JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

# OPTIMIZATION OF CELLULASE PRODUCTION FROM *Apergillus flavipes* BY SUBMERGED AND SOLID STATE FERMENTATION

*Dina S.*[1*], *Thankamani V.T.*[2], *Emilia Abraham T.*[3]

***Address(es):***
[1] Scientific Research Solutions[1], Karnataka, India.
[2] Department of Biotechnology, University of Kerala, Trivendram, India.
[3] National Institute for interdisciplinary Science and Technology (CSIR)Thiruvanathapuram-695019.

*Corresponding author: drsdina@yahoo.co.in

https://doi.org/10.55251/jmbfs.4754

ARTICLE INFO

Received 11. 5. 2021
Revised 18. 1. 2023
Accepted 19. 1. 2023
Published 1. 4. 2023

Regular article

OPEN ACCESS

**ABSTRACT**

Cellulase are important class of enzymes that has potential applications in textile, biofuel, food and feed industry. Fungi such as *Aspergillus*, *Trichoderma* are reliable sources of cellulase production. Optimisation of multiple parameters is required for efficient cellulase production in high titers in both SmF and SSF. Carbon sources such as lactose, maltose, cellulose, nitrogen sources including ammonium dihydrogen phosphate and beef extract enhance cellulase activities. Thiamine, Nicotinic acid and all metal chlorides except $CuCl_2$ enhanced cellulase production. Based on the one factor at a time model, optimum medium was designed which produced cellulase of 0.767 FPU/ml by SmF. Maximum CMCase, FPase activity and protein were produced at pH 4.5 at 150 rpm and with a working volume ratio (wvr) of 0.2. The production of cellulase was 4.6 fold higher than the un-optimized media. SSF is an ideal fermentation method for cellulase production by fungi. Maximum CMCase, FPase and pNPG activities were recorded at 50% moisture level, pH 5.0 and below 40 °C. Wheat bran acted as a best carbon source with higher FPase, pNPG and CMCase (5.6 IU/g, 17 IU/g and 14 IU/g carbon source) activities. *A. flavipes* thus acts a perfect fungal source for cellulase production both by SmF and SSF.

**Keywords:** Cellulase, solid state fermentation, submerged fermentation, *A. flavipes*, endoglucanase, exoglucanase, β-glucosidase

## INTRODUCTION

Cellulases are important biocatalyst having applications in textile (wet processing as biopolishing agents), food and feed processing (eg.,cellooligosaccharides), biofuel production (lignocellulosic ethanol) and enzyme based deinking of fibers. Proteases, amylases and cellulases are the widely used enzymes in the industry **(Bajaj and Mahajan, 2019)**. A wide range of aerobic microorganism including *Bacillus*, *Pseudomonas*, *Cellulomonas* or fungi such as *Aspergillus*, *Penicillium*, *Cladiosporium*, *Fusarium* are used to metabolize complex carbohydrates to produce simpler molecules. Anaerobic microbes such as *Fibrobacter*, *Clostridium* and *Ruminococcus* also produce cellulases **(Behera and Ray, 2016)**. Cellulases from *Trichodermareseei* are widely used for biofuel-cellulosic ethanol production **(Bischof *et al.*, 2015)**. Species belonging to *Aspergillus* are important producers of cellulase in industrial sector. Largely enzymes are used in its crude form, since the substrates need to be cleaved by a complex of enzymes. For instance, lignocellulosic biomasses the cheaper sources for biochemical and biofuel industries that are cleaved by a cocktail of enzymes such as exoglucanases, endoglucanase, β-glucosidases, xylanases, ligases and laccases **(Bajaj and Mahajan, 2019; Vieira *et al.*, 2021)**. Cellulases hydrolyze cellulose at the 1,4-β-D-glucan linkages to produce cellobiose, other cellooligosaccharides (cellohexaose, cellotetrose etc.,) and glucose. Cellulases by themselves are composed of exoglucanase, endoglucanase and β-glucosidases. Endoglucanases primarily act on the endo β,1-4 bonds of complex cellulose to release reducing and non-reducing ends that are acted upon by exoglucanases to liberate cellooligosaccharides, including cellobioses. β-Glucosidases finally act to hydrolyze cello-oligosaccharides into glucose molecules **(Sukumaran *et al.*, 2021)**.

Lignocellulosic materials have varying amount of cellulose percentage and varying degrees of crystallinity and recalcitrant nature which hampers the bioconversion process and pretreatment of these substrates also make the process expensive. A thorough knowledge of factors influencing production and stability of enzymes is required. For the commercial exploitation of cellulases, production of high titres of the enzymes, screening for better microorganism, critical optimization strategies and genetic engineering, which intern helps in cost effective production and application. Large insight is being laid on production of cellulases that are thermo stable, active at different ranges of pH and harsh conditions. Also enzyme systems that can complement and work in synergism with enzyme systems from other organisms would facilitate the bioconversion of lignocellulasesThe current study aims to understand the potential of a novel cellulolytic strain, *Aspergillus flavipes* isolated in our laboratory(Dina etal 2021) for bioconversion and to ascertain the culture parametres that influence the production of cellulases under submerged and solid state fermentation process. Nutritional and culture parameters are optimized to enhance cellulase production from *A. flavipes*.

## MATERIALS AND METHOD

Czapekdox medium, potato dextrose agar,Sabourad dextrose agar (SDA), Malt extract agar and potato carrot agar(PCA), pure cellulose used were microcrystalline cellulose from Lobo, CMC(Aldrich) and cellodextrins

### Microorganism

The strain was isolated from soil containing decaying paper waste from the campus of National Institute for Interdisciplinary Science and Technology (NIIST) Trivandrum. This was identified as *Aspergillus flavipes* in our laboratory **(Dina *et al.*, 2021).**

### Influence of type, age and concentration of inoculums on submerged cellulase production

The effect of different types of inocula (slant, mycelia mat and a seed inoculum) on cellulase production was analyzed.Spores from the slant were brought into suspension using distilled water containing 0.1% tween 80. For raising seed inoculum of 10 ml, 9ml of the fermentation media was inoculated with 1 ml of spore suspension. The suspension was filtered using a sterile funnel filled with glass wool to separate mycelia. Seed cultures of different ages 48, 72 and 96 hours and subsequently inoculated. The spore count was taken using a Neubeur's counting chamber to get a spore suspension of $10^6$, $10^7$ and $10^8$/ml concentrations and cultured for 72 hours. Fungus was repeatedly sub cultured on different slants for three months (potato sucrose agar (PSA), potato dextrose agar (PDA), potato carrot agar (PCA), sabourad dextrose agar (SDA) and malt extract agar (MEA)) and analyzed for cellulase production. All the different types of inocula were suspended in 100 ml of media in 500 ml flasks and fermentation was carried out for up to 72 hours at an agitation speed of 110 rpm in an orbital shaker.

