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Optimization of the medium for the production of cellulases by *Aspergillus terreus* and *Mucor plumbeus*

***Padmavathi. T, Vaswati Nandy and Puneet Agarwal**

Department of Microbiology, Centre of PG Studies, Jain University, 18/ 3, 9th Main, Jayanagar 3rd Block, Bangalore, India

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ABSTRACT

Fungal cellulases are well-studied, and have various applications in industry, health or agriculture. The present paper investigates the isolation of marine fungi (Aspergillus terreus and Mucor plumbeus) for the production of cellulase using submerged fermentation technique. Ten different substrates such as rice bran, wheat bran, bamboo leaves, banana leaves, peepal leaves, sugar cane leaves, lantana leaves, ragi straw, maize leaves and eucalyptus leaves were collected from different parts of rural Bangalore (India) and were used as substrates for the cellulase production; of which lantana leaves gave best result. The fermentation experiments were carried out in Erlenmeyer flasks using pretreated Lantana leaves. Lantana leaves gave best enzyme activity of 213. 3IU/ ml and 206 IU/ ml by Aspergillus terreus and Mucor plumbeus respectively. Various parameters such as carbon source, nitrogen source, pH and incubation temperatures were studied for the production of cellulases. Incorporation of lantana as carbon source (660 IU/ ml), ammonium sulphate as nitrogen source, pH 3 (240. 07 IU/ ml) and incubation temperature at 37° C (100 IU/ ml) gave good enzyme yield with Aspergillus terreus and Mucor plumbeus respectively. The degree of saccharification was also assayed on the basis of amount of reducing sugar released. The percentage of saccharification with respect to lantana leaves in presence of Aspergillus terreus and Mucor plumbeus were found to be 56% and 28% respectively.

Keywords: Cellulases, Lantana, Marine fungi, *Aspergillus terreus, Mucor plumbeus*

INTRODUCTION

In a time of rising energy prices, utilization of abundantly available biomass as an alternative resource is one of the thrust area of research. Utilization of renewable, economical, abundantly available agro waste for the production of useful products is an increasing trend in recent years. Cellulose is the most abundantly available biomass on earth. It is the main product of photosynthesis in terrestrial environments, and most abundant renewable biosource produced in biosphere. Cellulose is commonly degraded by an enzyme cellulase. Cellulases are widely used in the food, textile, laundry, baking, brewing, pulp and paper industries from biomass and genetic engineering [1- 5]. Microbial conversion of cellulose/ lignocellulosic biomass into useful byproducts is a complex process involving combined action of 3 enzymes namely endogluconase, exogluconase and β- glucosidase. Although cellulases are distributed throughout the biosphere, they are manifested in fungi, bacteria and a few actinomyctes [6, 7]. These microorganisms produce cellulases to release sugar for cell growth and product formation under certain

environmental conditions. More than 14,000 species of fungi have been found to be active in cellulose degradation [8- 11]. Among all the fungi, marine fungi are most common in decomposing wood and plant detritus in coastal water [12- 15].

Marine fungi are also common in calcareous animal shells, algae and corals. Most of the marine fungi belong to ascomycetes and few belong to basidiomycetes. Marine fungi usually influence the biotechnological production because of their special adaptations to their environment. The physical factors that usually influence the marine fungi are salinity, pH, low water potential, high concentration of sodium ions, low temperature, oligotrophic nutrient conditions and high hydrostatic pressure.

Many agricultural by products from agricultural activities and agro based processing litter the environment and constitute waste problems. Agro wastes such as rice straw, wheat bran, corn stover, sugar cane bagasse, pomace, corn cobs etc are used as substrate in solid state fermentation [16, 17]. The use of agro wastes as the basis for the cultivation media is a matter of great interest, aiming to decrease the costs of energy production and meeting the increase in awareness on energy conservation and recycling. Cellulase production by different organisms in submerged fermentation state has received more attention and is found to be cost prohibitive because of high cost of process engineering [18- 20].

