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EFFECT OF DIFFERENT NUTRITIVE SOURCES FOR ENHANCING CELLULASE PRODUCTION IN *ASPERGILLUS TERREUS*

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ABSTRACT
The influence of various carbon sources, nitrogen sources, metal ions, vitamin sources on cellulase activity was analyzed. The effect of dosage of glucose, groundnut meal, wheat bran, mouldy bran and inhibitors on cellulase activity was also determined. The other factors like effect of volume of substrate, v/s volume of culture vessel, storage behavior up to 25°, 25-30° and 40°C was also analyzed. From this, it is observed that glutamic acid, DL Galacturonic acid, Gum acacia, D mannose, Sucrose, CMC significantly increases the growth of *Aspergillus terreus* Cellulase activity is increased by starch, cellulase, Gum tracacanth, D galacturonic acid, Ascorbic acid, ethanolamine and glutamic acid. Out of 11 nitrogen sources tested, Ammonium chloride, Ammonium phosphate, Ammonium oxalate, Ammonium sulfate and gelatin increases the mycelial dry weight. Ammonium sulfate, gelatin and organic nitrogen sources gave better cellulase activity. Cu^{++}, Fe^{++}, Zn^{++}, Mo^{++}, Mn^{++}, Ca^{++}, shows insignificant activity. When vitamins are added together shows better cellulase activity and calcium pantothenate shows insignificant activity. 0.05 to 0.5% of groundnut meal increases the cellulase activity. Rice bran and orange peel have been used for good cellulase production. The cellulase highest activity was observed in mouldy bran extract with activated charcoal, $CaCl_2$, $MgCl_2$, benzoic acid,Sodium benzoate shows maximum inhibition of cellulase. When cellulase was stored at 5°C the activity remains constant, even after 12 month.When it is stored at 25-30°C, the activity reduces slowly from 90-40%.

KEY WORDS: *Aspergillus terreus*, CMC, Carbon sources, Nitrogen sources, Groundnut meal.

INTRODUCTION

Cellulase is an abundant renewable biological resources and a low cost energy based on energy content (3-4/G5, Lind *et al*., 2008, Zhang, 2009). The production of biobased products and bioenergy from less costly renewable lignocellulosic materials bring benefits to the local economy, environment and national energy security (Zhang, 2008). Cellulases is a costly enzyme and is important for their commercial use. High cost of cellulases are the main obstacles for commercialization of biomass, biorefinaries because a large amount of cellulases is consumed for biomass saccharification, for example -100g enzymes /gallon of cellulosic ethanol produced (Zhang *et al*., 2006b, Zhu *et al*., 2009), In order to decrease cellulases use, increase volumetric productivity and reduce capital investment, consolidated bioprocessing, (Cbp) has been propose by integrating cellulase production, Cellulase hydrolysis and ethanol fermentation in a single step (Lind *et al*., 2002, 2008). The use of expensive substrate is one of the main problems in cellulase production. Reduction of the cost of the substrate can be done by the modification of cellulolytic material using microorganism that have the ability to produce high activity of cellulase (Kotchoni and Shonucan, 2002). Reduction in the production cost and improvement in the cellulase yield can be achieved using appropriate and low cost carbon and nitrogen sources in the formulation of fermentation medium (Beg *et al*., 2000, Senthilkumar *et al*., 2005). Large volume lignocellulosic materials by the palm oil plantation, rice plantation, Sugarcane plantation, coir industry waste, oil industry waste has not been effectively utilized. It appears to be a viable alternative and a chief source of substrate for cellulase fermentation processes. For example, the generation oil palm empty fruit bunch (OPEFB) obtained after stripping the oil palm fruit from the bunch is about 7.3×10^6 tons annually (Chua, 1991). Since, OPEFB is available in large quantities and has fairly high cellulases content with an average of 50% based on Owan raid basis (Kume *et al*., 1990, Husin *et al*., 1985). It appears to be a potential biomass for Cellulase production like that coir waste it used as a substrate (Soma mirudula, 2011). Like that various cheaper sources like straw, Sugarcane waste, Wheat bran, ground nut cake *etc*., are also used as a substrate. The objective of this study is to investigate the effective medium component and culture condition on cellulase production by *A. terreus* using cheaper carbon sources, Nitrogen sources, Vitamin sources, metal ions and non-metal ions, enhances and inhibitors and various cheap sources like Groundnut meal, Mouldy bran, Wheat bran *etc*., The use of different types and concentration of these elements on the enhancement of cellulase production is investigated and presented. Subsequently the effect of culture condition like volume of the substrate, volume of the enzyme effect of temperature, and stability of cellulase enzyme was also analyzed.

