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Cellulase Production By Aspergillus Niger Using Lignocellulosic Substrates And Standardization Of Fermentation Process And Parameters

Om Parkash[1], Rupinder Tewari[1], Amita Sharma[1], Sanjeev Kumar Soni[2]*
[1]Deptt. of Microbial Biotechnology, Panjab University, Chandigarh-160014, India
Email: parkash.ed@gmail.com; rupinder@pu.ac.in; amita.sharma22@gmail.com
[2]Deptt. of Microbiology, Panjab University, Chandigarh-160014, India
Email: sonisk@pu.ac.in
Corresponding author: Sanjeev Kumar Soni, sonisk@pu.ac.in

***Abstract*—Cellulases {carboxymethylcellulase (CMCase), Filter paperase (FPase) and β-glucosidase (BGL)} are hydrolytic enzymes capable of degradation of cellulose for production of variety of value added products. In the present study a novel strain of fungus identified as Aspergillus niger RSO-1 was used to examine cellulase production by using wheat bran (WB), rice bran (RB) and saw dust (SD) as substrates in submerged (SmF), surface (SF) and solid state fermentations (SSF). Time course studies were performed and considerable amount of all three enzymes was observed after 4 days of incubation (CMCase, 2.9 IU/ml; FPase, 1.49 IU/ml; BGL, 3.29 IU/ml) using WB as substrate in SSF. Optimization was carried out with WB using SSF for physio-chemical parameters including incubation temperature, inoculum size, moisture content, exogenous addition of carbon sources, nitrogen sources, surfactants and mineral sources.**

Keywords—Cellulase; Wheat bran; Aspergillus niger; Solid state fermentation; Surface fermentation; Submerged fermentation; Optimization

I. INTRODUCTION

Lignocellulosic biomass is considered as the most abundant, inexpensive and renewable source on earth and it can be utilized to get industrially important compounds by bioconversion. The complex composition of lignocellulose and crystalline structure of cellulose make its degradation difficult. Lignocellulose is composed of lignin, hemicellulose and cellulose. Cellulose comprises the largest fraction (30-50%) of total biomass. Composition of hemicelluloses and lignin is variable. It is comprising of chains held in place by hydrogen bonding and β-1,4 linkages tightly to prevent even entry of water and making degradation difficult [1]. Cellulase system is capable of hydrolysing cellulose completely and require three major components: (i) Endoglucanases or 1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4), (ii) Exoglucanases including 1,4-β-D-glucan glucanohydrolases or cellodextrinases (EC 3.2.1.74) and 1,4-β-D-glucan cellobiohydrolases or cellobiohydrolases (EC 3.2.1.91), and (iii) β-glucosidases or β-glucoside glucohydrolases (EC 3.2.1.21) [2]. Endoglucanases nick the amorphous region of glucose and crystalline region is attacked by cellobiohydrolases or exoglucanases [3]. This leads to production of cellobiose units which are further acted upon by β-glucosidase. Under natural conditions a variety of cellulose degrading microorganisms have been reported from bacteria and fungi [4], [5]. Fungal cellulases consist of all the three components of cellulase enzyme system but bacterial cellulase system lacks one of the cellulolytic enzymes i.e. Filter Paper activity (FPase) [6]. Most commercial cellulases are produced by *Trichoderma* species as they are most efficient producers of CMCase and FPase but lack BGL [7], [8], [9], [10]. Production from other organisms like *Aspergillus niger* which is good producer of BGL is gaining attention [11], [12].

Cellulases have potential uses in various industries such as pulp and paper industry for deinking, animal feed by improving feed quality, brewing and wine industry by improving extraction, agriculture by improving soil quality, textile and laundry in terms of biopolishing and biostoning [13], [14], [15]. In times of fuel shortages, enzyme based biofuel production is gaining importance [16]. Currently some countries with higher ethanol and fuel prices are producing ethanol from cellulosic feedstock. Three components i.e. CMCase, FPase and BGL act synergistically for bioconversion of cellulose.

The global market for industrial enzymes has reached $4.4 billion by 2015 with cellulases ranking 3rd in terms of global enzyme market by dollar volume. India has a share of US$ 387.30 million in global enzyme industry [14], [15]. High enzyme cost is majorly due to low yield and poor hydrolytic efficiency [17]. Using cheaper substrates for production along with microbes capable of producing cellulases with improved activity and high titres are being targeted for a cost-effective enzyme technology [18], [19].

