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Optimization of Cellulases and Xylanase Production by *Aspergillus niger* JD-11 under Submerged Fermentation

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Abstract

In this study, an attempt has been made to optimize the cultural and nutritional conditions for cellulases and xylanase production by *Aspergillus niger* JD-11 under submerged fermentation. The alkali pretreated banana leaf waste was used as carbon source, in the ratio of 1 to 4% (w/v), for cellulases and xylanase production. Whole fermentation process was carried out in 250mL Erlenmeyer flasks with agitation speed of 170rpm. The maximum titers of cellulases (FPase, CMCase, β-glucosidase) and xylanase were obtained on 5th and 4th day respectively when *Aspergillus niger* JD-11 was grown at initial medium having pH 6.0 at 30°C using 2.0% (w/v) banana leaf waste biomass as substrate.

Introduction

Substantial research efforts have been made for biochemical conversion of agricultural lignocellulosic biomass (LB) into ethanol. The general process for converting LB into bioethanol take in steps like feedstock pretreatment, enzymatic hydrolysis, sugar fermentation, separation of lignin residue, and the recovery and purification of ethanol to meet fuel specifications (Arslan and Eken-Saracoglu, 2010). However, the cost of ethanol production from LB is relatively high based on current technologies, and the major challenges are the low yield and high cost of the enzymatic hydrolysis process (Govumoni *et al.,* 2013) which is achieved by a sequence of reactions with the main components of cellulase complex enzymes [endoglucanases (EG), cellobiohydrolases (CBH), β-glucosidases (BGL)] responsible for converting cellulosic part into fermentable sugars. But, in annual plants and hardwoods, xylan, the most abundant non-cellulosic

polysaccharide accounting for 20-35% of the total dry weight in biomass (Elegir *et al.,* 1994), must also be hydrolysed in the presence of decomposing enzyme, viz., xylanase. Thus, the complex enzymes 'cellulases and xylanase' play crucial role in the degradation of lignocellulosic materials. Currently the cost of enzymes is also too high and research is continuing to bring down the cost of enzymes as in many bioconversion strategies, the cellulases required for biomass conversion may account for as much as 40% of the total process cost (Ahamed andVermette, 2008). Therefore, large-scale low cost production of cellulases is very important through microorganism selection and improved fermentation process conditions (Delabona *et al.,* 2012). Moreover, the ability of filamentous fungi to secrete large amounts of cellulolytic proteins has motivated their extensive use for the production of industrial enzymes (Domingues *et al.,* 2000).Among the cellulolytic fungi, *Trichoderma* spp. and *Aspergillus* spp. have been widely studied for their ability to secrete high levels of cellulose degrading enzymes (Zhou *et al.,* 2008). Hence, the filamentous fungus *Aspergillus* was envisaged to be one of the major agents of decomposition and decay for the present study owing to its potential to produce a broad range of enzymes including cellulases as well as xylanase (Pelizer *et al.,* 2007).

The production of cellulases and xylanase is greatly influenced by media components, especially carbon and nitrogen sources, minerals and physical factors such as pH, temperature and moisture (Lynd *et al.,* 2002). In order to obtain maximum enzyme production, development of a suitable medium and culture conditions is necessary. Although the use of expensive substrate is one of the major problems in cellulases production by fermentation but the choice of substrate for enzyme production ultimately governs the cost of production.

Reduction in the production cost and improvement in cellulases yield could also be achieved using appropriate and low cost carbon and nitrogen sources in the formulation of fermentation medium (Senthilkumar *et al.,* 2005). Therefore, the use of abundantly available and cost-effective agricultural by products must allow reduction of the overall production cost of the decomposing enzymes which could be resolved by isolating cellulolytic strains with high levels of cellulases as well as xylanase productivity, optimizing fermentation conditions while making use of cheaper agricultural and industrial substrates as carbon or nitrogen source inthe fermentation medium (Zhang and Sang, 2012; Irfan *et al.,* 2016). Hence, the optimization of all the process parameters was being considered as pre-requisite to make the process of enzyme production cost-effective at large scale and submerged fermentation process was preferably selected because of more nutrients availability, sufficient oxygen supply and less time required for the fermentation than other fermentation techniques (Gouda, 2000).