MATERIALS & METHODS

1. Effect of addition of different carbon sources on cellulase activity was analysed according to the method of B. Annadurai and D. B. Motlag (1987).
2. Effect of dosage of glucose on Cellulase activity was carried out by adopting the method of Annadurai *et al*. (2001).
3. Effect of Nitrogen sources on cellulase production was done according to the method of Annadurai *et al.* (2004).
4. Estimation of protein was carried out by the method of Lowry *et al*. (1951).
5. Estimation of cellulase was done according to the method of Ghosh 1987.
6. Influence of the vitamins on Cellulase activity was adopted by the method of Annadurai (2000).
7. The influence of groundnut meal on Cellulase activity was carried out according to the method of Annadurai *et al*. (2004).
8. Effect of different combinations of wheat bran on cellulase activity in culture filtrate was studied according to the method of Annadurai *et al.* (2004)

RESULTS & DISCUSSION

The Effect of various carbon sources on mycelial dry weight, Protein content and cellulase activity is vividly presented in Fig1. Among different carbon tested for effect on cellulase activity the mycelial growth significantly increase by glutamic acid, DL Galacturonic acid, Gum acacia, D. Mannose, Sucrose, Ascorbic acid (P<0.001), Cellulase and DL-Galacturonic acid (P<0.02), Carboxy methyl cellulase (P<0.05) and Gum tracacanth (P<0.02). Other carbon sources do not influence the growth of *A. terreus*.

FIGURE 1. Effect of Various Carbon Sources on Mycelial dry Weight determination

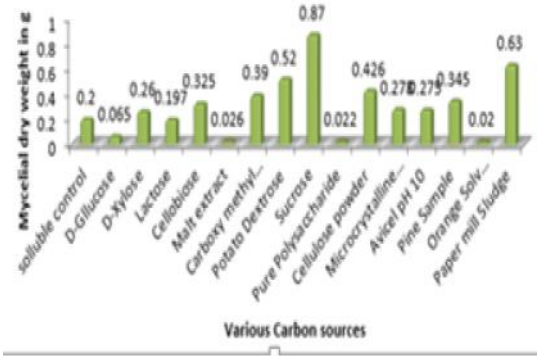
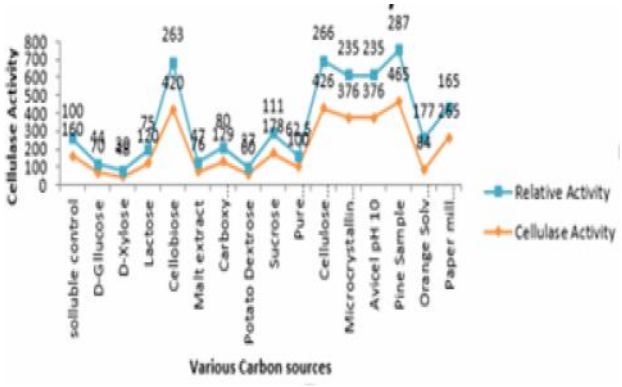
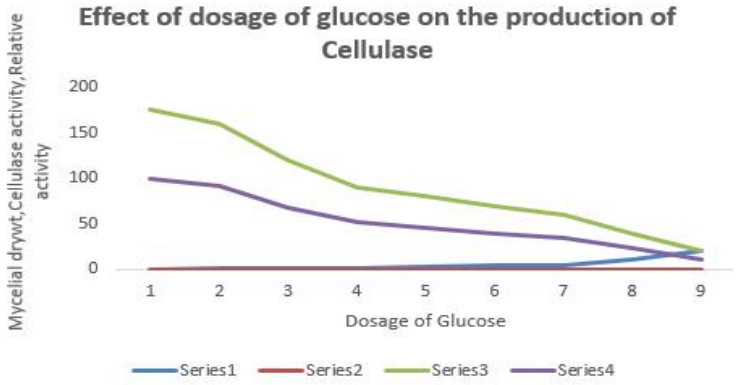


