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CELLULASES PRODUCTION BY *ASPERGILLUS NIGER* GROWN ON MEDIUM CONTAINING ACID PRETREATED AGRO WASTES AS CARBON SOURCE.

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ABSTRACT:

In this study agricultural wastes such as sugarcane peeler bagasse, sugarcane bagasse of industry, banana fruit stalk, sorghum husk and rice husk were hydrolyzed with 0.6N HNO3 and the fermentable sugars were incorporated in mineral medium as a carbon source for the growth of *Aspergillus niger and production of cellulases using submerged fermentation method.* The maximum production (10.89 units/ml) of CM-cellulase was obtained at 240 hours of incubation when banana fruit stalk hydrolysate was used as a carbon source. The cellobiose and salicinase maximum production 4.855 and 4.198 units/ml were obtained at 24 and 240 hours respectively when sugar cane peeler and rice husk hydrolysate used as a carbon source. Maximum cell growth 0.402g/100ml of *Aspergillus niger* was observed at pH 7.62 in case of hydrolyzed rice husk used as a carbon source. The total sugar and reducing sugars were decrease with the growth of Aspergillus niger in all carbon sources..

Key Words: Cellulase, *Aspergillus niger*, Agro wastes.

INTRODUCTION

Complete cellulose hydrolysis to glucose demands the action of exoglucanases, endo-glucanases and β-glucosidases. Exoglucanases (1,4-β-D-glucancellobiohydrolase EC 3.2.1.91) are usually active on crystalline cellulose and cleave disaccharide units either from non-reducing or reducing end. Endoglucanases (1,4-β-D-glucan-4-glucanohydrolase, EC 3.2. 1.4) are more active against the amorphous regions of cellulose and they can also hydrolyze substituted celluloses, such as carboxymethyl cellulose (CMC) and hydroxyethyl cellulose (HEC) internally. β-Glucosidases (EC 3.2.1.21) cleave cellobiose and other soluble oligosaccharides to glucose (Bhat, 1997). These enzymes find potential applications in the production of food, animal feed, textile, fuel, chemical, pharmaceutical industries and in waste management (Beguin, 1990).

The production of cellulase has been reported from a wide variety of bacteria and fungi (Anita, 2009). However, filamentous fungi are preferred for commercial enzyme production, because the level of the enzymes produced by these cultures is higher than those obtained from yeast and bacteria .Almost all fungi of genus *Aspergillus* synthesize cellulase, therefore this genus has the potential to dominate the enzyme industry. *Aspergillus* and *Trichoderma* spp. are well known efficient production of cellulases (Peij, 1998). with low water contents. There are several reports describing use of agro industrial residues for the production of cellulose such as wheat straw, wheat bran and rice straw, banana fruit stalk, sugarcane peeler bagasse and rice husk as substrates (Singhania, 2010). The other advantages of fermentation include superior productivity, simple technique; low capital investment (Souza and Magalhaes, 2010) and better product recovery (Tarek, 2007). Industrially important enzymes have traditionally been obtained from submerged fermentation because of the ease of handling and greater control of environmental factors such as temperature and pH (Fadel, 2000).

Looking to above studies, the organism was isolated, identified and used for the production of cellulases using agricultural waste as substrate after acid pretreatment.

MATERIALS AND METHODS

Microorganisms: *The Aspergillus niger was* isolated from Soil of Khairpur District and it was identified in the High Technology Research Laboratory, Shah Abdul Latif University Khairpur. The stock culture was maintained on Czepaks agar. The sterilized slants were inoculated with *A. niger*. After inoculation the slants were incubated at 27°C to obtain luxuriant growth.

Chemicals: Carboxymethyl cellulose (CMC) Salicin and cellobiose were purchased from BDH, Sodium Potassium tartrate from E.Merck and 3, 5-dinitrosalicylic acid was supplied by Sigma Chemicals. All other reagents used were of analytical grade

Culture Medium: The following ingredients as reported by Burrel et al., (1966) without changing the chemical composition using G/L of $(NH_4)_2SO_4$ 2.5 g/L; fumaric acid 2.0 g/L; KH_2PO_4 1.0 g/L; $MgSO_4.7H_2O$; 0.5 g/L; $(NH_4)_2Fe(SO_4)_2.12H_2O$; 0.2mg/L: $ZnSO_4\ 7H_2O$ 0.2 mg/L; $MnSO_4, 5H_2O$,0.1mg/L and thiamine hydrochloride 0.1 mg/L. The pH of the culture medium was adjusted to 6.0 (Schwermann, 1994).

