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Production, Purification and Characterization of Exoglucanase by Aspergillus Sp. Isolated from Mirpur Azad Kashmir

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ABSTRACT

Every organism in this world interacts with the biological environment and also with a biological environment. This interaction depends upon the physical and physiological properties of an organism under which it is restrained to a particular habitat. In that particular habitat an organism not only interacts with the other population of the same species but also with the other species in terms of reproduction, competition, predation etc. In the present study, the main concern was to understand or probe those ideal conditions under which an organism thrive and shows its maximum potential. In biological studies it is always an advantage for the researcher to have a better understanding of organism's life cycle and its optimized conditions. In our current study, different parameters were used to understand the maximum productivity of Aspergillus niger. The optimum growth of Aspergillus niger was observed on different temperatures, varying pH levels, size of inoculum and also the different concentration of carbon nitrogen sources. After wards the growth was correlated by the enzyme (exoglucanase) it produced. Obvious reason behind this was that at optimum growth the enzyme produced was also optimum. The enzyme exoglucanase was taken under observation because of high economic value of fungi in our daily life not only their uses in our industries but also because of its notorious nature in deteriorating our crops, lumber and human and plant diseases. There after enzyme was further purified and its activity was observed by using different parameters so that it's characterization could be done. Maximum activity of exoglucanase was observed at 80 percent moisture level (2.26 IU/mL/min, 6.0 pH (8.19 IU/mL/min), 37 ^{0}C growth temperature (3.15 IU/mL/min) and 1 ml inoculum size (1.266 IU/mL/min). Addition of 0.3 percent fructose as a carbon source and glucose gave maximum activity (3.10 IU/mL/min).The exoglucanase produced by Aspergillus niger under optimum conditions was partially purified using ammonium sulphate precipitation method. The maximum exoglucanase (3.46 IU/mL/min) was obtained by adding 0.2 percent ammonium sulphate. Partially purified enzyme was further purified by gel filtration chromatography using 5 percent silica gel. The maximum exoglucanase activity (1.604 IU/mL/min) was observed in elution number 23. There was 1.106-fold purification observed after ammonium sulphate purification and 1.066 fold after gel filtration chromatography.

Keywords: *Aspergillus niger, exoglucanase activity, enzyme optimization, fungal biotechnology*

INTRODUCTION

Lignocellulose is the most abundant carbohydrate on earth and easily available in most of the cases in the form of waste material such as wheat straw, sugar cane sludge *etc*. it is also being consider as a renewable source in this respect that fermentable sugars can also be obtain. As a waste material, it is produced in the pulp industry, forestry, agricultural practices and also in forestry. One of the major aspect of the lignocellulosic material obtained from different industries as the waste can be used in the production of various valuable products such as nutrients, biofuel, animal feed by a simple enzymatic action. (Saleem *et al.*, 2008: Han and Chan, 2010). It has been estimated that there is total production of fifteen thousand billion tons of cellulose and lignocellulosic products all over world per year (Rajeev *et al.*, 2005). With the courtesy of cellulosic degrading enzyme, which provides opportunity of obtaining various valuable products from this renewable resource (Ahmed *et al*., 2009). By the action of these hydrolytic enzymes various important chemicals can be obtained like methanol, ethanol, and other types of fuels. (Carere *et al*., 2008: Dashban *et al*., 2009). There is need to focus on these types of alternative fuels if to combat the fuel shortage problem in the near future. These biofuels are not only cost effective but also source of elimination of hazardous elements which are produced from the burning of customized fuels, which are not only the source of pollution but an environmental threat of global warming. As a biopolymer, it has linear chain structure of D- Glucose linked by β (1-4) glycosidic linkage (Jarvis, 2003). The chemical structure of cellulose made it harder to hydrolyse this polysaccharide by normal processes. In spite of these difficulties it is the best source as a substrate which can be used in different biotechnological processes. (Malherbe and Cloete. 2003). Most of the reaction of cellulose hydrolyses is achieved by the help of cellulytic enzymes, some of them and important in them are exo-glucanase, endo-glucanase and β-glucosidase (Bhat, 2000)

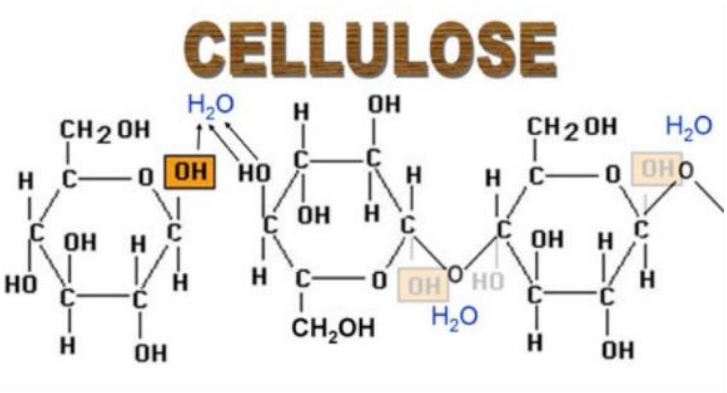


Fig-1 Structure of cellulose

Cellulose is present in the primary cell wall of the plant cell in a criss cross pattren, cellulose is aslo accomanied be hemicellulose and pectin. These microfibrils are linked in the bond by β-1-4-glucan bonds,which cause further stenghening (Trathewey *et al*.,2005). These type of bonding is important in determining the physiological properties of cell wall. These type of bonding need dissociation is necessary by glucanases for the conversion of lignocellulosic material into energy feed stock (Casado *et al*.,2008). Various types of microorganisms produce cellulases, which can be use in the production of glucose or simple sugar. These cellulases in their order of reactions are endoglucanase, exoglucanase, and β-glucosidase, these enzymes can be used at industrial scale for the production of various valuable products (Asad *et al*., 2006: Gao *et al*., 2008: Dashban *et al*., 2009). Cellubiose units can be obtained by degradation of cellulosic fibres from the non-reducing end by the help of exoglucanase. The way exoglucanase act on its substrate by viewing its three-dimensional structure which shows it attacks its substrate by forming

hydrogen bonding (Sinnott, 1997).Cellulases play a pivotal role in the world of industrialization in which these enzymes can be seen in these industries such as baking, textile, processing food, paper industry, for the production of essential oil, baby food jams, juices and for the animal feed lot (Lynd *et al.,* 2002: Shafique *et al*.,2004., Zhou *et al.,* 2008). The importance of these enzymes can be estimated by this that these enzymes are widely used in many industries (Rajoka and Malik.1997). For the maximization of the production of these enzymes different parameters can be used such as percentage of cellulose in different substrates, preparation of media and various other factors that control their growth and production (Gosh,1987).

Enzymologist cut the prizes down of cellulases by using different approaches one of them is the use of lignocellosic material. The lignocellulosic materials used by different enzymologists are bagasse (Guerra *et al*., 2006), wheat straw (Norma and Guillermo, 2005: Yang *et al*., 2006), saw dust (Lo *et al*., 2005), corn cobs, rice straw (Betini *et al.,* 2009).

Efforts are being made in producing cellulase from these types of substrates all around the word because these enzymes are not only cheaper in their cost but also environmental friendly (Bergeron, 1996). The impediment in the use of these enzymes, in the fermentation of cellulosic material is the low activity of native celluloses (Himmel *et al.,* 1999).To obtain better quality of the product, the processes of solid state fermentation (SSF) is used because they are easily available in nature and cost effective too (Sherief *et al*., 2010). Presence of carbon in the substrate is the basis on which they are selected in solid state fermentation(SSF), So carbon percentage provides the base on which substrate is selected (Beg *et al.,* 2000, Senthikumar *et al*,2005).

Sugar cane straw is used for the growth of *Aspergillus niger*, as a substrate. Sugar cane was planted on more than 1000 hectare to produce 67 metric tons of production during the year 2016-17 (crop survey of Pakistan 2016-17). Total area under sugar cane cultivation in Pakistan is 5.5 percent. Only in Punjab 31percent of the total area under cultivation is of sugar cane. Sugarcane's share in GDP of Pakistan is 3.7 percent (Economic survey of Pakistan 2015-16). After extraction of sugar molasses tons of wastes of sugarcane straw is produced every year. Usage of sugarcane straw as a substrate can be use in the production of several enzymes e.g. exoglucanase.It has the percentage of cellulose between 45-55 percent and that of hemicellulose and lignin is 20-25 percent and 18-24 percent respectively (Ahmed *et al*., 2009). These high percentages made the sugar cane straw are suitable for the production of exoglucanases (Csoka *et al.,* 2008).

Aspergillus niger has a saprotrophic mode of nutrition which belongs go genus *Aspergillus*, phylum Ascomycota, class *Eurotiomycetes*, order *Eurotiales* and Family *Trichocomaceae*. In ecosystem, it plays an important role in operating different nutrient cycle such as carbon and nitrogen cycles, while decomposing the organic matter; it secretes different types of enzymes such as exoglucanases. The main study of *Aspergillus niger* here will be in the production of exoglucanse while using sugar cane straw as a substrate. While considering the importance of celluloses we have the following objectives: Cellulose is the most abundantly found polysaccharide in nature. Cellulose is a renewable resource consist of linear chain of glucose joined by β-1-4-glycosidic bond to form long fibre. Cellulose can easily be hydrolysed by different enzymes such as exoglucanse, endoglucanse and β-glucosidases and important is that each enzyme has its own specificity. To understand the mechanism of hydrolysis focus should be on the isolation and characterization of individual enzymes (Jarvis,2003: Gao *et al.,* 2008).