FIGURE 2. Effect of Various Carbon Sources on Cellulase Activity





Cellulase activity is increased by starch, cellulase, gum tracacanth, D galacturonic acid, Ascorbic acid, ethanolamine and glutamic acid (P<0.001). Other carbon sources are ineffective. Mandel and Reese (1957) reported maximum yield of cellulase at 1% concentration with different carbon sources in *Trichoderma viride*. Sucrose was best effective carbon source for cellulase production (13.32 U/ml), followed by glucose (5.16 U/ml) and lactose (4.10 U/ml). Protein content is increased by D-Arabinose, D-Fructose, Lactose, Maltose, starch, Pectin, Gum tracacanth, DLGalacturonic acid, Ascorbic acid, Sorbose and Ethanolamine (P<0.001). Other carbon sources are ineffective. When glucose, fructose, maltose, sucrose, lactose, galactose, arabinose and starch which were separately added to the culture medium for maximum CMCase production. The results in Fig.1 indicates that the maximal cellulase activity (260 U/ml) was observed when lactose used as carbon source, and the lower CM Case production was recorded when glucose, galactose or starch were used as carbon sources, this result is agreed with Sherief *et al*., 2010; Solomon *et al*., 1997; Lee *et al*., 2010 Ahmed, Bashir, Saleem, Saadia, and Jamil (2009) where he found that carboxymethyl cellulase (CMC) induced cellulase production by *T.harzianum* whereas glucose repressed the synthesis of cellulase. This might be due to the lactose induce the enzyme activity, or may increasing

the penetration rate of lactose through the cell membrane Miyamoto, Ooi, and Kinoshita, 2000).

Fig 2 presents effect of various doses of glucose concentration on cellulase activity. From this it is understood that in 0.5% the mycelia dry weight was significant ($p<0.001$). Out of various concentration of glucose even single concentration has not increases the cellulase activity. All concentration and not significant. The effect of glucose on Cellulase activity is shown in Fig 2. It indicates that it has an effect of mycelial growth significantly at 0.1 % ($P<0.001$) level. Out of various concentration of glucose not even a single concentration has increases the endoglucanase activity. Infact all concentrations are not significant. Although 0.1g and 0.3g of glucose does not ($P<0.002$) but 1.0, 2.0 and 4.0g of glucose concentration increased the Protein content ($P<0.001$).

Effect of different Nitrogen sources

To detect the appropriate nitrogen source for cellulase production by *A. terreus*, the culture medium was supplemented with five inorganic (ammonium sulfate, ammonium nitrate, ammonium chloride, sodium nitrite and ammonium dihydrogen phosphate) and four organic (urea, yeast extract, beef extract and peptone) nitrogen sources. The nitrogen sources (2.5g/l) used were separately added to the culture medium for maximum CMCase production (Fig 3), it shows the influence of nitrogen sources on Cellulase. Out of 11 nitrogen sources tested, Ammonium chloride, Ammonium phosphate, ammonium oxalate, Ammonium sulphate, Gelatin, groundnut flour and peptone shows increase mycelial dry weight. When compare to control. The protein activity slightly increases and the influence of ammonium nitrate, ammonium phosphate, Ammonium sulphate Magnesium nitrate, Aspargine, Gelatine, Groundnut and Peptone whereas cellulase activity significantly increases with the influence of Ammonium sulphate, Gelatin and Groundnut.and generally it was observed that organic nitrogen sources gave better CMCase activity than inorganic nitrogen sources. These results agreed with the results of Deswal, Khasa, and Kuhad (2011) who found that urea caused maximum CMCase production and inorganic nitrogen sources did not exhibit any significant effect on increase in enzyme production. On the contrary, Sasi, Ravikumar, and Kani (2012) found that *A. flavus* showed the highest production of cellulase enzyme utilizing Ammonium sulfate as nitrogen source than yeast extract. Kocher *et al.* (2008) reported that the best nitrogen source of *T. harzianum* MTCC8230 when grown on rice straw was $(NH_4)_2SO_4$ (0.5 g L_1) as nitrogen source.