**Effect of physical parameters on submerged cellulase production**

Seed cultures of $10^7$/ml spore concentration cultured for 72 hour on PCA slants were inoculated into fermentation flasks. One factor at a time model was applied, where the medium was maintained at different pH 3.5, 4.5, 5.5, 6.5, 7.5 and 10. Different agitation speeds of culture flasks at 50,100,150 and 200 rpm were also optimized. The effect of aeration was studied by varying the working volume ratio (wvr). Medium volumes of 50,100,150 and 200 ml media were taken in 250 ml flasks which correspond to wvr's of 0.2,0.4, 0.6 and 0.8.

**Effect of carbohydrate sources on submerged cellulase production**

The effect of cellulose and sugars like glucose, fructose, lactose, maltose,xylose, alcohols such as mannitol, sorbitol and starchy material maltodextrin were also studied. Carboxymethyl cellulose (CMC) was used at 1,2,3 and 4%. Before the enzyme assay, the samples of high initial concentration of CMC were dialysed overnight in 100 volumes of 0.05 M citrate buffer pH 4.9.

**Effect of nitrogen sources on cellulase production**

Effect of organic nitrogen sources urea, yeast extract, beef extract and peptone at 0.2% concentrations on the three enzymes produced were studied. Beef extract was also tried at different concentrations (0.1, 0.2, 0.3, 0.4, 0.5 and1%). Inorganic compounds like ammonium dihydrogen phosphate ($NH_4H_2PO_4$),sodium nitrate ($NaNO_3$), ammonium nitrate ($NH_4NO_3$), potassium nitrate ($KNO_3$) ammonium sulphate($(NH_4)_2SO_4$) were used at 0.05M concentration. A combination of beef extract and urea (combination I); beef extract, urea and ammonium dihydrogen phosphate (combination II) and beef extract, urea and ammonium sulphate (combination III)were also compared.

**Effect of metal ions and growth factors on cellulase production**

The effect of metal ions (manganese, cobalt, copper, nickel, zinc and calcium) was studied by using their respective chlorides at 1mM concentration in the fermentation media. The role of growth factors biotin, nicotinic acid and thiamine at 0.02% concentration and cysteine HCl at 0.01% on cellulase production was studied.
Optimum parameters generated from one factor at a time model were combined and cellulase production was studied for the optimized media.

**Solid state fermentation**

The medium was the same as that of the optimised medium used in submerged fermentation. The fermentation was carried out at 30± 2 ºC in sterile 250 ml conical flasks. The substrate was dewaxed cotton. At an interval of 24 hours the each flasks were harvested up to 96 hrs. Enzyme extraction was carried out using citrate buffer of 0.05M, pH 5 containing 0.1% Tween 80. The cotton was squeezed using a glass rod. The extract was then centrifuged at 5000 rpm for 15 minutes. Enzyme assay was done according to standard methods using FP, CMC and pNPG. The enzyme activity is expressed as IU/gm dry weight of the substrate. Dry weight was found out after drying the contents at 110ºC till constant weight was obtained. The total soluble protein was estimated by Lowry method. Inoculum was a spore suspension of concentration $10^8$/ml prepared from a six day old PSA slant.

**Effect of solid to liquid ratio**

The moisture content was varied to 50 %, 60 % and 70% by varying the ratio of substrate medium ratio. The flasks were shaken thoroughly. After inoculation the flasks were incubated at ambient (30± 3 ºC) temperature up to 96 hours. The growth profiles from 24 to 96 hours were studied and parameters like total soluble protein, dry weight, enzyme activity on FP, CMC and pNPG were found out.

**Incubation temperature**

The different incubation temperatures were 20± 5, 30± 3 and 40 ºC. The pH of the medium was 5 and initial moisture level was 50%. The fermentation was carried out as above.

**Initial pH of the medium**

The pH of the medium was adjusted using 1N HCl and 1N NaOH, prior to autoclaving. The different pHs studied were 4.0, 5.0 and 6.0

**Carbon sources**

The different native substrates like cotton, rice straw wheat bran and green gram hull were dried and powdered. The initial moisture level determined and was sieved through a 125 µm mesh. The initial moisture content was adjusted to 60% and the pH of the medium to 5 and the fermentation temperature was ambient.

The culture filtrate after fermentation was used to calculate cellulase activity (Filter Paper Units (FPU)/ml), the endoglucanase and β-glucosidase through pNPG activity was calculated. Enzyme assay was done according to standard methods using FP, CMC and pNPG. The enzyme activity is expressed as IU/gm dry weight of the substrate. Dry weight was found out after drying the contents at 110°C till constant weight was obtained. The total soluble protein was estimated Lowry method (Gokhale *et al*., 1991).
Statistical analysis: All data were recorded in Microsoft excel sheet. All the experiments were done in triplicates and mean, standard deviation were calculate.

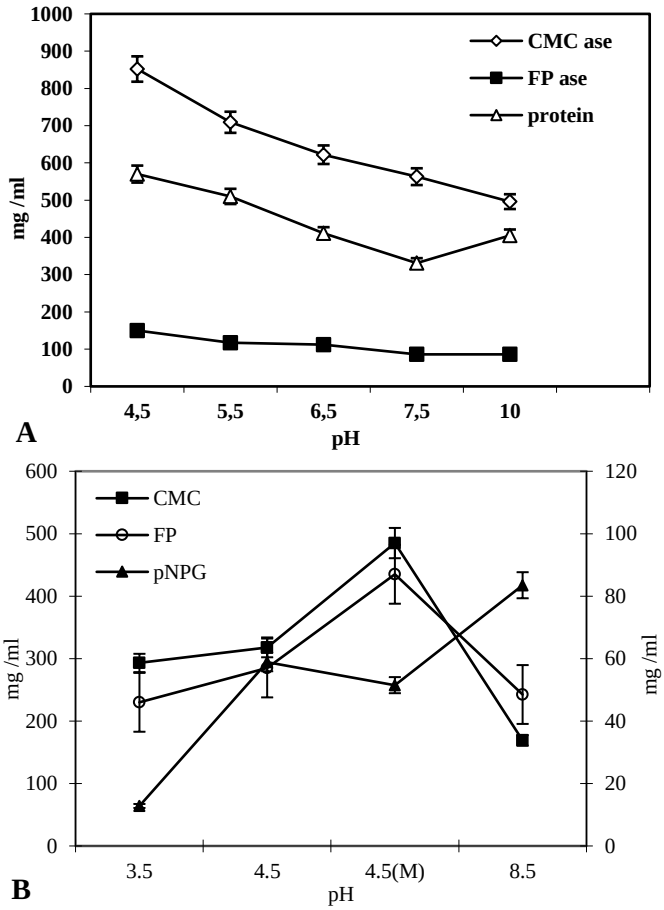
**RESULTS**

**Influence of type, age and concentration of inoculum**

The results showed maximum endoglucanase and filter paper activity in the flasks inoculated with 72 hour old cultures. The 96 hour seed culture produced a very small amount of biomass and enzyme activity was negligible. Spore concentration of $10^7$/ml was the best inoculum, as the endoglucanase activity was increased 1.7 times and filter-paper activity by 2.67 fold compared to $10^6$/ml spore inoculum (Table 1). Five different medium (potato sucrose agar (PSA), potato dextrose agar (PDA), potato carrot agar (PCA), Sabourad dextrose agar (SDA) and malt extract agar (MEA) were tested for enzyme production and their relative activity compared to inoculum from PCA slants. The relative activity was PSA 67%, PDA 80%, SDA 42 % and MEA 60 %.

