnSO_4.7H_2O$, 1.4; $COCl_2$, 2.0. Enzyme activity was measured in the broth. The aim was to isolate strains of fungi with improved cellulase production and had potential industrial application.

Substrate profiling for SSF: All four substrates were collected from local fields and used as a substrate for enzyme production. Substrates were ground and sieved by 5–7mm mashes separately. Then these substrates were pre-treated with different percentage of NaOH i.e. 5%, 10%, 15% and 20% and were incubated at room temperature for 12h.

Then the slurry was filtered through muslin cloth and the biomass was washed completely under tap water till neutrality and was dried in oven at 80ºC till moisture was evaporated. The completely dried pre-treated biomass was then used for the optimization of enzyme production and saccharification.

Cellulose content estimation from substrates:

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

The inoculum was kept in shaker (200 rpm) at 30°C for 48 hours before it was used for the fermentation process.

Fermentation process: Fermentation was performed in 250mL Erlenmeyer flask containing 5g of pre-treated substrate. The moistening agent used was Mandel and Weber's medium used for secondary screening. The medium and the trace elements were autoclaved separately. The flask was cooled down at room temperature and a known amount of sterilized trace elements were added.

The flasks were then inoculated with 1×10^6 spores/mL and incubated for 6 days at the ambient temperature (28±1ºC). At the end of fermentation time 0.05 M citrate buffer of pH 5.3 was added to give total 50mL/flask. The flasks were shaken at 150 rpm for 1hr and then filtered with muslin cloth to obtain clear filtrate for determining enzyme activity.

Determination of reducing sugar and cellulase activity

Miller's method of DNSA

Endoglucanase assay: Activity of endoglucanase in the culture filtrates was quantified by carboxy-methyl cellulase (CMCase) method (Ghosh, 1987). The reaction mixture with 0.5 mL of crude enzyme, 1.0 mL 0.05 M sodium citrate buffer having pH 5.3 and 0.5 mL substrate CMC prepared in the same buffer was incubated in the water bath at 50°C for 30 minutes. The released reducing sugar was determined according to Miller's method of DNSA (Miller, 1959). Appropriate control without enzyme was simultaneously run.

FPase assay: Total cellulase activity was measured by FPase assay. The reaction mixture with 0.5 mL of crude enzyme, 1.5 mL 0.05 M sodium citrate buffer having pH 5.3 and a strip of Whatman filter paper #1 (50 mg, 1 X 6 cm) was incubated in the water bath at 50˚C for 1 hour. The released reducing sugar was determined according to Miller's method of DNSA. Appropriate control without enzyme was simultaneously run.

Optimization of culture conditions for enzyme production

Influence of various agro-wastes as a carbon source on cellulase production: Four lignocellulosic agro-wastes were selected on the basis of their cellulose content and were used to check their influence on the enzyme production. Fermentation was carried out for 6 days and cellulase production was measured.

Influence of various alkali pre-treated substrates on cellulase production: Substrates which gives the maximum production during the study of their influence on the enzyme production were selected for this study. Different alkali pre-treated substrates i.e. 5%, 10%, 15% and 20% NaOH, were used for the production optimization to check their effect on the enzyme production.

Influence of incubation period on enzyme production: Fermentation period is an important parameter for enzyme production. In this study, fermentation experiment was carried out up to 7 days and production rate was measured at every 24 hour.

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 and 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.

Influence of temperature on enzyme production: To check the effect of temperature on the enzyme production, fermentation was carried out at two different temperature 28°C and 30°C and the enzyme activity was determined.

Influence of spent wash as moistening agent on cellulase production: Effect of distiller waste spent wash is checked on the enzyme production by using it with various concentration ratio of MW media like 3mL MW media, 2:1 (MW media: spent wash), 1:2 (MW media: spent wash) and 3mL spent wash.

Partial purification: All procedures of the cellulase purification were carried out at 4°C. The culture supernatant was separated by centrifugation process, by using buffer like buffer A, 50mM citrate buffer (pH 5.3), followed by fractional ammonium sulphate precipitation by adding solid ammonium sulphate to the culture filtrate from 60% to 80% saturation. After 48 h the resulting precipitate was collected by centrifugation at 10,000 × *g* for 30 min and dissolved in buffer A and dialysed overnight against three changes of the same buffer. Insoluble material was removed by centrifugation at 10,000 × *g* for 10 min. The clear supernatant was filter sterilized and stored at 4^0C.