While trying to achieve cheap and high production of cellulases, strategies including use of low cost substrates and optimization can be utilized [20]. Selection of a fermentation process is vital as optimization of production parameters is based on it. Three types of fermentation methods for the production of cellulases including solid state fermentation (SSF), submerged state fermentation (SmF) and surface culture fermentation (SF) can be employed. SmF and SF are carried out in the presence of free flowing liquid. SSF is carried out with moisture level just enough to support growth. Although SSF is more suited for fungal culture while other modes can also be used. SSF has the advantage of low cost technology with ease of handling. On the other hand SmF has well established control parameters but is costlier than SF [21].

In this study we have investigated cellulase production using different lignocellulosic substrates in three types of fermentation processes including SSF, SmF and SF and optimization of factors affecting enzymes production in selected fermentation strategy.

II. Materials and methods

A. *Microorganism*

The culture, *A. niger* RSO-1, already available in laboratory was morphologically characterized after cultivation on potato dextrose agar medium at 30ºC for 96 h. This was identified on the basis of various microscopic, macroscopic and molecular characteristics involving 18S rRNA sequence.

B. *Substrates*

5 Wheat bran (WB), rice bran (RB) and saw dust (SD) procured from local sources were used in this study. Six sets of the media used in the enzyme production consisted of i) WB+water, ii) RB+water, iii) WB+RB(1:1)+water, iv) SD+water, v) WB+nutrient broth(NB) and vi) WB+NB+0.1% v/v Trace element solution (0.005 mg/L $FeSO_4$, 0.002 mg/L $MnSO_4.H_2O$, 0.001 mg/L $CuSO_4.5H_2O$, 0.002 mg/L $ZnSO_4.7H_2O$, 0.001 mg/L $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.002 mg/L $CoCl_2.6H_2O$ and 0.001 mg/L $NiCl_2.6H_2O$) in 50 mM Na-tartrate buffer (pH 4.0).

C. *Inoculum preparation*

Inoculum was prepared by harvesting spores in 0.1% Tween 80 and adjusting the count to 1×10^8 spores/ml.

D. *Experimental design for SSF*

Erlenmeyer flasks (250ml) containing 5.0 g of either of substrates including WB, RB, WB+RB and SD moistened with 6.5 ml of distilled water or nutrient broth with or without trace element solution were autoclaved, cooled and inoculated with 1 ml *A. niger* RSO-1 spore suspension. This was followed by incubation at 30°C for 10 days under stationary conditions. The flasks were withdrawn after regular interval of 24 h of incubation, enzymes were extracted and used to check for cellulase activity.

E. *Experimental design for SmF and SF*

Five g of either of the substrates dispensed in 250 ml Erlenmeyer flasks were mixed with 100 ml of distilled water or nutrient broth with or without trace element solution. These were autoclaved, cooled and inoculated with 1 ml spore suspension of *A. niger* RSO-1 having spore count of 1×10^8 spores/ml, incubated at 30°C for 10 days at 220 rpm for SmF and under static conditions for SF. The flasks were withdrawn at regular intervals of 24h and the enzymes were extracted and assayed for cellulase activity.

F. *Extraction of cellulases*

Cell free supernatants obtained after centrifugation of cultures from SmF and SF were used for enzyme assays. In case of SSF, production medium was mixed with 100 ml of distilled water, churned in a mixer and filtered using mechanical sieve. The filtrate was then subjected to centrifugation for 10 min (10000 rpm, 4°C) and the supernatant thus obtained was used for enzyme assays.

G. *Enzyme assays*

The activities of cellulases were determined in terms of CMCase, FPase and β-glucosidase and expressed in terms of International Units, equivalent to µmoles of glucose liberated in 1 min at 50°C, pH 4.5. CMCase activity was determined using CMC (0.5% in acetate buffer pH 4.2) as the substrate which was incubated with appropriately diluted enzyme at 50°C for 30 min. For FPase assay, appropriately diluted cultural filtrate was added to Whatman No. 1 filter paper strip (1 × 6 cm) weighing approximately 50 mg followed by incubation at 50°C for 30 min. B-glucosidase activity was determined after incubation of appropriately diluted enzyme with salicin (0.5% in acetate buffer pH 4.2) at 50°C for 30 min. The reducing sugars released were determined using DNSA method [22].