MATERIAL AND METHODS

Substrate

Sugarcane bagasse was used as a source of lignocellulosic substrate in order to grow *Aspergillus niger* for the production of exoglucanase. Sugarcane bagasse was used because it is easily available and it contains high content of cellulose it has the percentage of cellulose between 45-55 percent and that of hemicellulose and lignin is 20-25 percent and 18-24 percent respectively (Ahmed *et al*., 2009).

Preparation of Substrate

Sugarcane bagasse was collected from the surroundings of Jhelum and Dina. Than it was air dried for ten days. In order to remove further moisture the contents of the substrate was oven dried for 24 hours at 60 ºC. The substrate was grounded to powder in department of biotechnology, Mirpur University of Science Mirpur, Azad Kashmir. After making the powder substrate was packed in plastic jars.

Fermentive Organisms

Aspergillus niger were selected from the surroundings of Mirpur Azad Kashmir, from agricultural research farm situated at Hamlet for the production of exocellulase enzyme. *Aspergillus niger* was picked on the loop and it was gently transfer to the medium of potato dextrose agar (PDA) to obtain pure cultures (Sherief *et al.,* 2010). The pure culture was prepared at 37 ºC and was preserved at 4 ºC in refrigerator for further use.

Table 1 Composition of growth media

Sr. No.	Component	Concentration (g)
1.	Potato-starch	10.00
2.	Dextrose	10.00
3.	Agar	10.00
4.	Urea	1.50
5.	KH2PO4	0.04
6.	ZNSO4.7H20	0.005
7.	KCl	0.075
8.	MgSO4.7H2O	0.250
9.	Distilled water	Up to 500 Ml

Preparation of Inoculum

The flask having the inoculum medium (Table 2.1) was adjusted at 5.5 pH with the help of 1M NaOH/HCl and then it was autoclaved at 121 ºC and 15 psi for 15 minutes. After cooling it the spores were transferred with the help of loop from fungal slants and then the flask was placed in shaking incubator at 150 rpm at 37 ºC for 72 hours. Suspension of conidial spores was adjusted at 10^7-10^8conidia/ml, for this haemocytometer (Zarofonetis, 1959).

Process of Fermentation

For the production of exoglucanase the process of solid state fermentation (SSF) was used, by taking sugarcane bagasse as the carbon source. The flask containing 5gm of grinded sugarcane bagasse was moisten with 3.5 ml distilled water having the pH of 5.5; pH was maintained by the help of 1 M HCl/ 1 M NaOH. Autoclave was followed by inoculating the substrate with *Aspergillus niger*. Flasks were prepared in duplicate and then incubated at 37 ºC.

Harvesting of the Sample

After specified day each flask was harvested for the isolation of exoglucanse by contact method (Kleman *et al.*, 1996). After harvesting each flask 50 ml of distilled water was added having the pH of 5.5. The flasks were placed in the shaking incubation at 150 rpm for 30 minutes. The main aim for this is to dissolve the extracellular enzymes in water, if any. The dissolved solution obtained taken from the shaking incubator was filtered and filtrate was collected in each falcon tube (Shafique *et al*., 2006).

Optimization

For optimization, different conditions were given in order to obtain maximum quantity of exoglucanase from *Aspergillus niger*. The conditions which were optimized are as under.

Table 2 Composition of inoculum media for *A.niger*

Sr. No.	Component	Concentration(g)
1	Potato starch	10
2	Dextrose	10
3	Urea	1.50
4	KH_2PO_4	0.04
5	$ZnSO_4.7H_2O$	0.005
6	KCl	0.075
7	$MgSO_4.7H_2O$	0.250
8	D.W	up to 500 ml

Optimization of Fermentation Period

The period of incubation was optimized by maintain the fungal cultures for variable days, in order to know the maximum production of enzymes (table 3). The enzymes were collected after the interval of 24 Hours. and then these enzymes were subjected to assay. The substrate showing maximum enzyme production was isolated and preserved for further studies. For the optimization of fermentative period the fungus was grow in 250 ml flasks in duplicate.

Optimization of Moisture Level

Five different moisture concentrations were used using mineral salt solution, in duplicate. Incubation of fungus was studied at different moisture level at 37^0C for 72 hours. Composition of growth media for the optimization of moisture level is in table 4

Table-3 Optimization of fermentation period

Treatments	T1	T2	T3	T4	T5	T6
Substrate (g)	7	7	7	7	7	7
Temperature C^0	37	37	37	37	37	37
Incubation period (Hrs)	00*	24	48	72	96	120

*Positive control

Table-4 Optimization of moisture level

Treatments	T1	T2	T3	T4	T5	T6
Substrate (g.)	7	7	7	7	7	7
Incubation period(Hrs)	72	72	72	72	72	72
Temperature C^0	37	37	37	37	37	37
Moisture (%)	00*	50	60	70	80	90

*Positive control

Optimization of pH

By optimization of pH it is meant that pH at which fungi produce maximum enzyme and ultimately maximum growth. *Aspergillus niger* produce maximum enzyme at its appropriate pH which is called its optimum ph. For these five different flasks were taken having different pH values have been selected in duplicate. The composition of growth media for the optimization of pH is in table 5

Optimization of Temperature

Five different flasks were taken in duplicate and each flask was adjusted at pH 5.5 placed them for incubation at different temperature levels, in order to find optimum temperature. Composition of growth media for the optimization of temperature is given below in table 6

Inoculum Size Optimization

Inoculum size was adjusted in order to know the best size for the maximum production of exoglucanase which is related to the best growth of fungus. Different volumes 1 ml, 2 ml, 3 ml, 4 ml, 5 ml were taken. Each of the inoculum size was assayed induplicate and incubate at 37 ºC for 72 hrs. Moisture level was

maintain at 80 percent and pH was adjusted at 5.5. The composition of growth media for the optimization of inoculum size is given in table 7

Carbon Source Optimization

Aspergillus niger also consume carbons source compounds and hydrolysed these compounds, which is necessary for its growth. Different compounds having different carbon ratios can be used and having different impact on the growth of *Aspergillus niger* and the production of enzymes.

Glucose Optimization

Different glucose concentrations can be observed in the optimum growth of *Aspergillus niger* and it is obvious that this optimum growth is related to the production of optimum enzyme production. Different concentrations of glucose percentages are given in table 8

Fructose optimization

Different Fructose concentrations can be observed in the optimum growth of *Aspergillus niger* and it is obvious that this optimum growth is related to the production of optimum enzyme production. Different concentrations of fructose used are given in table 9

Nitrogen Source Optimization

By varying the amounts of nitrogen sources can be used in the observation of different growth patterns of *Aspergillus niger* and also the maximum production of enzymes. Three different nitrogenous compounds were used during the current study and its effects were checked these compounds are Ammonium sulphate, Urea and Ammonium phosphate.

Table 5: Optimization of pH

Treatments	T1	T2	T3	T4	T5	T6	T7
Substrate(g)	07	07	07	07	07	07	07
Moisture (%)	80	80	80	80	80	80	80
Temperature(C^0)	37	37	37	37	37	37	37
Incubation period	72	72	72	72	72	72	72
pH	4	4.5	5	5.5	6	6.5	7

Table 6 Optimization of temperature

Treatments	T1	T2	T3	T4	T5
Substrate(g)	07	07	07	07	07
Incubation period (Hrs.)	72	72	72	72	72
Moisture level (percent)	80	80	80	80	80
pH	6	6	6	6	6
Temperature (ºC)	35	37	39	41	43

*Positive control was incubated at room temperature

Table 7 Optimization of size of inoculum

Treatments	T1	T2	T3	T4	T5	T6
Substrate(gm.)	07	O7	07	07	07	07
Incubation period (Hrs)	72	72	72	72	72	72
Moisture (%)	80	80	80	80	80	80
pH	6	6	6	6	6	6
Temperature (C^0)	37	37	37	37	37	37
Inoculum size (mL)	1	2	3	4	5	6

Table 8 Optimization of glucose

Treatments	T1	T2	T3	T4	T5	T6
Substrate (gm.)	07	07	07	07	07	07
Moisture (%)	80	80	80	80	80	80
Inoculum size	2	2	2	2	2	2
Glucose (mM)	0.0*	2	4	6	8	10

*Positive control

Urea optimization

Another organic compound was used for the determination of maximum production of exoglucanase was urea. Different concentrations of urea were used in order to find out the optimum one. Different percentages were used which are of 0.1 percent, 0.2 percent, 0.3 percent, 0.4 percent, 0.5 percent respectively. Optimization of urea is shown in table 10

Table 9 Fructose optimization

Treatments	T1	T2	T3	T4	T5	T6
Incubation period (Hrs)	72	72	72	72	72	72
Moisture (%)	80	80	80	80	80	80
Temperature (C^0)	37	37	37	37	37	37
Inoculum size (mL	2	2	2	2	2	2
Fructose (%)	0.0*	0.1	0.2	0.3	0.4	0.5

*Positive control

Table 10 Urea optimization

Treatments	T1	T2	T3	T4	T5	T6
Moisture (percent)	80	80	80	80	80	80
Inoculum size (Ml)	2	2	2	2	2	2
Fructose (percent)	0.3	0.3	0.3	0.3	0.3	0.3
Urea (percent)	0.0*	0.1	0.2	0.3	0.4	0.5

Carbon-Nitrogen Optimization

Different carbon and nitrogen concentrations were used to find out the optimum combination of carbon and nitrogen for the maximum production of exoglucanase from *Aspergillus niger*. Different carbon sources which were used were glucose, fructose, and sucrose. The weight of each carbon compound was 0.03 gm. The nitrogen compounds which were selected were ammonium sulphate, urea, and ammonium phosphate. The weight used was 0.015 gm. The ratio selected is as follows C: N was 0.5:1 respectively. Table 11 and 12

C = substrate (6 gm) X 0.5/100 = 0.03 gm

N= substrate (6 gm) X 0.35/100 = 0.015 gm

Table- 11 Carbon-nitrogen ratio

Source	Glucose(C_1)	Fructose(C_2)	Sucrose(C_3)
Ammonium sulphate (N_1)	C_1 N_1	C_2 N_1	C_3 N_1
UREA (N_2)	C_1 N_2	C_2 N_2	C_3 N_2
Ammonium phosphate (N_3)	C_1 N_3	C_2 N_3	C_3 N_3

Table-12 Calculation of carbon-nitrogen ratio

Source	Glucose(C_1)gm	Fructose(C_2)gm	Sucrose(C_3)gm
Ammonium sulphate (N_1)gm	0.03+0.015= 0.045	0.03+0.015= 0.045	0.03+0.015= 0.045
UREA (N_2)gm	0.03+0.015= 0.045	0.03+0.015= 0.045	0.03+0.015= 0.045
Ammonium phosphate (N_3)gm	0.03+0.015= 0.045	0.03+0.015= 0.045	0.03+0.015= 0.045

Standard Curve

Sugarcane bagasse was used as a standard because exoglucanase react to cellulose and convert it into disaccharides units (cellobiose). Different concentration of standard was prepared according to the table 14. Standard factor would be calculated by measuring absorbance of each parameter at 540 nm table 13. Standard curve was plotted between conc. Of standards against absorbance at 540 nm.