Effect of pH on the enzyme activity: The optimum pH for the crude enzyme was determined by incubating crude enzyme with substrate (1% CMC) prepared in appropriate buffers; 0.05 M citrate buffer (pH 3.0 to 8.0). Crude enzyme mixture in those buffers was incubated for 30 min at 50°C. Cellulase activity was assayed by DNS method.

Effect of temperature on the enzyme activity: The effect of temperature on activity of endoglucanase was determined by incubating crude enzyme with 1 % CMC in 0.05 M citrate buffer (pH 3.0 to 8.0) at temperatures between 30 and 80°C. Enzyme activity was assayed by DNS method at different temperatures as described above.

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. Each metal ion was used at concentration of 5mM.

Enzymatic hydrolysis: Alkali pre-treated and untreated rice straw and banana stem, carried in different 250mL of Erlenmeyer flask, mixed with extracted enzyme having 1.2 U/mL activity and purified enzyme having 1.2 U/mL activity in each flask. The total volume of solution is adjusted to 50mL by addition of 50mM sodium citrate buffer of pH 5.3. This Enzymatic solubilization was performed in an orbital shaker at 50°C, 150 rpm for 24 h. The content of reducing sugar solubilized into hydrolysate was determined by DNSA method at every 2 hours.

Characterization of released reducing sugar by Thin Layer Chromatography (TLC): Enzymatically saccharified samples were spotted on readily available TLC plate of silica, 60 $F_{254,}$ Merck, Germany, and allowed to run by capillary action in the mixture of n-Butanol, n-Propanol, Ethanol and Water in the ratio of 4:6:6:4 followed by heating the TLC plate at 100°C for 10 min in the oven. The plate was sprayed with the solution containing DPA spraying solution. Then the plate was dried in oven at 100^0C to develop the colour. Glucose (0.1 mM) was used as standard.

Result and Discussion

Isolation and screening of cellulolytic organism

The fungi were isolated and screened from the samples collected from local compost pits of Bakrol village, Anand, Gujarat, India, for cellulase production by Congo red assay. Among 20 different isolates from the soil sample. We selected nine different fungi and they were named as N_1, N_2, N_7, N_{10}, N_{14}, 2b, NBTry, NBN_8, and Asp. The isolated strains were identified by morphological characteristics including colour of the colony and growth pattern studies. Some of the microscopic characteristics examined under the microscope include spore formation and colour. Isolated fungal strains were subjected to screening. The diameter of hydrolytic zone on solid plate containing 1% CMC was measured. Isolate 2b showed highest zone of hydrolysis of 12 mm (Fig. 1). Further screening is carried out by growing all the isolated strains in submerged fermentation by using MW media. The aim was to isolate strains of fungi with improved cellulase production and had potential industrial application. Cellulase production by each isolated strain is indicated in figure 2.

Isolate 2b was observed with highest CMCase activity as 4.39 U/mL and Fpase activity as 5.01 U/mL. Therefore this isolated strain was selected for further studies for cellulolytic enzyme production on solid state fermentation.

Identification of fungal isolate 2b

Colony morphology: The selected strain was subcultured on PDA agar medium. Initially the colonies were white and changed to black, as culture matured. When immature, the colonies were covered with white fluffy aerial mycelia, while mature colonies were covered with black spores and reverse of the colony was buff coloured (Fig. 3).

Microscopic examination of the isolate 2b

The matured colonies were subjected to lacto phenol cotton blue staining and

microscopic examination was made. From the microscopic observation it was clear that the colony showed the hyaline septate hyphae. The conidial head was large and appeared black to brownish black. The conidiophores were hyaline or brownish near the vesicle. Each vesicle appeared globose in shape and cover with brownish sterigmata on the entire surface in two series (Fig. 4). Based on the colony morphology and microscopic observation the genus was confirmed as *Aspergillus.*

Substrate profiling for SSF

Various agro industrial residues like rice husk, wheat bran, rice straw, banana stem, banana leaves, and sugarcane bagasse were used for SSF. All these agro residues vary in their cellulose content. To use these agro wastes as a substrate for cellulase production, estimation of their cellulose content is necessary.

Cellulose content measurement from various lignocellulosic agrowastes

Cellulose is the major component of biomass (30–60% of dry biomass) (Lee, 1997). Among vast variety of lignocellulolytic agro wastes, we have selected four agro waste and measured their cellulose content and the results are shown in table 1.

From selected four agro waste, in banana stem (BS) and rice straw (RS), cellulose content was estimated higher than other two substrates. Coweling (1975) reported that lignocellulosic biomass contain 40–60% cellulose content.