H. *Optimization of parameters for production of cellulases*

As the solid state fermentation of wheat bran resulted in the highest enzyme productivities, the process parameters of solid state fermentation including the incubation temperature, substrate to moisture ratio, inoculum size, supplementation of various nitrogen sources, supplementation of carbon sources, surfactants, nitrogen sources and minerals were standardized for augmenting the cellulase yields by varying one factor at a time keeping other factors constant. The effect of all other factors was

evaluated by keeping the incubation period constant to 4 days of incubation at 28°C. Effect of temperature was studied by incubating the SSF flasks at different temperatures including 10, 20, 28, 30, 37, 45 and 55ºC. Effect of Inoculum size was studied by using the inocula with counts ranging from 10^7 to 10^{12} spores/ml. Effect of moisture content was studied by employing various substrate to moisture ratios of 1:0.5, 1:0.75, 1:1.0, 1:1.25, 1:1.5, 1:1.75, 1:2.0 and 1:2.25. The effect of carbon sources was studied by exogenous supplementation of various sugars and cellulosic residues including fructose, salicin, dextrose, starch, carboxymethycellulose (CMC), xylose, sucrose, lactose, maltose, composite, saw dust, baggasee, corn stover, wheat straw and rice husk, separately, at a concentration of 1% w/v. Meat extract, urea, soyabean meal, ammonium sulphate, yeast extract, sodium nitrate, malt extract, tryptone, peptone, ammonium chloride, calcium nitrate, Bovine serum albumin (BSA) and ammonium molybdate tetrahydrate were supplemented, separately at a concentration of 1% w/v to check the effect of various nitrogen sources. Copper sulphate, magnesium sulphate, zinc sulphate, ammonium chloride, tin chloride, manganeese sulphate, sodium dihydrogen orthophosphate, dipotassium hydrogen phosphate, potassium di hydrogen phosphate, ammonium sulphate, aluminium sulphate, cobalt chloride, calcium chloride, sodium chloride and sodium molybdate were supplemented, separately, at a level of 0.1% w/v to check effect of metal salts. Effect of various surfactants including Triton X-100, Tween 40, sodium dodecyl sulphate (SDS) and Tween 80 was analyzed by supplementing the same, separately, in the wheat bran based solid media at a concentration of 0.1% w/v.

III. RESULTS AND DISCUSSION

Agricultural and agro-industrial wastes act as substrates for growth of various microorganisms in nature. These have a potential for inducing the commercial production of a wide range of lignolytic, hemicellulolytic as well cellulolytic enzymes in view of their vast abundance. Use of lignocellulosic waste for production of cellulases has advantage of these being near zero cost substrate. The recalcitrant nature of lignocellulose prevents its degradation. Cellulases on the other hand are the enzymes produced by microbes that are capable of hydrolyzing cellulose present in lignocellulosic material. However, because of their high cost, there is a need to develop strategies for cheap production of cellulases.

Majority of studies for enzymes production have been conducted either on one type of fermentation or two in few cases. Production and optimization of cellulases from cheap sources is a way to reduce the cost and to improve the enzyme yield. In the current study we have analyzed three types of fermentations for production of cellulases by already available strain of *Aspergillus niger* RSO-1. The colony morphology of the strain revealed that it was whitish yellow during mycelia growth and got covered with mat of black spores with time. Microscopical examination on lactophenol cotton blue staining hyphae was septate and hyaline, biseriate phialides emerged from metulae. Based on colony morphology and 18S rRNA sequence analysis, it was confirmed as *A. niger* RSO-1. After selecting fermentation approach producing the highest enzyme yields, optimization of physio-chemical parameters was carried out.