Twenty fungal isolates were isolated from marine water. Most of the isolates, obtained from marine source, were *Aspergillus* spp., *Mucor plumbeus* which was very dominant in marine environment. The present paper investigates the isolation of marine fungi, effect of different substrates, pH and different incubation temperature on the production of cellulase by fungi.

MATERIALS AND METHODS

Isolation: Marine fungi were isolated from marine source (Pondicherry and Mangalore) using standard microbiological techniques. A sample of 10 gram was placed in a graduated cylinder, sterilized distilled water was added to make a total of 100 ml. Serial dilution was done till 10^{-4}. 1 ml of desired dilution (10^{-3}and10^{-4}) was transferred aseptically into a potato dextrose agar (PDA) prepared from marine water adding one drop of 20% lactic acid to suppress bacterial growth. Plates were incubated at room temperature for 5 days. After incubation, small portion of mycelium from each fungal colony was transferred into PDA slants.

Identification: Identification of the tested fungal isolates was done depending on the morphological characters of fungi and comparing them with those that are present in the identification references [21]. Twenty fungal isolates were isolated from marine water. Most of the isolates, obtained from marine source, were *Aspergillus* spp. which was very dominant in marine environment. Among all the fungi *Aspergillus terreus* and *Mucor plumbeus* were selected as it showed high cellulase production on liquid state fermentation.

The potential fungal isolates were assayed enzymatically for cellulases. The assay methods employed were:
(a) Cellulolytic enzyme Assay: Test fungi were cultivated on basal medium supplemented with 0.4 % glucose and solidified with 1.6 % w/v agar. A single agar disc cut from the actively growing colony margin of culture was used to inoculate each assay medium. Basal media with concentration of 0.1- 0.2% w/v are sufficient to support cellulose degrading fungi.

(b) Filter paper degrading method: Filter paper used in this assay was be almost 100% cellulose. Cellulolytic basal medium (CBM) was prepared, 10 ml aliquots was transferred to glass culture bottles and was autoclaved. One 25x 5mm strip of sterile filter paper was added aseptically to each bottle making sure that all filter paper stripes were completely submerged. The test fungus was inoculated and uninoculated bottles were retained as control. Care was taken that bottle caps were loosely fitted to allow adequate gas exchange. The bottles were incubated at 25° C in darkness and were examined daily for 10 days. Degradation of filter paper was assessed based on increased opacity and physical degradation in comparison with uninoculated controls [22].

(c)Cellulase agar clearance (cellulose agar) method: This assay uses ball- milled acid swollen or microcrystalline cellulose. Generally microcrystalline cellulose is degraded more slowly than ball-milled or acid swollen cellulose. CBM medium was prepared by incorporating 4% w/v cellulose, 1.6% w/v agar and was autoclaved. It was transferred aseptically to petri dishes (agar was cooled until viscous and was mixed gently before pouring to ensure

uniform distribution of cellulose in the agar medium). The test fungus was inoculated. The culture was incubated at 25° C in darkness and was examined daily for 10 days. Cellulolysis was assessed based on clearance zones of the opaque agar around growing colonies.

(d)Dye staining of Carboxy methyl cellulase (CMC): CMC is a substrate for endogluconase and so can be used as a test for β glucanase activity. This assay is a good indicator to check the Cellulolytic ability of fungi.

CBM was prepared, supplemented with 2% w/v of low viscosity CMC and 1. 6 % w/v agar, autoclaved and transferred to petri dishes aseptically. Test fungus was inoculated and incubated at 25° C in darkness. When the colony diameter is approximately 30 mm, agar plates were stained using Congo red and left for 15 mins. Plates were decanted and agar surface was washed with distilled water. The plates were flooded with 1M Nacl for 15 mins to destain.

Inoculum Preparation: The spore suspension was used as inoculum in the present studies. It was prepared from a 7 days old slant by adding 10 ml of sterilized marine water to it. The spores were scratched with the help of a sterilized wire loop to make a homogeneous suspension of spores. Spore count was measured with the help of Haemocytometer.