From results it was clear that mild NaOH pre-treatment increased accessible cellulose content (Zhang *et al.,* 2007) while it causes weight loss of substrate. In another study by Damisa *et al.,* 2008, pretreating of substrate with NaOH may have resulted in the swelling of particles and causing easy removal of lignin which may be the possible reason of weight loss.

Optimization of cellulase production by SSF

Cellulase production depends upon the composition of the fermentation medium. Optimization for over production of the enzyme is an important step and involves a number of physico-chemical parameters such as the incubation period, pH, temperature and moistening agent, and availability of substrate from various agro wastes in solid state fermentation. For the initial optimization of the medium, the traditional method of "one variable at a time" approach was used by changing one parameter at a time. The selected cellulolytic strains were grown in selected media consisting of selected substrates for enzyme production. Studies were performed for different fermentation conditions for hyper cellulase production.

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

Among the 4 substrates (banana stem, banana leaves, rice straw, and sugarcane bagasse) screened, banana stem gave the maximum cellulase production when fermented with selected isolate 2b under SSF (figure 6). Maximum activity found was 8.6 U/mL and 7.4 U/mL of Fpase and CMCase, respectivly. Considerable amount of enzyme production was observed on rice straw and sugarcane bagasse. Comparatively less enzyme production was observed with the banana leaves on SSF. Since banana stem and rice straw showed maximum production of cellulase, it was selected for further optimization studies for SSF

systems. In a study conducted by Ojumu *et al.,* (2003) on *Aspergillus flavus* reported that the saw dust, rice straw and sugarcane bagasse were found to be the best substrates for the production of cellulase. Sugarcane bagasse, tea production waste, coconut coir pith, rice husk, wheat bran, rice bran *etc*., have been employed for production of cellulase using a variety of microorganisms such as *Trichoderma, Aspergillus, Penicillium, Botrytis, Neurospora etc*., (Fig.5) (Pandey *et al.,* 2011). Ananda Muniswaran *et al.* (1994) used banana stalk and coconut coir for production of cellulases. *T. lignorum* was grown on the banana waste based medium for production of cellulolytic enzymes and assays were carried out as reported earlier (Baig *et al.,* 2003)

Influence of various alkali pre-treated substrates on cellulase production

The effect of different concentration of NaOH on enzyme releases from alkali treated banana stem and rice straw fermented with fungus 2b and WR is presented in table 3. The highest CMCase enzyme activity from 2b was achieved using banana stem pre-treated with 5% NaOH (12.25 U/g) followed by 10% (6.44 U/g), 15% (4.39 U/g), and 20% (3.25 U/g). The results revealed that low concentration of alkali is the best treatment for the maximum cellulase production. Bjerre *et al.* (1996) found that dilute NaOH was an effective pre-treatment for lignocellulosic materials. In another study by Damisa *et al.* (2008) revealed that the use of NaOH pre-treated substrate gives higher cellulase yield.

Influence of incubation period on cellulase production

The incubation period is directly related with the production of enzyme. Up to a certain extent isolate 2b and WR showed the most active cellulolytic species along with different incubation period. The incubation period to achieve peak enzyme activity 12.25 U/mL and 13.26 U/mL of CMCase and FPase, respectively by the isolate 2b, in SSF with 5% NaOH treated both substrates, was 144 h which was suitable for commercial point of view (Table 4). It might be due to the depletion of nutrients which stressed the fungal physiology resulting in the inactivation of secretory machinery of the enzyme (Nochure *et al.,* 1993).

Influence of pH on cellulase production

Among physical parameters, pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion. Initial pH of the medium has strong influence on the enzyme production. Production of most fungal cellulases is reported in the pH range of 4.5-5.0 (Latifian *et al.,* 2007; Wen *et al.,* 2005). The pH change observed during the growth of microbes also affects product stability in the medium.

The cellulase enzyme production by the isolates 2b and WR at different pH showed maximum CMCase activity of 12.21 U/g and 9.32 U/g respectively while maximum FPase activity of 16.03 U/g and 11.4 U/g respectively at pH 4.8. Das *et al.* (2008) also observed cellulase activity was optimum at pH 4.8 (Fig. 9 and Table 5).

Further increase or decrease in pH from 4.8, reduced all the enzyme activity. Similar observation was made for cellulase production by *A. niger* in SSF by Doppelbauer *et al.* (1987), whereas pH 7 was reported by Krishna (1999) for the production of bacterial cellulases by using banana wastes in SSF.