**Effect of physical parameters on submerged cellulase production**

The final pH of the media was found enhance by 0.5 units in cultures maintained at pH 3.5, 4.5 and 5.5 whereas in pH 6.5 and 7.5, no modifications were noted. The final pH reduced to 9.0 in cultures maintained at pH 10 after 72 hours. Maximum CMCase, FPase activity and protein was produced at pH 4.5 (Fig 1A). In the above experiments, the culture pH was not maintained. In a separate experiment, higher levels of enzyme and protein were observed when the pH was maintained at 4.5 throughout the fermentation process (Fig 1B). β-glucosidase activity was found to be higher at initial growth pH 8.5 and when culture was maintained at pH 8.5. The enzyme activities in the cultures increased progressively as the agitation was increased up to 150 rpm, but decreased at 200 rpm (Fig. 1C). A reduction of 22.8 % in activity was observed. An agitation speed of 150rpm was the optimum for enzyme production. The effect of aeration was studied by varying the culture volumes at constant agitation speeds, where the smallest working volume ratio 50 ml culture volume to 250 ml (volume of flask) was found to be the best (Fig. 1D).



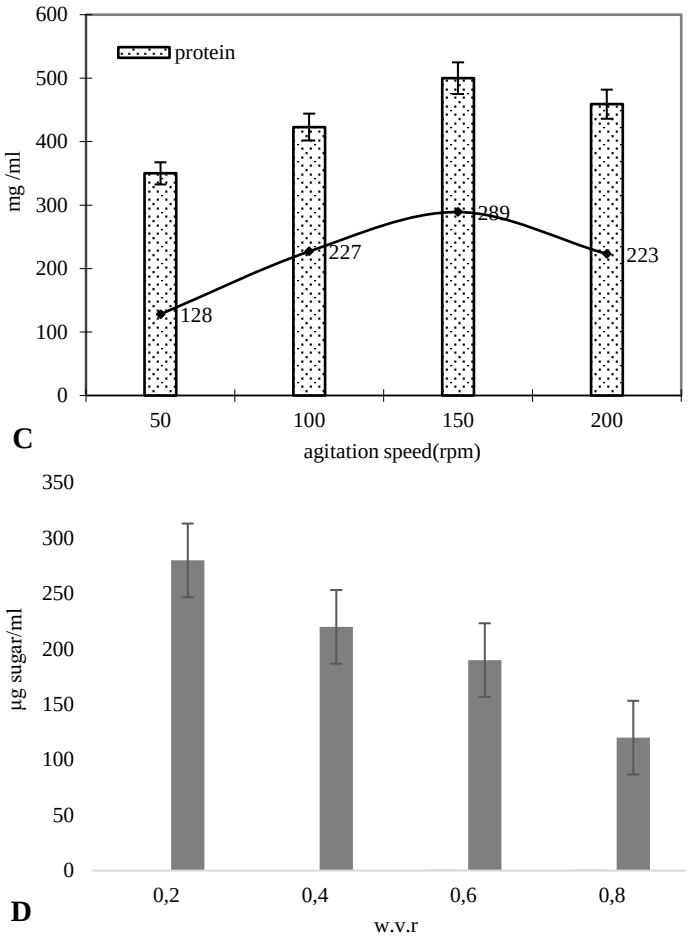


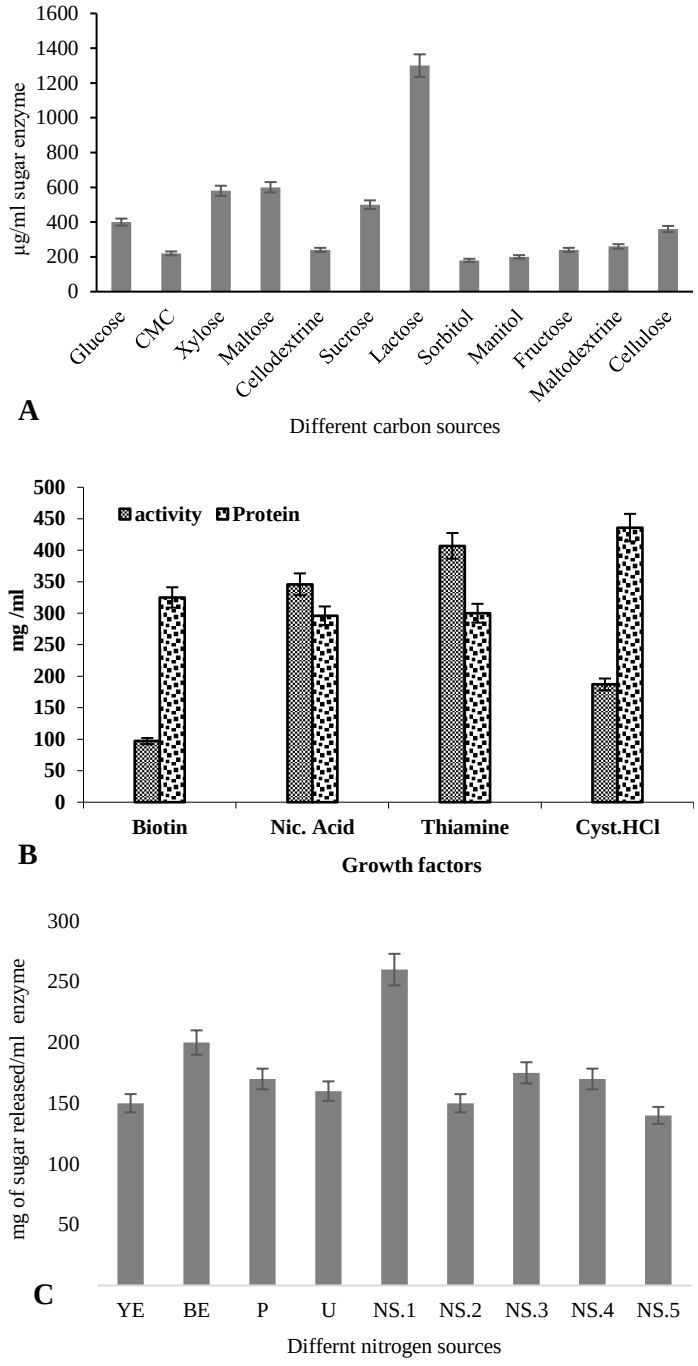

**Figure 1** Effect of physical parameters on submerged cellulase production

**A. Effect of initial growth PH:** The enzyme activity is expressed as µg sugar released/ml enzyme during the assay period and total soluble protein as µg /ml. The pH was not maintained throughout the fermentation. The final pH was also recorded.
**B. β-glucoasidase activity:** The reducing sugar released as µg /ml of enzyme is given on the Y axes. pNPG activity is given as µgpNP released/ml enzyme on the secondary Y axis.
**C. Effect of agitation:** The enzyme activity is expressed as µg sugar released/ml enzyme and the soluble protein produced as µg protein/ml culture filtrate.
**D. Effect of aeration**The working volume ratios are calculated as ratios of culture volumes to the volume of culture flask. The enzyme activity is expressed as µg reducing sugar released/ml enzyme.

