BIOSCIENCE JOURNAL

# PROCESS OPTIMIZATION FOR ENHANCED PRODUCTION OF CELLULASES FORM LOCALLY ISOLATED FUNGAL STRAIN BY SUBMERGED FERMENTATION

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**How to cite:** ABDULLAH, R., et al. Process optimization for enhanced production of cellulases form locally isolated fungal strain by submerged fermentation. *Bioscience Journal*. 2021, **37**, e37021. https://doi.org/10.14393/BJ-v37n0a2021-53815

## Abstract

Cellulase has myriad applications in various sectors like pharmaceuticals, textile, detergents, animal feed and bioethanol production, etc. The current study focuses on the isolation, screening and optimization of fungal strain through one factor at a time technique for enhanced cellulase production. In current study sixteen different fungal cultures were isolated and the culture which quantitatively exhibits higher titers of cellulase activity was identified both morphologically and molecularly by 18S rDNA and designated as *Aspergillus niger* ABT11. Different parameters like fermentation medium, volume, temperature, pH and nutritional components were optimized. The highest CMCase and FPase activities was achieved in 100ml of M5 medium in the presence of 1% lactose and sodium nitrate at 30 °C, pH5 after 72 hours. The result revealed *A. niger* can be a potential candidate for scale up studies.

**Keywords:** *Aspergillus niger*. Cellulase. CMCase. FPase. Molecular Characterization.

## 1. Introduction

Cellulose is an insoluble crystalline polysaccharide of repeating units of glucose that is linked through β-1, 4-glucosidic bonds. The group of cellulases possess the ability of completely hydrolyse the cellulose and needed three main components i.e. Endoglucanases, Exoglucanases and β-glucosidases. Endoglucanases act on the amorphous region of cellulose while Exoglucanase acts on crystalline region, resulting in the production of cellobiose units which are further attacked by β-glucosidase and ultimately production of glucose (Oliveira et al. 2019). Cellulases has multifarious applications in different sectors like textile, pharmaceuticals, detergents and animal feed , food and paper etc. (Bhat 2000).

The chief skeletal constituent of plant cell wall is cellulose, which is found along with hemicelluloses, pectin and lignin. Cellulases can be obtained from microorganisms, including fungi and bacteria. However, filamentous fungi are preferred over bacteria because of their ability to penetrate deep in cellulosic substrates by using their hyphal extensions and thus showing their enzyme systems in confined cavities within the cellulosic particles as compared to bacteria that cannot penetrate deep in to the substrate (Lynd et al. 2002). In addition to this fungal cellulases has the capability to act on both amorphous and crystalline structures of cellulose in contrast to bacteria (Damisa et al. 2011). Most commonly used fungal species for the production of cellulases are *Aspergillus niger, Penicillium dupontii, Fusarium oxysporum* and *Trichoderma viride* etc. (Hussain et al. 2012).

Presently the production of cellulase has extensively been studied, but the relatively high productivity costs have hindered the frequent use of enzyme for industrial applications. So there is a dire need to improve the economics of cellulase production either by reducing the productivity cost or enhancing the enzyme activities. The production cost is basically associated with the enzyme productivity of microbial strain as well as the type of fermentation media used (Akula and Golla 2018). Keeping in view the above consideration the current work focused on the process optimization for enhanced cellulase production by locally isolated fungal strain.

## 2. Material and Methods

### Isolation of cellulolytic fungi

Different fungal strains having cellulolytic potential were isolated from different natural sources such as the compost and different soil samples were collected from different areas of Punjab, Pakistan. The isolation of fungi was carried out using serial dilution method (Reddy et al. 2014). The primary screening was performed on the basis of the zone of cellulose hydrolysis (Kim et al. 2006), while secondary screening was performed via submerged fermentation. The strain showed highest cellulolytic potential was identified both morphologically and molecularly.

### Molecular characterization

Molecular characterization was carried out according to Nisar et al. (2020).