Preparation of Spore Suspension: To stock culture *Aspergillus niger*, 10.0 ml of sterilized water was added and the surface was gently rubbed with sterilized wire loop. The spore suspension was further diluted to 100 ml with sterilized water (Park and Almeida, 1991).

Hydrolysis of Agriculture wastes: 10.0 g of each agricultural wastes such as sugarcane peelar bagasse, sugarcane bagasse, banana fruit stalk, sorghum husk and rice husk were hydrolyzed with 800 ml of 0.6N HNO_3 for two hours on flame, maintaining the level of slurry constant. The digested slurry was autoclaved for 30 minutes at 1.5 kg/ cm^2. The slurry was filtered through whatman No.1 filter paper after cooling at room temperature. The filtrate of solubilized agricultural waste was incorporated into mineral medium as a carbon source. The loss in weight of agricultural waste was determined after drying at 105°C to constant weight (Noomrio 1992].

Cultivation Condition: 50ml of solubilized agri-cultural waste incorporated with mineral medium was taken in 250 ml conical flasks plugged with cotton wool and autoclaved at 1.5 kg/ cm^2 for 20 minute. The sterilized media cooled at room temperature, inoculated with 1.0 ml of *A. niger spore*s. The flasks were incubated in cooled orbital shaking incubator at 28 ±2°C adjusted at 200 revolutionary per minute. The culture broth was separated from mycelia after an interval of 24 hours incubation period by filtration through whatman No.1 filter paper. The enzyme activities of CM–cellulase, β-glucosidase and salicinase were examined in the culture broth. The mycelium was dried at 105°C in an oven to constant weight (Park, 1986).

Assay of CM-Cellulase activity: CM-cellulase activity was determined as reported method by Mandels (1976). 1.0 ml of enzyme sample (culture broth) was mixed with 1.0 ml of 1% CM – cellulose and 2. 0 ml of sodium acetate buffer pH 4.6. The reaction was carried out at 35°C for one hour. Reducing sugar released was estimated by the dinitrosalicylic acid method CM- cellulase activity is calculated from Glucose standard.

were used for the preparation of culture medium

One unit of CM–cellulase activity is defined as the amount of the enzyme that liberate one mgl/ml of reducing sugar as glucose from CM cellulose under the assay conditions.

Assay of β-glucosidase (cellobiase and Salicinase) activity: Cellobiase and salicinase activities were determined by the method of Stenberg (1977), 1.0 ml of enzyme sample (culture broth) was mixed with 1.0 ml of 1% cellobiose (for cellobiase) or Salicin (for Salicinase) and 2.0ml of Sodium acetate buffer pH 4.6. The reaction was carried out at 35 °C for One hour. The reducing sugars produced were estimated by dinitrosalicylic acid method with glucose as a standard.

One unit of Cellobiase and Salicinase activities are defined as the amount of the enzyme that liberate one mg/ml of reducing sugar as glucose from cellobiose or salicin under the standard assay condition.

Determination of protein: The protein content of culture broth was determined by Lowry et al., (1951) method and the results were calculated from bovine serum albumin as a standard.

Determination of total carbohydrate: The concentration of carbohydrate in the agricultural wastes hydrolysate and culture broth was measured by Montgomery method (1961) and the results were calculated from standard curve of glucose.

Determination of reducing sugars: The concentration of reducing sugars in the hydrolysate of Agro wastes and culture broth was determined by dinitrosalicylic acid (DNS) method (Miller, 1959) and results were calculated from glucose as a standard.

Statistical analysis: The data is presented as means ± SD. Analysis of the data was done by one- way ANOVA.

RESULTS AND DISCUSSION

In this study, attempts were made to hydrolyze Agro waste to fermentable sugars by chemical acid method and results are presented in Tables 1. It is quite evident from this tables that banana fruit stalk solubilized more with 0.6 N HNO_3. However, the production of total sugar and reducing sugar were found higher in acid hydrolysate. The term total sugar refers to all sugars dissolved in the liquid and it is determined by converting all sugars to monomers. The reducing sugar accounts for all sugar moieties with a free reducing end group.

Table–1: Effect of 0.6N HNO_3 on hydrolysis of agricultural wastes and the yield of percentage of hydrolysis total Carbohydrate and reducing sugar.