Substrates: Ten different substrates such as rice bran, wheat bran, bamboo leaves, banana leaves, peepal leaves, sugar cane leaves, lantana leaves, ragi straw, maize leaves and eucalyptus leaves were collected from different parts of rural Bangalore (India) and were used as substrates for the cellulase production. Among all the above mentioned substrates, utilization of lantana leaves gave good results hence lantana leaves alone was used for the production of cellulase by both the fungi i. e *A. terreus and M. plumbeus.*

Pre treatment of substrates: The collected raw materials were air dried for 3- 5 days depending on the moisture content. Moisture content of the substrates was removed. It was powdered. The same substrates were used for the media preparation.

Fermentation Technique: 2.5 g of cellulosic substrate was taken in a 250 ml Erlenmeyer flask as a fermentation media for the production of cellulase. The major constituents of media were ammonium nitrate (0.8%) and 50 ml of distilled water. The initial pH value of the medium was adjusted to 5 before sterilization at 121 ºC and 15.0 lbs/inch^2 pressure for 15 min. The autoclaved medium was inoculated with 1 mL of freshly prepared spore suspension of fungi and incubated at room temperature for 7 days. After 7 days, the fermented broth was centrifuged at 10000 rpm for 10 min., and the supernatant was assayed for enzyme activity [23].

Different experimental set up were maintained to check the effect of carbon source, nitrogen source, incubation temperatures and effect of pH on the fungi for the production of cellulase [24].

Effect of Carbon and Nitrogen sources on enzyme production: Experiments were carried out to investigate the effect of different carbon and nitrogen sources on the production of cellulase enzyme. The enzyme activity was determined by DNS method [25] and the protein was estimated by Bradford’s method [26].

Effect of pH and Incubation temperature on enzyme production: An experiment was conducted to determine the most suitable pH of the fermentation medium by adjusting the pH of the culture medium at different levels in the range of pH 3 to 9 using different buffers.

In order to determine the effective temperature for cellulase production by *A. terreus* and *M. plumbeus*; fermentation was carried out at 5° C intervals in the range of 15 to 55° C

Enzyme Activity: Cellulase activity was measured by the DNS (3, 5-dinitrosalicylic acid) method [25], through the determination of the amount of reducing sugars liberated. Glucose was used as a standard; 0.5 ml of extract was reacted with phosphate buffer (pH 5). Reaction was stopped by the addition of DNS solution. The reaction mixture was boiled for 10 min, cooled in water for colour stabilization, and the optical density was measured at 540 nm. The cellulase activity was determined by using a calibration curve for glucose. One unit of enzyme activity was defined as the amount of enzyme that releases 1 mol of glucose per minute per ml.

Protein estimation: Protein concentration was determined according to the method described by Bradford, M.M [26]. 5ml of Bradford reagent was added to 0.5 ml of the extract and the extinction was measured after 5 min at 600nm. Different concentrations of bovine serum albumin (BSA) were used as a protein standard: 10, 20, 40, 60, 80, and 100 ug/ ml distilled water.

Saccharification conditions: To determine the optimum saccharification conditions the reaction mixture was incubated at 37 ^{o} C temperature for 7 days and it was centrifuged at 10000 rpm for 10 mins. After this reducing sugar was analyzed by DNS method and was calculated using the formula given below.

$$\textbf{Saccharification (\%)} = \frac{\textbf{Glucose (mg/ ml) x 100}}{\textbf{Substrate (mg/ ml)}}$$

RESULTS AND DISCUSSION

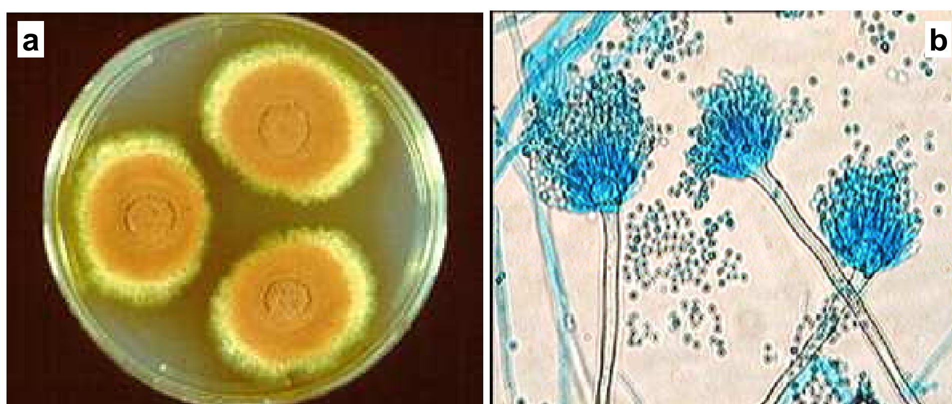

Fig. 1a Morphology and characteristics of *A. terreus* colony & 1b Microscopic view

