STUDIES OF SUBMERGED FERMENTATION FOR ENHANCED CELLULASE PRODUCTION FROM *ASPERGILLUS NIGER* (VAN TIEGHEM, 1867).

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ABSTRACT: *The present study aims to inspect a locally isolated fungus, Aspergillus niger (van Tieghem 1867) which might be capable of producing cellulases effectively and feasibly by utilizing cellulosic wastes in submerged fermentation. Maximum quantity of cellulase (7.89 U/mL) was produced when the strain was grown on 50 mL of culture medium containing sugar can bagasse as cellulosic substrate after 96 h, at temperature of 30° C, at initial pH 5.0, agitation rate 100 rpm and inoculum size 6x10^6 conidia. The strain was pH (upto 9.0; 0.13 U/mL) and thermo stable (up to 60° C; 0.09 U/mL) and its value (3.95 U/mg at optimized conditions) of product yield coefficient ($Y_{p/s}$) favours its feasibility for the use in industries.*

Key words: cellulosic wastes, submerged fermentation, *Aspergillus niger (van Tieghem 1867)*

1. INTRODUCTION

Cellulose a linear polymer of glucose units which are associated together by beta-1, 4-glycosidic linkage [1]. A large number of bacteria and fungi can utilize cellulose by converting this insoluble substrate into soluble compounds with the help of group of enzymes, cellulases, which are released by them. The term cellulases means a group of enzymes which includes; Endocellulase (EC 3.2.1.4), it randomly splits internal bonds creating new chain ends; Exocellulase (EC 3.2.1.91), it splits 2-4 units from the ends produced by endocellulase; Cellobiase (EC 3.2.1.21), it splits the exocellulase product into glucose units; Oxidative cellulose (EC 1.1.99.18) depolymerize cellulose by radical reactions; Cellulose phosphorylase (EC 2.4.1.20), depolymerize cellulose using phosphates. They all synergistically convert cellulose into glucose and therefore are used in industries [2]. On a commercial scale, the use of cellulases for the conversion of cellulosic biomass is costly process. Therefore it is necessary to study a wide range of microorganisms which could be used for cellulose production effectively and feasibly [3].

Large quantities of cellulosic wastes are created through agricultural, industrial processes and forestry which accumulate in the environment causing pollution problem. Cellulosic wastes in Pakistan are usually disposed of by environment non-friendly manner. So there may be two objectives which can be achieved by using agricultural wastes as sources of carbon. On one hand they can be used as raw materials for the production of valuable enzymes and other useful products while on the other hand pollution problem can be reduced [4, 5, 6]. In the present work specific interest has been focused to the utilization of cellulosic wastes for the production of cellulases from locally isolated strains of *Aspergillus niger* (van Tieghem 1867) in submerged fermentation.

2. MATERIALS AND METHODS

2.1 Strain

Strain of *Aspergillus niger* (van Tieghem 1867) was isolated from the soil of NED University of Engineering & Technology Karachi and culture was maintained as followed by Dahot [7]. Number of conidia was counted by haemocytometer. Spore suspension was maintained about 4x106 conidia and they were added to 50 mL of fermentation media in 250 mL flask.

2.2. Enzyme Activity

Cellulase activity was determined by following [3] using carboxymethyl cellulose (Sigma-Aldrich, USA) as a substrate. The reaction mixture contains 0.5 mL of 1 % (w/v) substrate in 0.1 M sodium citrate buffer (pH 4.8) and 0.5 mL of culture broth in a test tube which was then incubated at 40° C for 30 min. The reaction was terminated by adding 1.0 mL of 3, 5-dinitrosalicyclic acid (DNSA) and boiled the contents of test tube in water bath for 15 minutes. The absorbance was noted at 540 nm at UV-Visible spectrophotometer. One unit of cellulase activity was expressed as the amount of enzyme required to release 1 µmol reducing sugars per mL per minute under the assay condition by using glucose as a standard curve.

2.3. Kinetic parameter

Kinetic parameter, product yield coefficient (Yp/s) was determined by the method of Pirt [8]. It is the amount of enzyme which is produced per mg of substrate consumed.

2.4. Optimization of Enzyme Production Parameters

All experiments were done in such a way that the parameter optimized in one experiment was fixed in the subsequent experiments for the production of cellulose splitting enzyme. First of all the most suitable culture medium containing various substrates from cellulosic wastes (Brassica campestris, Brassica nigra, Pomegranate peel, Coconut, Malta peel, Apple peel, sunflower waste, cotton stalk, wheat bran, rice husk, banana peel and sugar can bagasse) was determined. The composition (in g/L) of culture medium [9], in which dextrose was replaced by same quantity of cellulosic wastes, was Dextrose 10, Peptone 5, Epsom salt 5, KH_2 PO_4 5, Common salt 2.5, ferrous sulphate hepta hydrate 0.01, $ZnSO_4.7H_2O$ 0.002, $MnSO_4.H_2O$ 0.001 and thiamine hydrochloride 0.001.