This study was aimed at evaluating growth conditions that affect the production of cellulases and xylanase by using *Aspergillus niger* JD-11 and optimizing their cultivation conditions under submerged fermentation (SmF) using alkali treated banana leaf waste as substrate.

Materials and Methods

Collection and pretreatment of substrate: *Musa paradisiaca*L. leaves (= banana leaf waste) were collected from new fruit and vegetable market, Rohtak city which is located at a latitude of 30°1`N and longitude of 75°17`E. The collected banana waste was washed with tap water and finally with distilled water and then oven-dried at 70°C till constant weight. Then the feedstock was grounded and its size reduced to pass through a 1.0mm sieve. The dried biomass was soaked in 0.1N NaOH in the ratio of 1:10 (w/v). The mixed slurry was incubated at 121°C for one hour. The solid residue was collected by filtration and washed extensively with tap water and finally with distilled water until pH became neutral. The pretreated biomass was dried at 70°C to constant weight and stored in airtight plastic bags at room temperature for further use. The alkali pretreated LB of banana waste, with particle size between 0.2 to 1.0 mm, was used as substrate for the production of cellulolytic enzymes in SmF.

Microorganism: The experimental microorganism *Aspergillus niger* JD-11 was isolated from soil samples, collected from Rohtak district, Haryana. The strain was cultivated and maintained on potato dextrose agar (PDA) at 4^0 C.

Inoculum preparation: Sterilized normal saline (10mL) containing Tween-80 (0.1% v/v) was added to five days old slants of fungal culture grown on PDA medium. The spores were scratched by sterile wire loop and shaken vigorously for preparing a homogenous spore suspension. A standard spore count was done using a hemocytometer. One milliliter of spore suspension containing $1x10^7$ spores was used as inoculum for submerged fermentation.

Fermentation Technique: Basal medium, composition described by Mandels and Sternburg's (MS) (1976), was used for submerged fermentation. The media contained (g/l) proteose peptone, 1; $(NH4)_2SO_4$, 1.4; KH_2PO_4, 2.0; urea, 0.3; $CaCl_2$, 0.3; $MgSO_4.7H_2O$, 0.3; $FeSO_4.7H_2O$, 0.005; $MnSO_4.H_2O$, 0.0016; $ZnSO_4.7H_2O$, 0.0014; $CoCl_2$, 0.002; Tween-80, 0.1 % (v/v), supplemented with 0.1% (w/v) glucose) and 1 to 4% (w/v) banana leaf waste as substrate. Then, 50mL of the liquid medium was added to 250mL Erlenmeyer flasks and autoclaved at 121^{o}C for 20 minutes. After sterilization, the media was allowed to cool and inoculated with 1.0mL spore suspension of the fungus containing approximately $1x10^7$ spores. The inoculated flasks were incubated at 30^{o}C for seven days in an orbital incubator shaker with the agitation speed of 170 rpm. The effect of different variables, viz., pH, incubation period, temperature and inducer substrate concentration and agitation speed were studied on cellulases and xylanase production by 'one variable at one time' approach.

Enzyme Extraction: One millilitre of the culture filtrate was withdrawn under aseptic conditions at desired intervals and centrifuged at 10,000rpm for 10 minutes at 4^{o}C to remove unwanted particles and spores. The supernatants obtained after centrifugation

were used as enzyme source and assayed for filter paperase (FPase), carboxymethyl cellulase (CMCase), β-glucosidase and xylanase.

Enzyme assay: The FPase and Carboxymethyl cellulase (CMCase) activities were measured according to IUPAC method of Ghose (1987). Xylanase and β-glucosidase assays were performed according to the method of Ghose and Bisaria (1987). The reducing sugar concentration was estimated by DNSA method (Miller, 1959).

