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Study on the Cellulase Activity of Fungi by Solid State Fermentation Using Cellulosic Wastes

Komal U. Chaudhary[1], Anjali Padhiar[2]

[1]Department of Biotechnology, KSV University, Gandhinagar, India

[2]Department of Biotechnology, KSV University, Gandhinagar, India

Abstract: ***The present study aims to use of fungi for the degradation of cellulosic wastes like Waste Paper, Cotton ginning, Wheat Bran, Sugarcane bagasse and Cellulose using solid state fermentation for cellulase production. Total 34 isolates were obtained from various sources by the primary screening technique from which 14 isolates were showing higher cellulase activity. Potential isolates were obtained from wood furnishing region and paper industry waste. Different fungal strains were tested to find their ability to produce cellulase, which catalyses the degradation of cellulose, which is a linear psolymer made of glucose subunits linked by 1-4 glyosidic bonds. Selected 14 fungi strains were inoculated on different agriculture waste for the production of cellulase enzyme. Among these, wheat bran gave maximum zone of hydrolysis of carboxy-methyl cellulose and shown higher activities of the cellulase, which were determined by Filter paper assay (FPA) and Carboxy-methly cellulase assay (CMCase). Maximum production of cellulolytic enzymes was found in wheat bran. A4, A5, A7, A8 and A10 produced higher endoglucanase activity 5.36 U/g, 5.32 U/g, 7.53 U/g, 6.53 U/g, 5.26 U/g respectively as well as higher FPase activity 2.45 U/g, 0.95 U/g, 1.86 U/g, 1.98 U/g, 1.06 U/g respectively.***

Keywords: Fungi, Solid stat fermentation, FPase, CMCase, Cellulosic wastes

1. Introduction

Agricultural and industrial wastes are among the major causes of environmental pollution. Their conversion into useful products may ameliorate the problems they causes. These wastes which include mainly leaves, straws, cereals, corncobs etc., in many countries, these materials are generally used as animal feeds. Cotton waste and discarded paper also used as a cellulosic waste. A huge quantity of cotton gin waste is generated in cotton mills. Globally, India is the second largest cotton producing country which produces undesired cotton gin waste (Sahu S. and Pramanik K, 2015). A huge amount of these materials are left on farmlands to be decomposed by microorganisms such as Bacteria and fungi (Jadhav*et al*., 2013).

Cellulose is commonly degraded by an enzyme called cellulase. Fungi are the main cellulase producing microorganisms, though a few bacteria and actinomycetes have also been reported to yield cellulase activity. For complete hydrolysis of cellulose to glucose, cellulase systems must contain endo-1, 4-glucan (1, 4 - D-glucanohydrolase' EC 3.2.1.4), exo -1, 4 -glucan (1, 4 - D - cellobiohydrolase' EC 3.2.1.9) and - glucosidase (- D - glucohydrolase' EC 3.2.1.91) or cellobiase. Thus the hydrolysis of cellulose is completed by the synergistic action of endo- and exoglucanases(Fadel*et al*., 2000).

The enzymes of industrial importance have traditionally been produced in submerged fermentation (SmF) because of the ease of handling and good control of environmental factors such as temperature, aeration, agitation and pH (Singh *et al*., 2007). However, solid state fermentation (SSF) techniques are better adapted to enhance the yield, which reduces the cost of enzyme production because of the ability of filamentous fungi to grow well on solid substrates (Ghildyal*et al*., 1985; Pandey, 1992; Hui *et al*., 2010). The other advantages of SSF include maximum productivity; ease of technique; low capital investment, low energy requirement and less water output, better product recovery and lack of foam build up and reported to be most appropriate process for developing countries (Zeng and Chen, 2009 and Souza and Magalhaes, 2010).

Cellulases have enormous potential in industries and are used in food, beverages, textile, laundry, paper, waste management, medical/pharmaceutical industry, protoplast production, genetic engineering and pollution treatment (Jahangeer *et al*., 2005) and pulp industries etc.