**Role of medium components on submerged cellulase production**

Among the different carbohydrate sources studied for efficient cellulase production, lactose produced more cellulase followed by maltose, xylose and sucrose (Fig 2A). Due to hydrolysis the reducing sugar in the medium increased with higher initial concentration of CMC. Up to 2.3 % CMC concentrations both enzyme activities were uninhibited.
Nitrogen sources enhanced cellulase production visualized through higher activity (Fig 2C), where ammonium dihydrogen phosphate (NS1) followed by beef extract was superior. The other nitrogen sources ammonium nitrate (NS3), potassium nitrate, ammonium sulphate and peptone enhanced the cellulase activity. Beef extract at 0.1% was better nitrogen source than at higher concentrations (Fig 2D). The combinations yielded cellulase showing higher enzyme activity, where combinations of beef extract, urea and ammonium dihydrogen phosphate was better (Table 2).
Thiamine and nicotinic acid enhanced the enzyme activity and cysteine HCl enhanced the protein concentration (Fig 2B). Copper reduced the enzyme activity with enhanced protein concentration. The other metal chlorides enhanced both enzyme activity and protein concentration (Fig 2E). Optimum parameters generated from one factor at a time model of optimization were combined. CMC was replaced by a combination of 1% lactose and 1% cellulose, nitrogen source combinations (beef extract 0.1%,urea 0.05 M and ammonium dihydrogen phosphate 0.14%).The metal ions ($CaCl_2$, $MgSO_4$, $FeSO_4$, $CoCl_2$, $MnSO_4$, $ZnCl_2$) which had positive effects were included in the medium. These metal ions were incorporated in the same concentrations as those given in the Reese and Mandels medium for *T. viride*.

Thiamine and nicotinic acid were also added as positive growth factors. The culture filtrates of the above optimized medium yielded 0.767FPU/ml. This was 4.6 times more than the initial medium prior to optimization. Cellulase production by *T. reesei* Q9414 on 10 g/l of lactose, cultured for 336 hours and yield of 0.31 FPU/ml with a productivity of 0.92 FPU/ml/h was observed. In comparison, *A. flavipes* on 10g/l of lactose, cultured for 144 hours yielded 0.225 FPU/ml and productivity of 1.56 FPU/ml/h.



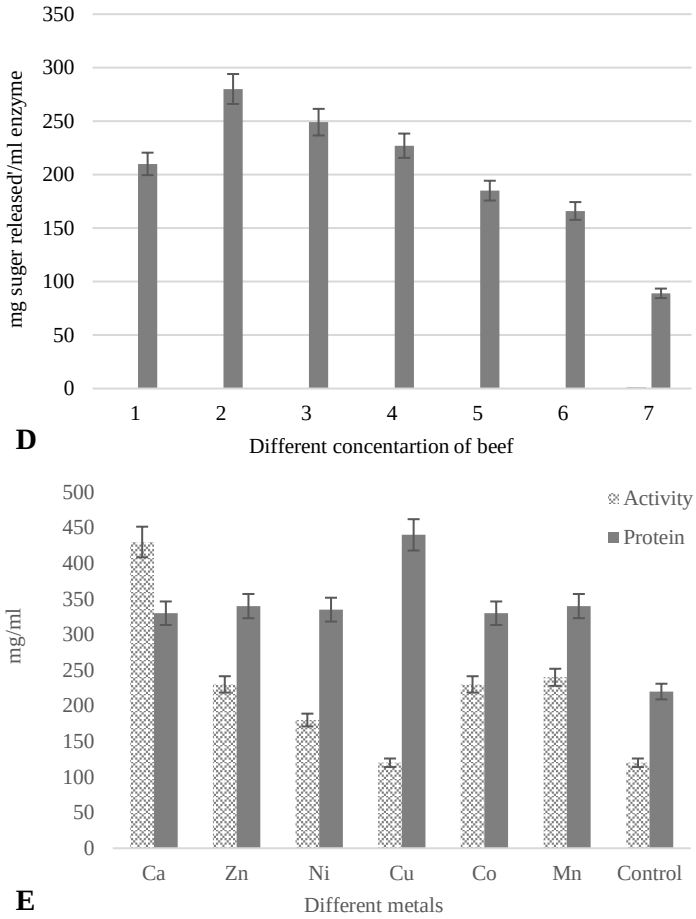



**Figure 2** Effect of nutritent components on submerged cellulase production. **A. Role of Carbon sources:** The different carbon sources like glucose, CMC, xylose, maltose, cellodextrin, sucrose, lactose, sorbitol, mannitol, fructose, maltodextrin and cellulose were used at 1% concentration in the medium. **B. Role of Growth factors C. Role of Nitrogen sources**: The various organic nitrogen sources are yeast extract(YE), beef extract(BE), peptone(P) and urea(U) at 0.1% concentrations, Inorganic nitrogen sources (0.05M) NS.1 toNS.5 are $NH_4H_2PO_4$, $NaNO_3$, $NH_4NO_3$, $KNO_3$ and $(NH_4)_2SO_4$. **D. Effect of Beef extract:** Five concentrations of beef extract (expressed as %) were tested. The enzyme activity is expressed as µg sugar released/ml enzyme during the assay period. **E. Role of metal ions:** The chlorides of metals (Ca, Zn, Ni, Cu, Co and Mn) were incorporated into the medium (1mM). The control flasks lacked all the above metals.

**Solid state fermentation of cellulase production**

**Effect of Solid to Liquid Ratio (Moisture content)**

The maximum levels of CMC, FP and pNPG activities (5.2, 0.912 and 2.8 IU/gm dry weight of the substrate respectively) were obtained after 72 hour in all the initial moisture levels. pNPG activity was obtained as early as 24 hours where as FP and CMC activity was not observed. The best initial level of moisture was found to be 50%. At 70% moisture, the activities were lowered with pNPG activity was only 35% of that at 50%, FP activity 8.8% and CMC activity 19%.