Enzyme Assay

Avicel, as a Substrate, was selected to react with the exoglucanase (Sherief *et al*., 2010), Avicel is the microcrystalline powder obtained from cellulose, used mostly for filtration chromatography. 1 percent Avicel solution in distilled water was used for enzyme assay.

Table 13 Cellobiose Absorbance at 540 nm

No.	Concentration (µM)	Absorbance at 540
1.	0.0	0.00
2.	0.5	0.288
3.	1.0	0.388
4.	1.5	0.620
5.	2.0	0.975
6.	2.5	1.318
7.	3.0	1.667
8.	3.5	1.782
9.	4.0	1.954

DNS Reagent

The hydrolytic products produced after the action of exoglucanase on enzyme, makes coloured complexed with dinitro salicylic acid. DNS solution was prepared by dissolving 5 g of NaOH, 0.25 g Na_2SO_4, 1g of phenol, 91 g Rohelle salt, 5 g 3,5-dinitrosalicylic acid in distilled water up to 500 ml.

Sodium Citrate Buffer

Buffer which was used to control the pH is sodium citrate. Buffer was prepared by mixing 3.88 g of citric acid and 1.91 g of tri-sodium citrate in distilled water up to 500 ml. by using NaOH AND HCl the pH is adjusted at 4.8.

Enzyme Assay Principle

Hydrolysed products produced by the action of exoglucanase, reacts with DNS to produce coloured complex. By using spectrophotometer at 540 nm concentration of these complexes can be detected. There is a direct relationship between the concentration of enzyme and hydrolysed products.

Enzyme Assay Procedure

For the estimation of enzyme, exoglucanase, 1 mL of 1 percent Avicel (substrate) was taken in a test tube. Buffer was taken to maintain the pH of the mixture by adding sodium citrate, which was adjusted at pH 4.8 (Shafique *et al.*, 2004). Test tubes were placed in the incubator at 37^0C for 30 minutes. After removing the test tubes from the incubator 3 ml DNS (Dinitrosalicyclic acid) was added in each test tube so that the reaction can be stopped and then test tubes were placed in boiling water bath. The digested products which were produced by the action of exoglucanase, now react with DNS, to form complexes. These complexes can be determined by determining the absorbance at 540 nm in spectrophotometer.

Enzyme Activity Calculation

Enzyme activity can be calculated by using the formula:

$$\text{Enzyme activity (IU/ml/min)} = \frac{\text{Absorbance X standard factor X Dilution factor}}{\text{TIME OF INCUBATION (min.)}}$$

$$\text{Standard factor} = \frac{\text{Concentration of standard (µM/mL)}}{\text{Absorbance of standard at 540 nm}}$$

Taking the value from table 2.8

$$\text{Standard factor} = \frac{\text{Conc. Of substrate}}{\text{Absorbance of solution}}$$

$$\text{Standard factor} = \frac{2+4+6+8+10}{9.51} = 3.15$$

Standard factor = 3.15

Time of Incubation = 30 min

Dilution factor= 50

So, enzyme activity of different samples was calculated as follow:

$$\text{Enzyme activity (IU/ml/min)} = \frac{1.75 \text{X } 3.15 \text{ X } 50}{\quad}$$

Estimation of Protein

Estimation of protein was performed in crude enzyme sample according to Bradford reagent.

Preparation of Bradford reagent.

Bradford reagent was prepared by mixing 50 mg comessie brilliant blue R-250, 25ml ethanol (100 percent), 50 ml phosphoric acid (98 percent) and added distilled water to achieve the final volume up to 500 ml.

Bradford protein assay

Bovine serum albumin (BSA) was taken as a standard for the estimation of protein by Bradford protein method. Different concentrations were used (3.10). Each concentration was estimated at spectrophotometer at 540 nm absorbance. The absorbance of enzyme action was also been recorded at the end. The protein concentration was observed from linear regression equation (3.14)

Interpretation of Results

Absorbance, which was taken at 540 nm, will increase with the increase production of exoglucanase enzyme. The products formed by the action of exoglucanase react with the DNS (Dinitrosalicylic acid) and as the consequence complex products formed. Complex compounds have the direct relation with the absorbance i.e. more the compounds formed more will be the absorbance.

EXOGLUCANASE PURIFICATION

Enzyme was purified, which was produced under optimized condition, for further characterization. The methods used are as under;

Ammonium sulphate precipitation

Proteins dissolved in solution become insoluble while dissolving ammonium sulphate. Different concentrations of ammonium sulphate were used e.g.20%, 30%, 40%, 50%, and 60% in 10 ml of crude enzyme. The enzyme purified was focused to find the activity and Bradford assay in order to know the outcome of protein concentration.

Gel Filtration Chromatography

Ammonium sulphate purified enzyme was than subjected to gel filtration chromatography for further purification. 5 percent silica gel column was used for the purification of exoglucanase, dissolve in sodium citrate buffer having pH 4.8. The elution of cellulose of cellulases was maintained at a linear flow rate 30 cm/h (Ahmed *et al*., 2009). The different elution was subjected to enzyme activity assay and biuret assay to detect the concentration of protein. Elution having maximum activity was further used for characterization of different kinetic parameters (Kocher *et al*., 2008).

CHARACERIZATION OF EXOGLUCANASE

Production of enzyme, exoglucanase, from Aspergillus niger can be subjected to purification through gel filtration chromatography and ammonium sulphate for its characterization. Different parameters which were used are as follows:

Optimization of Fermentation Period

The period of incubation was optimized by maintain the fungal cultures for variable days, in order to know the maximum production of enzymes (table 2.3). The enzymes were collected after the interval of 24 Hours and then these enzymes were subjected to assay. The substrate showing maximum enzyme production was isolated and preserved for further studies. For the optimization of fermentative period the fungus was grow in 250 ml flasks in duplicate.

Optimization of Moisture Level

As discussed earlier solid state fermentation required less moisture as compared to liquid state fermentation. Five different moisture concentrations were used using mineral salt solution, in duplicate. Incubation of fungus was studied at different moisture level at 37^0C for 72 hours. Composition of growth media for the optimization of moisture level is in table 2.4

Optimization of pH

By optimization of pH it is meant that pH at which fungi produce maximum enzyme and ultimately maximum growth. *Aspergillus niger* produce maximum enzyme at its appropriate pH which is called its optimum pH. For these five different flasks were taken having different pH values have been selected in duplicate. The composition of growth media for the optimization of pH is in table 2.5

Optimization of Temperature

Five different flasks were taken in duplicate and each flask was adjusted at pH 5.5 placed them for incubation at different temperature levels, in order to find optimum temperature. Composition of growth media for the optimization of temperature is given below in table 6

Inoculum Size Optimization

Inoculum size was adjusted in order to know the best size for the maximum production of exoglucanase which is related to the best growth of fungus. Different volumes 1 ml, 2 ml, 3 ml, 4 ml, 5 ml were taken. Each of the inoculum size was assayed induplicate and incubate at $37C^O$ for 72 Hours. Moisture level was maintained at 80 percent and pH was adjusted at 5.5. The composition of growth media for the optimization of inoculum size is given in table 7.

Carbon Source Optimization

Aspergillus niger also consume carbons source compounds and hydrolysed these compounds, which is necessary for its growth. Different compounds having different carbon ratios can be used and having different impact on the growth of *Aspergillus niger* and the production of enzymes.

Glucose Optimization

Different glucose concentrations can be observed in the optimum growth of *Aspergillus niger* and it is obvious that this optimum growth is related to the production of optimum enzyme production.

Fructose Optimization

Different Fructose concentrations can be observed in the optimum growth of *Aspergillus niger* and it is obvious that this optimum growth is related to the production of optimum enzyme production.

Nitrogen Source Optimization

By varying the amounts of nitrogen sources can be used in the observation of different growth patterns of *Aspergillus niger* and also the maximum production of enzymes. Three different nitrogenous compounds were used during the current study and its effects were checked these compounds are Ammonium sulphate, Urea and Ammonium phosphate.