Results and Discussion

Effect of pH on Enzymes Production: The effect of initial pH of culture medium on the cellulases and xylanase production by *Aspergillus niger* JD-11 under SmF was investigated using alkali pretreated banana leaf waste as substrate. A gradual increase in cellulases and xylanase production was observed with the increase in pH from 5.0 to 6.0. The highest titer of FPase (0.14 U/mL), CMCase (0.21 U/mL), β-glucosidase (0.30 U/mL) and xylanase (12.39 U/mL) was obtained when *Aspergillus niger* JD-11was grown in medium with initial pH of 6.0 at 30°C, 170rpm and using 1.0% (w/v) banana waste biomass as substrate (Figure 1). The decline in production of cellulases and xylanase was observed when pH of fermentation medium was further increased. Corroborative results were also obtained by many researchers, for example, Sarkar and Aikat, 2014 found that *Aspergillus fumigates* exhibited maximum cellulases and xylanase activity at 4.0 and 5.0 pH respectively, Knob and Carmona (2008) found maximum xylanase production by *Penicillium sclerotiorum* at pH 6.5 while Bakri *et al*. (2008) found highest xylanase production by *Aspergillus niger* at initial pH 7.0. This could have been owing to the change in morphology of microorganism at a particular pH which in turn might have affected the extracellular secretion of enzymes (Papagianni, 2004) as the cellulases and xylanase are inducible enzymes and induction efficiency of substrates depends on their chemical composition and morphological structure.

Effect of Incubation Period on Enzymes Production: *Aspergillus niger*JD-11 was inoculated into MS basal medium (pH 6.0) in 250mLconical flasks and incubated at 30°C and 170rpm for a period of six days. Figure 2 shows the effect of incubation period on FPase, CMCase, β-glucosidase and xylanase production at regular intervals. The FPase, CMCase, β-glucosidase and xylanase activity (U/mL) on banana waste ranged from 0.08 to 0.29, 0.09 to 0.31, 0.11 to0.47 and 7.09 to 12.31 respectively. Maximum activity (U/mL) of FPase, CMCase and β-glucosidase on banana waste on 5^{th} day was found to be 0.29, 0.31 and 0.47 at pH 6.0, 170 rpm and 30°C. The maximum activity of xylanase (12.31 U/mL) was obtained on 4^{th} day of fermentation period at pH 6.0, 170rpm and 30°C. The maximum production of cellulases (FPase, CMCase and β-glucosidase) and xylanase on 5^{th} and 4^{th} day respectively declined slowly thereafter (Figure 2). Corroborative results were obtained by many researchers like Ahmed *et al*. (2009) could achieve maximum production of FPase, CMCase and β-glucosidase from *T. Harzianum* at 120 hours while Kang *et al*. (2004)and Dedavid *et al*. (2008)obtained maximum

cellulases production from *A. niger* and *A. phoenix* respectively at 120 hours incubation period and Shah and Madamwar (2005) reported maximum xylanase production from *Aspergillus foetidus* on the fourth day while maximum xylanase Production by *Penicillium sclerotiorum* during 5 days was reported by Knob and Carmona (2008). Substantiating the results, other researchers found different incubation times for maximum cellulases production as the time-course required to reach maximum level of cellulases and xylanase activity might be affected by several factors, including the presence of different ratios of amorphous to crystalline cellulose (Ogel *et al*., 2001).