Influence of temperature on cellulase production

Incubation temperature plays an important role in the metabolic activities of a microorganism. In the present study, the optimum temperature for maximum enzyme production by isolates 2b was 12.25 U/mL and 13.26 U/mL for the activity of CMCase and FPase, respectively recorded at 28ºC. In general the temperature maintained in SSF system is in the range of 25 to 35ºC and depends on the growth kinetics of the microorganism employed rather than on the enzyme produced (Lonsane *et al.*, 1985). Ali *et al.* (1991) reported maximum yield of cellulase from *Aspergillus niger* at 40ºC, respectively in SSF. Singhania *et al.* (2006) studied 28°C temperature was suitable for cellulase production (Fig. 8 and Table 6).

Influence of spent wash as moistening agent on cellulase production

Moisture content is a critical factor in SSF processes because this variable has influence on growth, biosynthesis and secretion of enzyme. In the present study distiller spent wash was used as moistening agent in various combinations with MW media. The maximum enzyme production was obtained 3mL of spent wash concentration (Table 7). Distiller spent wash possess high nutrient amount required for the growth of microorganisms. Presence of organic carbon and nitrogen as well as some metal ions like calcium, magnesium, zinc, cobalt, etc.

Partial purification

Enzyme produced by solid state fermentation of banana stem treated with 5% NaOH by isolate 2b is further purified by ammonium sulphate fractionation method and then by performing dialysis of obtained fraction of 60-80%. During 60–80% ammonium sulphate fractionation our desired protein gets precipitated which is further dialyzed against 0.05M citrate buffer having pH 5.3. Following table shows the efficiency of purification method (Table 8). By analysing the above table we come to know that we got 7.45 fold purified enzyme. This purified enzyme was stored at 4°C and further used for its kinetic studies.

Enzyme characterization

Study of enzyme characterization is necessary to understand the behaviour of enzyme in various physical and chemical conditions. In this section we study the effects of various parameters like pH, temperature and effect of various metal ions on the activity of enzyme.

Effect of temperature on purified enzyme activity

The optimal temperature for the purified celluase is 50˚C at pH 4.8. It shows 4.05 U/mL activity. The purified cellulase was stable at temperatures under 30˚C to 50˚C,

while the temperature I_{50} was 60℃.

According to previous studies, cellulases are active at the pH range of 6.0–7.0 from *A. Niger* (Akiba *et al.*, 1995) from 30˚C enzyme activity increased but activity started to decline as temperature increased above 60°C and became completely denatured at 100°C.

Effect of pH on purified enzyme activity

cellulase exhibited highest activity at pH 5.3 and was stable between pH 5 to 6. According to previous studies, cellulases are active at the pH range of 6.0 to 7.0 from *A. Niger* (Akiba *et al.*, 1995).

Effect of various metal ions on purified enzyme activity

The present study revealed that metal ions Ca^{2+} and Mn^{2+} activated cellulases of isolated fungi 2b, which was moderately inhibited by Co^{2+} and Fe^{2+} and strongly inhibited by Cu^{2+}. The major action of these metal ions is to work as cofactor of the enzyme (Fig. 11). Activation of cellulase occurs when storage or purification results in aggregation and auto oxidation by reducing agents which cause the reduction of disulfide bonds, similar to the findings of Smriti et al in 1999 for *Catharanthus roseus.*

Enzymatic hydrolysis

Enzymatic hydrolysis is essential to complete cellulose degradation. Action of cellulase leading to the production of glucose would make the production of bioethanol from lignocellulosic materials more profitable. In the present study, saccharification of agro-residues like rice straw and banana stem was carried out by crude cellulase enzyme and partially purified cellulase enzyme from the isolate 2b. Figure 10 shows the profile of reducing sugars obtained upon enzymatic hydrolysis of rice straw and banana stem with 5% NaOH treatment and untreated substrates. After an initial phase of rapid sugar formation, there was decrease in the rate of hydrolysis. This could be due to enzyme inactivation or depletion of an easily hydrolysable fraction of cellulose in the mixture. From the observed results, it was found that the sugar yield was maximum from the pre-treated banana stem as compared to pre-treated rice straw. Enzymes synthesized on banana agro-waste medium released more glucose (Baig *et al.,* 2003). Untreated substrates were found less accessible towards enzymatic hydrolysis as evidenced by very low amount of reducing sugar released during enzymatic hydrolysis. Many authors used microbial enzymes for hydrolysis of lignocellulosic materials. Fadel (2000) used cellulase for wheat straw saccharification for ethanol production. Cellulase from *T. reesei* and *A. niger* were used for hydrolysis of pine parks (Parajo *et al.,* 1988).

Thin layer chromatography

Detection of sugars from the hydrolysates by thin layer chromatography showed remarkable presence of glucose in the hydrolysate of pre-treated substrates (Figure 10).