2. Materials and Methods

2.1 Isolation and Screening of Cellulose-Degrading Fungi

Samples including paper industry waste, municipal waste, sugarcane farm, garden, and wood furnishing were collected from different site of Gandhinagar and Kadi in sterile polythene bags. Isolation and Screening of cellulolytic fungal species was done on Carboxyl agar medium. The presence of cellulase was tested using the media proposed by Hart *et al.,* (2002). The individual microorganism grown on basal salt media supplemented with 1% Carboxy methylcellulose (CMC) used as carbon source. The pure cultures were inoculated in the center with almost equal amounts and incubated at 37ºC until substantial growth was recorded. The petri plates were kept at 50ºC for 30 min. Plates were flooded with 1% Iodine solution and allowed it to stand for 5-10 minutes. The clear zone was observed around the colony due to cellulose hydrolysis.

2.2 Characterization and Identification of fungal Isolates

Fungal colonies were isolated form different samples for cellulase producing microorganisms by serial dilution method. 100µl of samples diluted up to 10^{-5} dilutions, were spread on respective solidified PDA plates. The inoculated

petri plates were incubated at 30°C for 48 hours. The isolates were inoculated on sterile PDA plates by four flame method and incubated at 30°C for 48 hours in order to obtain pure cultures. Colony characteristics, morphological characteristics and microscopic examinations of the various isolates were determined and the reproductive and vegetative structures were also studied (Devenathan*et al*. 2007).

2.3 Substrate preparation for enzyme production

Five different types of Cellulosic waste based medium was used for production of cellulolytic enzymes. The Cellulose, Wheat bran, Sugarcane bagasse, Cotton waste and discarded paper were used as substrate for the production of cellulase. Cotton Waste and discarded paper were pretreated with 3% H_2SO_4. This 2.0 gm of substrates were supplemented to the basal medium. The composition of Basel medium includes KH_2PO_4: 2gm, $(NH_4)_2PO_4$: 1.4 gm, Urea: 0.3 gm, $CaCl_2.2H_2O$: 0.3 gm, $MgSO_4$: 0.3 gm, Peptone: 1.0 gm, $FeSO_4.7H_2O$: 5.0 mg, $MnSO_4.7H_2O$: 1.6 mg, $ZnSO_4.7H_2O$: 1.4 mg, $CoCl_2.6H_2O$: 2 mg, Tween 80: 0.2% (v/v), D/W 1000 ml and pH: 5.5. Mandel and Resse (1957)

2.4 Experimental design of solid state fermentation (SSF)

Inoculum preparation was carried out using Sabouraud dextrose broth (150 ml), prepared in 500ml Erlenmeyer flasks and autoclaved at 15 lbs for 15 min. The medium were inoculated with isolated fungus of 3 mycelial disc (7 mm diameter) punched out from the edges of its 8 days old colonies from Petri plates. The flasks were incubated at 30 ± 2 °C for 72 hrs.

The fungus was inoculated into substrates flasks for enzyme production. Enzymes were extracted from substrate flask by addition of 5 ml of cold 0.05 M acetate buffer (pH 4.8). The homogenate was filtered through muslin cloth and the filtrate was centrifuged at 5000 rpm at 4°C for 15min. The supernatant was analyzed for carboxyl methyl cellulase (CMCase) and filter paper activity (FPase).

2.5 Enzyme Assay

2.5.1 Filter paper assay (FPase)

Filter paper activity of the culture filtrates was determined according to the method of Mendels and Weber (1969). Whatman filter paper strips containing 50 mg (1cm X 6cm) weight was inoculated in 1 ml of 0.05 M sodium acetate buffer (pH 4.8) and kept at 50 °C in a water bath. Suitable aliquots of enzyme source were added to the above mixture and incubated for 60 minutes at 50°C. After incubation, the liberated reducing sugar was estimated by the addition of 3, 5-dinitrosalicylic acid (DNS). The color developed in tubes were measured at 540 nm using spectrophotometer. Appropriate control without enzyme was simultaneously run. Activity of cellulase was expressed in filter paper units. One filter paper unit (FPU) was defined as the amount of enzyme releasing 1 mole of reducing sugar from filter paper /ml /min.