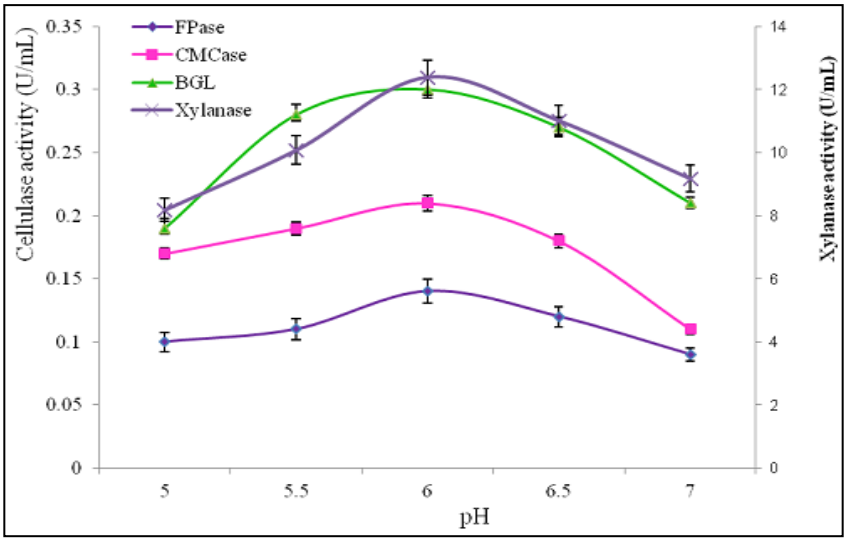

Figure 1: Effect of pH on cellulases and xylanase production by *Aspergillus niger* JD-11 under SmF

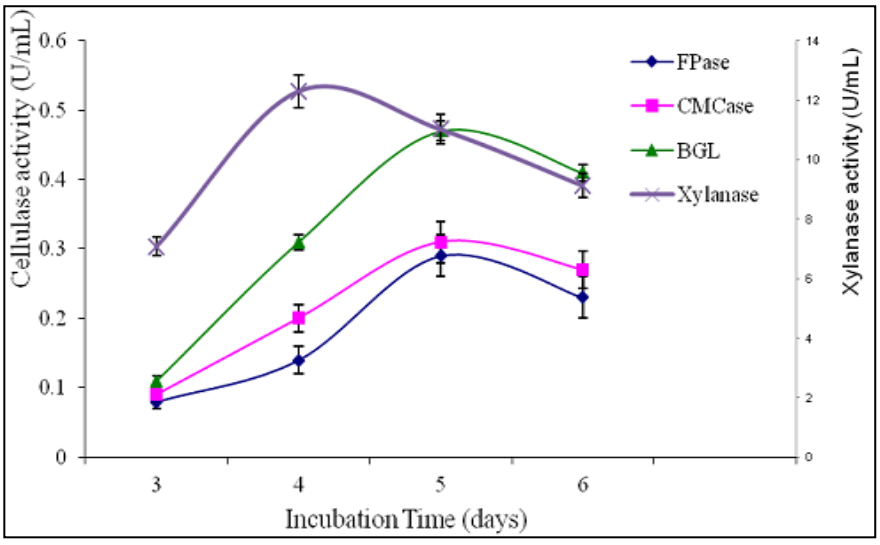

Figure 2: Effect of incubation time on cellulases production by *Aspergillus niger* JD-11 under SmF

Effect of Temperature on Enzymes Production: Incubation temperature is a very important physical factor which influences the metabolic activities of microorganisms. The effect of temperature on cellulases production was determined by incubating the culture flasks of *Aspergillus niger* JD-11 at different temperatures of 27, 30, 33, 37 and 40°C. The initial pH of fermentation medium was adjusted to 6.0 and culture flasks were incubated at 170 rpm. Figure 3 shows the effect of different incubation temperatures (27-40°C) on cellulases production by *Aspergillus niger* JD-11 on banana waste. The FPase, CMCase, β-glucosidase and xylanase activities of *Aspergillus niger* JD-11 on banana waste were found to be in the range of 0.17 to 0.29, 0.11 to 0.31, 0.13 to 0.47 and 6.13.16 to 12.31 U/mL respectively.