After the determination of the most suitable composition of culture medium, incubation time periods (24-240 h), temperatures (20-70° C), pH (3-9), inoculum sizes (4x106-8x106 conidia) and agitation rates (50-300 rpm) were checked in step by step as mentioned in our earlier papers [4, 5, 6]. The initial pH of a medium has an effect on growth and productivity of microorganism. A range of pH between 4.0-9.0 was checked for optimum production.

3. RESULTS AND DISCUSSIONS

3.1. Effect of various substrate of culture medium

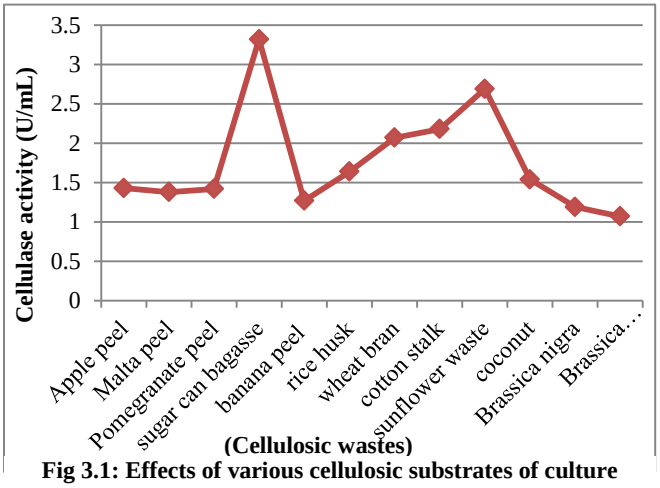

Fig 3.1: Effects of various cellulosic substrates of culture medim on cellulase production by *Aspergillus niger* (van Tieghem 1867) after 24 h, at 30° C, initial pH 6.0, inoculum size 4x10^6 conidia and agitation rate 50 rpm.

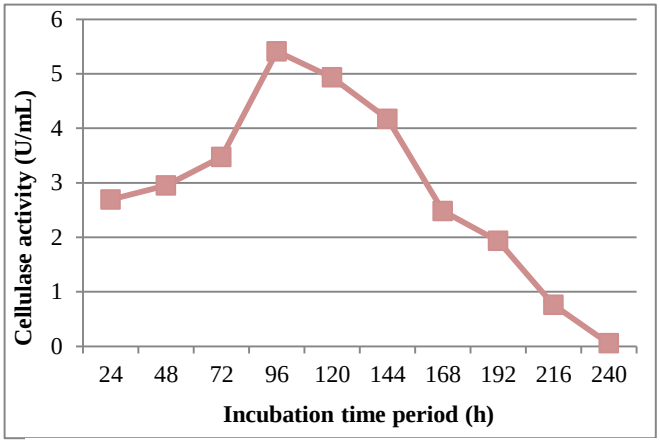

Fig 3.2: The effects of incubation time periods (24-240 h) on cellulase production by *Aspergillus niger* (van Tieghem 1867) in culture medium containing sugar can bagasse at 30° C, initial pH 6.0, inoculum size 4x10^6 conidia and agitation rate 50 rpm.

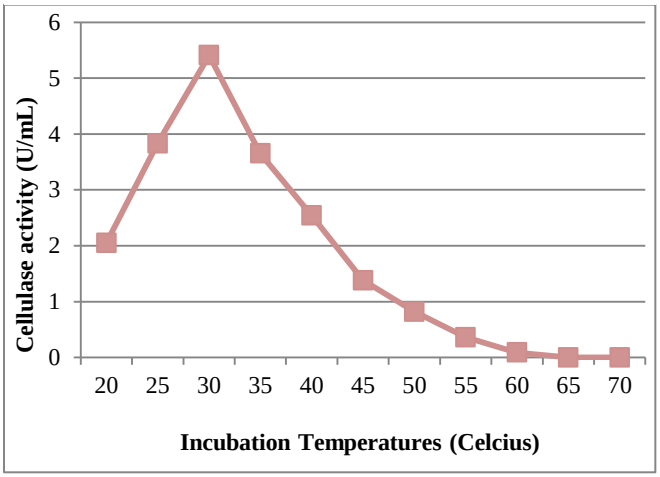

Fig 3.3: The effects of incubation temperatures (20-70° C) on cellulase production by *Aspergillus niger* (van Tieghem 1867) after 96 h in culture medium containing sugar can bagasse at initial pH 6.0, inoculum size 4x10^6 conidia and agitation rate 50 rpm.