FIGURE 3: Effect of Nitrogen Sources on Mycelial dry weight determination

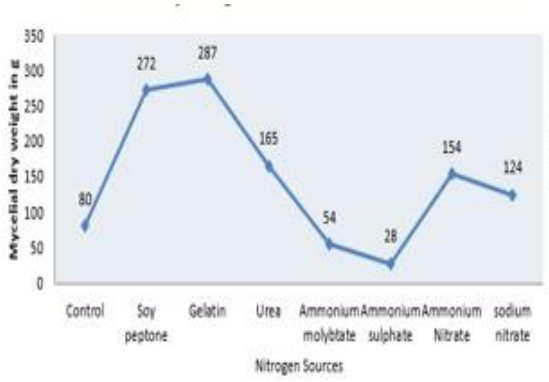


FIGURE 4: Effect of Nitrogen Sources on Cellulase activity

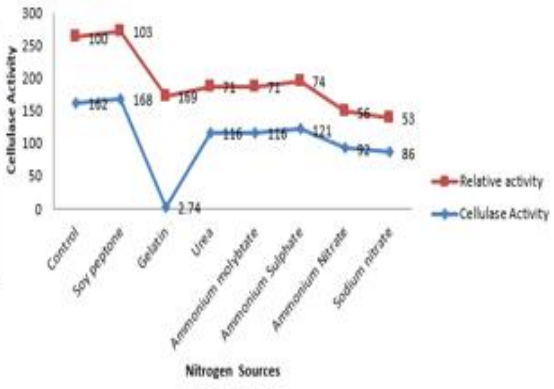


FIGURE 5: Effect of Nitrogen Sources on protein level

FIGURE 6: Effect of Nitrogen dosage on production of Cellulase

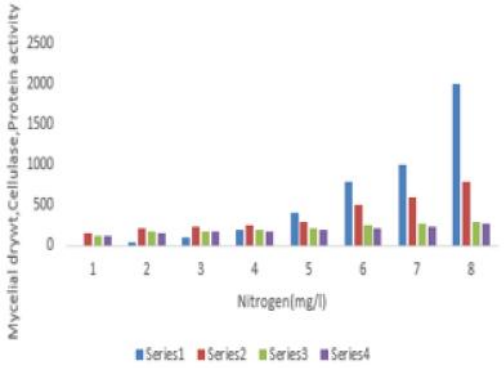


Fig 6 shows the effect of various dosage of nitrogen on Cellulase production almost all dosage from 50ml gram/litre to 200ml gram/litre. Increases the mycelial dry weight RVU results and Cellulase activity.

Effect of Metal ions on Cellulase Activity

Fig 7 & 8 shows the influence of inorganic elements like Cu^{++}, Fe^{++}, Zn^{++}, Mo^{++}, Mn^{++}, Ca^{++} shows insignificant mycelial dry weight protein activity and cellulase activity.

FIGURE 7: Effect of Metal ions on Mycelial dry weight determination

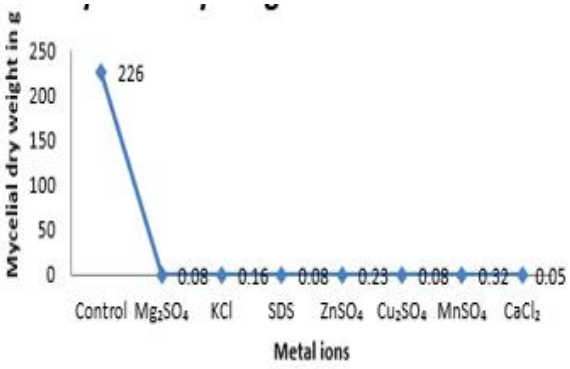
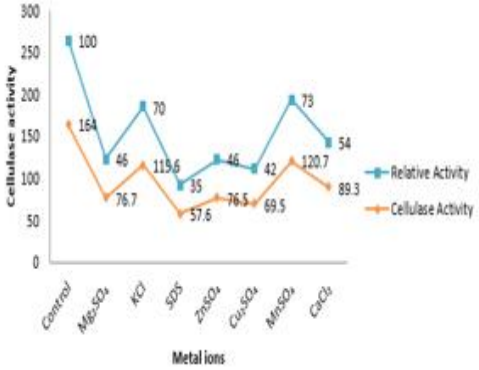