SmF is first choice for industrial enzyme production due to ease of control but SSF and SF pose as low cost alternative [23]. During present study, production profile was analyzed in SSF, SmF and SF in form of time course of enzyme production. In solid state fermentation, maximum CMCase activity was observed after 5th day on RB with 3.21 IU/ml (Fig. 1 b). BGL activity was maximum on WB and RB in 50:50 ratio after 7th day with 4.39 IU/ml (Fig. 1h). FPase activity was found maximum after fourth day on RB with 1.57 IU/ml (Fig. 1e). In submerged fermentation maximum CMCase activity was observed after 7th day in WB + RB with 3.29 IU/ml (Fig. 1a). BGL activity was maximum on WB after 6th day with 1.49IU/ml (Fig. 1g). FPase activity was found maximum after sixth day on RB with 1.74 IU/ml(Fig. 1d). In SF maximum CMCase activity was observed after 5th day on RB with 2.66 IU/ml (Fig. 1c). BGL activity was maximum on WB after 9th day with 3.34 IU/ml (Fig. 1i). FPase activity was found maximum after 7th day on WB with 1.57 IU/ml (Fig. 1f). Also when NB supplemented with and without trace element solution was used with WB, production of cellulases was low. Time course studies for coproduction of cellulases peaked at different days but appreciable yields of all the three enzyme components were observed after 4th day of incubation in SSF using WB as substrate. The enzyme productivities corresponding to 2.9IU/ml,1.49 IU/ml and 3.29 IU/ml of CMCase, FPase and BGL respectively were obtained (Fig. 1). A study by Mrudula *et al.,* [23] has compared the production using SmF and SSF, the maximum production of cellulase was obtained after 72 h of incubation in SSF and 96 h in SmF. Production of high titres and specific activity in SSF for fungal culture in comparison to SmF [24] has been reported followed by SF [6]. In the present study SSF and SmF gave far better performance in comparison to SF. CMCase and BGL production was highest in SSF whereas FPase was found maximum in SmF. Among the WB, RB and SD; WB was found to produce good amount of cellulases and as its also readily available in the region, it was used for further studies. Reddy *et al.,* [25] has also compared the production of cellulase in SmF and SSF using RB and WB, singly and in combination. They were able to get 2.632, 2.478 and 2.984 U/mL of FPase on RB, WB and WB+RB respectively in SmF. In case of SSF FPase on RB, WB and WB+RB were 29.81, 25.2 and 32.18 U/gds respectively and suggested SSF as a better choice in terms of cost and yield. On the other hand, *A. heteromorphous* grown on wheat straw for 5 days of submerged fermentation at 30ºC has been reported to yield 3.2 IU/ml and 83

IU/ml, filter paper activity and CMCase respectively [26]. Acharya *et al*., [6] used saw dust for production of cellulases, but in our study we found no production of cellulases using saw dust as substrate. The absence of pre-treatment might be a probable cause for it. SSF using WB as substrate was selected in the present study for optimization studies using one factor at a time approach although good production was also observed in RB.