**Effect of initial pH**

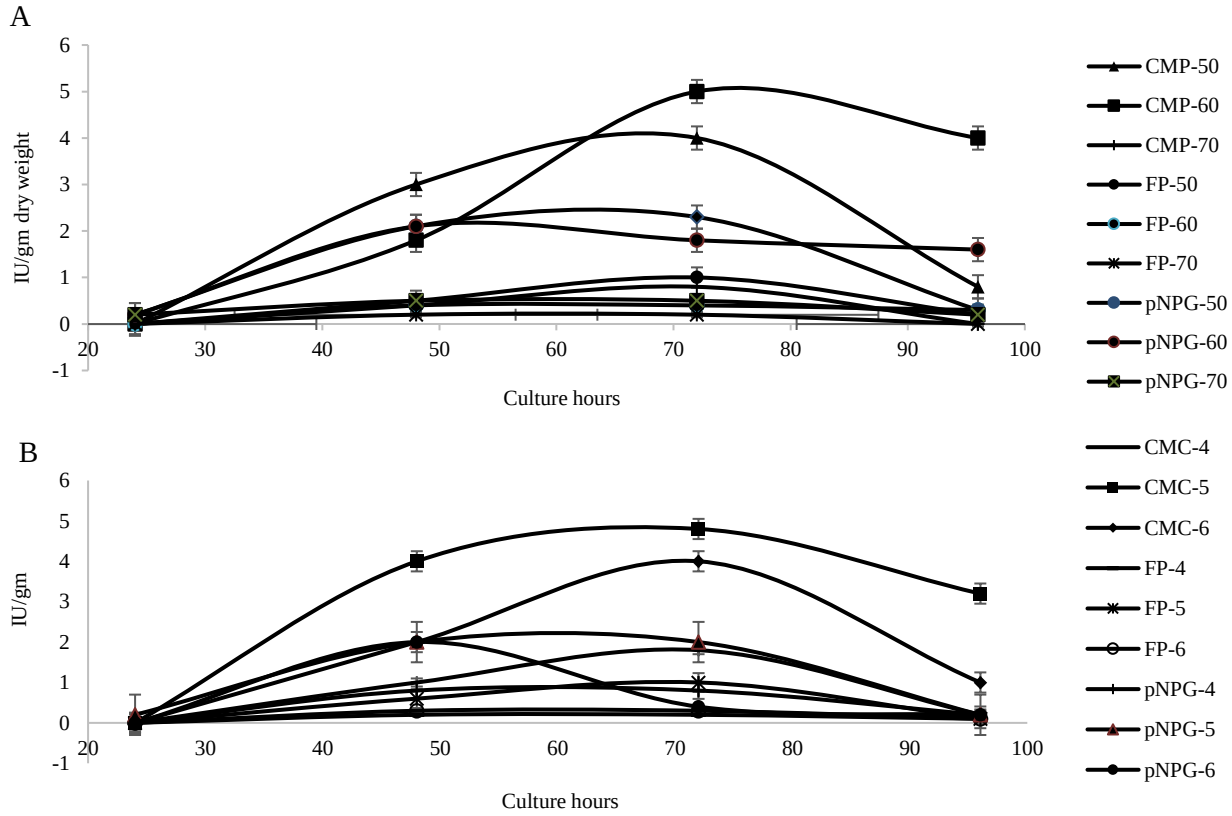
The CMC and FP activities were maximum 5.2 IU and 0.79 IU per gram dry weight of the substrate) after 72 hours of fermentation at an initial medium pH 5. However pNPG activities were more at higher medium pH studied. At pH 6, the maximum pNPG activity was (2.3 IU/gm dry weight of the substrate) produced at 48 hour which was equal to that produced at pH 5 at 72 hour. Of the different pH studied the best pH for β-glucosidase production was pH 6 which resulted in the earlier production of maximum levels of the enzyme. pH higher than 6 was found to be better for pNPGase production also in submerged fermentation.

**Effect of Growth Temperature**

Lower temperature favoured CMCase, FPase and β-glucosidase production by *A. flavipes*. Temperature at 30°C yielded higher enzyme followed by cultures fermented at 20°C. At 40°C low levels of pNPG activity and trace amounts of FP activity were observed at 48 hour, beyond 48 hour no activity was obtained on any of the substrates.

**Effect of Carbon Source**

The best carbon source of all the native substrates studied (bagasse, cotton, green gram hull, wheat bran and rice straw) was wheat bran produced higher cellulase activities. Enzyme activities measuring 5.6 IU, 17 IU and14 IU per gram dry weight of the carbon source for FP, pNPG and CMC were obtained respectively **(Fig** 3 and 4).



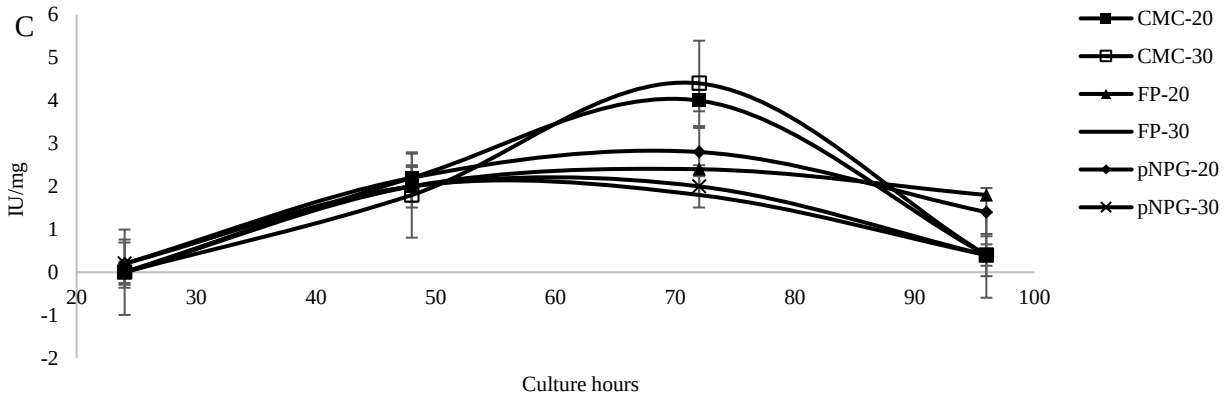



**Figure 3** Solid State fermentation for cellulase production by *A. flavipes*: Effect of moisture, pH and temperature.
**A. moisture (50, 60 and 70%); B. pH (5, 6 and 7); C. temperature (20±5ºC and 30±2ºC) on CMC, FP and pNPG production is depicted in the graph. The IU of cellulase activity/gm dry weight of the substrate is plotted on the Y axis against culture hours on the X axis**