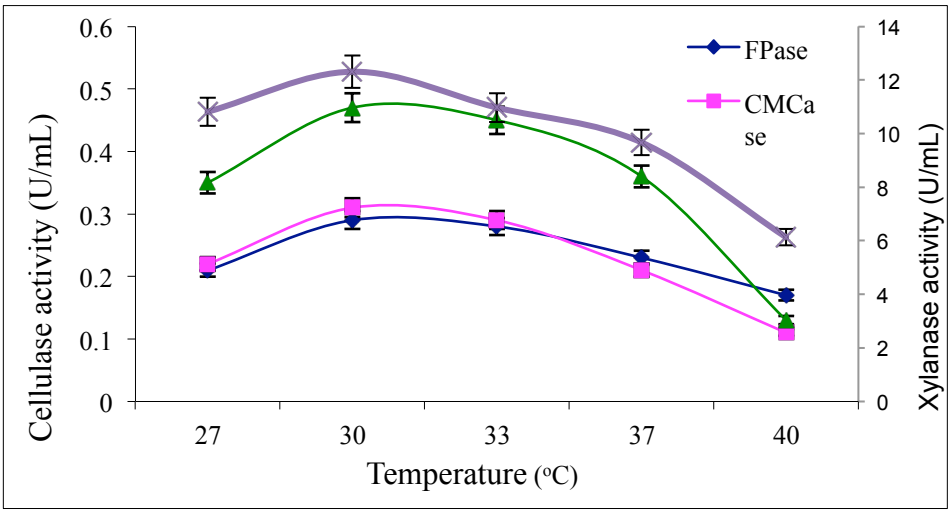

Figure 3: Effect of temperature on cellulases and xylanase production by *Aspergillus niger*JD-11 under SmF

The cellulases production gradually increased when incubation temperature increased from 27 to 30°C. Further increase in incubation temperature above 30°C resulted in decreased production of cellulases and xylanase. The maximum activity of FPase, CMCase and β-glucosidase was found to be 0.29, 0.31 and 0.47 U/mL respectively on 5th day at 30°C. Similarly the maximum activity of xylanase 12.31 U/mL was observed at 30°C but on 4th day. Contrary to the above results, different cellulolytic fungi were found to produce maximum cellulases at 28°C, for example, *T. Viride* (Zhou *et al*., 2008),*T. Harzianum* (Ahmed *et al.,* 2009), *T. Asperullum* (Bara *et al*., 2003), and *A. niger* (Kang *et al*., 2004) yielded maximum cellulases at 28°C. On the other hand, Deshpande *et al.* (2009) reported maximum cellulases production by *T. Reesei* at 30°C while Sarkar and Aikat (2014) found maximum cellulases and xylanase activity by *Aspergillus fumigates* at 30°C. However, as many researchers had reported on variety of optimum temperatures, it could be suggested that the optimal condition for cellulases production depended not only upon the variation of the microorganism (Murao *et al.*, 1988) but also the temperature variation of the culture conditions.

Effect of Substrate Concentration on EnzymesProduction: In the present study, the alkali pretreated banana leaf waste was used as carbon source for cellulases and xylanase production in the ratio of 1 to 4% (w/v). The initial pH of medium was adjusted to 6.0 and culture flasks were incubated at 30°C and 170rpm. Figure 4 represents the cellulases and xylanase production by *Aspergillus niger*JD-11 at varying concentrations of banana waste under SmF. The results revealed that the maximum activity of FPase, CMCase, β-glucosidase and xylanase were found to be 0.32, 0.33, 0.51 and 13.47 U/mL respectively at 2% substrate concentration. Further increase, >2%, in substrate concentrations resulted in decreased cellulases and xylanase production. The lowering of cellulases yield with increase in substrate concentration might be due to the adsorption of cellulases on the surface of substrate and its resistance to mass transfer (Soni *et al*., 1999).