2.5.2 Endoglucanases enzyme assay (CMCase):

Endoglucanase activity of Carboxy methyl cellulase (CMCase) was carried out as described by Ghosh (1987). The reaction mixture of 0.5 ml enzyme, 0.5 ml of 1% carboxy methyl cellulose in 0.05 sodium acetate buffer (pH 5.0) and 1ml DNSA were incubated at 50 °C in a water bath for 20 minute. The release of glucose due to the enzyme activity was assayed by 3, 5 Dinitro salicylic acid measured at 540 nm using spectrophotometer (Miller, 1959).

3. Results and Discussion

In this investigation, we have navigated the isolation of cellulolytic fungi from paper industry waste, municipal waste, sugarcane farm, garden, and wood furnishing. Samples were collected from different site of Gandhinagar and Kadi. Total 34 different isolates were obtained by the primary screening technique from which 14 isolates were showing higher cellulase activity. Potential isolates were obtained from wood furnishing region and paper industry waste shown- in table no 1. In the Primary screening, cellulase activity was determine by ditch method as well as spot inoculation method. Cellulase activity was measured after 72 hrs of incubation.

The fungal strains were tested for their cellulolytic ability on plate clearing assay by using Carboxy methyl cellulose and iodine. Formation of clear zone diameter on CMC agar were measured in mm. 14 out of 34 isolates were selected based on cellulose hydrolysis ranging from 5 mm to 15mm diameter of clear zone shown in Figure 1. Morphological, Microscopic observation and cellulose hydrolysis zone were shown in table 1.

Table 1: Colony morphology and microscopic observation of selected culture

Isolated Culture	*Isolation from*	*Colony Morphology*	*Cellulase Zone (mm)*
A1	Soil	White to green, dark green spore	7
A2	Cotton waste	White to green, dark green spore	14
A3	Cotton waste	Green to black	12
A4	Vegetable waste	Green to Black, Black mycelium	21
A5	Wood	White to Green, Green spore	18
A6	Cotton waste	White to green, dark green spore	13
A7	Soil	Green to Black	21
A8	Plant	White mycelium, Greyish green	12
A9	Soil	White to green, dark green spore	12
A10	Paper waste	White to Green	18
A11	Soil	White to light Green, light and dark green mycelium	25
A12	Plant	White to light Green	22
A13	Plant	White to green, dark green spore	13
A14	Plant	White to light green, light green mycelium	8

Experiment was design using solid state fermentation for optimum cellulase production. Among five different cellulosic substrate, four were different lignocellulosic waste (waste paper, cotton, wheat bran sugar cane bagasse) and fifth was pure cellulose. Approx. $1x10^7$ spores of different 14 fungus were inoculated in the solid state substrate flask. The enzyme activity was analysed only after 2nd day of incubation to allow the optimal fungal growth. It was reported earlier that the enzyme production by the fungi started after a lag period of 24 hr or more, and the activities reached to maximal levels within 3-5 days of incubation (Gomes I *et al*., 2006). FPase activity and CMCase activity of each cultures from different substrates were carried out at the interval of 24 hrs at 30°C.

Figure 2: Inoculum for development

Figure 3: Solid State Fermentation for cellulase production

Maximum FPase activity was achieved after 3 days of incubation by utilizing different cellulosic substrate like Wheat bran, Sugarcane bagasse, Cotton waste, discarded Paper and cellulose. But both CMCase and FPase activity was decline after the 5th day of incubation which shown in all graph.

FPase and CMCase activity on Paper waste of all 14 fungal cultures were shown in Figure 4. After 96 hrs.of incubation A5, A7 and A8 cultures gave higher FPase activity 0.986 U/g. 0.713 U/g and 0.586 U/g respectively compare to other fungal cultures. While CMCase activity of A4, A5, A7 and A8 cultures were 1.14 U/g, 3.56 U/g, 3.79 U/g and 4.35 U/g respectively after 96 hrs. Cellulase enzyme production was also studied by Charitha *et al*., (2012) using fungal strain with lignocellulosic bio wastes like sawdust, paper cellulose and maximum enzyme production 3.9 IU was achieved using Paper.