Table.1 Cellulose content measurement from various lignocellulosic agrowastes

Substrate	**Cellulose content (%)**
SB	23%
BS	**36%**
BL	25%
RS	**32%**

Table.2 Effect of pretreatment on substrate

		Banana stem		Rice straw	
		Cellulose (%)	Weight (g)	Cellulose (%)	Weight (g)
Before pretreatment		36	5	32	5
After pretreatment	5%	59.6	4.1	46.3	3.6

	10%	30.2	3.6	28.7	3.01
	15%	26.3	2.4	20.3	2.16
	20%	11.2	1.7	10.2	9.76

Table.3 Influence of various alkali pretreated substrates on cellulase production by WR

WR				
Alkali Pretreatment	Banana Stem		Rice Straw	
	Enzyme activity (U/g)		Enzyme activity (U/g)	
	CMCase	FPase	CMCase	Fpase
5%	11.65	12.02	9.31	10.39
10%	5.43	7.13	4.75	5.24
15%	3.26	5.87	1.89	2.01
20%	2.01	4.31	1.23	2.67

Table.4 Influence of incubation period on cellulase production

Incubation period (hours)	**2b**		WR	
	Enzyme activity (U/g)		Enzyme activity (U/g)	
	CMCase	FPase	CMCase	Fpase
96	7.83	8.43	5.32	7.92
120	9.62	10.7	7.19	8.62
144	**12.25**	**13.26**	11.39	12.98
168	10.36	11.32	10.04	12.48

Table.5 Influence of pH on cellulase production by isolate 2b and WR

pH	**2b**				WR			
	CMCase (U/g)		FPase(U/g)		CMCase(U/g)		FPase(U/g)	
	BS	RS	BS	RS	BS	RS	BS	RS
4.0	9.7	8.17	10.6	9.61	7.34	7.02	6.95	6.32
4.8	**12.21**	10.14	**16.03**	13.27	11.41	10.01	10.03	9.57
5.0	9.04	7.19	8.03	10.39	8.02	6.89	8.12	7.89
5.3	7.31	6.35	7.72	9.85	6.98	6.01	7.02	6.43
6.0	5.55	4.31	7.17	9.07	4.32	3.98	6.89	6.12
6.5	4.12	3.53	5.43	4.79	3.98	3.12	4.03	3.39

Table.6 Influence of temperature on cellulase production

Substrate	Enzyme activity (U/g)	Temperature			
		28^0C		30^0C	
		2b	WR	**2b**	WR
Banana stem	Fpase	**13.26**	11.98	12.16	10.02

	CMCase	**12.25**	11.32	11.01	9.78
Rice straw	CMCase	10.49	9.87	9.23	8.65
	Fpase	12	10.98	11.23	10.21

Table.7 Influence of spent wash as moistening agent on cellulase production

Substrate	Enzyme activity (U/g)	**Spent wash with various concentration of MW media**			
		MW media (3mL)	**2:1**	**1:2**	**Spent wash (3mL)**
Banana stem	Fpase	7.12	8.62	9.72	**10.01**
	CMCase	6.26	8.3	10.77	**11.11**
Rice straw	CMCase	6.01	7.23	8.85	9.25
	Fpase	7.21	9.1	12.73	12.88

Table.8 Partial purification of crude enzyme

Procedure	**Total Activity (U)**	**Total protein (mg)**	**Specific activity (U/mg)**	**Purification (fold)**	**Yield (%)**
Crude enzyme extract	1254.16	0.079	15875.44	1	100
Ammonium sulphate precipitation (60-80%)	2118.4	0.123	17222.76	1.08	98.52
Dialysis	1918	0.162	11839.50	7.45	44.61