Urea Optimization

Another organic compound was used for the determination of maximum production of exoglucanase was urea. Different concentrations of urea were used in order to find out the optimum one. Different percentages were used which are of 0.1 percent, 0.2 percent, 0.3 percent, 0.4 percent, 0.5 percent respectively. Optimization of urea is shown in table 3.10

Statistical Analysis

Statistical analysis was implemented on all the data available by using analysis of variance (ANOVA), under completely randomized design (CDR) and treatment mean will be compared by Duncan's range (DMR) test (steel *et al*., 1997).

RESULTS AND DISCUSSION

Cellulose is most abundant polysaccharide in the world, and is easily available in the form of agricultural waste. Cellulosic material is easily degraded by exoglucanase enzyme into simpler units. The current study is about the production of exoglucanase and its various aspects of optimization, under which more enzymes can be produce and these enzymes is used in the degradation of cellulose into simpler sugars. These sugars can be further fermented to produce various biological products which are not only cost effective and also environment friendly. The different parameters used for the optimization of the enzymes are discussed below.

Fermentation Period Optimization

This is shown in table 4.1. It was observed that with the increasing time of fermentation, increase in the enzyme activity was seen, however after 72 Hours it reached its maximum than show small decrease afterwards up to 168 hrs. with the passage of time the concentration of sugarcane bagasse decreased, that's why with the reduction of substrate thee production of enzyme i.e. exoglucanase also decrease because not enough substrate is left behind for the growth of *Aspergillus niger*. Varying optimization periods beginning from day second to eighth day was observed in the case of *Aspergillus niger*. The *Aspergillus* showed optimized period after 72 Hours on cellulolytic material (Narashima *et al*., 2006). Optimized growth of *Aspergillus* after four days was also confirmed by Sherief *et al*., 2010. Shafique *et al.,* in 2004 also confirmed the 72 Hours as optimized period of *Aspergillus*.

Fig.1 activity of exoglucanase produced by *Aspergillus niger* under varying fermentation period

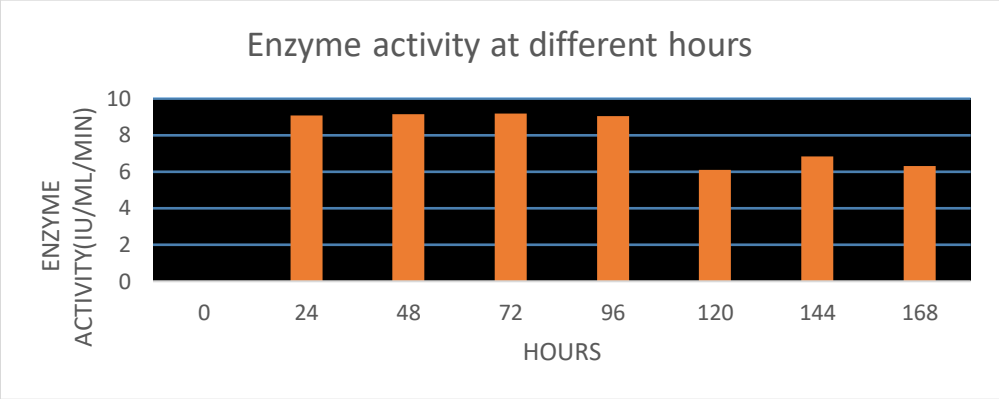


Table 1: Statistical analysis table

Statistical Analysis

S. No.	Sum of squares	df	Mean square	f	P value
1.	Enzyme activity vs. time				
	23741.4	1	23741.4	13.4	0.00237
2.	Enzyme activity vs. moisture				
	9645.8	1	9645.8	18.97	0.00143
3.	Enzyme activity vs. pH				
	11.8008	1	11.8008	14.83	0.00321
4.	Enzyme activity vs. temperature				
	3358.06	1	3358.06	652.1	5.927
5.	Enzyme activity vs. inoculums size				
	16.2634	1	16.2634	9.279	0.01233
6.	Enzyme activity vs. glucose concentration				
	7.70884	1	7.70884	85.69	0.00019
7.	Enzyme activity vs. fructose concentration				
	14.1372	1	14.1372	146.6	2.005
8.	Enzyme activity vs. urea concentration				
	15.376	1	15.376	56.85	0.00114
9.	Enzyme activity vs. ammonium sulphate concentration				
	9.76144	1	9.76144	19.85	0.00212

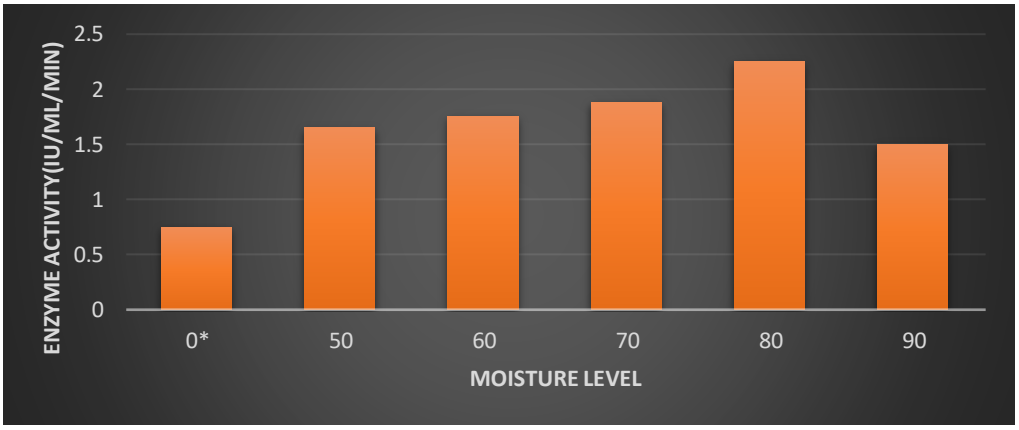
Statistical analysis of time of incubation and production of enzyme shows that the SP value is less than 0.5 which shows that results are significant (Table 1).

Moisture Level Optimization

Different moisture levels of the substrate (sugarcane bagasse) were used in order to obtain the optimum moisture level for having maximum growth and obviously more exoglucanase enzyme. The production of exoglucanase was analysed from enzyme activity assay, showing maximum activity (2.26 IU/ml/min.) at 80 percent (Fig.2). Beyond 80 percent moisture level a declining activity was seen. In SSF (Solid state

fermentation) it was also reported by Shafique *et al*., 2004 that maximum production of exoglucanase by *Aspergillus* at the moisture level of 70 percent -80 percent. The logical reason in the decrease production of exoglucanase is due to the fact in the poor aerating condition in the solid-state fermentation.

Fig 2: Activity of exoglucanase produced by *Aspergillus niger* under varying moisture level



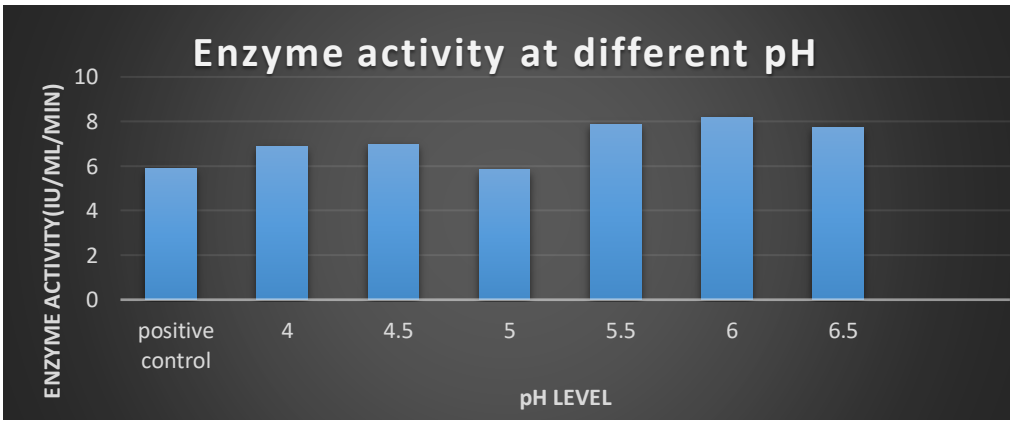
Positive control has zero % moisture level

Statistical analysis of size of inoculum and the production of enzyme shows that the SP value is less than 0.5 which shows that results are significant (Table 1).

Optimization of pH

pH of the medium is adjusted in such a way to obtain maximum production of exoglucanase from *Aspergillus niger*, because it effects the ionic strength of the medium. *Aspergillus niger* was grown on seven different pH mediums but the maximum activity was recorded at pH 6. Above this pH decline in the activity was seen (Fig.3). These optimum pH (6) was also confirmed by Mukerjee *et al*.,2011, Asad *et al*., 2006. The maximum activity of Aspergillus can be seen at the pH five and ranging up to nine was also been observed by Goyal and Soni in 2011.

Fig 3: Activity of exoglucanase produced by *Aspergillus niger* under varying pH



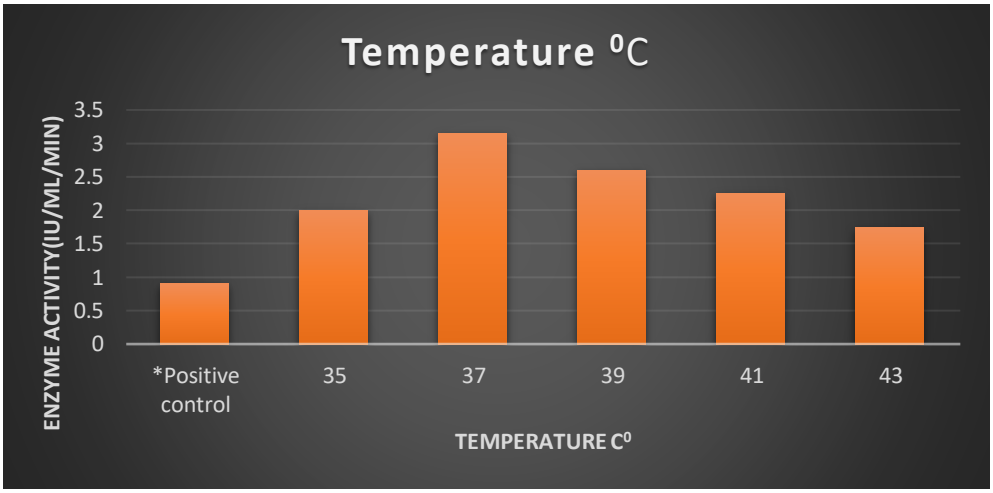
Positive control was at neutral pH

Statistical analysis of various pH and the production of enzyme shows that the SP value is less than 0.5 which shows that results are significant (Table 1).