FIGURE 8: Effect of Metal ions on Cellulase activity

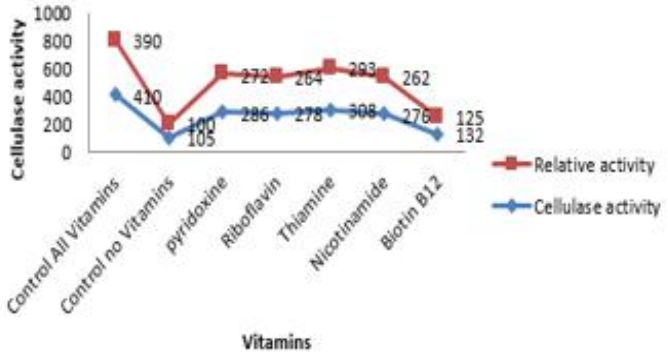


According to Spiridonov and Wilson (1998), all the microorganisms which have an important industrial application can utilize inorganic and organic nitrogen sources. The results depicted in Fig 7 & 8 showed that in *Aspergillus spp*. The maximum enzyme activity (200-300U/ml) was obtained with Mg_2SO_4, $MnSO_4$ and KCl. Sun *et al*., 1999 reported that the addition of inorganic nitrogen source Mg_2SO_4 resulted in increased growth and cellulase production. These findings are more or less similar to Narasimha *et al*., 2006 who reported that in *Aspergillus spp*., Mg_2SO_4 showed maximum cellulase activity.

Effect of Vitamin Sources on Cellulase activity

Figure 9 indicates the effect of B complex on Cellulase enzymes. All vitamins increases the mycelial dry weight ($p<0.001$) but Cellulase activity was very less when compare to the control in which all vitamins are added together. The influence of vitamins on endoglucanase activity is shown in Fig 9. All vitamins of the B complex group increase the growth of mycelium ($P<0.001$). Except calcium pantothenate, other vitamins increase the activity of Cellulase when all the vitamins are added together, maximum Cellulase activity is observed. These results concur with the observations of other researchers (Keshka, M.A.S. 2014; Rajendra B. Kakde and Ashok M. Chavan, 2011; Scott H. W. and Dehority, B. A. 1965).

FIGURE 9: Effect of Vitamin sources on Cellulase activity



Effect of Groundnut on Cellulase activity

In northern and middle Tamilnadu groundnut is one abundant lignocellulosic material that could be used for biorefinery industrialization (Yinbo *et al*., 2006). In this work, pretreated groundnut was used as the model material for enzymatic hydrolysis by cellulase produced by *A.terreus*.

The effect of groundnut meal on endoglucanase activity is indicated in Figure 10, 11 & 12. All concentration of groundnut meal from 0.05% to 0.5% increases the mycelial growth whereas endoglucanase activity is not significant. Protein content significantly increased by 0.2% yo 0.5% at 0.1% level.

FIGURE10: Effect of Groundnut meal on Mycelial dry weight determination

FIGURE 11: Effect of Groundnut meal on cellulose activity

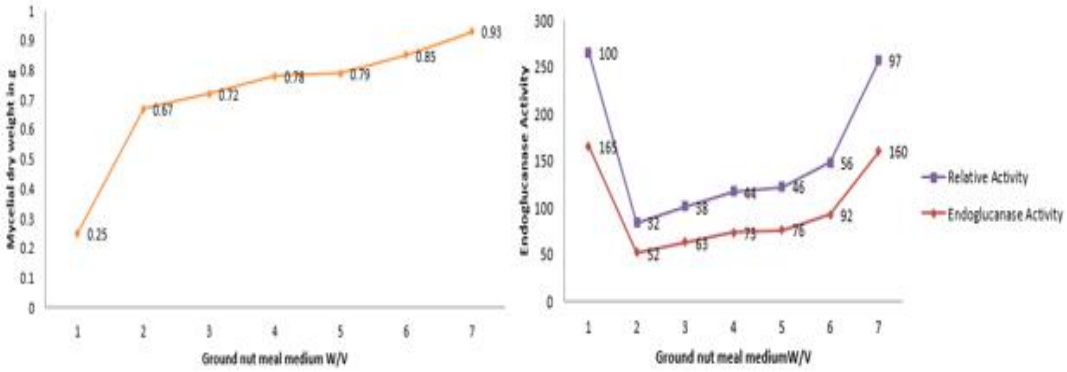
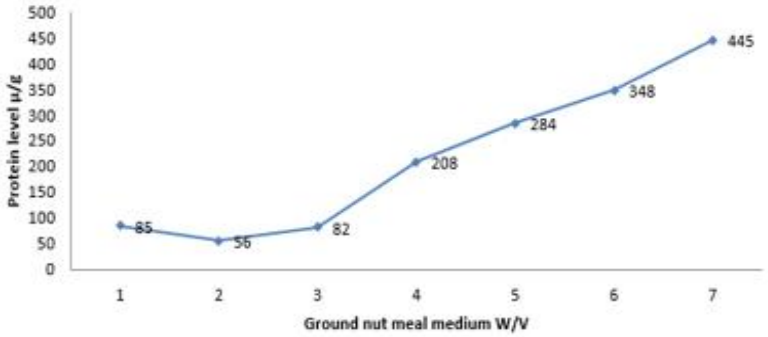