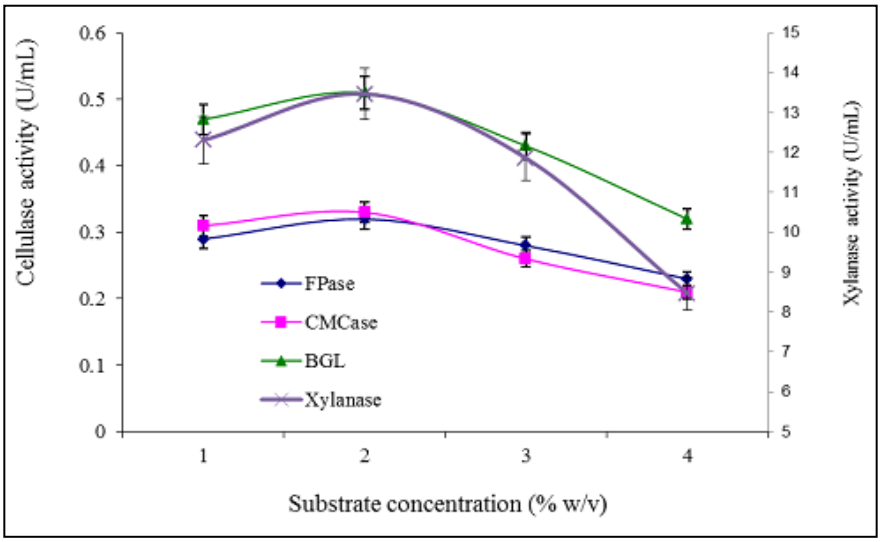

Figure 4: Effect of substrate concentration on cellulases and xylanase production by *Aspergillus niger* JD-11

A study by Parry and Slater (1984) also suggested that increasing the substrate concentration did not improve the level of cellulases activities due to the enzyme inactivation as cellulases production was regulated by the induction and repression mechanism. Moreover, increase in the percentage of substrate concentration also reduced the porosity of the medium and subsequently decreased the oxygen transfer rate (Gomathi *et al*., 2012). However, Gautam *et al*. (2010) reported the maximum production of cellulases at 4% substrate concentration under SmF by *T. viridae* but Deshpande *et al.* (2009) reported a comparable cellulases production in the culture media with 1.0 to 4.0% substrate concentration and decrease at 6.0% and above by *T. reesei.* Ibrahim *et al*. (2013) studied the cellulases production by *T. asperellum* and *A. fumigates* by using pretreated oil palm empty fruit branch as a substrate in the range of 0.5–2.0% and found the maximum activity at 1% substrate concentration. The fungal strain *T. atroviridae*676 isolated from Amazon forest soil (Brazil) by Grigorevski-Lima *et al*. (2013) was found to

produce maximum cellulases and xylanase when grown in medium containing 3% (w/v) sugarcane bagasse at 30°C. Likewise, Gomathi *et al*. (2012) found maximum production of CMCase by *A. flavus* under SmF using 4% wheat bran as substrate.

Effect of Agitation on Enzymes Production: Agitation plays an important role in determining the fungal morphology as well as physiology which in turn affect the cell growth and influence enzyme production. The culture flasks with 2% (w/v) substrate concentration and initial medium pH 6.0 were incubated at 30°C. The effect of agitation on cellulases and xylanase production was evaluated by incubating the flasks at 70, 120, 170 and 220 rpm. Figure 5 exhibits that the maximum activity of FPase 0.32 U/ml, CMCase 0.33 U/mL, β-glucosidase 0.51 U/mL on 5th day and xylanase 13.47 U/mL on 4th day were obtained at 170 rpm (Figure 5). The cellulases and xylanase production was significantly decreased at higher or lower agitation.