Parameters	Sugarcane peeler bagasse (gram)	Sugarcane bagasse	Banana fruit stalk	Sorghum husk	Rice husk
Initial weight of sample grams	10.00	10.00	10.00	10.00	10.00
Final weight of sample grams after hydrolysis	7.31	6.78	8.71	7.91	8.61
Loss of weight grams	2.69	3.22	1.29	2.09	1.39
% of hydrolysis	26.9	32.2	12.9	20.9	13.9
Total carbohydrate mg/ml soluble filtrate	3.55	3.41	3.91	3.41	3.61
Reducingsugar mg/mlsoluble filtrate	2.31	2.32	2.41	2.40	2.67

An acid hydrolysate of Agrowastes were supplemented with mineral medium for the growth of *Aspergillus niger* and cellulolytic enzymes production. Maximum production of cellulases was obtained from the substrate of sugarcane peeler bagasse it was observed 24 and 240 hours respectively as shown in Table - 2. It was 2.348, 4.198 and 5.612 units/ml. The pH of the culture medium was observed increasing in order. The maximum yield of fungal mycelial biomass obtained at 240 hours. The concentration of total sugar and reducing sugar were decreased in the culture broth of *A. niger* grown in pretreated sugarcane peeler bagasse.

Table-2: Effect of 0.6N HNO_3 pretreated sugarcane peelar bagasse waste as a carbon source on the growth of *Aspergillus niger* and production of cellulases. Culture was incubated in cooled orbital shaking incubator adjusted at 200 rev/min with initial pH 6.0 at 28 □ 2 ^{0}C.

Time Period Hours	Final pH	Weight of mycelia g/100ml	Total sugar mg/ml broth	Reducing Sugar mg/ml broth	Enzyme activity units/ml broth		
					C1	C2	C3
24	5.81	0.068	590±1.156	409±1.156	3.267±0.001	2.348±0.007	4.198±0.001
48	5.88	0.082	586±0.883	353±1.734	3.387±0.002	2.277±0.001	2.76±0.015
72	6.16	0.128	552±1.766	330±0.883	3.753±0.004	2.221±0.005	2.18±0.020
96	6.23	0.167	534±2.030	314±1.455	3.769±0.201	1.909±0.006	2.12±0.008
120	6.41	0.219	510±1.734	276±2.030	4.11±0.006	1.713±0.004	2.11±0.069
144	6.48	0.265	483±2.312	261±1.766	4.185±0.003	0.902±0.019	2.11±0.048
168	6.67	0.278	459±1.203	257±2.312	4.212±0.005	0.735±0.002	2.09±0.006
192	6.84	0.286	435±1.455	244±1.203	4.508±0.131	0.541±0.008	2.08±0.009
216	7.28	0.288	403±2.336	223±1.858	4.638±0.031	0.485±0.003	2.07±0.007
240	7.39	0.292	372±2.732	204±2.336	5.612±0.007	0.367±0.009	2.06±0.010
C1 = Cellulase				C2= cellobiase		C3= Salicinase	

It was noted that maximum amount of CM-Cellulase, cellobiase and salicinase was produced by *Aspergillus niger* at 24 and 240 hours in pretreated sugarcane bagasse. As shown in Tables 3, the CM-cellulase, production by *Aspergillus niger* reached highest at 240 hours whilst cellobiase and salicinase were found at 24 hrs with 2.892, 3.946 and 3.868 units /ml broth respectively, when grown on treated sugarcane bagasse. The maximum production of CM-cellulase, cellobiase and salicinase were observed 10.89 2.102 and 2.778 unists/ml at 240 hours when *A. niger* grown on pretreated Banana fruit stalk (Table -4) The final pH values of culture medium of *Aspergillus niger* grown on acid pretreated sugarcane bagasse increasing in order while in banana fruit stalk found decreasing in order with the increase of growth period. The fungal mycelial biomass increasing in order due to increase of time period. Total sugars and reducing sugars were found continuously decreased with the increased of fermentation time period.

Table-3: Effect of 0.6N HNO_3 pretreated sugarcane bagasse waste as a carbon source on the growth of *Aspergillus niger* and production of cellulases. Culture was incubated in cooled orbital shaking incubator adjusted at 200 rev/min with initial pH 6.0 at 28± 2 ^{0}C.