The selected organisms *A. terreus* and *M. plumbeus were* isolated from marine water by enrichment technique in basal cellulose medium. The cellulolytic ethanologenic isolates were identified as *Aspergillus terreus* and *Mucor plumbeus by* morphology.

Colonies of *A. terreus* on potato dextrose agar at 25°C are beige to buff to cinnamon. Reverse is yellow and yellow soluble pigments are frequently present. Moderate to rapid growth rate. Colonies become finely granular with conidial production as depicted in Fig. 1a. Microscopic observation of *A. terreus* is as depicted in fig. 1b. Hyphae are septate and hyaline. Conidial heads are biseriate and columnar. Conidiophores are smooth-walled and hyaline terminating in mostly globose vesicles. Conidia are small, globose, and smooth.

Colonies of *M. plumbeus* grow rapidly at 25-30°C and quickly cover the surface of the agar. Its fluffy appearance with a height of several cm resembles cotton candy. From the front, the colour is white initially and becomes grayish brown in time. From the reverse, it is white as depicted in Fig. 2a.

Microscopic features of *M. plumbeus* are shown in Fig. 2b and are nonseptate or sparsely septate, broad hyphae, sporangiophores, sporangia, and spores are visualized. Intercalary or terminal arthrospores (oidia) located through or at the end of the hyphae. Apophysis, rhizoid and stolon are absent. Sporangiophores are short, erect, taper towards their apices and may form short sympodial branches. Columella are hyaline or dematiaceous and are hardly visible if the sporangium has not been ruptured. Smaller sporangia may lack columella. Sporangia are round, 50-300 µm in diameter, gray to black in color, and are filled with sporangiospores.

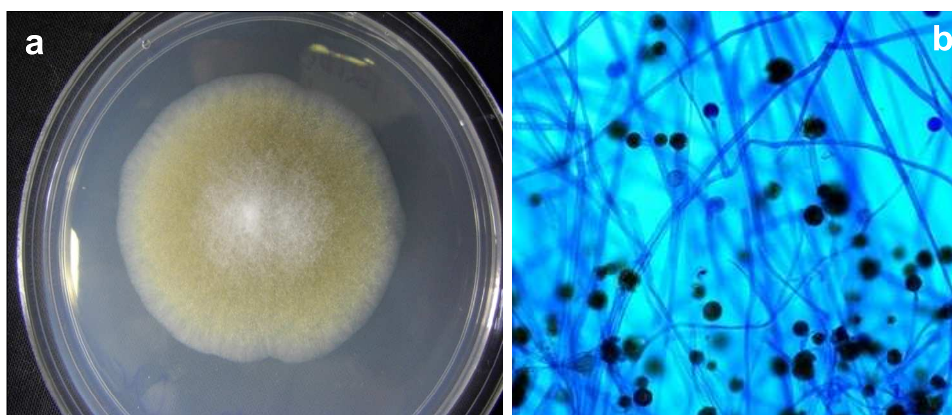

Fig. 2a Morphology and characteristics of *M. plumbeus* colony & 2b Microscopic view

Substrate optimization with lantana leaves: *A. terreus* and *M. plumbeus* were found to degrade various cellulosic agro waste in liquid state fermentation condition of the ten substrate used. Lantana leaves showed very promising result in liquid state fermentation. Lantana leaves gave best enzyme activity 213. 3 IU/ ml and 206 IU/ ml by *A. terreus* and *M. plumbeus* respectively.