Optimization of Growth Temperature

Temperature plays a vital role in the optimized growth of *Aspergillus niger*. It has been observed that optimum growth of *Aspergillus niger* was at 37 ^{0}C, having activity 3.15 IU/mL/min. The result can be deduced from this that maximum growth rate at 37 ºC. By increasing the temperature beyond this a decline in the activity was seen. (Fig.4).Temperature plays a critical factor in the production of enzyme (Exoglucanase) from the fungi.

Fig 4 Activity of exoglucanase produced by *Aspergillus niger* under varying incubation temperature



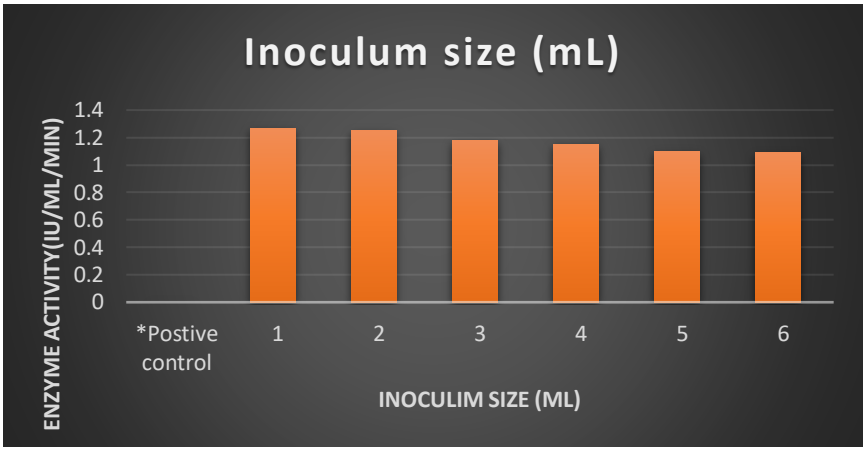
* Positive control was recorded at room temperature.

Statistical analysis of various temperatures and the production of enzyme shows that the SP value is less than 0.5 which shows that results are significant (Table 1).

Optimization of Inoculum Size

By increasing the size of inoculum, it is meant more number of spores. More number of spores will be responsible for more growth of the fungi if substrate is present. By increasing the size of inoculum leads to the maximum production of *Aspergillus niger* (Fig. 5). At the inoculum size of 1mL size, maximum enzyme activity was recorded, which was IU/mL/min. by adding more inoculum decrease enzyme activity was observed, showing the decreased production of exoglucanase from *Aspergillus niger*.The relation between the size of inoculation and the production of exoglucanase was also been observed by Shaafique *et al*., 2004 and Zhaang *et al*., 2006. They also narrated that different concentration of inoculum size cause varying degree of production of exoglucanase.

Fig 5 activity of exoglucanase produced by *Aspergillus niger* with varying inoculum size



* Positive control has 0 mL inoculum size

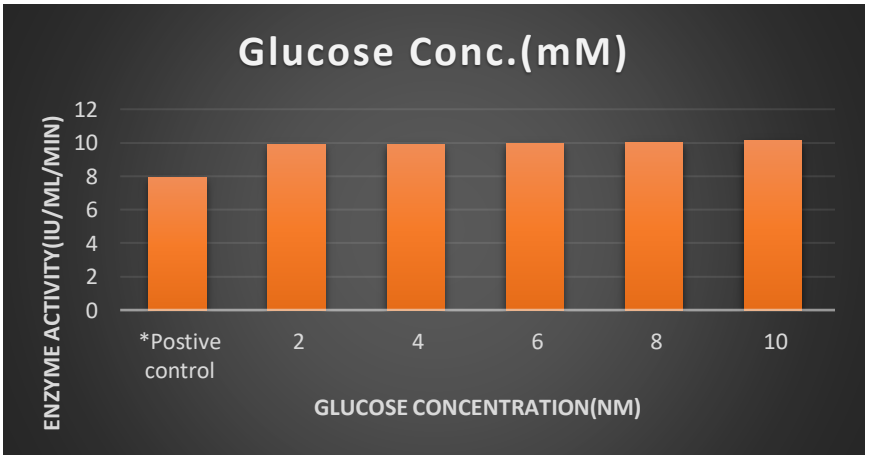
Fig.5: Varying activity levels of exoglucanase by *Aspergillus niger* with varying inoculum sizes

Statistical analysis of size of inoculum and the production of enzyme shows that the SP value is less than 0.5 which shows that results are significant (Table 1).

Carbon Source Optimization

Fungi is hetrotrotropic in its nutrition and obtain carbon source by fermenting the cellulose, so by adding different carbon sources in the substrate may increase the production of exoglucanase from *Aspergillus niger*. Fructose and glucose were used as the carbon source for the production of exoglucanase.Glucose is an important carbohydrate which is utilized by the cell as the source of energy, by increasing the concentration of glucose the production of exoglucanase also increase from *Aspergillus niger* (Fig. 6). At mM percent glucose concentration maximum enzyme activity was found which is 10.02 IU/mL/min. It was found that by further increasing the concentration of glucose the production of exoglucanase decreases. `

Fig 6: Activity of exoglucanase produced by *Aspergillus niger* with varying concentration of glucose



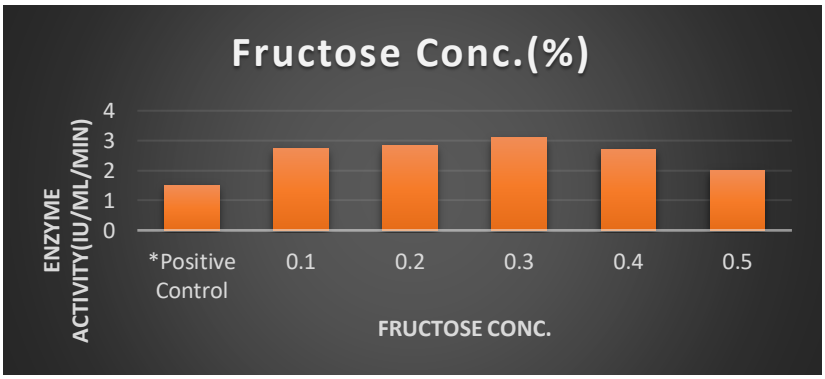
Positive control 0% glucose

Statistical analysis of various glucose concentrations and the production of enzyme shows that the SP value is less than 0.5 which shows that results are significant (Table 1).

Fructose optimization

Maximum enzyme activity was found at 0.3 % of fructose (0.021 g in 7 gm of sugarcane straw) which is 3.10 IU/mL/min. further increase of fructose concentration reduces the production of exoglucanase.

Fig7: Fructose concentration and Activity of exoglucanase from *Aspergillus niger*



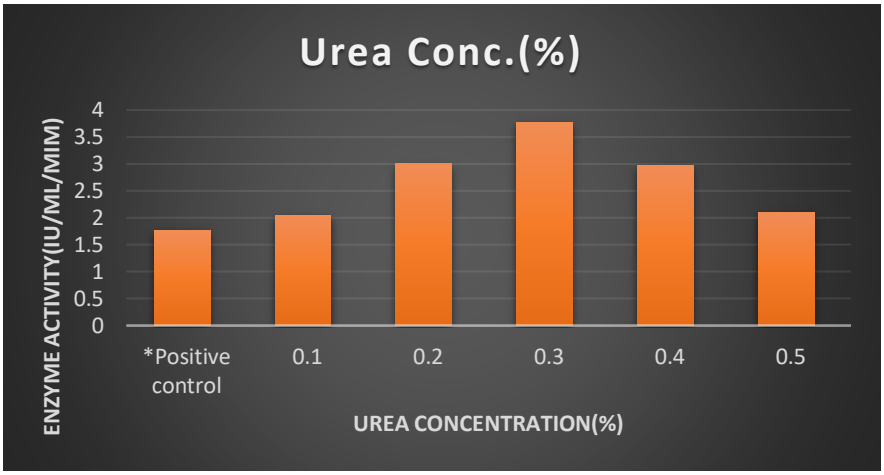
*Positive control has 0 % fructose concentration

Statistical analysis of time of incubation and the production of enzyme shows that the SP value is more than 0.5 which shows that results are insignificant (Table 3.1).Chinedu *et al.*, in 2007 proposed increased production of exoglucanase by adding fructose to substrate. They also stressed that fructose promote more production of enzyme as compared to glucose.

Optimization of Nitrogen Source

Nitrogen is present in urea, which is slightly soluble in water and also non-toxic in nature. By adding urea in different concentration in the substrate effects the growth of *Aspergillus niger* and also its growth and also the production of exoglucanase. The activity of enzyme is shown in fig 4.8. Highest activity of 3.77 IU/mL/min of enzyme was observed at 0.3 percent urea. Mantovani *et al.*, in 2007 also confirmed the increase in growth by adding nitrogen source.