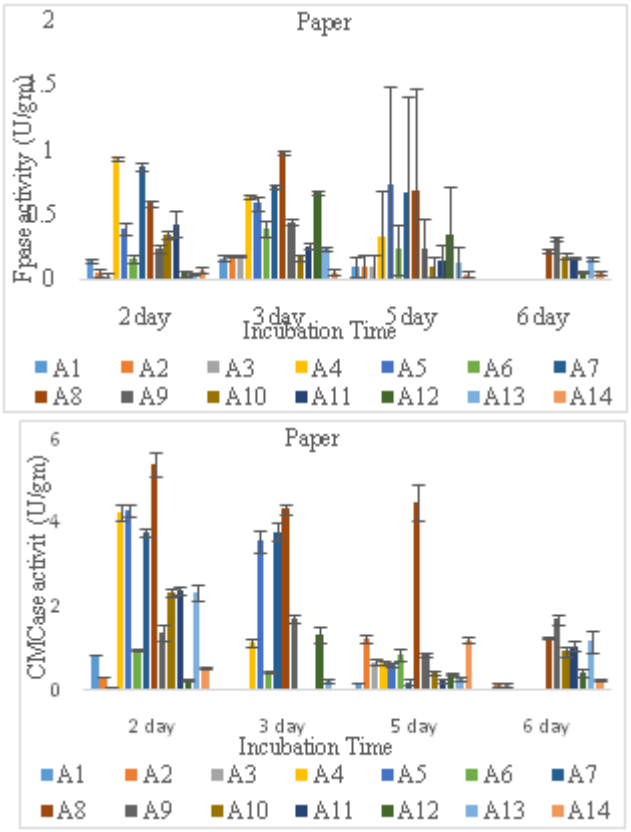
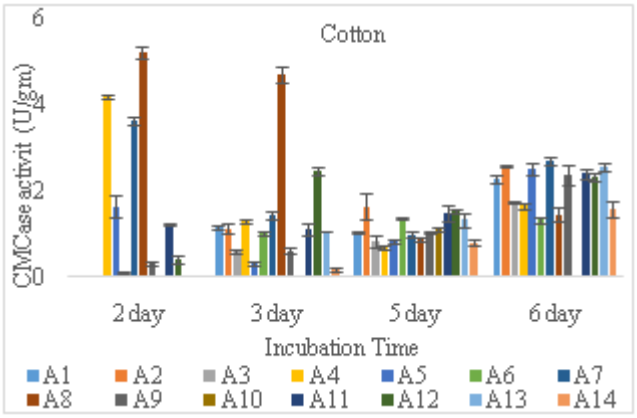


Figure 4: FPase and CMCase activity on Paper

FPase and CMCase activity on Cotton waste of all 14 fungal culture were shown in Figure 5. After 96 hrs. of incubation A4, A5, A7, A8 and A10 cultures gave higher FPase activity 1.22 U/g. 0.94 U/g, 1.0 U/g, 0.73 U/g, and 1.05 U/g respectively compare to other fungal cultures. While CMCase activity of A4, A7 and A8 cultures were 4.21 U/g, 3.64 U/g, and 5.21 U/g respectively after 24 hrs. These shows that cultures can be utilized for degradation of cotton ginning waste which was the major waste of India.