These fungal species showed best results in liquid state fermentation of various agro wastes studied, this might be due to the fact that the *Aspergillus terreus and Mucor plumbeus* sp prefer to grow and synthesize enzymes in submerged condition because the growth is restricted only on the surface [27]. Adsorption of enzymes and the formation of enzyme substrate complexes are considered to be critical steps in the enzymatic hydrolysis of cellulose. Fibers of cellulose contain both amorphous and crystalline regions and are considered to be more difficult to be degraded than the amorphous regions [28]. The highest productivity of cellulases on lantana leaves means that these enzymes were absorbed efficiently on lantana leaves also means that lantana leaves contains the two forms amorphous and crystalline cellulose [21].

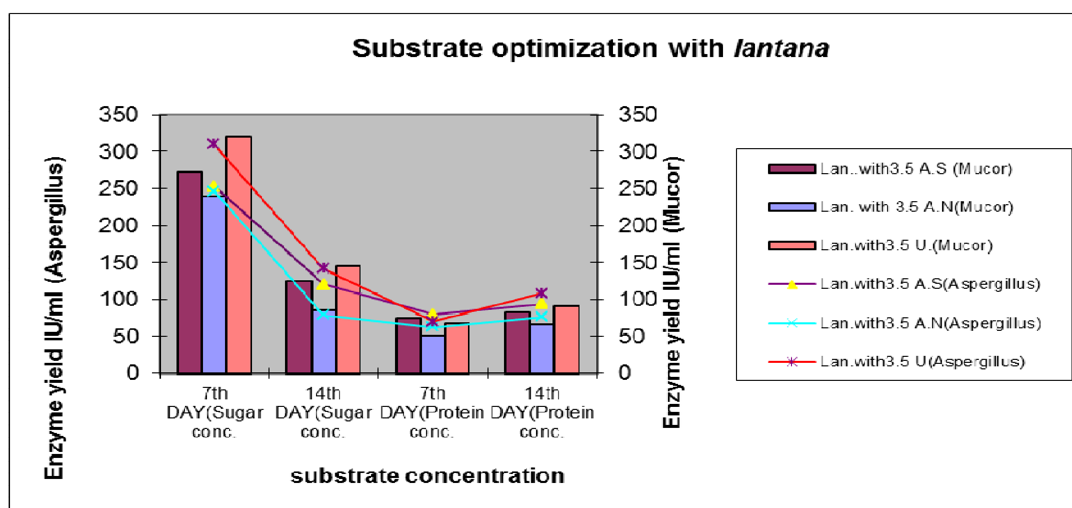

Fig. 3 Substrate optimization with lantana leaves.

Among the three concentrations of substrate optimized 3.5 gms found to be the best for Lantana leaves as depicted in Fig. 3. Higher concentration of Lantana leaves could restore the amount of enzyme production in the fermentation media. Reduced enzyme production in other agro waste substrates could be probably due to the adverse effect of concentrations of nutrient suppliments present in these substrates in enzyme production [29]. Hydrolysis rates of substrates decline with time due to depletion of the more amorphous substrates, product inhibition and enzyme

inactivation [30]. Narasimha *et al*. also gave similar time course reports of maximum glucose yield on 5th day of fermentation using *A. niger* [31].

Effect of Carbon and Nitrogen source on enzyme production:

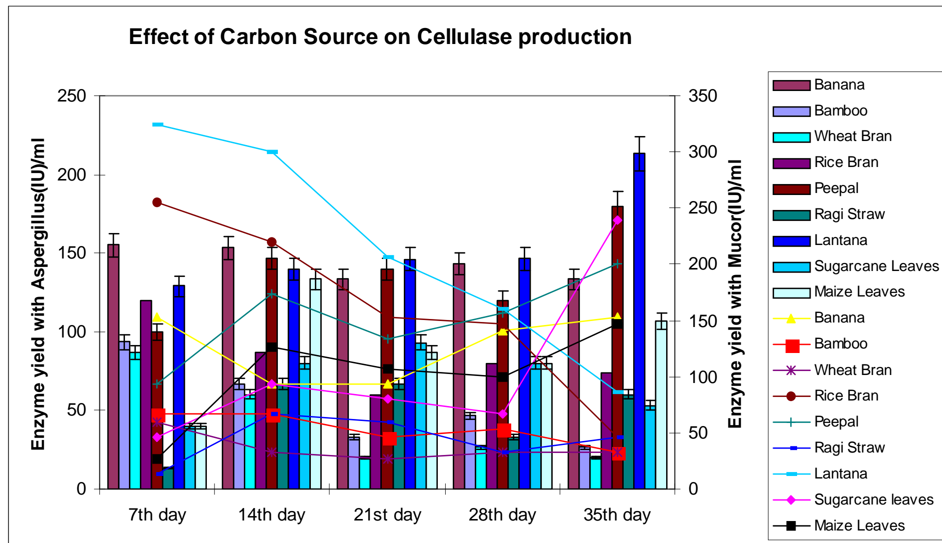

Fig. 4 Effect of Carbon source on cellulase production.

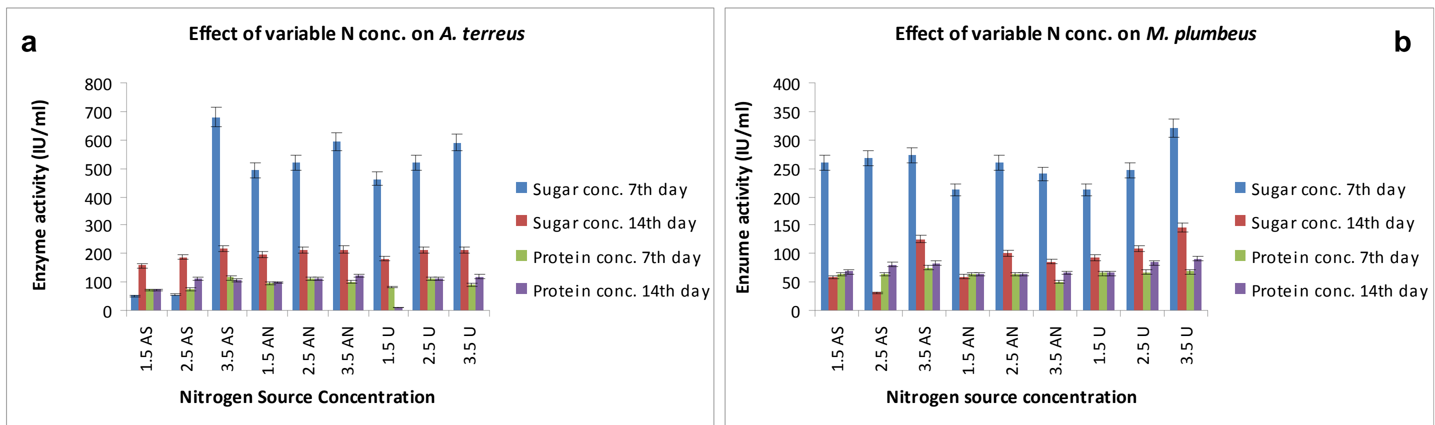

Fig. 5a & 5b Effect of Nitrogen source on cellulase production.

Cellulase synthesis by *A. terreus* and *Mucor plumbeus sp* was highest when cellulosic wastes were used as carbon sources compared to the use of sugars. High cellulase production was obtained using Lantana leaves as carbon source (660 IU/ml). Rice bran, maize leaves, ragi straw and sugarcane leaves has given the least cellulase values when used as carbon source (Fig. 4). Soluble enzyme inducers are reported to be generally weaker in their inductive power when compared to insoluble substrates [32]. The substrate not only serves as a carbon source but also produces the necessary inducing compounds for the organism [33]. Reduction in the cost of cellulase production can be achieved by the use of cheap and easily available substrates.

In fungi, the production of cellulolytic enzymes is subject to transcriptional regulation by available carbon sources. The cellulase genes are repressed in the presence of glucose. Earlier it has been reported that endoglucanase was induced by CMC but repressed by glucose [34]. In this study, we recorded the similar results with very less cellulases activities in the presence of glucose, while CMC proved to be a strong inducer of cellulase.