Time Period Hours	Final pH	Weight of mycelia g/100ml	Total sugar mg/ml broth	Reducing Sugar mg/ml broth	Enzyme activity units/ml broth		
					C1	C2	C3
24	4.11	0.083	542±0.883	477±0.578	1.559±0.001	3.946±0.002	3.868±0.001
48	4.25	0.102	527±1.203	454±0.883	1.775±0.003	3.855±0.003	3.743±0.372
72	5.71	0.152	4662.030	391±1.529	1.787±0.002	3.475±0.004	3.667±0.002
96	5.74	0.198	453±2.084	377±2.188	1.883±0.004	3.198±0.006	2.865±0.003
120	5.78	0.207	418±1.858	363±2.851	2.168±0.007	3.142±0.005	2.713±0.004
144	6.31	0.213	376±1.156	325±2.520	2.421±0.006	2.665±0.007	2.521±0.005
168	6.81	0.215	3541.529	271±2.966	2.431±0.005	2.206±0.008	2.471±0.006
192	7.16	0.218	319±1.766	257±1.766	2.497±0.008	2.076±0.001	2.207±0.007
216	7.32	0.221	281±2.407	180±2.649	2.824±0.009	1.976±0.009	1.835±0.008
240	7.48	0.225	271±2.188	159±1.858	2.892±0.010	1.733±0.010	1.642±0.009
C1= CM-cellulase			C2= cellobiase		C3= Salicinase		

Table-4: Effect of 0.6N HNO_3 pretreated banana fruit stalk waste as a carbon source on the growth of *Aspergillus niger* and production of cellulases. Culture was incubated in cooled orbital shaking incubator adjusted at 200 rev/min with initial pH 6.0 at 28 □ 2 ^{0}C.

Time Period Hours	Final pH	Weight of mycelia g/100ml	Total sugar mg/ml broth	Reducing Sugar mg/ml broth	Enzyme activity units/ml broth		
					C1	C2	C3
24	6.81	0.085	470±1.156	358±0.578	3.258±0.001	1.669±0.001	2.348±0.001
48	6.41	0.092	464±0.578	320±0.883	3.478±0.002	1.707±0.002	2.374±0.003
72	6.22	0.132	450±1.529	293±1.203	4.145±0.003	1.765±0.003	2.436±0.002
96	5.48	0.163	415±1.766	284±2.646	5.583±0.004	1.802±0.004	2.503±0.004
120	5.35	0.182	373±2.084	277±3.055	5.601±0.005	1.871±0.005	2.542±0.005
144	5.31	0.185	358±2.030	265±3.786	5.608±0.006	1.882±0.006	2.597±0.007
168	5.28	0.192	354±2.188	253±2.188	7.505±0.008	1.906±0.007	2.645±0.008
192	5.26	0.198	319±1.455	219±1.858	8.451±0.007	2.076±0.011	2.693±0.009
216	5.23	0.201	233±2.407	178±1.455	9.873±0.010	2.093±0.008	2.759±0.006
240	5.21	0.212	222±2.520	173±2.030	10.89±0.009	2.102±0.009	2.778±0.010
C1= CM-cellulase			C2= Cellobiase		C3= Salicinase		
±= error of standard deviation							

Tables 5 and 6 shows the growth pattern and cellulolytic enzyme synthesis by *Aspergillus niger* when 0.6N HNO3 pretreated sorghum husk and rice husk used as carbon source. The results illustrated in Table 5 and 6 indicates that higher yield of CM-Cellulase (24 hours in case of rice husk), cellobiase and salicinase were achieved at 240 hours with 3.639, 4.795, 3.931 and 5.103, 4.855, 3.931 units/ml respectively by using sorghum husk and rice husk, as a carbon source for the growth of *Aspergillus niger*. The final pH value and mycelial biomass were observed in increasing order throughout study period. It is observed from the results that total sugar and reducing sugar level found in decreasing order during fermentation period.

Table- 5: Effect of 0.6N HNO_3 pretreated sorghum husk as a carbon source on the growth of *Aspergillus niger* and production of cellulases. Culture was incubated in cooled orbital shaking incubator adjusted at 200 rev/min with initial pH 6.0 at 28 □ 2 ^{0}C.

Time Period Hours	Final pH	Weight of mycelia g/100ml	Total sugar mg/ml broth	Reducing Sugar mg/ml broth	Enzyme activity units/ml broth		
					C1	C2	C3
24	6.88	0.058	580±0.883	433±0.883	3.639±0.001	2.749±0.081	2.538±0.024
48	7.22	0.095	566±1.203	414±1.455	3.447±0.002	2.997±0.272	3.004±0.001
72	7.31	0.122	464±1.529	371±2.084	3.325±0.003	3.001±0.240	3.466±0.032
96	7.51	0.125	428±1.766	370±1.156	3.223±0.004	3.145±0.348	3.583±0.083
120	7.81	0.135	390±2.407	330±2.336	2.221±0.005	3.907±0.041	3.633±0.098
144	7.88	0.135	362±1.858	324±1.766	2.001±0.006	4.088±0.089	3.687±0.096
168	7.91	0.139	354±2.084	304±2.909	1.918±0.007	4.193±0.194	3.729±0.091
192	7.92	0.142	317±1.156	260±1.156	1.7860.008	4.276±0.059	3.801±0.115
216	7.93	0.152	296±2.407	222±2.851	1.473±0.009	4.623±0.019	3.876±0.122
240	7.94	0.186	289±2.851	182±0.578	1.221±0.010	4.795±0.007	3.931±0.150
C1= CM-cellulase			C2= Cellobiase		C3= Salicinase		
±= error of standard deviation							