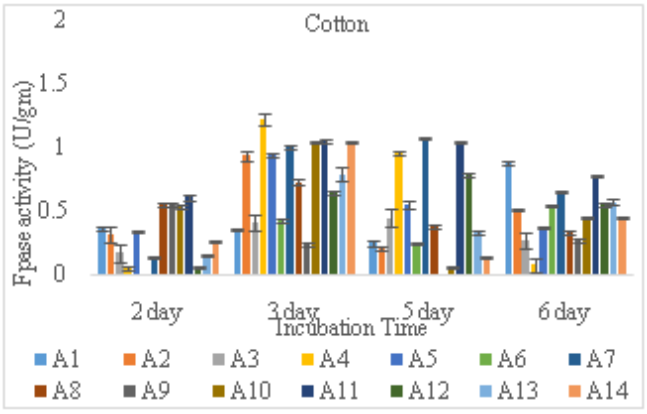


Figure 5: FPase and CMCase activity on Cotton Ginning

FPase and CMCase activity on Wheat Bran of all 14 fungal culture were shown in Figure 6. After 72 hrs. of incubation, A4, A7, A8, A10 and A12 cultures gave higher FPase activity 2.45 U/g. 1.86 U/g, 1.98 U/g, 1.05 U/g, and 1.14 U/g respectively compare to other fungal cultures. While CMCase activity of A4, A7, A8 and A10 cultures were 5.36 U/g, 7.53 U/g, 6.53U/g and 5.26 U/g respectively after 72 hrs. This higher activity indicate that wheat bran can be used as cheapest alternative source for cellulase production.

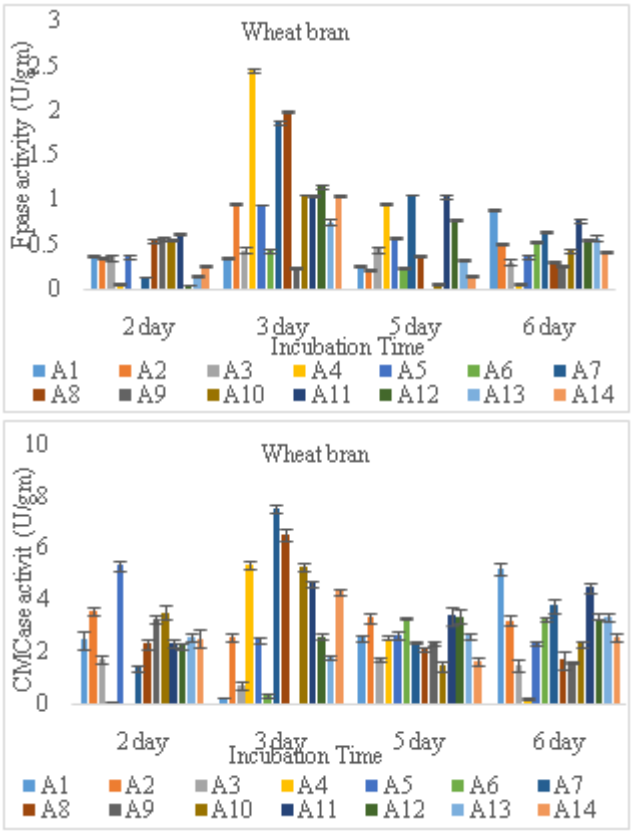


Figure 6: FPase and CMCase activity on Wheat bran

FPase and CMCase activity on Sugarcane of all 14 fungal culture were shown in Figure. 7. After 72 hrs. of incubation A4, A5 and A7 cultures gave higher FPase activity 0.53 U/g. 0.50 U/g and 0.53 U/g respectively and A8 gave 1.35 U/g after 120 hours of incubation compare to other fungal cultures. While CMCase activity of A4, A5, A7, A8 and A10 cultures were 5.59 U/g, 5.2U/g, 5.32 U/g, 5.31 U/g and 4.81 U/g respectively after 72 hrs.