Fig. 5a & b depicts that the supplementation of organic and inorganic nitrogen sources stimulated the cellulase yield and activity. Results indicate that the sources of nitrogen greatly affected the production of cellulase enzyme. Ammonium sulphate (AS) was the best nitrogen source for *M. plumbeus* and *A. terreus* for lantana leaves. Our results are in accordance with the work of Enari *et al*. who reported that good cellulase production can be obtained with the organic nitrogen sources such as yeast extract and peptone [35]. But enzyme production was remarkably decreased in presence of urea (U) and ammonium nitrate (AN), a report contrary to that of by *Aspergillus niger* [36]. Many papers have reported that ammonium compounds are the most favorable nitrogen sources for protein and cellulase synthesis.

Effect of pH and Incubation temperature on enzyme production:

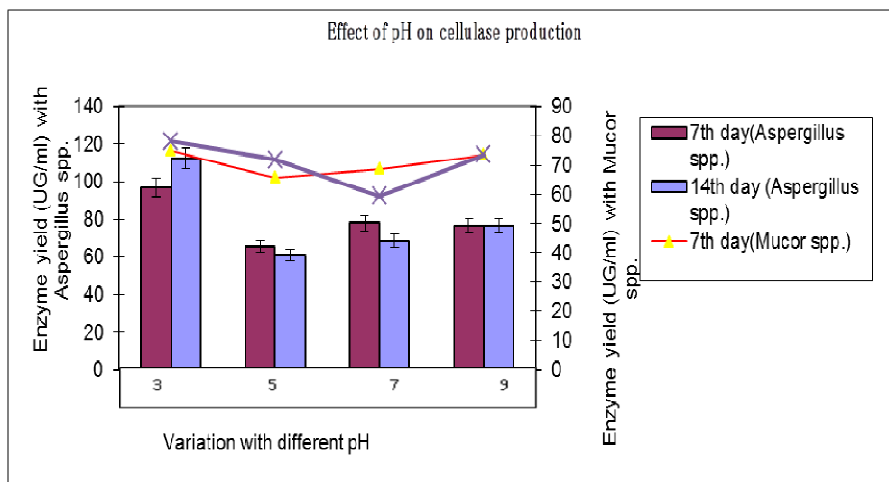

Fig. 5 Effect of pH on cellulase production on Lantana.

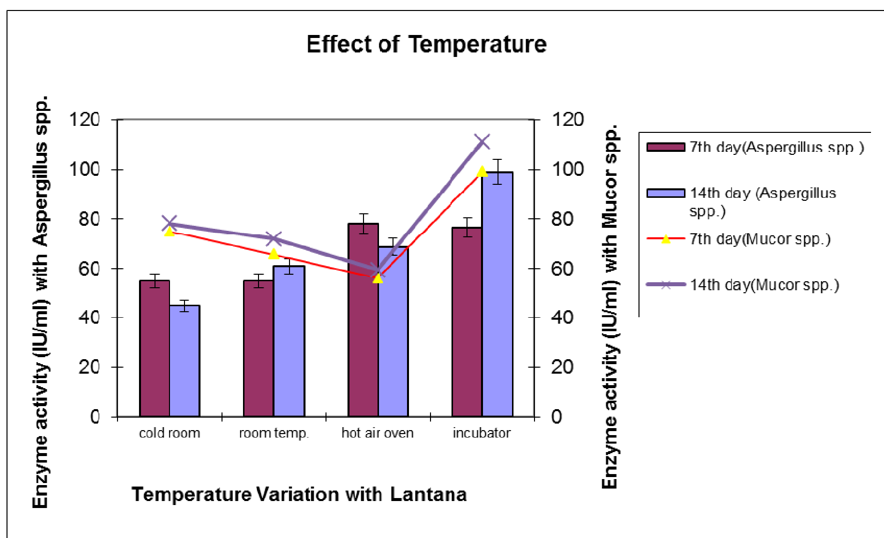

Fig. 6 Effect of incubation temperature on cellulase production on Lantana.