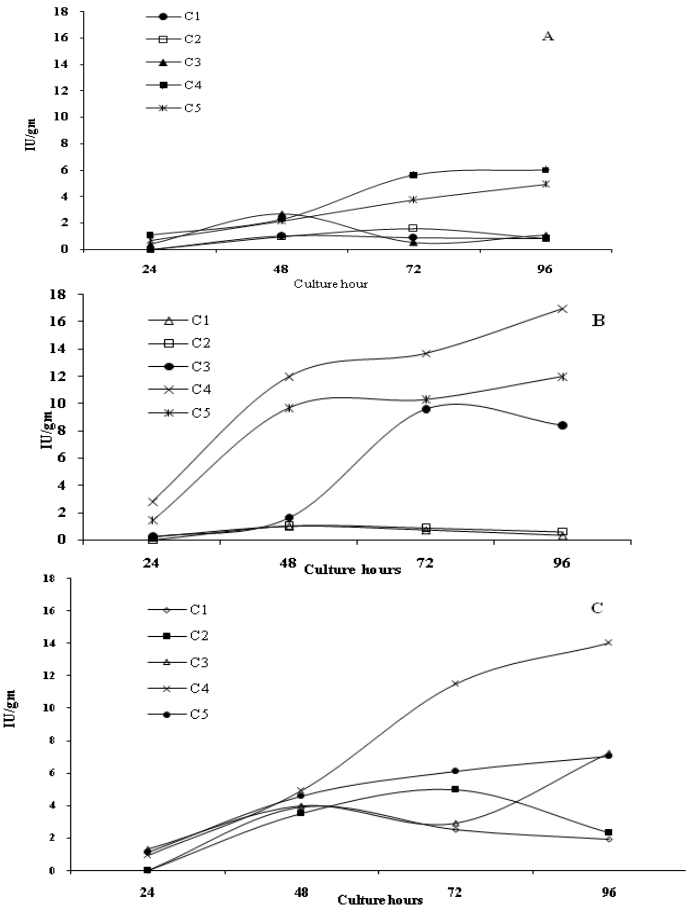



**Figure 4** Solid State fermentation for cellulase production by *A. flavipes*, role of carbon sources. Figures A, B and C show the effect of different carbon sources on FP, pNPG and CMC respectively. The carbon sources C1 to C5 are bagasse, cotton, green gram hull, wheat bran and rice straw respectively. The IU of cellulase activity/gm dry weight of the substrate is plotted on the Y axis against culture hours on the X axis.

## DISCUSSION

The cellulase enzyme cost is expensive for commercial applications such as large scale cellulosic ethanol production or in textile industry. Screening for microorganism with high cellulase production and implementation of submerged or solid state fermentation for effective production can fetch cost reductions. Multiple parameters need to be optimized for efficient cellulase production.

A recent study demonstrated improvement of cellulase production by genetically disrupting the protease genes to construct the *T. reesei* strains with low extracellular protease secretion. It also proved an efficient approach for the strain improvement by precise genetic engineering for the industrial enzymes to reduce the cost of lignocellulose bioconversion **(Qian *et al.*, 2019)**. In an another study on *T. reesei* new strategy based on novel fusion transcription factors was successfully established in *T. reesei* to improve cellulase titers without impairing fungal growth **(Wang *et al.*,2019)**. The influence of type, age and concentration of inoculum affects the cellulase enzyme production, where inoculum size of $10^7$/ml aged for 72 hours from a PCA slant generates a good quantity of cellulase in submerged fermentation conditions. Nutrient availability is expected to decrease with an increase in the inoculums size. Beyond a threshold, the enzyme production decreases, as with *Acinetobacter. junii* beyond 8% inoculum size a decrease in cellulase production was observed **(Banerjee *et al.*,, 2020)**. Similarly higher activity was observed with 4% inoculum which decreased at higher inoculums size **(Afzal *et al.*, 2012)**. Maximum cellulase activity was observed with 24 hr old *B. cereus* inoculum with an inverse relationship existing between cellulase production and inoculum age **(Afzal *et al.*, 2012)**. Wild type and mutant strains of *A. terreus* produced better cellulase production with 30% inoculum level optimum for β-glucosidase and 40% optimum for CMCase and FPase activity **(Isaac and Abu-Tahon, 2015)** with SSF. Lesser inoculums size (4%) was required for *A. niger*, *S.cerevisiae* and *T. longibrachiatum* when cultured on plantain peel **(Omajasola and Jilani, 2009)** or using 7.35 x $10^5$ spores/ml of *Aspergillus* sp on agricultural and plant residues **(Kulkarni, *et al.*, 2018)**. It has been reported that for *T. koningi*, PCA medium slants were the best for repeated sub-culturing and maintaining of the cultures **(Wood and Bhat, 1988)**. PCA medium supports spore production, as they do not support profuse growth due to its very low nutrient content compared to the other medium used in the study.

Maximum CMCase, FPase activity and protein was produced at initial pH 4.5 and when medium was maintained at pH 4.5. β-glucosidase activity was found to be higher at initial growth pH 4.5 and when culture was maintained at pH 8.5. During β-glucosidase production in *Trichoderma* cultures the pH values dropped from 5 to below 3, where β-glucosidase is inactivate. The pH profile is not stable and fluctuates, therefore a continuous adjustment and a specific pH profile has to be followed for optimum enzyme production **(Tangnu *et al.*, 1981)**. An agitation speed of 150 rpm and smallest working volume ratio of 0.2 was the optimum for enzyme production. The results of both agitation and aeration suggest that better yields of cellulase from this fungus are dependent on oxygen supply. β-glucosidase production by submerged fermentation of three strains of *A. niger* and one *A. terreus* strain was higher at an optimum parameters of 30 °C, pH 6.0, 200 rpm and 0.5% sucrose **(Alarid-García and Escamilla-Silva, 2017)**. On the contrary Yu *et al*. **(Yu, *et al.*, 2012)** showed an improved cellulase production by *A. niger* (3.5 IU/ml) with increased agitation (400 rpm) with a two fold increase compared to 100 rpm.

Among the different medium parameters, lactose followed by maltose, xylose and sucrose were better carbohydrate sources, ammonium dihydrogen phosphate (NS1) followed by beef extract were superior nitrogen sources, thiamine and nicotinic acid, metals other than $CuCl_2$ were best for efficient cellulase production. A combination of beef extract, urea and ammonium dihydrogen phosphate was better with CMC below 2.3 % concentration did not alter both enzyme activities. Lactose gave higher cellulase yields in *A. niger* biofilm (370 U/g lactose), SSF (212 U/g lactose) and SmF (217 U/g lactose) cultures **(Gamarra *et al.*, 2010)**. When genetically engineered strain of *T. reesei* is used, cellulase production is induced by the carbon source, lactose since it activates MET3 promoter **(Bischof *et al.*, 2015)**. Substrate addition (lactose, peptone and coir waste) enhanced cellulase productivity in *A. niger* SmF and SSF cultures **(Mrudula and Murugammal, 2011)**. $MnCl_2$, $FeCl_2$ and $FeCl_3$ enhanced cellulase activity of thermophilic *A. terreus* RWY in SSF **(Sharma *et al.*, 2014)**. Addition of divalent metal cations $HgCl_2$, $BaCl_2$, $MgCl_2$, $FeCl_2$ (2 mM) did not enhance cellulase production by *A. flavus*, whereas it was marginally increased by CaCl2 and $ZnCl_2$ (2 mM) **(Bano *et al.*, 2019)**.