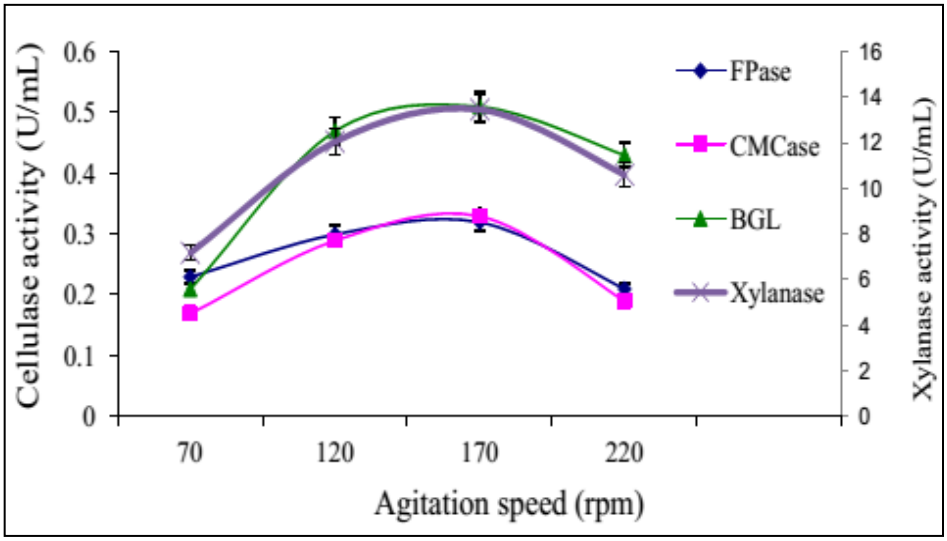

Figure 5: Effect of agitation on cellulases and xylanase production by *Aspergillus niger* JD-11 under SmF

As shown in Figure 5, the decrease in cellulases production was observed at higher or lower agitation other than 170rpm. Likewise, Matkar *et al.* (2013) reported maximum production of cellulases by *A. sydowii* under SmF at 120rpm while Grigorevski-Lima *et al*. (2013) carried out SmF for cellulases and xylanase production at 200rpm and obtained maximum activities of CMCase 1.9 U/mL, FPase 0.25 U/mL, β-glucosidase 0.17 U/mL and xylanase 61.3 U/mL by using *T. atroviridae*676. Similarly, Ibrahim *et al.* (2013) carried out submerged fermentation at 150rpm and 0.8 U/mL of FPase, 24.7 U/mL of CMCase and 5.0 U/mL of β-glucosidase were produced by *T. asperellum* and 1.7 U/mL of FPase, 24.2 U/mL of CMCase and 1.1 U/mL of β-glucosidase were produced by *A. fumigatus*. Although, Gomathi *et al.* (2012) carried out SmF at 125rpm using *A. Flavus* and achieved maximum CMCase activity of approximately 12 U/mL but Sarkar and Aikat (2014) reported maximum production of

cellulases and xylanase by *Aspergillus fumigates* at 150 rpm. The effect of agitation on enzyme production could have occurred owing to its role not only in uniform distribution of nutrients and proper aeration of culture medium but also determining the fungal morphology influencing the rheology and cell growth (Matkar *et al.*, 2013).

Conclusions

There is a growing demand for cellulases and xylanases in the market for a variety of applications, among which the bioconversion of lignocellulosic biomass for ethanol production is the foremost one. The results of present study revealed that *Aspergillus niger* JD-11 gave maximum production of FPase 0.32 U/mL, CMCase 0.33 U/mL, β-glucosidase 0.51 U/mL on 5thday and xylanase 13.47 U/mL on 4th day at 2% (w/v) substrate concentration, 30°C, 170 rpm and initial pH 6.0 of the basal medium under SmF. Use of low cost banana waste as substrate make the process of enzyme production more cost effective. Further investigations are required to enhance the cellulolytic enzyme activity by means of carbon and nitrogen sources from submerged fermentation.

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Authors contributions: *Dr. Meenakshi Suhag (Assistant Professor) wrote the manuscript on her Ph.D. work; she is also cooresponding author of manuscript and Dr. Joginder Singh (Associate Professor) helped in designing and Mr. Anil kumar (Assistant Professor) in editing of the manuscript.*

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