Fig.1 Hydrolytic diameter of fungal isolate 2b

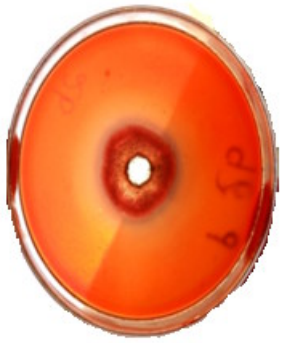


Fig.2 Cellulase production by each isolated strain

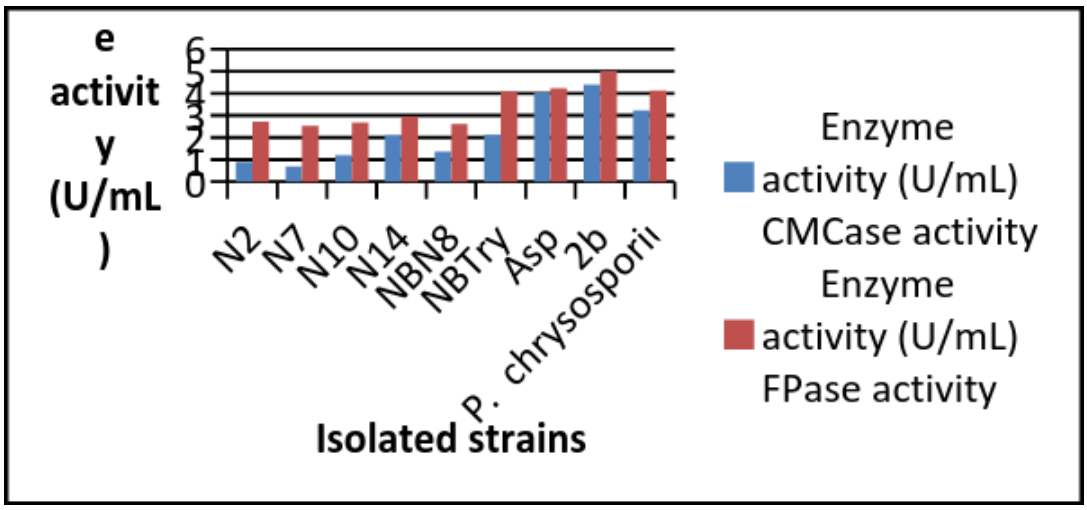


Fig.3 Identification of fungal isolate 2b

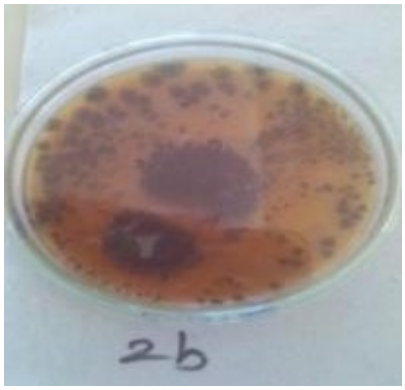


Fig.4 Microscopic appearance of the isolate 2b

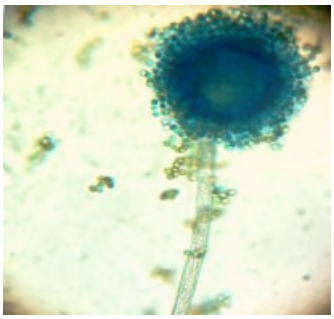