### Pretreatment of substrate

The small pieces of sugar cane bagasse were sun dried and after this treated with sodium hydroxide (4%) by dipping in the solution for 24 hours. The sample was then washed with water and dried in oven for 3-4 h at 60°C (Haq et al. 2006).

### Fermentation

The sterilized fermentation medium (100ml) was prepared in 250 ml Erlenmeyer flasks was inoculated with 1ml of conidial inoculum. All the flasks were incubated at 30$^{o}$C for 72 hours in a shaking incubator with 160 rpm agitation speed. Later, the fermented broth was centrifuged for 15-20 min at 6000 × g. The clear supernatant was used for the determination of CMCase and FPase activity (da Silva et al. 2016).

### Evaluation of different fermentation media used for cellulase production

Following media (g/l) was evaluated to produce cellulases:

M1: 3 sugar cane bagasse; 1.4 $(NH_4)_2SO_4$; 0.3 urea; 2.0 $KH_2PO_4$; 0.3 $CaCl_2$;0.3 $MgSO_4.7H_2O$; 0.75 tryptone; 0.005 $FeSO_4.7H_2O$; 0.0014 ZnSO4; 0.0016 $MnSO_4.3H_2O$; 2 Tween-80 (Tao et al. 2010).

M2: 1.0 $KH_2PO_4$; KCl 0.5; $(NH_4)_2SO_4$ 0.5; $MgSO_4.7H2O$ 0.2; L-asparginine 0.5; $CaCl_2$ 0.1; yeast extract 0.5; 10 Wheat bran (Haq et al. 2005).

M3: Urea 0.3; 1.4 $(NH_4)_2SO_4$; $KH_2PO_4$ 2.0; 0.4 $CaCl_2{\cdot}2H_2O$; 0.3 $MgSO_4{\cdot}4H_2O$; 1.0 peptone; 0.005 $FeSO_4{\cdot}7H_2O$; 0.0016 $MnSO_4{\cdot}4H_2O$; 0.0014 $ZnSO_4{\cdot}7H_2O$; 0.002 $CoCl_2{\cdot}6H_2O$; 2.0 Tween 80; 10 Whatman No.1 (Karnchanatat et al. 2008).

M4: 50 Wheat bran, ammonium nitrate 8 (Padmavathi et al. 2012).

M5: 2g Wheat straw, 50 ml of Mandel and Sternberg's mineral medium containing (g/l): 1.4 $(NH_4)_2SO_4$; 2.0 $KH_2PO_4$; 0.3 $CaCl_2$; 0.0003 $MgSO_4.7H_2O$; 0.005 $FeSO_4.7H_2O$; 0.0016 $MnSO_4.H_2O$; 0.0014 $ZnSO_4.7H_2O$; 0.002 $CoCl_2$ (Singh et al. 2009).

M6: $KH_2PO_4$ 2.0; $MgSO_4.7H_2O$ 0.3; $CaCl_2.2H_2O$ 0.3; $(NH_4)2SO_4$ 1.4; $FeSO_4.7H_2O$ 0.005; $MnSO_4.H_2O$ 0.0016; $ZnSO_4.7H_2O$ 0.0014; $CoCl_2.6H_2O$ 0.002; peptone 1.0; Tween 80 1.0; 10 CMC (Karnchanatat et al. 2008).

### Assay of FPase and CMCase

FPase and CMCase assay was performed following the Gao et al. (2008). For the determination of FPase 50 mg of Whatman filter paper (1×6 cm) along with 0.5ml of supernatant and 0.5ml of 0.1M citrate buffer (pH, 5.0) was incubated at 60°C for half an hour. Blank was also run side by side. After incubation the

reducing sugar was measured by DNS method (Miller 1959). The absorbance was noted at 546nm using spectrophotometer. For the determination of CMCase above mentioned procedure was followed except Whatman filter paper strip was replaced by 0.5ml of CMCase (1%) prepared in citrate buffer (0.1 M; pH5).