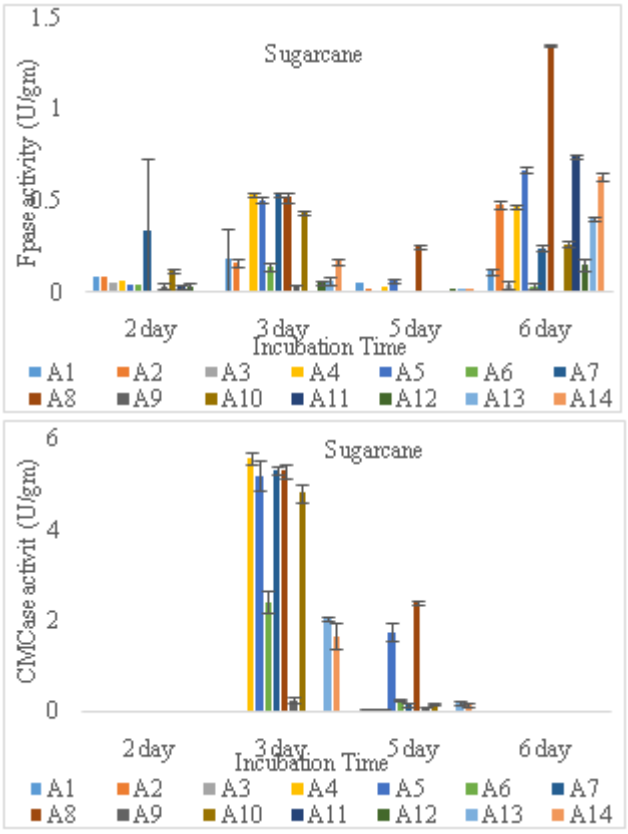
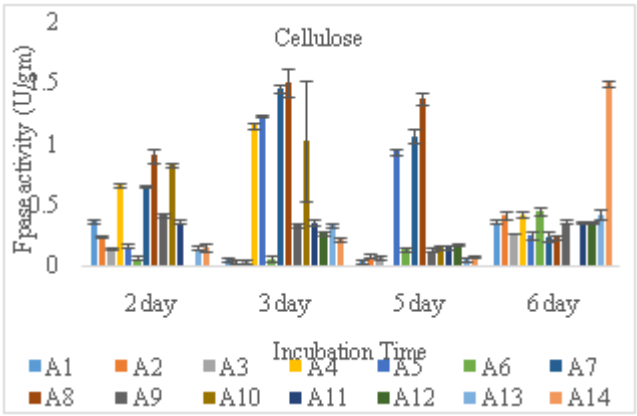


Figure 7: FPase and CMCase activity on Sugarcane bagasse

FPase and CMCase activity on Cellulose of all 14 fungal culture were shown in Figure 8. After 72 hrs. of incubation A4, A5, A7, A8 and A10 cultures gave higher FPase activity 1.15 U/g. 1.24 U/g, 1.46 U/g, 1.50 U/g and 1.02 U/g respectively and A14 gave 1.5 U/g after 120 hours of incubation compare to other fungal cultures. While CMCase activity of A4, A7, and A8 cultures were 4.61 U/g, 7.47 U/g and 7.56 U/g respectively after 120 hrs. and A5 gave 4.46 U/g after 72 hrs. of incubation.



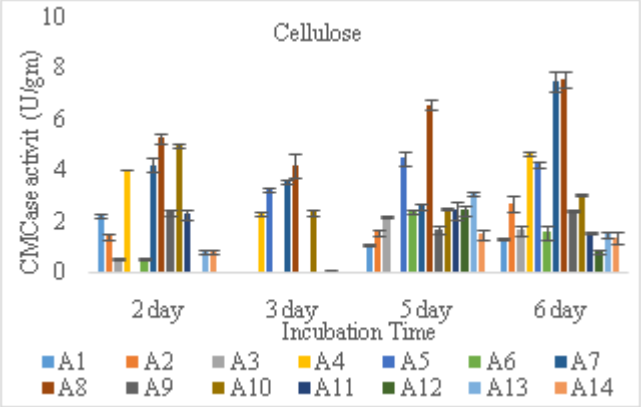


Figure 8: FPase and CMCase activity on Cellulose

Among five different cellulosic substrates waste paper, cotton, wheat bran, sugar cane bagasse and cellulose tested in solid state fermentation (SSF) by different 14 fungi, wheat bran yielded maximum enzyme activity of FPase and endoglucanase after 96 hours of growth. A4, A5, A7, A8 and A10 produced high endoglucanase 5.36 U/g, 5.32 U/g, 7.53 U/g, 6.53 U/g, 5.26 U/g and FPase 2.45 U/g, 0.95 U/g, 1.86 U/g, 1.98 U/g, 1.06 U/g respectively.