Fig.8 Exoglucanase activity by *Aspergillus niger* at varying concentration of Urea



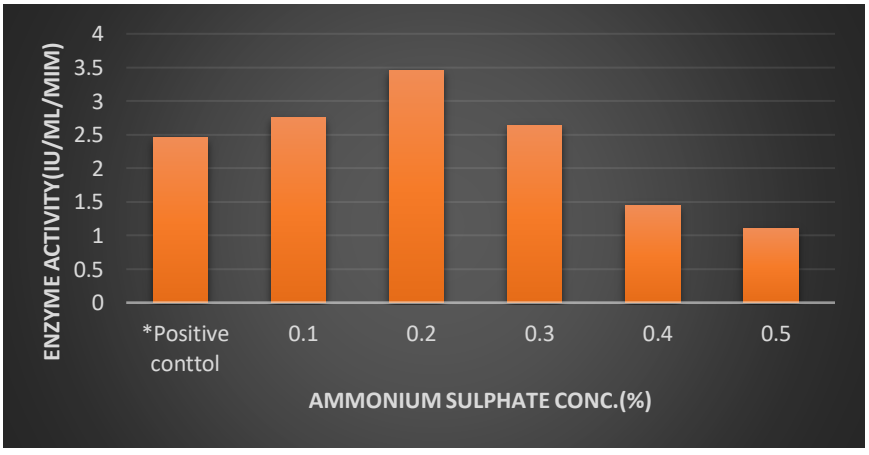
* Positive control 0 % Urea conc.

Statistical analysis of various urea concentrations and the production of enzyme shows that the SP value is less than 0.5 which shows that results are significant (Table 1).

Optimization of Ammonium Sulphate

Ammonium sulphate is an inorganic salt its different concentrations were used in the analysis of maximum production of exoglucanase from *Aspergillus niger*. Five different concentrations of ammonium sulphate were used in order to optimize it. Maximum activity was observed at 0.2 percent which was 3.44 IU/mL/min. The data obtained showed that ammonium sulphate has a positive effect not only on the growth of *Aspergillus niger* but also in the production of exoglucanase. The biological reason behind this is that nitrogen is necessary not only for various metabolic activities but also for the synthesis of enzymes.

Fig 9: Activity of exoglucanase produced by *Aspergillus niger* under varying concentration of ammonium sulphate

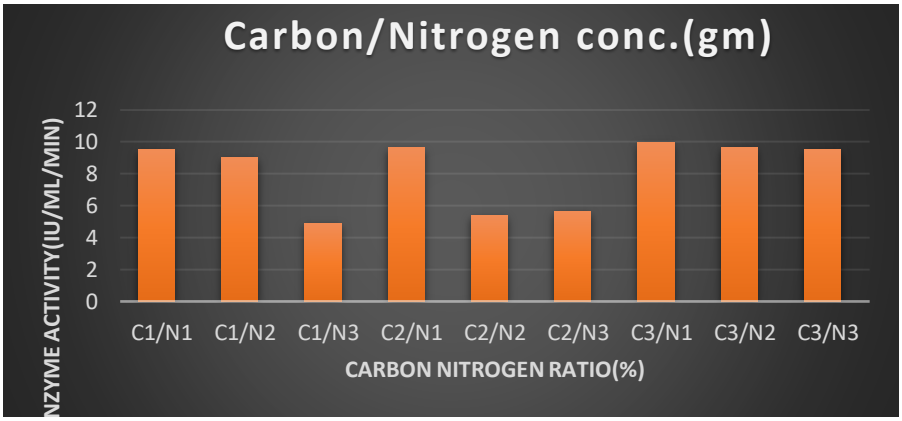


*Positive control has 0.0 percent Ammonium sulphate

Statistical analysis of various ammonium sulphate concentrations and the production of enzyme shows that the SP value is less than 0.5 which shows that results are significant (Table 1).

Carbon-Nitrogen Optimization

Fig 10: activity of exoglucanase produced by *Aspergillus niger* under varying concentration of carbon-nitrogen source

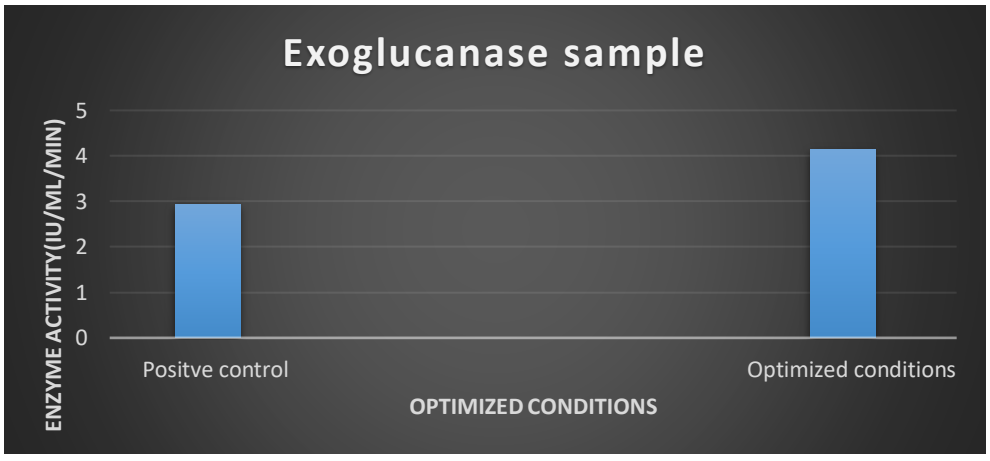


Different concentration of carbon and nitrogen were used. The ratio between carbon and nitrogen were fixed at 0.5:1 ratio. The carbon sources used were Glucose (C1), Fructose (C2), and Sucrose (C3) respectively. Various nitrogen sources used as a substrate were Ammonium sulphate (N1), urea (N2), and Ammonium bicarbonate (N3). Maximum activity was observed at C3/N1 which is 9.634 IU/mL/min. the combination belonging to C3/N1 shows maximum enzyme activity.

Optimized Conditions

After optimizing all the conditions *Aspergillus niger* was grown on the growth media having all the optimized factors. Thereafter the production of exoglucanase was estimated and its enzyme assay activity was monitored. The optimized conditions which were taken as standard are, 37 ^{0}C temperature, 72 Hours period of incubation, pH 6.0, 1 ml inoculum size, carbon source (Fructose) 0.3 percent. The results which was deduced that enzyme (Exoglucanase) production at optimized condition shows more activity

Fig 11 Enzyme activity at optimized conditions



Positive Control without Optimization

10 percent 30 percent, 40 percent, 50 percent, 60 percent, 70 percent, 80 percent, ammonium sulphate was added in 10 ml of enzyme. Sample was left over for overnight. Sample containing the enzyme was centrifuged at 5000 rpm for 5 min and after completing centrifuge, supernatant was used for enzyme activity. At 40 percent of ammonium sulphate concentration maximum enzyme activity was recorded (2.135IU/mL/min), by further increasing the activity the decrease in enzyme activity was observed.

Fig 12 Showing different conc. Of Ammonium sulphate and enzyme activity

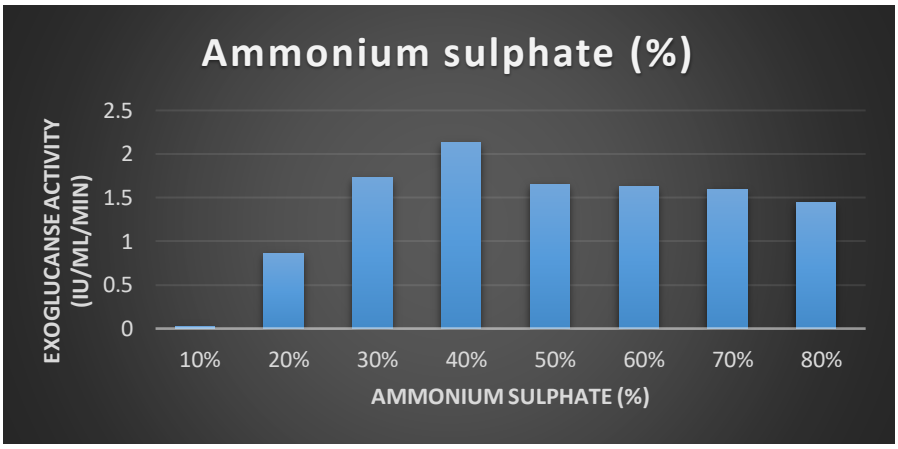


Fig. 12 Graph showing different ammonium sulphate conc.

Gel Filtration Chromatography

Exoglycanase (Enzyme) partially purified is more purified by gel filtration chromatography, 5 % silica gel column was used in this procedure. Enzyme was added in column followed by citrate buffer whose pH was adjusted at 4.8 in order to obtain different elution, obtained were subjected to exoglucanase activity assay. Total thirty elutions were taken and first seven elutions were discarded. Twenty-three elutions were taken and it was observed that elution number twenty-three showed maximum enzyme activity (8.42 IU/ml/min) and reduces to 1.097 IU/ml/min up to elution number twenty-three. (Fig 13).