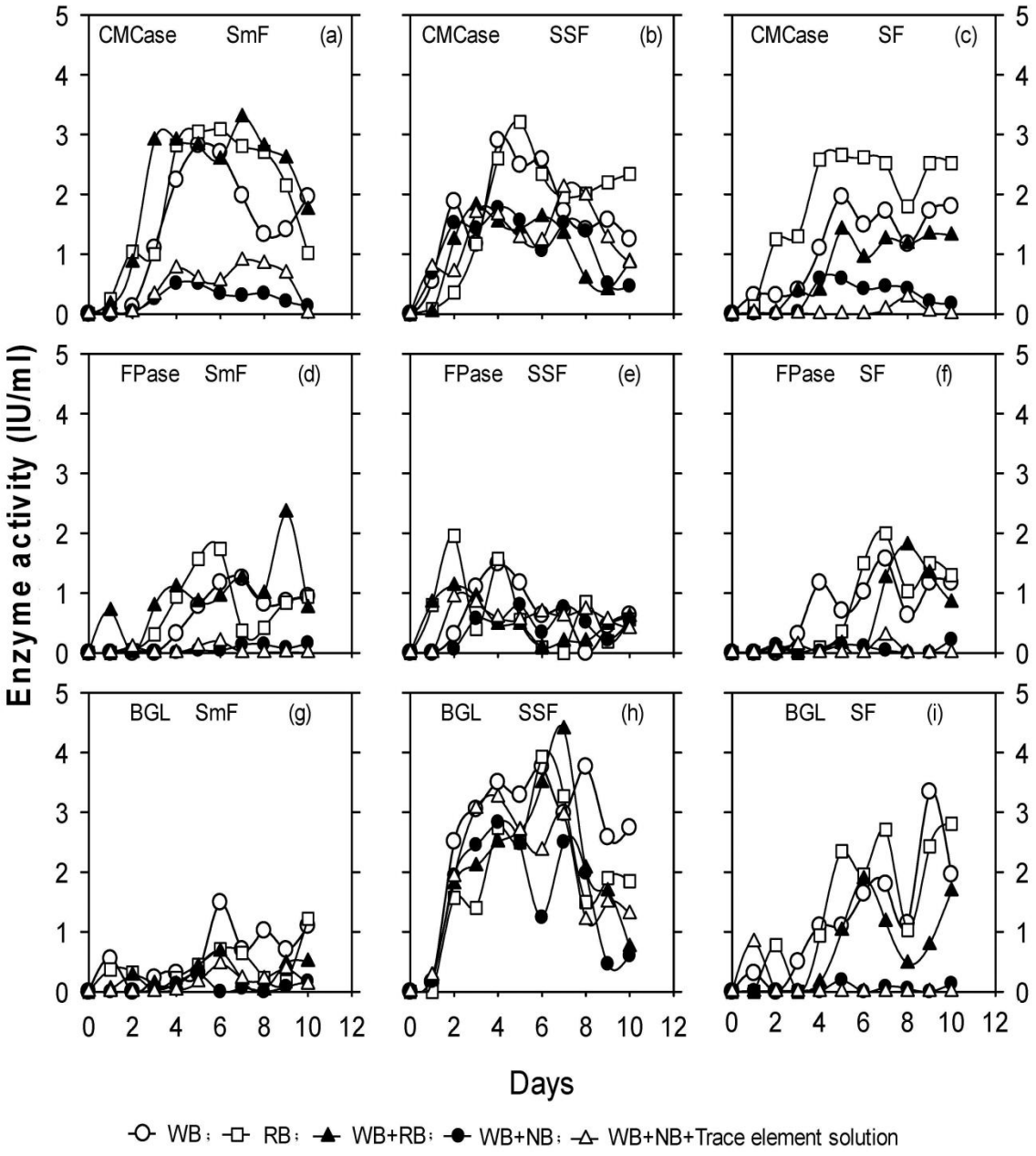

Fig. 1: Time course of CMCase (a, b, c) FPase (d, e, f) and BGL (g, h, i) production by *Aspergillus niger* RSO-1 in SmF (a,d,g), SSF (b,e,h) and SF (c,f,i**).**

Among various environmental parameters, incubation temperature plays important role in enzyme production and usually varies with strain. While low temperatures inhibit growth, at higher temperature cell membrane alteration leads to protein catabolism affecting production. Incubation temperatures in SSF normally range from 25 to 30ºC [27]. Optimal enzyme yield by *A. niger* RSO-1, in the present study, was also achieved at 28ºC but appreciable enzyme production was also observed at 30-45^{o}C (Table 1). Inoculum size of 10^{8} spores/ml was found to be the best for enzyme productivities (Table 1). The low yields at lower inoculum size were probably due to the availability of less number of cells to utilize medium efficiently while the low productivities at high inoculum size are perhaps due to nutritional imbalance created by high growth [28].

Table 1: Effect of temperature, moisture content, inoculum size and various supplements including surfactants, carbon sources, nitrogen sources and mineral sources on cellulase production by solid state cultures of *Aspergillus niger* RSO-1