FIGURE12: Effect of Groundnut meal on protein level



Effect of wheat bran on endoglucanase production by *A.terraeus*

The effect of Mouldy bran extract on Cellulase enzyme production is observed in Fig 14. Cellulase activity is fairly more when compared to other enzyme production. Rice bran and orange peel have been used in production of cellulase. Rice bran and orange peel supported the production of cellulase by *A. niger*. The results highlight the potentials of the substrates as possible raw materials for cellulase production using *A.niger*, with rice bran being a more suitable substrate because of its ability to support production of more cellulase activity than the other substrate used.The treatment of Mouldy bran extract with activated charcoal and raw starch on Cellulase is presented in Fig 15.The results indicates highest activity was observed in Mouldy bran extract with activated charcoal and raw starch on Cellulase activity, Protein content and Relative activity.

FIGURE 13: Effect of Wheat bran on Cellulase Activity

FIGURE 14: Enzyme makeup of CF and Mouldy bran extract

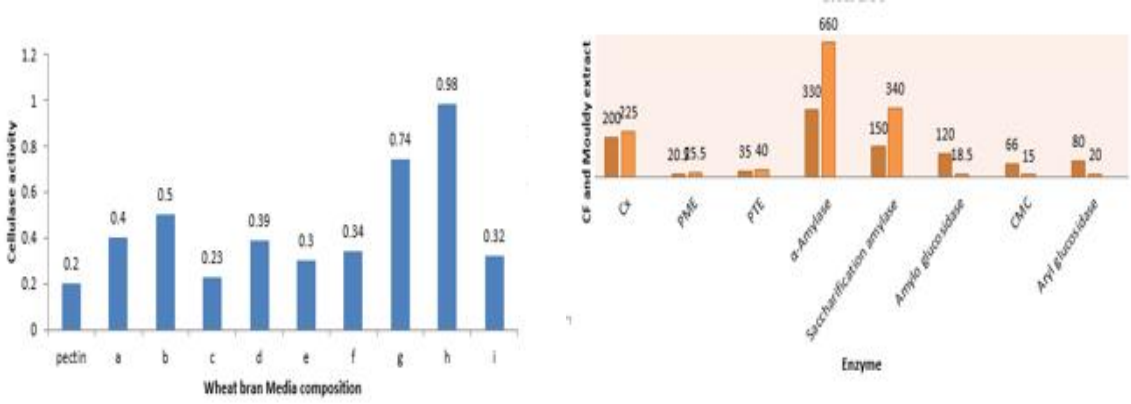


FIGURE 15: Treatment of Mouldy bran extract with activated Charcoal and raw strach

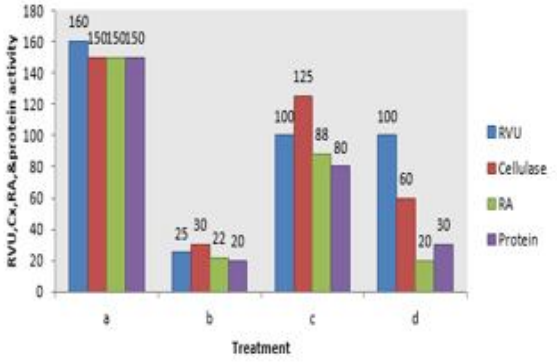


FIGURE 16: Effect of volume of substrate V/S volume of culture vessel on cellulase production

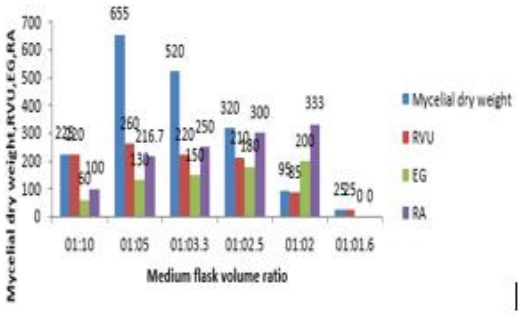


Fig 11 presents the effect of the volume of substrate and volume of culture vessel and growth and enzyme production. The enzyme production is increases with the increase with the volume of the vessel from 25 to 150ml capacity.