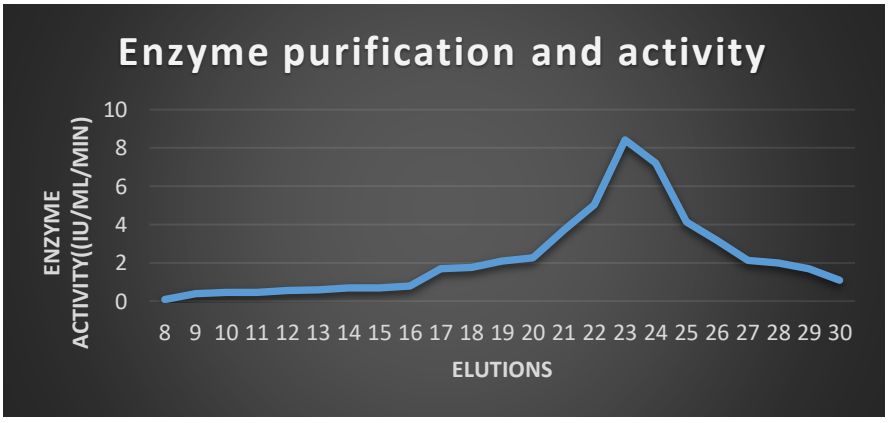


Fig.13 Gel filtration chromatography elutions

Bradford Test

Elution showing maximum activity was further analysed in bread ford test. After bread ford test a decreased enzyme activity (5.81 IU/ml/min) is seen showing reacting enzyme with the bradford (5.99 IU/ml/min) taken as a standard.Fig.14

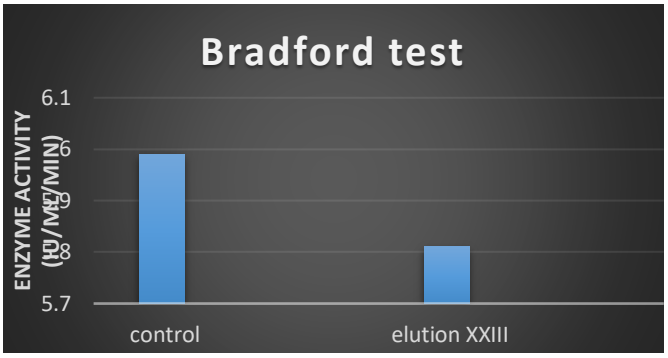


Fig. 14 Decreasing enzyme activity in Bradford test

Protein Estimation by Bradford

Protein is estimated from various standardized samples by Bradford method. Different parameters which were taken in protein estimation were 3gm, pH 6, 5ml, C_3N, 6 gm(r), 4gm(s), and 168 hours. OD was taken at 595, varying enzyme activities were measured. Fig 3.15. Maximum activity was seen in the sample of 3 gm (1.604 IU/ml/min). Linear equation was obtained from BSA test from which protein amount can be calculated. The equation obtained from BSA is as follows:

Y= 0.2401X+0.1789, where Y is the optical density of biuret reagent which is 1.142

And X is the quantity of protein obtained.

The OD reading of bred ford reagent obtained was 1.142

Putting the value of bread ford reagent in the linear equation the protein was estimated to be 4.57µg/ml. in ammonium sulphate precipitation the amount which was estimated to be 6.08 µg/ml and that of ammonium sulphate concentration was 6.08 µg/ml, and of C_3N_1 is 6.180 µg/ml and that of crude enzyme the protein estimated was 6.50 µg/ml.

Table 2: Purification Table for Exoglucanase by *Aspergillus niger*

Purification Step	Volume (ml)	Activity (IU/ml/min)	Protein (mg/ml)	Total Activity (IU)	Total Protein (mg)	Specific Activity (IU/mg)	Purification Fold
Crude enzyme	50	7.896	6.50	394.8	325.0	1.21	1.00
Optimized conditions	10	7.922	6.284	79.22	62.8	1.26	1.04

Ammonium sulphate purified	5	7.925	6.08	39.62	30.04	1.29	1.06
Gel filtration chromatography	2	8.421	6.50	16.84	13.0	1.29	1.07

Protein Estimation by Bradford

After gel filtration chromatography and ammonium sulphate precipitation the concentration of protein was estimated. Bovine Serum Albumin (BSA) was used as a standard in biuret assay. A graph showing different concentration of Bovine serum album was sketched at the Absorbance 540 nm (fig 3.35). The concentration of protein in sample after ammonium sulphate precipitation and gel filtration chromatography was determined from the regression equation obtain from the graph. Protein concentration increased in 1.10 folds after ammonium sulphate precipitation and 1.066 folds purification after gel filtration chromatography (Dariot *et al.*, 2008; Jabbar *et al.*, 2008). The enzyme obtained after gel filtration chromatography was used for further exoglucanase characterization (Ahmed *et al*.,2009). 2.53 folds increase in concentration of cellulose after purification by ammonium sulphate and gel filtration was also reported by Iqbal *et al*., 2011.

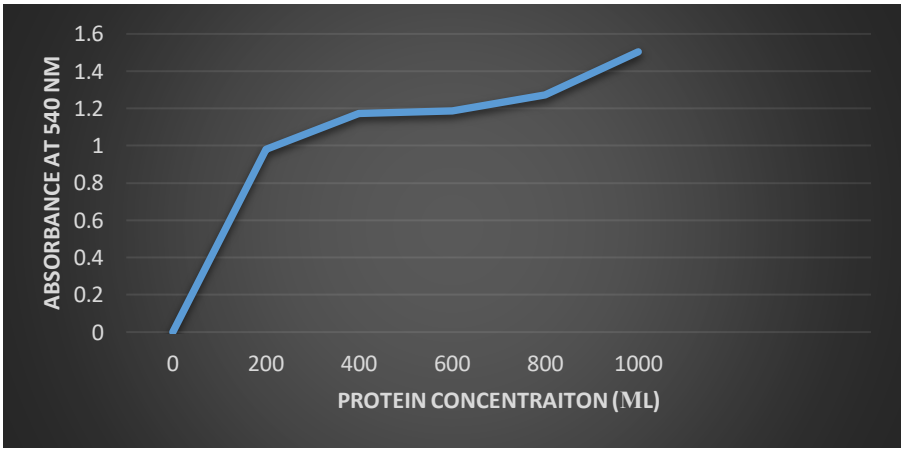


Fig 15. Standard curve of bovine serum (BSA) at 540 nm

Ammonium Sulphate Precipitation

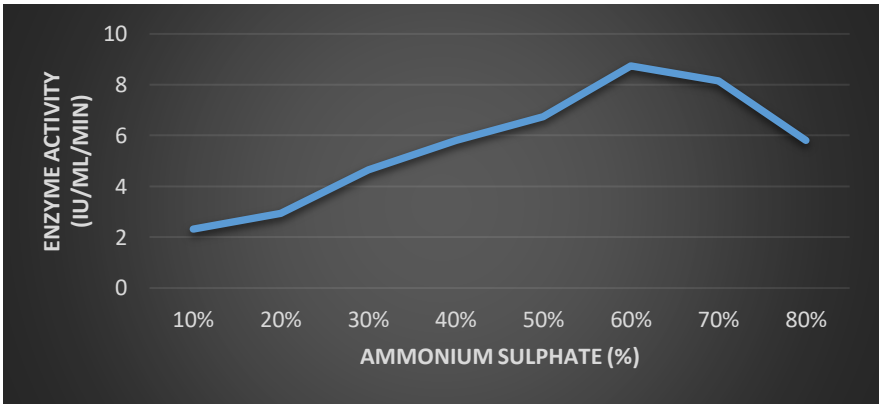
Ammonium sulphate precipitation principle is based on the phenomenon that various proteins have different composition of amino acids and some are dissolved in aqueous solution but when different concentration of salts are added to the solution various proteins are precipitated out from the solution. In our study ammonium sulphate was used for salting out principle and these precipitates were isolated after centrifuging the samples at 5000 rpm.

Table 3: Ammonium sulphate concentration (%) and enzyme activities

Ammonium sulphate (%)	Supernatant(IU/Ml/Min)	Residual(IU/ML/Min)
10	0	2.32
20	0.866	2.94
30	1.65	4.64

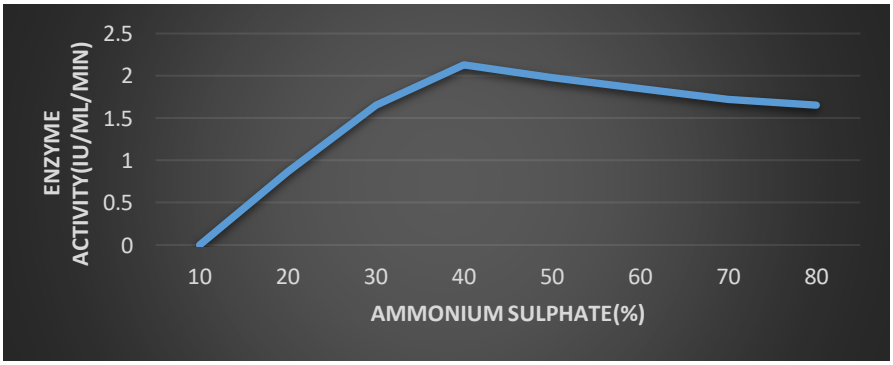
40	2.13	5.80
50	1.98	6.74
60	1.85	8.74
70	1.72	8.16
80	1.65	5.82

Fig 16: Enzyme activity at different ammonium sulphate percentages (Residual)



At the increasing concentration of ammonium sulphate percentage enzyme activity is also increased till it reaches its maximum of 8.74 IU/Ml/min at 60 percent of ammonium sulphate thereafter there is a gradual decrease in enzyme activity.

Fig 17: Enzyme activity at different ammonium sulphate percentages (supernatant)



At the increasing concentration of ammonium sulphate percentage enzyme activity is also increased till it reaches its maximum of 1.98 IU/Ml/min at 50 percent of ammonium sulphate thereafter there is a gradual decrease in enzyme activity.