Among all 14 selected fungal culture A7 and A8 shows high FPase and CMCase activity in all cellulosic substrates which is shown in Figure 9. From the present study it was conclude that, among five cellulosic substrates Wheat Bran gave higher FPase and cellulase activity by using A8 culture yielding 1.98U/g and 7.53 U/g during 3 day incubation. Haque *et al*., (1989, 1990, and 1991) and Subhosh Chandra *et al*., 2007 also studied solid substrate fermentation by different fungi using different cellulosic biomass and they also found maximum enzymes production using wheat bran.

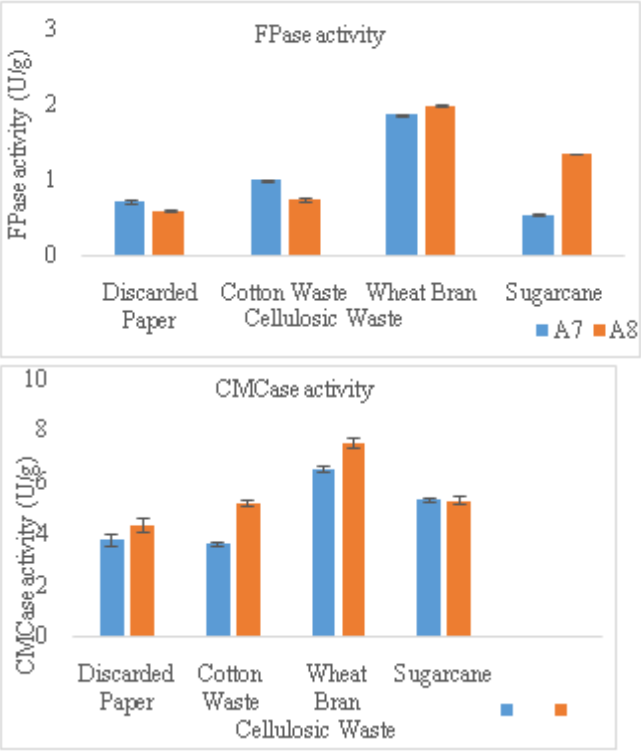


Figure 9: FPase and CMCase activity by A7 and A8on different cellulosic waste

4.Conclusion

Cellulosic wastes are most abundant renewable resources in the biosphere which have been shown to be used in the production of valuable products by microorganisms specially fungi. Total 34 fungi were isolated from different Agriculture waste. According to clear hydrolysis zone of cellulose, 14 wild type strains have been selected for cellulase producion. Production of cellulases on Cellilosic wastes under Solid state fermentation was studied by different 14 strain of fungal have the potential of converting cellulose in a single step fermentation process. Among different agriculture waste wheat bran was show higher activities of the cellulase were determined by Filter paper assay (FPA) and Carboxy-methly cellulase assay (CMCase) assay. Cellulase production with A7 and A8 were maxium on 3 day of incubation and in presence of substrates (Wheat bran) at 37ºC. These two cultures were producing higher cellulase which can be used for future bioethanol prospects.

5. Future Scope

The isolated microorganism and selected lignocellulosic waste can be used for further bioethanol prospects.

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7. References

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Author Profile

Ms. Komal Chaudhary received the B.Sc degree in Biochemistry from Gujrat University, Ahmedabad. M.Sc. degrees in Biotechnology from KSV University, Gandhinagar. Ph.D continued in Biotechnology from KSV University, Gandhinagar, Gujarat, India. She has an experience in research as JRF for more than 2 years in GSBTM Project. She has also attended national and International conferences.

Dr. Anjali Padhiar, M.Sc., in Food Biotechnology from Sardar Patel University, Ph.D., in Life Sciences from HNGU, is working as Assistant professor in the Department of Biotechnology, KSV University, Gandhinagar, India since 2001. She has 16 years of teaching and research experience, teaching both UG & PG students. She has published more than 7 International research papers in peer reviewed journals. Is a life member of The Association of Microbiologists of India (AMI). Has deliberated her research findings in International conferences and attended various workshops, seminars, guest lectures. She is also Board member of Microbiology, KSV University.