Figure.5 Cellulase production by each isolate in SmF

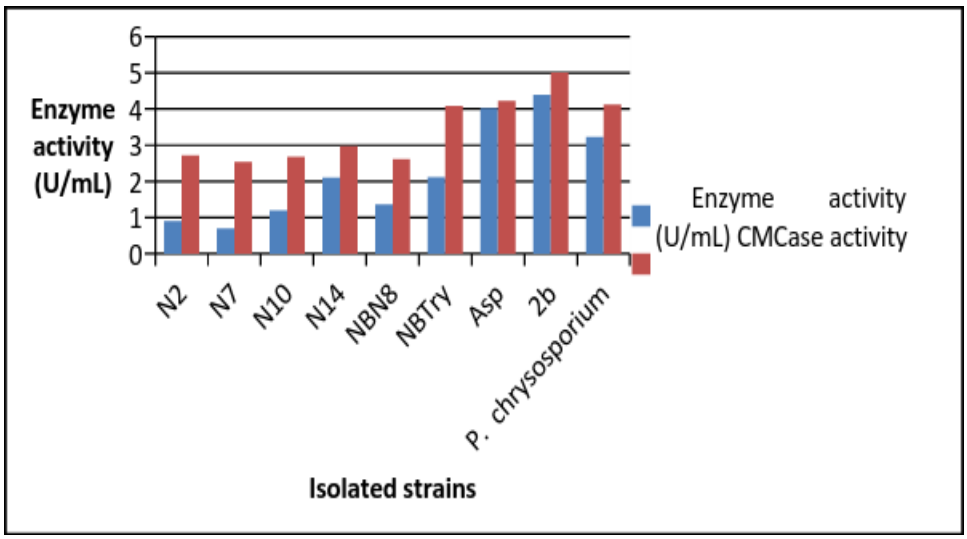


Figure.6 Influence of various substrates on enzyme production

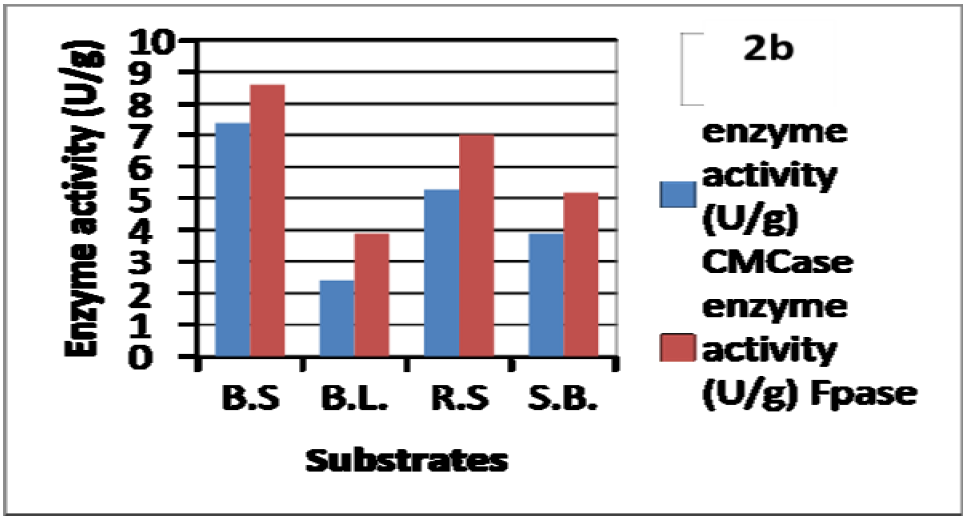


Figure.7

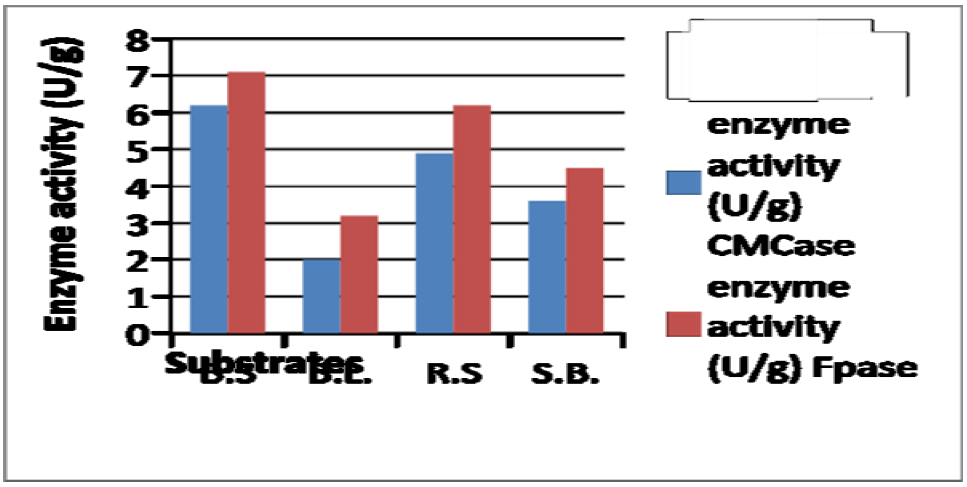


Figure.8 Effect of temperature on enzyme activity