CONCLUSION

The current study was carried out to produce, purify and enhance exoglucanase by *Aspergillus niger*. The agricultural waste was used as a lignocellulosic substrate for the fermentation process. To obtain maximum yield of enzyme different conditions were optimized including, moisture level, pH, temperature, inoculum size, nitrogen source, carbon source and different surfactants. The enzyme thus produced was partially

purified with ammonium sulphate and its specific activity was calculated. Partially purified enzyme was further purified with gel filtration chromatography and its activity was calculated. Protein contents of different elution were found with the help of biuret assay method. Purified enzyme was than characterized for pH, temperature, and other parameters. Maximum activity of exoglucanase was observed at 80 percent moisture level (2.26 IU/mL/min, 6.0 pH (8.19 IU/mL/min), 37^0C growth temperature (3.15 IU/mL/min) and 1 ml inoculum size (1.266 IU/mL/min). Addition of 0.3 percent fructose as a carbon source and glucose gave maximum activity (3.10 IU/mL/min).The exoglucanase produced by *Aspergillus niger* under optimum conditions was partially purified using ammonium sulphate precipitation method. The maximum exoglucanase (3.46 IU/mL/min) was obtained by adding 0.2 percent ammonium sulphate. Partially purified enzyme was further purified by gel filtration chromatography using 5 percent silica gel. The maximum exoglucanase activity (1.604 IU/mL/min) was observed in elution number 23. There was 1.106-fold purification observed after ammonium sulphate purification and 1.066 fold after gel filtration chromatography.

REFERENCES

Ahmed, S. A., Bashir, H., Saleem, saadia, M., and jamil, A., Production and purification of ceelulosedgrading Enzymes from a filamentous fungus *Trichoderma harzianum*. Pak. J. Bot. 41(3), 1411-1419 (2009).

Asad M.J., M. Sageer, M. A. Sheikh and J. I. Sultan. Production of *Neurospora sitophila* cellulases in Solid sate Cultures. Jour. Chem. Soc. Pak., 28:6. (2006).

Beg Q.K., B. Bhushan, M. Kapoor and G. S. Hoondal. 2000. Enhanced production of thermostable xylanase from *streptomyces* sp. QG-11-3 and its application in bioleaching of eucalyptus Kraft pulp. Enzyme Microb. Technol., 27: 459-466(2000).

Bergeron, P C. E. In handbook on Biotechnology. Wyman, Taylor and Francis. Washington DC, 89-103 (1996).

Betini, J. H. A., M. Michelin, S. C. Peixoto-Nogueira, J. A. Jorge, H. F. Terenzi and M. L. T. M. Polizeli. Xylanases from *Aspergillus niger*, *Aspergillus niveus* and *Aspergillus ochraceus* produced under solid-state fermentation and their application in cellulose pulp bleaching. Bioproc. Biosyst.Eng, 32:819-824 (2009).

Bhat, M. K. Celluases and related enzymes in biotechnology. Bioteehnol. Adv., 18:355:383 (2000)

Carere, C, R., R. Sparling, N. Cicek and D. B. Levin. Third Generation Biofuels Via Direct Cellulose Fermentation. Int. J. Mol. Sci., 9:1342-1360. (2008).

Casado, L. G., B. R. Urbinnowicz, C. M. B. Damasceno and J. K. C. Rose. Plant glycosyl hydrolases and boifuels: a natural marriage. Cruu. Opin. Plant Biol., 11:329-337 (2008).

Chinedu, N. S., V. Okochi, H. Smith, U. Okafor,O. B. M. Okerenta and O. Omidiji. Effect of carbon sources on cellulase (EC 3. 2. 1. 4) production by *Penicillium chrysogenum* PCL501. Afri. J. Biochem. Res., 1:66-10 (2007).

Csoka, L., a. Lroincz and A. Winkler. Sonochemically modified wheat straw For pulp and papermaking to increase its economic performance and Reduce environmental issues. BioSource, 3(1): 91-97(2008).

Dariot, D. J., A. Simonetti, f. Plinho, and A. Brandeli. Purification and characterization of extracellular B-glucosidase from *Monascus ouroureus*. J. Microbiol. Biotech., 18:933-941(2008).

Dashban M., H. Schraft and W. Qin Fungal bioconversion of lignocellulosic residues: Opportunities and prospective. Int. J. Biol. Sci., 5: 578-595 (2009)

Gao, J., H. Weng, D. Zhu M. Yuan, f. Guan. And Y. Xi. Production and characterization of cellulolytic enzyme form thermoacidophilic fungal. *Aspergillus terreus* M11 under solid state cultivation of cornstover. Biosource. Technol., 997623-76 29 (2008).

Gosh, T. K. Measurement of cellulase activities. Pure App. Chem., 59: 267-288 (2007).

Goyal, M. and G. soni, Production and characterization of cellolytic enzymes by *Pleurotus florida*. Afri. J. Microbiol. Res., 5(10): 1131-1136 (2011).

Han, Y., and H. Chen Biochemical characterization of maize stover B-Exoglucanase and its use in lignocellulose conversion. Biosource. Technol., 101:6111-6117 (2010)

Himmel M. E., M. F. Ruthand C. E. Wyman Cellulase for commodity products from cellulosic biomass. Curr. Opin. Biotechnol., 10:358-364 (1999).

Iqbal, H. M. N., I. Ahmed, A. Z. Muhammad and I. Muhammad. Purification and characterzation of the kinetic paramemter of ecllulase Produced from wheat straw by *Trihnoderma virde* under SSF and its detergent compatibility. Adv. Biosci. Biotech., 2:149-165 (2011).

Jabbar, A., M. H. Rashid, M. R. Javed. R. Perveen and M. Aslam Malana. Kinetics and thermodynameics of a novel endoglucanase (CMCase) form *Gymnoascella citrine* Produced under solid state condition. J. Ind. Microbiol. Biotechnol., 33:515-524 (2008).

Jarvis, M. Cellulose stacks up. Nature, 426: 611-612. (2003).

Kocher, G., Kalra and G. Banta Optimization of celluloase production by submerged fementation of rice straw by *Trichoderma harzianum* Rut – C 8230. Internet J. Microbiol., 5-11 (2008).

Lo, C. Q. Zhang, P.Lee and L., Ju Cellulase prodction by *trichoderma reesel* using sawdust hydrolysate. Applied Biochem. Biotechnol., 122:561-573 (2005).

Lynd, L., R, P. J. Welmer, H. Van Zyl and L. S. Pertorius Microbial cellulse utilization: fundamentls and biotechnology. Microbiol. Molbiol Rev., 66-(3): 566-577 (2002).

Malherbe, S, and T. E. Cloete. Lignocellulse biodegradation:fundamentls and application: A rev. Environ. Sci. Biotechnol., 1:105-114 (2003).

Mantovani T. R. D., G. A. Linde and N. B. Colauto. Effect of the addition of nitroggen sources to cassava fiber and carbon – to nitrogen rations on *Agaricus brasiliensis* growth. Can. J. Microbiol., 53: 139-143(2007).

Mukherjee, S., M. Karmakar and R. R. Ray. Production of extra cellular exoglucanase by *rihizopus oryzae* from submerged fermention of agro wastes. Rec. Res. Sci. Tech., 3: 69-75 (2011).

Rajoka, M. l. and K. A. Malik. Production of cellulases by four native species of *cellulmonas grown* on different cellulosic and agricultural substrates. Folia Microbiol., 42: 59-64 (1997).

Saleem, F., S. Ahmed and A. Jamil. Isolation of xylan degrading gene from genomic DNA library of thermopillic fungus *Chaetomium thermophile* ACCT 28076. Pak. J. Bot., 40 :1225-1230 (2008).

Senthikumar, S. R., B. Ashokkumar , R. K. Chandra and P. Gunasekaran. Optimization of medium composition for alkali- stable xylanase producation ny *Aspergillus fischeri* Fxn I in solid – State fermentation using central composite rotary design. Bioresour. Technol., 96: 1380-1386 (2005).

Sinnott, M. Study of hemocellulytic enzymes form the fungi Phanerochaete *chryosparium and Trichoderma ressie*. J. Baceriol., 133: 465-471 (1997).

Shafique, S., M. Asghar, M. A. sheikh. And M. J. Asad .Solid State Fermentation of Banana Stalk for Exoglucanase Producation. IntI J. Agric. Biol., 3: 488491 (2004).

Sherief , A. A., Ab. AI Tanash, and N. Atia. Cellulase producation by Aspergillus fumigatus grown on mixed substrate of rice straw and rice bran. Res. J. Microbial., 199-211 (2010)

Steel,R. G. D., J. H. Torrie and D. A, Dickey. Principles and Procedures of Statistics: A Biometrical Approach," 3rd Edition, McGraw-Hill (1997).

Stricklen, M . 2006. Plant genetic engineering to improve biomass characateristics for biofuels. Curr. Opin. Biotechnol. 17: 315-319. (2006)

Trathewey, j. A. K., L. M. Campbell and P. J. Harris. 2005. (1-3), (1-4)- B- D- gulcans in the cell walls of the poales (sensu lato): an immunogold labelling study using a monoclonal antibody I. Amric, J. Bot., 92 : 1660-1674.(2005).

Yang, S. Q., Q. J. Yan, Z. Q. Jiang, L. T. Li. H. M. Tian and Y. z. Wang. 2006. High Level of xylanase producation by the thermophilic *Paeoilomyces thermophila* J18 on weat straw in solid-fermentation Bioresour. Technol., 97: 1794-1800. (2006).

Zhou, J., Y. H. Wang, j. Chu, Y. P. zhuang, S. L., zhang and P. Yin. Identification oa purification of the main components od cellulases from a mutant strain of *Trichoderna Virde* T 100-14. Biosource. Technol., 99:6826-6833 (2008).

Zerofonrtis, G. Approved laboratory techniques. Appleton. Century crafts inc. New York, 5: 54-59 (1959).