Parameter	Enzyme activity (IU/ml)			Parameter	Enzyme activity (IU/ml)		
	CMCase	FPase	BGL		CMCase	FPase	BGL
Temperature (^oC)				**Nitrogen sources**			
10	0.3856	0.0761	0.1521	None	0.4853	0.1587	0.3908
20	0.4039	0.0826	0.2413	BSA	0.5325	0.1482	0.7843
28	0.5062	0.2085	0.7948	Meat extract	0.9049	0.3751	1.1620
30	0.5403	0.1285	0.7187	Urea	0.8446	0.3410	1.7941
37	0.4879	0.0931	0.5325	Soyabean meal	0.9207	0.4328	1.4033
45	0.4800	0.1207	0.5639	Ammonium sulphate	0.8918	0.4485	1.6315
55	0.0157	0.0879	0.0000	Yeast extract	0.9810	0.3712	1.4741
Substrate: moisture ratio				Sodium nitrate	0.9259	0.3659	1.4977
1.0:0.50	0.7371	0.2308	0.4564	Malt extract	0.9390	0.3371	1.2722
1.0:0.75	0.9207	0.3764	1.1699	Tryptone	0.9417	0.3003	1.4295
1.0: 1.00	1.0125	0.3856	1.1594	Peptone	0.9207	0.3646	0.0000
1.0:1.25	0.9233	0.4131	1.1909	Ammonium chloride	0.9889	0.3371	1.9043
1.0:1.50	0.9417	0.5377	1.4846	Calcium nitrate	0.8761	0.3266	1.7259
1.0:1.75	0.9705	0.4026	1.3377	Ammonium molybdate	0.8944	0.3253	1.0623
1.0:2.00	0.9023	0.4407	1.3640	**Mineral sources**			
1.0:2.25	0.9023	0.4328	1.3587	Copper sulphate	0.5535	0.1561	0.6426
Inoculum size (spores/ml)				Magnesium sulphate	0.5089	0.1521	0.6190
10^{12}	0.5220	0.2544	0.9653	Zinc sulphate	0.4695	0.1443	0.5508
10^{11}	0.5771	0.2649	0.8997	Ammonium chloride	0.4748	0.1875	0.6453
10^{10}	0.5718	0.2125	1.0046	Tin chloride	0.4826	0.0997	0.6846
10^{9}	0.5928	0.2702	1.0754	Manganese sulphate	0.4905	0.0800	0.3043
10^{8}	0.6321	0.2518	1.3404	Sodium dihydrogen orthophosphate	0.5220	0.1416	0.8105
10^{7}	0.5744	0.1810	0.7790	Dipotassium hydrogen phosphate	0.5194	0.1902	0.6636
Carbon sources				Potassium di hydrogen phosphate	0.5771	0.1666	0.8604
Fructose	0.7948	0.4223	1.0387	Ammonium sulphate	0.5167	0.1180	0.8367
Salicin	1.0335	0.5823	1.7574	Sodium sulphate	0.5403	0.2164	0.7292
Dextrose	0.8105	0.4735	0.9548	Aluminium sulphate	0.5246	0.1587	0.6767
Starch	0.8682	0.4669	1.0151	Cobalt chloride	0.5220	0.1810	0.6085
CMC	0.9364	0.5862	1.0518	Calcium chloride	0.5167	0.1141	0.6899
Xylose	0.9522	0.4512	1.0125	Sodium chloride	0.5115	0.1338	0.7135
Sucrose	0.8761	0.4276	0.8971	Sodium molybdate	0.4617	0.1430	0.4695
Lactose	0.8472	0.4249	0.9863	**Surfactants**			
Maltose	0.9023	0.4551	1.0308	Triton X 100	0.5194	0.1193	0.7581
Composite	0.8892	0.4695	1.0099	Tween 40	0.5849	0.2098	0.7790
Saw dust	0.9023	0.463	0.9154	SDS	0.4354	0.1980	0.1757
Bagasse	0.8604	0.4603	0.9574	Tween 80	0.5299	0.1797	0.6531
Corn stover	0.8997	0.4394	0.9810				
Wheat straw	0.9233	0.5430	1.0387				
Rice husk	0.9023	0.5272	1.1174				

Moisture content is dependent on the choice of substrate selected. Water holding capacity of a substrate decides initial moisture content to be used [29]. Low moisture content leads to reduced solubility of nutrients and high moisture content causes reduction in porosity and change in particle size [30]. It was found that optimum production of the enzymes in the present study was at substrate to moisture ratio of 1:1.5 (Table 1). Productivities were also appreciable at high moisture content but got reduced at low moisture content. Sources of essential carbon, nitrogen, phosphates, sulphates etc affect the production as cellulases are inducible enzymes [11], [31]. The CMCase productivity in the present study was enhanced by xylose, CMC, salicin, copper sulphate and KH_2PO_4. FPase was induced in presence of pectin, ammonium sulphate, soyabean meal, K_2HPO_4, salicin and CMC. BGL production was enhanced by urea, ammonium sulphate, ammonium chloride, calcium nitrate, ammonium sulphate and salicin (Table 1). Peptone and KH_2PO_4 are well known to enhance cellulase production [32], [33], [34]. It has been reported that ammonium compounds and peptone are also good inducers for cellulase production [35], [36]. Among the surfactants Triton X 100 and Tween 40 enhanced the production of cellulases(Table 1). This may be attributed to increase in availability of nutrients by reduction in surface tension and alteration in cell membrane [37]. The optimisation studies in the present study ultimately led to enhance the cellulase productivities by 1.46, 2.8 and 1.9 fold for CMCase, FPase and BGL respectively.

IV. CONCLUSION

The present study suggests WB to be a good substrate producing appreciable amount of all the three components of complete cellulase system by SSF of *Aspergillus niger* RSO-1. Co-production of these three enzymes may remove the need of adding them individually thus reducing the cost of cellulose hydrolysis. Further optimization, scale up studies and kinetic characterization of the complete cellulase system may be carried out before their commercial exploitation for the production of second generation biofuel and other value added components.

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