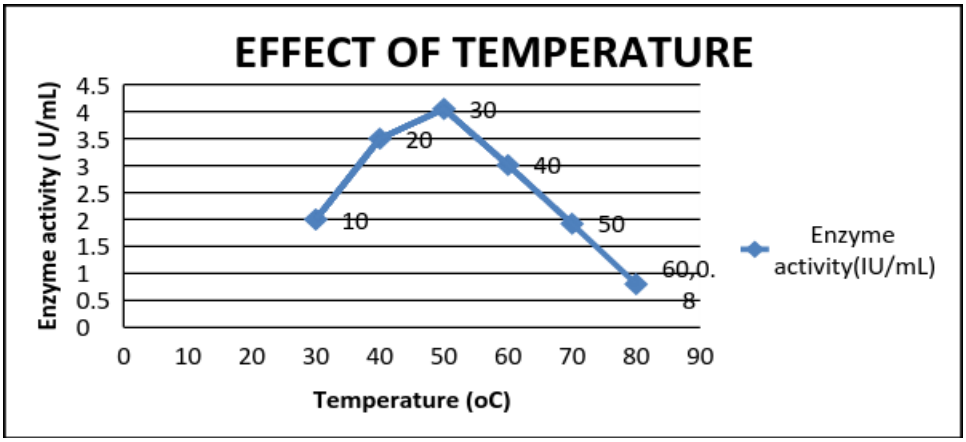


Figure.9 Effect of pH on purified enzyme activity

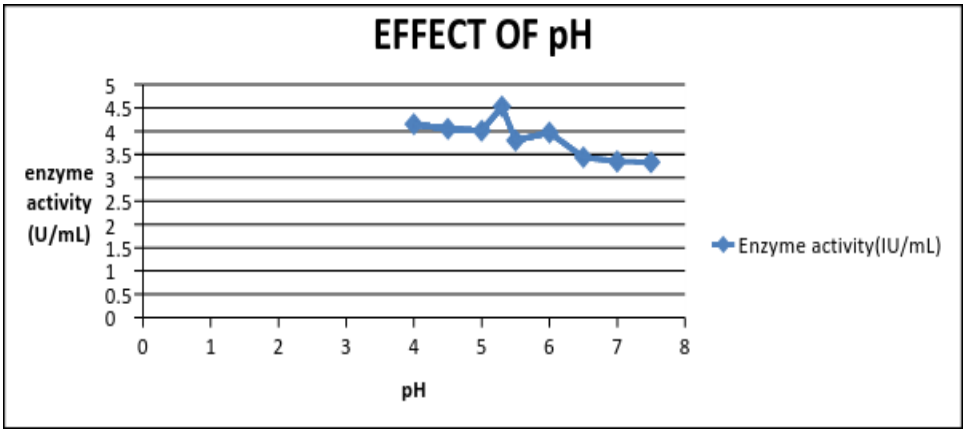


Figure.10 Enzymatic hydrolysis

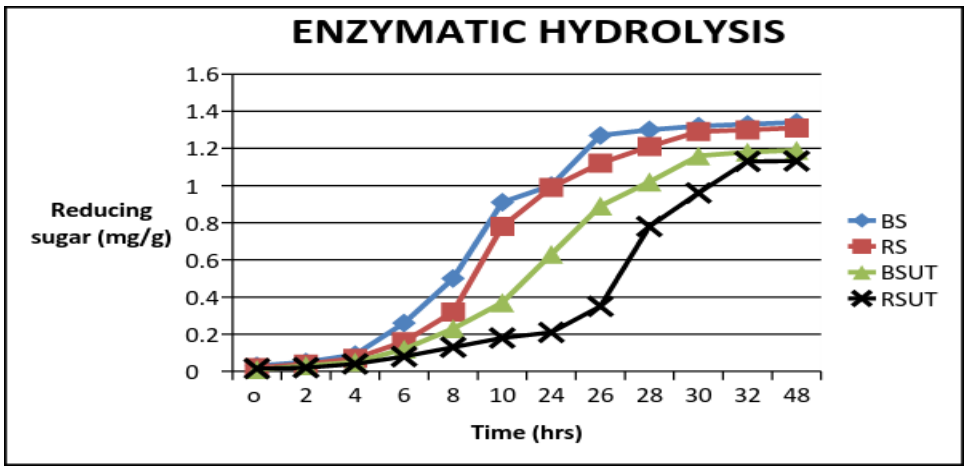
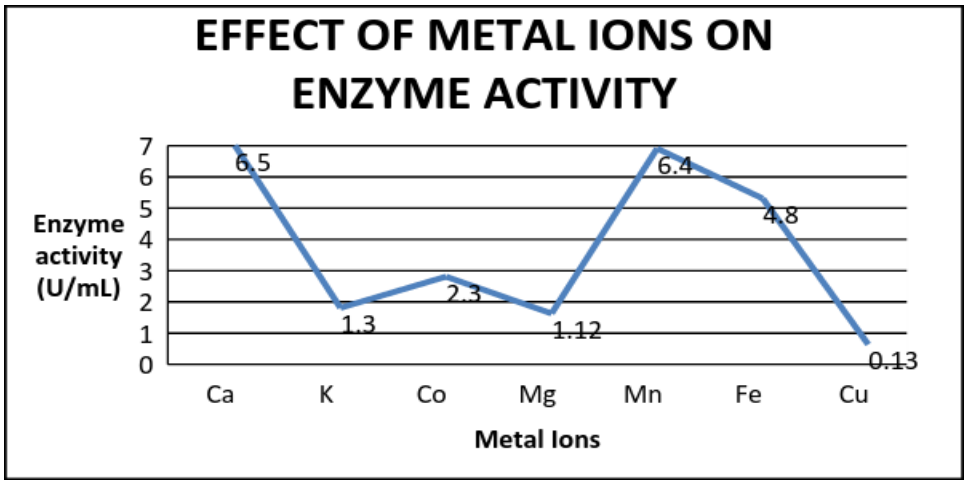


Figure.11 Effect of metal ions on enzyme activity



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