Lantana leaves gave maximum cellulase activity of 240. 07 IU/ ml at pH 3. Further increase or decrease on pH from this level retarded the enzyme activity. According to Ahmed et. al, the optimum pH for the fungal growth; for maximal exoglucanase (EXG), endoglucanase (EG) and Beta glucosidase production was found to be 5.5 at 28°C [34]. An optimum pH for fungal cellulases varies from species to species; though in most cases the optimum pH ranges from 3.0 to 6.0 [37, 38].

Cellulase production declined upon further increases in pH. Cellulase synthesis at pH 3.0 and 9.0 agreed with the observation of Raghukumar et al that low or high pH values inactivates the enzyme and may affect its production [39]. The optimum pH for the growth of fungi has been reported to vary from one organism to another.

Incubation temperature plays an important and significant role in the metabolic activities of microorganisms. It was observed that enzyme activity was high in the flasks that were kept in incubator compared to that kept in cold room, room temperature and hot air oven.

Saccharification of Substrates:

A. tereus and M. plumbeus synthesized cellulases and were used for saccharifaction of agrowaste. The cellulytic enzyme complex when incubated with agro waste released sugars the degree of saccharification was assayed on the basis of release of reducing group the amount of reducing sugar increased with time of incubation in the presence of enzyme the maximum amount of percent saccharifaction was found to be 56% (*A. terreus*) and 28% (*M. plumbeus*) for lantana leaves as tabulated in Table 1 and 2. Enzymatic conversion of cellulose to food, fuel and chemical feedstock is a well-established process. However, high cost of cellulases production has hindered use of this enzyme in industry. The enzymatic conversion of the carbohydrate part of lignocellulosic material has received considerable interest during recent years. This source of raw material is available in abundance and generally free of cost.

Table 1: Saccharification percentage of ten agro wastes by *A. terreus*

Substrates	7th day	14th day	21th day	28th day	35th day	42th day	49th day
	PERCENTAGE SACCHARIFICATION						
Banana	66	46	40	52	40	42	54
Bamboo	28	20	10	14	8	16	18
Wheat Bran	26	18	6	8	6	14	22
Rice Bran	18.6	26	18	24	22	24	26
Eucalyptus	22.2	44	36	44	30	88	64
Ragi straw	4	20	20	10	18	10	10
Lantana	36	42	66	42	64	50	56
Sugarcane	12	22	28	22	16	20	22
Maize	12	40	24	22	24	34	30
peepal	30	44	42	36	54	36	40

Table 2: Saccharification percentage of ten agro wastes by *M. plumbeus*

Substrates	7th day	14th day	21th day	28th day	35th day	42th day	49th day
	PERCENTAGE SACCHARIFICATION						
Banana	46	28	28	42	46	38	36
Bamboo	20	20	14	16	10	16	14
Wheat Bran	18	12	8	10	10	16	20
Rice Bran	36	16	46	44	14	22	18
Eucalyptus	22.2	38	38	46	38	86	64
Ragi straw	4	20	18	10	14	12	12
Lanatna	42	52	62	48	26	22	28
Sugarcane	14	28	24	20	72	62	66
Maize	8	38	32	28	44	28	30
Peepal	28	48	40	46	60	72	76

CONCLUSION

Production of cellulases on agro wastes under submerged fermentation was studied by *A. terreus* and *M. plumbeus*. *A. terreus* and *M. plumbeus* have the potential of converting cellulose in a single step fermentation process. Among the ten different substrates used lantana leaves gave best enzyme activity of 213. 3IU/ ml and 206 IU/ ml by *A. terreus* and *M. plumbeus* respectively. Lantana leaves is a cheap residue which can be used as a substrate for enzyme production which reduces the cost of enzyme production and enzymatic conversion of carbohydrate part of lantana leaves into fermentable sugar. In conclusion, attempt was made to find the optimum fermentation conditions for successful cultivation of *A. terreus* and *M. plumbeus* and also towards an enhanced production of third most demanded industrially important cellulase. However, the suitability of the enzymes for biotechnological applications can be investigated through kinetic characterization of the purified enzymes as thermo-stability is a desired characteristic of an enzyme for its possible use in industry.

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