Table-6: Effect of 0.6N HNO_3 pretreated rice husk as a carbon source on the growth of *Aspergillus niger* and production of cellulases. Culture was incubated in cooled orbital shaking incubator adjusted at 200 rev/min with initial pH 6.0 at 28 □ 2 ^{0}C.

Time Period Hours	Final pH	Weight of mycelia g/100ml	Total sugar mg/ml	Reducing Sugar mg/ml broth	Enzyme activity units/ml broth		
					C1	C2	C3
24	6.31	0.052	456±1.156	426±0.883	3.389±0.001	3.042±0.374	2.871±0.024
48	6.71	0.084	429±1.203	374±1.156	3.507±0.062	3.124±0.419	3.004±0.001
72	6.81	0.131	406±1.766	341±1.456	4.427±0.002	3.315±0.554	3.466±0.032
96	7.13	0.176	379±1.058	314±1.766	4.725±0.003	3.485±0.688	3.583±0.083
120	7.31	0.179	367±2.084	270±2.030	4.803±0.004	4.171±0.305	3.633±0.098
144	7.48	0.183	353±1.455	242±2.084	4.872±0.005	4.188±0.248	3.687±0.096
168	7.52	0.252	354±1.766	230±1.203	4.918±0.006	4.23±0.338	3.729±0.091
192	7.55	0.255	295±0.883	195±1.734	4.938±0.007	4.276±0.160	3.823±0.115
216	7.58	0.309	278±1.734	163±2.407	4.996±0.008	4.726±0.122	3.876±0.122
240	7.62	0.402	270±2.030	138±2.336	5.103±0.009	4.855±0.267	3.931±0.150
C1= CM-cellulase			C2= Cellobiase		C3= Salicinase		
±= error of standard deviation							

DISCUSSION

Cellulolytic enzymes are very useful in many industuries. Cellulase produced by fungi, bacteria or actinomycetes, but the most common producer is fungi (Arriffin, et al., 2006). There is no definite relationship between time of growth and cellulolytic enzyme production but nature of carbon source, pH of culture medium and medium composition also play an essential role in enzyme synthesis by microorganisms (Macris et al., 1985, Shoemaker et al., 1981). It is known that production of hydrolytic enzyme by fungi is subject to catalytic repression by high pure sugar concentration affecting inducible and constitutive enzymes (Aguillar and Huitron, 1987, Guevara et al., 1997). On the other hand, the consistence and size of particles in media component interfered during fermentation (Mitchell et al., 2000). In this study sugarcane peelar bagasse, sugarcane bagasse of industry, banana fruit stalk, sorghum husk and rice husk were hydrolyzed with 0.6N HNO_3 and the fermentable sugars were incorporated in mineral medium as a carbon source for the growth of *Aspergillus niger and production of cellulases using submerged fermentation method.* Mostly dilute acids are utilized to degrade hemi-cellulose, cellulose and other non-crystalline polymer to simple sugars (glucose). Acid hydrolysis produces minimal decomposition of monosaccharide and conventional neutralization is not necessary (Fanta 1994). The chemical pretreatment method is less expensive and more effective (Varino, 1989). The ratio of total sugar or reducing sugar reflects the average degree of polymerization (DP) of sugar moieties in solution. It is important that this ratio is close to 1.0. Han et al., (1997) have reported the effectiveness of acid treatment depends on the substrate and other optimal conditions.

Lyayi, (2004) and Kang et al., (2004) reported that higher amount of cellulase production achieved by indigenous strains. In present study confirm that the maximum production (10.89 units/ml) of CM-cellulase was obtained at 240 hours of incubation when banana fruit stalk hydrolysate was used as a carbon source. Whereas, cellobiose and salicinase maximum production 4.855 and 4.198 units/ml were obtained at 24 and 240 hours respectively when sugar cane peeler and rice husk hydrolysate used as a carbon source by indigenous strain Aspergillus niger. This production rate is higher than the results of Siddiqui et al., (2015) in case of CM-cellulase production (0.499 units/ml) by Aspergillus niger when 4% wheat bran used as a carbon source.

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