*A. flavipes* on 10g/l of lactose, cultured for 144 hours yielded 0.225 FPU/ml and productivity of 1.56 FPU/ml/h. In submerged fermentation *A. flavipes* on all the native substrates (bagasse, cotton, green gram hull, wheat bran and rice straw)

produced higher activities, with wheat bran yielding higher cellulase activity. The yield of the enzyme expressed as IU/gm substrate is comparable to those reported **(Shamala and Sreekantiah, 1986)**. The production of β-glucosidase was higher in solid state fermentation than in submerged fermentation. The enhanced production of β-glucosidase and FP activity is important for the complete and extensive saccharification. The high units of activity produced with native substrates other than cotton and bagasse indicate that the fungus may be a potent producer of enzymes other than a complete cellulase system useful in the bioconversion of lignocellulosics. The fungal strain also produces significant amounts of xylanase which will enhance the degradation of the hemicelluloses in the native substrates (unpublished data). This in turn will enhance the accessibility of the enzymes to the cellulose fibres and the need for extensive pretreatment methods will be reduced. The average cellulose content in pretreated substrate is only around 50% **(Persson *et al.*, 1991)**.

The optimum moisture level for cellulase enzyme production was found out to be 50% at 72 hrs, better CMC and FP activities at pH 5, best β-glucosidase production was at pH 6 and at low temperatures of 30°C. The limiting levels of water activity (aw) for growth is characteristic to each fungus. High moisture content is reported to produce decreased substrate porosity which vary with the substrate used, pretreatment and the particle size) and subsequent resistance to oxygen penetration, high rate of mycelial growth and low sporulation **(Pandey, 1994)**. Along with moisture the particle size porosity crystallinity the methods of pretreatment etc are also reported to have influence on the enzyme production **(Pandey *et al.*,1999)**. On the other hand 70 and 75% **(Tai *et al.*, 2019)**of moisture content enhanced the FPase and CMCase titers produced by *A. niger* using oil palm frond as substrate. Optimum moisture content is required for SSF mediated productivity. Lower than optimum moisture level leads to decreased solubility and availability **(Dutt and Kumar, 2014)**. Initial medium pH 5.5 was optimal for maximum cellulase production by *A. niger* DWA8 **(Tai *et al.*, 2019**). The optimum pH range varied with respect to the species, for instance in cellulase production by *A. phoenicus* (pH 4.8-5.5) and *A. ornatus* and *T. reesei* (pH 4.6-5.5) for better CMCase, FPase and β-glucosidase activity **(Gottschalk *et al.*, 2010)**. Room temperature reduces the cost of SSF fermentation. *A. niger* 38 and *A. niger* USM AI1 also produced 14.8 U/ml and 62.6 U/ml of CMCase activity at optimal temperature of 30°C**(Lee *et al.*, 2011)**.

Through response surface methodology optimization of cellulase production at optimal pH, temperature, inoculums size, moisture content and substrate concentration for *A. niger* resulted in increased CMCase and xylanase production by 124.5% and 78.5% **(Tai *et al.*, 2019)**. Corn-steep liquor, ammonium dihydrogen orthophosphate and ammonium sulfate were the best nitrogen sources for the production of cellulase by *A. niger* **(Gokhale *et al.*, 1991)**. Organic and inorganic nitrogen sources including cornsteep liquor, urea, cattle urine, ammonium nitrate, ammonium sulfate, sodium nitrate, ammonium iron sulfate and ammonium chloride were analyzed for cellulolytic enzyme production by *A.terreus* GN1, which showed ammonium iron sulfate andcornsteep liquor were the best sources **(Garg and Neelakantan, 1982)**.

## CONCLUSION

Cellulases are of higher cost which limits its application in industries and increases the economy of final product production. Optimizing physical and nutrient parameters of the locally isolated indigenous strain of *A. flavipes* produce higher titers for cellulase production through SmF. SSF is preferred fermentation method for cellulase production by fungal species as it can produce concentrated enzyme economically. The isolate *A. flavipes* produces cellulolytic system that can well complement bioconversion processes of lignocellulosic wastes along with other enzyme systems and SSF using lignocelluosic waste is ideal for cost effective production of the various components of cellulase and favor the management and exploitation of wastes. Application of statistical analysis such as response surface methodology would help in determining the interaction between factors that affect cellulase production. Optimizing physical and nutrient parameters of the locally isolated indigenous strain of *A. flavipes* produced 4.6 fold higher titres for cellulase production through SmF. The maximum enzyme production was recorded at earlier fermentation hours making the productivity higher compared to other commercial strains. The isolate *A.flavipes* produces cellulases in high titres under SSF using lignocelluosic wastes, under temperatures much lower than required by bacteria makes it ideal for cost effective production of the various components of cellulase and favor the management and exploitation of wastes.

**Acknowledgements:** The first author is indebted to the CSIR, India for providing financial assistance for the study in the form of research fellowship.

**Conflict of interest:** There is no conflict of interest amongst the authors

## REFERENCES

Afzal I, Shah AA, Makhdum Z, Hameed A, Hasaan F (2012) Isolation and characterization of cellulase producing *Bacillus cereus* MRLB1 from soil. *Minerva Biotecnol* 24:101–109

Alarid-García C, Escamilla-Silva EM (2017) Comparative study of the production of extracellular β-glucosidase by four different strains of *Aspergillus* using submerged fermentation. *Prep Biochem Biotechnol* 47:597–610. http://dx.doi.org/10.1080/10826068.2017.1286598

Bajaj P, Mahajan R (2019) Cellulase and xylanase synergism in industrial biotechnology. *Appl Microbiol Biotechnol* 103:8711–8724. http://dx.doi/10.1007/s00253-019-10146-0

Banerjee S, Maiti TK, Roy RN (2020) Production, purification, and characterization of cellulase from *Acinetobacter junii* GAC 16.2, a novel cellulolytic gut isolate of *Gryllotalpa africana*, and its effects on cotton fiber and sawdust. *Ann Microbiol* 70:28. http://dx.doi.org/ 10.1186/s13213-020-01569-6

Bano A, Chen X, Prasongsuk S, Akbar A, Lotrakul P, Punnapayak H, Anwar M, Sajid S, Ali I (2019) Purification and Characterization of Cellulase from Obligate Halophilic *Aspergillus flavus* (TISTR 3637) and Its Prospects for Bioethanol Production. *Appl Biochem Biotechnol* 189:1327–1337. http://dx.doi.org/10.1007/s12010-019-03086-y

Behera SS, Ray RC (2016) Solid state fermentation for production of microbial cellulases: Recent advances and improvement strategies. Int *J BiolMacromol* 86:656–669. http://dx.doi.org/ 10.1016/j.ijbiomac.2015.10.090