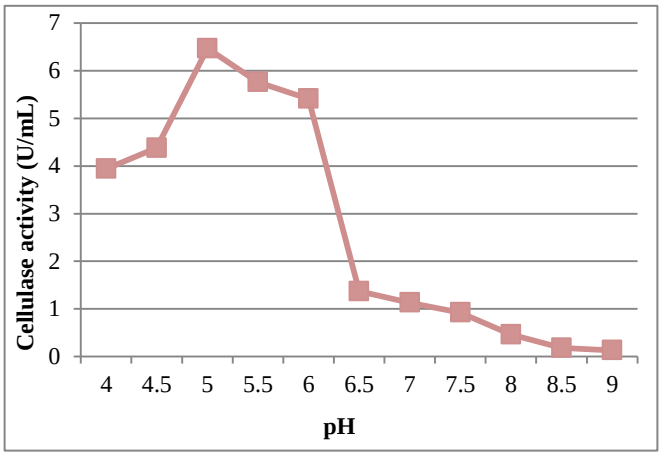

Fig 3.4: The effects of initial pH of fermentation medium on cellulase production by *Aspergillus niger* (van Tieghem 1867) after 96 h in culture medium containing sugar can bagasse at 30° C, inoculum size 4x10^6 conidia and agitation rate 50 rpm.

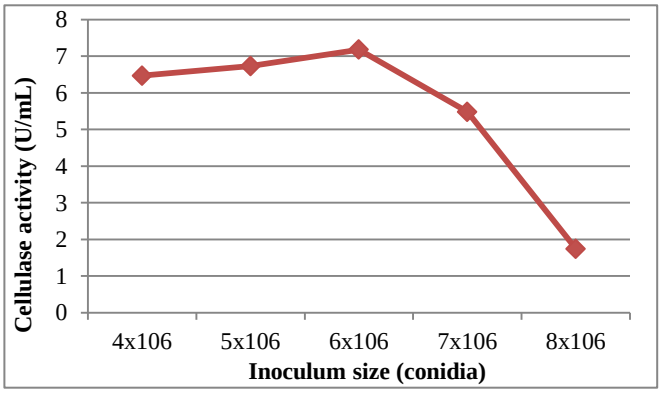

Fig 3.5: The effects of inoculum sizes on cellulase production by *Aspergillus niger* (van Tieghem 1867) after 96 h in culture medium containing sugar can bagasse at 30° C, initial pH 5.0 and agitation rate 50 rpm.

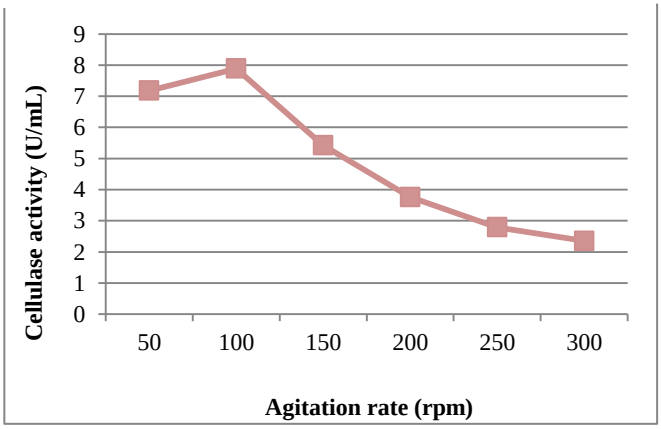

Fig 3.6: The effects of agitation rates on cellulase production by *Aspergillus niger* (van Tieghem 1867) after 96 h in culture medium containing sugar can bagasse at 30° C, at initial pH 5.0 and inoculum size 6x10^6 conidia.

Table 1: Product yield coefficient ($Y_{p/s}$) at various stages of submerged fermentation process.

Incubation Time (h)	$Y_{p/s}$ (U/mg)	T (Celsius)	$Y_{p/s}$ (U/mg)	pH	$Y_{p/s}$ (U/mg)	Inoculum Size (Conidia)	$Y_{p/s}$ (U/mg)	Agitation rate (rpm)	$Y_{p/s}$ (U/mg)
24	0.05	20	0.49	4	0.85	$4x10^6$	2.19	50	3.07
48	0.17	25	0.67	4.5	1.37	$5x10^6$	2.89	100	3.95
72	0.28	30	1.28	5	2.19	$6x10^6$	3.07	150	2.59
96	0.79	35	1.05	5.5	2.06	$7x10^6$	2.03	200	1.32
120	1.29	40	0.83	6	1.28	$8x10^6$	1.01	250	0.47
144	1.12	45	0.46	6.5	0.74			300	0.08
168	0.83	50	0.19	7	0.26				
192	0.73	55	0.01	7.5	0.01				
216	0.41	60	0.00	8	0.01				

Effects of various cellulosic substrates (*Brassica campestris*, *Brassica nigra*, pomegranate peel, coconut, orange peel,apple peel, sunflower waste, cotton stalk, wheat bran, rice husk, banana peel and sugar cane bagasse) of culture medim on cellulase production by *Aspergillus niger* (van Tieghem 1867) after 24 h, at 30° C, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rpm are presented (Fig 3.1). It was observed that enzyme activity is the highest (3.32 U/mL) when sugar can bagasse was used as cellulosic substrate. Acharya *et al.* [10] reported saw dust as the good cellulosic substrate for cellulose production from *Aspergillus niger*.