Effect of inhibitors on Cellulase activity

Fig 16, 17 & 18. The effect of inhibitors on Cellulase activity is showed. From this it is evident that $CaCl_2$, $MgCl_2$, Benzoic acid, Sodium Benzoate and 5',5'Dithiobis shows maximum inhibition on cellulase activity, Mycelial dry weight and Protein content on all type of concentration

FIGURE 16: Effect of inhibitors ($1x10^{5}$M Conc) on Cellulase activity

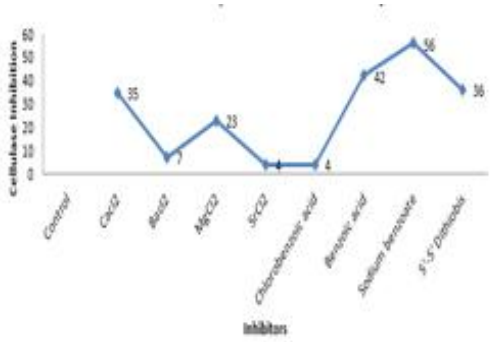


FIGURE 17: Effect of inhibitors ($1x10^{10}$M conc) on Cellulase activity

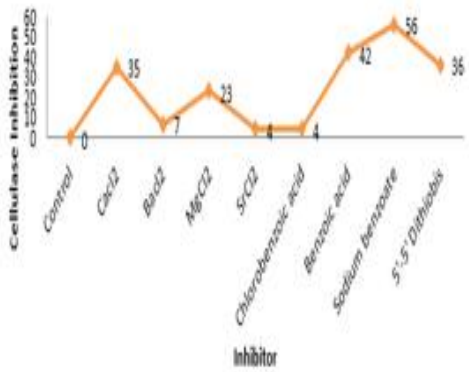
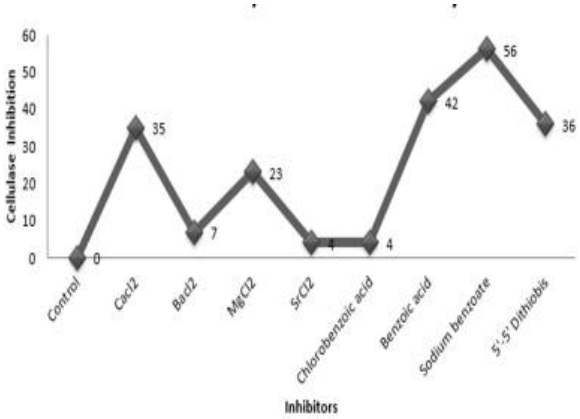


FIGURE 18: Effect of inhibitors ($1x10^{20}$M conc) on cellulose activity



Time course profile (Fig.18) for the stability of cellulases was evaluated between 1 to 6 months (*i.e.* 180 days), and at different temperatures an interval of 24 h. Figure 18. Time course of the cellulases production by *Aspergillus niger* using 5% pawpaw peels as single carbon source Cellulase activities were expressed in terms of percentage relative activity for the incubation periods as (24, 48, 72, 96 and 120 h) 61.69 %, 76.62%, 100%, 85.70%, 76.62%,

respectively. Enzyme activity increased with increase in fermentation period and reached maximum (0.96 µmol/ min/mL) at 72 h of fermentation. Further increase in the incubation period beyond the optimum time (72h) resulted in the decreased production of cellulases. The decrease in the percentage relative activity of *A. niger* after 72h of incubation might be due to the depletion of nutrients and accumulation of other by products like proteases in the fermentation medium initiating autolysis of cells (Gautam *et al*., 2010; Olaniyi *et al*., 2013).

Effect of Storage behaviour of Cellulase activity

Fig.19 shows the storage behavior of Cellulase enzyme, when Cellulase was stored at 5ºC upto 75% remains even after 12 months. When it is stored at 25 to 30ºC the activity reduces slowly from 90% to 40%.