Bischof RH, Horejs J, Metz B, Gamauf C, Kubicek CP, Seiboth B (2015) L-Methionine repressible promoters for tuneable gene expression in *Trichoderma reesei*. Microb Cell Fact 14:120. http://dx.doi.org/10.1186/s12934-015-0308-3

Dina S, Thankamani VT, Emilia Abraham T.( 2021) A novel cellulolytic system from Aspergillus flavipes for lignocellulosic bio-conversion. Indian J Sci Technol. May 3;14(15):1219-32. DOI: 10.17485/IJST/v14i15.209

Dutt D, Kumar A (2014) Optimization of cellulase production under solid-state fermentation by *Aspergillus flavus* (AT-2) and *Aspergillus niger* (AT-3) and its impact on stickies and ink particle size of sorted office paper. *Cellul Chem Technol* 48:285–298

Gamarra NN, Villena GK, Gutiérrez-Correa M (2010) Cellulase production by *Aspergillus niger* in biofilm, solid-state, and submerged fermentations. *Appl Microbiol Biotechnol* 87:545–551. http://dx.doi.org/10.1007/s00253-010-2540-4

Garg SK, Neelakantan S (1982) Effect of nutritional factors on cellulase enzyme and microbial protein production by *Aspergillus terreus* and its evaluation. *Biotechnol Bioeng* 24:109–125. http://dx.doi.org/10.1002/bit.260240110

Gokhale D V, Patil SG, Bastawde KB (1991) Optimization of cellulase production by *Aspergillus niger* NCIM 1207. *Appl Biochem Biotechnol* 30:99–109. http://dx.doi.org/ 10.1007/BF02922026

Gottschalk L, Oliveira R, Bon E (2010) Cellulases, xylanases, β-glucosidase and ferulic acid esterase produced by *Trichoderma* and *Aspergillus* act synergistically in the hydrolysis of sugarcane bagasse. *Biochem Eng* J 51:72–78. http://dx.doi.org/ 10.1016/j.bej.2010.05.003

Isaac GS, Abu-Tahon MA (2015) Enhanced alkaline cellulases production by the *thermohalophilic Aspergillus terreus AUMC 10138 mutated by physical and chemical mutagens using corn stover as substrate. Brazilian* J Microbiol 46:1269–1277. http://dx.doi.org/ 10.1590/S1517-838246420140958

Kulkarni N, Vaidya T, Rathi G (2018) Optimization of cellulase production by *Aspergillus* species under solid state fermentation. *Pharma Innov* J 7:193–196. http://dx.doi.org/ 10.22271/tpi

Lee CK, Darah I, Ibrahim CO (2011) Production and Optimization of Cellulase Enzyme Using *Aspergillus niger* USM AI 1 and Comparison with *Trichoderma reesei* via Solid State Fermentation System. *Biotechnol Res Int* 2011:658493. http://dx.doi.org/ 10.4061/2011/658493

Mrudula S, Murugammal R (2011) Production of cellulose by *Aspergillus niger* under submerged and solid state fermentation using coir waste as a substrate. *Brazilian J Microbiol* 42:1119–1127. http://dx.doi.org/10.1590/S1517-838220110003000033

Pandey A (1994) Solid state fermentation: An overview. Solid State Ferment 3–10

Pandey A, Selvakumar P, Soccol C, Nigam P (1999) Solid State Fermentation for the Production of Industrial Enzymes. *Curr Sci* 77:149–162

Persson I, Tjerneld F, Hahn-Hägerdal B (1991) Fungal cellulolytic enzyme production: A review. *Process Biochem* 26:65–74. http://dx.doi.org/ https://doi.org/10.1016/0032-9592(91)80019-L

Qian Y, Zhong L, Sun Y, Sun N, Zhang L, Liu W, Qu Y, Zhong Y. Enhancement of cellulase production in *Trichoderma reesei* via disruption of multiple protease genes identified by comparative secretomics. Frontiers in microbiology. 2019 Dec 3;10:2784. https://doi.org/10.3389/fmicb.2019.02784

Shamala TR, Sreekantiah KR (1986) Production of cellulases and d-xylanase by some selected fungal isolates. *Enzyme Microb Technol* 8:178–182. http://dx.doi.org/10.1016/0141-0229(86)90109-2

Sharma R, Kocher GS, Bhogal RS, Oberoi HS (2014) Cellulolytic and xylanolytic enzymes from thermophilic *Aspergillus terreus* RWY. *J Basic Microbiol* 54:1367–1377. http://dx.doi.org/ 10.1002/jobm.201400187

Sukumaran RK, Christopher M, Kooloth-Valappil P, Sreeja-Raju AR, Mathew RM, Sankar M, Puthiyamadam A, Adarsh V-P, Aswathi A, Rebinro V, Abraham A. Pandey A (2021) Addressing challenges in production of cellulases for biomass hydrolysis: Targeted interventions into the genetics of cellulase producing fungi. *Bioresour Technol* 329:124746. http://dx.doi.org/ 10.1016/j.biortech.2021.124746

Tai WY, Tan JS, Lim V, Lee CK (2019) Comprehensive studies on optimization of cellulase and xylanase production by a local indigenous fungus strain via solid

state fermentation using oil palm frond as substrate. *Biotechnol Prog* 35:e2781. http://dx.doi.org/ 10.1002/btpr.2781
Tangnu SK, Blanch HW, Wilke CR (1981) Enhanced production of cellulase, hemicellulase, and β-glucosidase by *Trichoderma reesei* (Rut C-30). *Biotechnol Bioeng* 23:1837–1849. http://dx.doi.org/10.1002/bit.260230811
Vieira MM, Kadoguchi E, Segato F, da Silva SS, Chandel AK (2021) Production of cellulases by *Aureobasidium pullulans* LB83: optimization, characterization, and hydrolytic potential for the production of cellulosic sugars. *Prep Biochem Biotechnol* 51:153–163. http://dx.doi.org/ 10.1080/10826068.2020.1799393
Wang F, Zhang R, Han L, Guo W, Du Z, Niu K, Liu Y, Jia C, Fang X. Use of fusion transcription factors to reprogram cellulase transcription and enable efficient cellulase production in *Trichoderma reesei*. Biotechnology for biofuels. 2019 Dec;12(1):1-2. (2019) https://doi.org/10.1186/s13068-019-1589-2
Wood TM, Bhat KMBT-M in E (1988) Methods for measuring cellulase activities. In: Biomass Part A: Cellulose and Hemicellulose. Academic Press, pp 87–112
Yu L, Chao Y, Wensel P, Chen S (2012) Hydrodynamic and kinetic study of cellulase production by *Trichoderma reesei* with pellet morphology. *Biotechnol Bioeng* 109:1755–1768. http://dx.doi.org/ 10.1002/bit.24433