3.2. Effect of incubation time period

The effects of incubation time periods (24-240 h) on cellulase production by *Aspergillus niger* (van Tieghem 1867) in culture medium containing sugar can bagasse at 30° C, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rpm are plotted (Fig 3.2). Activity of cellulase was measured at regular interval of 24 h and it was found that the maximum activity (5.41 U/mL) was observed after 96 h of incubation. On prolonged incubation enzyme activity was decreased, which might be due to denaturing of enzyme or synthesis of inhibiting metabolite [6, 11, 12]. Khan *et al*., [12] also reported 96 h for the cellulase production from *Trichoderma harzianam*, *Trichoderma sp.* and *Phanerochaete chrysosporium.*

3.3. Effect of temperature

The effects of incubation temperatures (20-70° C) on cellulase production by *Aspergillus niger* (van Tieghem 1867) after 96 h in culture medium containing sugar can bagasse at initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rpm are exhibited (Fig 3.3). Activity of cellulase was the highest (5.41 U/mL) at about 30° C. The strain showed thermo stability up to 60° C (0.09 U/mL). Acharya *et al.*, [10] also reported 30° C as optimum temperature for cellulase production by *Aspergillus niger.*

3.4. Effect of initial pH

The effects of initial pH of fermentation medium on cellulase production by *Aspergillus niger* (van Tieghem 1867) after 96 h in culture medium containing sugar can bagasse at 30° C, inoculum size $4x10^6$ conidia and agitation rate 50 rpm are presented (Fig 3.4). The range of pH (4.0 to 9.0) was studied and found that initial pH of 5.0 was the best for maximum enzyme production (6.47 U/mL). Various initial pH of fermentation medium reported for cellulase prodction by different researchers [10, 12]. These differences may be observed even within the same genus of microorganism [12].

3.5. Effect of inoculum size

The effects of inoculum sizes on cellulase production by *Aspergillus niger* (van Tieghem 1867) after 96 h in culture medium containing sugar can bagasse at 30° C, initial pH 5.0 and agitation rate 50 rpm are plotted (Fig 3.5). Flasks were added with $4x10^6$-$8x10^6$ conidia and maximum cellulase activity (7.18 U/mL) was observed when $6x10^6$ conidia were added to the medium. Dominguesa *et al.*, (2000) also reported 10^6 spores as the optimized inoculum size for cellulose production from *Trichoderma reesei*. Literature survey revealed that researchers used various inoculum sizes [4, 5, 6, 10, 13]. Large inoculum size caused overgrowth and nutritional imbalance resulting less production of enzyme [6, 11].

3.6. Effect of agitation rate

The effects of agitation rates on cellulase production by *Aspergillus niger* (van Tieghem 1867) after 96 h in culture medium containing sugar can bagasse at 30° C, at initial pH 5.0 and inoculum size $6x10^6$ conidia are given (Fig 3.6). The fermentation medium was agitated at 50, 100, 150, 200, 250 and 300 rpm. Activity of cellulase was maximum (7.89 U/mL) at 100 rpm. Literature survey revealed that researchers reported various agitation rates (100-300 rpm) for enzymes production by different microorganisms (4, 5, 6, 10, 11, 12, 13].

3.7. Kinetic parameter

Kinetic parameter, product yield coefficient ($Y_{p/s}$) of cellulase production throughout the fermentation process is summarised in table 1. It can be observed that values are favourable (3.95 U/mg) with the optimzed conditions. Similar types of values of product yield coefficient ($Y_{p/s}$) were also reported by Kiran *et al.* [14].

3. CONCLUSION

From the study it was concluded that the strain, *Aspergillus niger* (van Tieghem 1867) produced maximum quantity of cellulase (7.89 U/mL) after 96 h in culture medium containing sugar can bagasse cellulosic waste at 30° C, at

initial pH 5.0, agitation rate and inoculum size 6x106 conidia. It was a pH (upto 9.0; 0.13 U/mL) and thermo stable (up to 60° C; 0.09 U/mL) strain whose product yield coefficient (Yp/s) is 3.95 U/mg which is a favourable value at optimized condition.

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