FIGURE 19: Storage behavior of cellulase enzyme conc (0-25^{o}C)

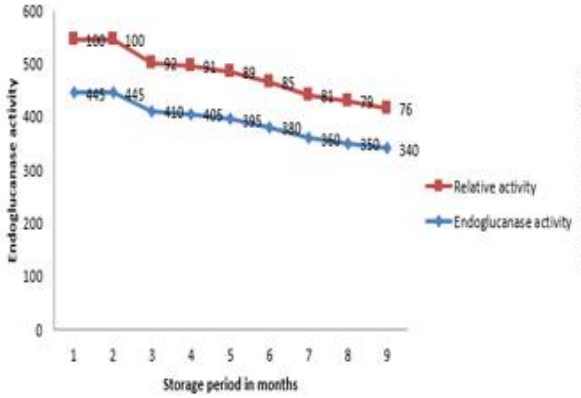


FIGURE 20: Stroage behavior of cellulase enzyme conc (25-30^{o}C)

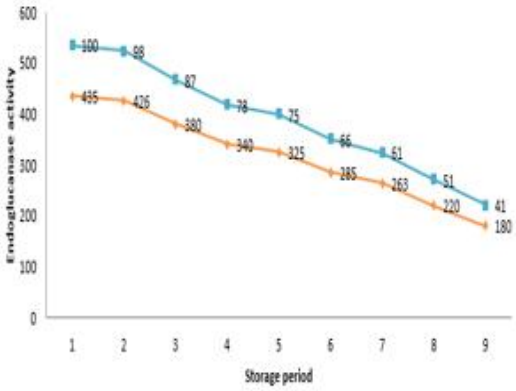
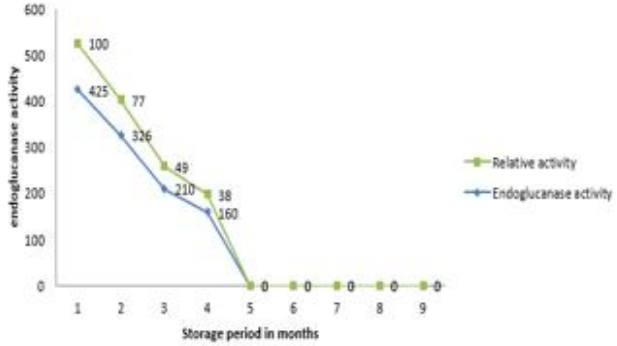


FIGURE 21: Storage behavior of cellulose enzyme conc (40^{0}C)



Values given are the mean (X) of residual activity of EG after storage at different temperatures ±SD d.f. = degrees of freedom = n-1 observations.

One unit of Cellulase activity was defined as the amount of enzyme releasing one µ mole of reducing sugar /ml /h.

Relative activity is expressed as percentage by taking initial activity as cent percent.

The medium, in which the microorganism grows, determines the types and quantities of cell wall degrading enzymes. Living organisms are known to utilize 40 elements among which carbon 912. As a component of both structural and functional cell constituents, carbon accounts for fifty percent of the total mycelial dry weight. All important components of the cell wall like cellulase, chitin and pectic substances contain carbon in varying forms and concentrations. The results of various carbon sources shown in Fig 3 suggest that starch, cellulase, gum tracacanth, DLgalacturonic acid, Ascorbic acid and glutamic acid are the sources of carbon which influence the increase of growth and endoglucanase. This catabolic repression of sugars was reported by (Hussain and Kelman, 1959; Mussel and Stransy, 1972; Wood, 1960; Zucker and Herkin, 1971 17, 24, 38, 39) the activity of endoglucanase. This effect, of glucose shown in Figure 4 also supports this view. Fungi requires vitamins for the production of Enzymes which are involved in various metabolic activities. Some fungi secrete vitamins by themselves while others require it from external sources. The results of the effect of various vitamins on cellulase activity and mycelial growth are presented in Figure 5. It suggests that when all vitamins are supplemented together there is luxuriant growth of the fungus and Cellulase production. When one vitamin is omitted from the medium, it is observed that except calcium pantothenate all other vitamins have no effect on the mycelial growth and cellulase activity when compared to the control. The precise role of vitamins in enzyme production is still a matter of controversy. Data presented in Fig 10 reveals the effect of groundnut meal on cellulase activity. It indicates

that there is a decrease in cellulase activity when the concentration of groundnut meal increases. The effects of various carbon sources like starch, cellulase, gum arabic, glutamic acid along with vitamins are favorable for the growth of *A.terreus* and Cellulase production.

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