One unit of FPase and CMCase activity was defined as the“ quantity of enzyme requisite to release 1 µmol of reducing sugars from appropriate substrate under standard assay conditions” (Chellapandi and Jani 2008).

**Determination of total protein and dry cell mass**

Dry cell mass was determined by following the method of Irfan et al. (2011) and Total protein was estimated by Bradford et al. (1976). The Bovine serum albumin was used as a standard.

**Statistical analysis**

All the experimental data was analyzed statistically using SPSS (17.0).

**Bioinformatics analysis**

Sequence was analyzed using BLAST and phylogenetic tree was constructed through MEGA 7 (Kumar et al. 2018).

**3. Results and Discussion**

Due to increasing demand of cellulases and consequently higher productivity it becomes essential to give more attention to explore the hyper productive microbial strain and process optimization strategies. In the current study sixteen different fungal strains were screened for the desire enzyme production (data not shown). The fungi possessing the highest cellulolytic ability was identified both on morphological and molecular basis (18S rDNA) by using universal primers ITS-1(F): TCCGTAGGTGAACCTGCGG and ITS-4 (R): TCCTCCGCTTATTGATATGC and found to be *Aspergillus niger*. The Blast analysis clearly indicates the isolate belong to *Aspergillus* genus and show 99% similarity with *Aspergillus niger* (Figure 1). This strain was designated as *Aspergillus niger* ABT11. The production of cellulases through fermentation is multivariable process and depends upon the genetic nature of organism, medium composition and physicochemical properties. All these factors dramatically affect the enzyme productivity. In this study six different media were tested (Figure 2) and M5 medium found to the best medium the reason could be that it contains wheat straw which serve as a carbon source for the growth of fungi and subsequently for the enzyme production. In addition to this it also contains low lignin contents. Our findings agree with the Gori and Malana (2010) who stated wheat straw as a best substrate for cellulase production.

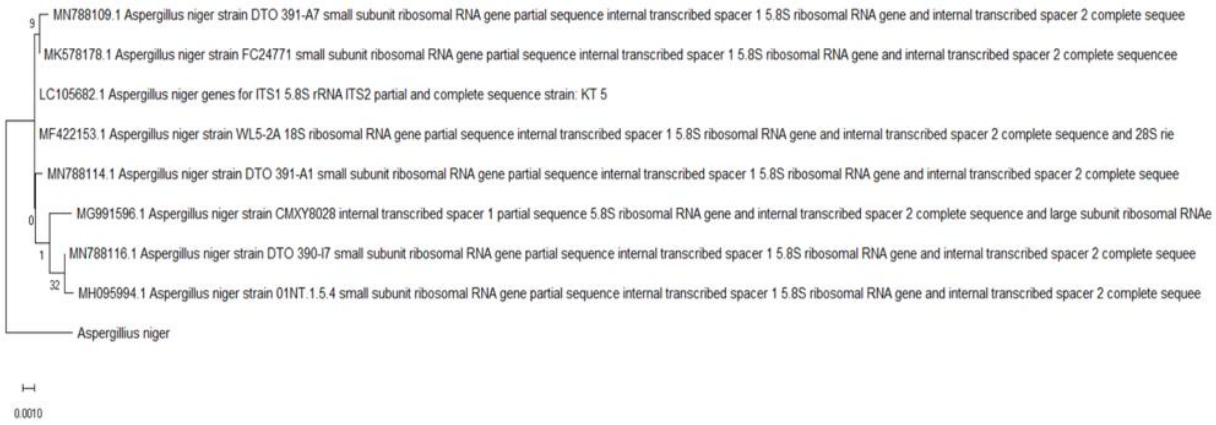


**Figure 1.** Phylogenetic relationship of *Aspergillus niger* and related filamentous fungi based on their ITS sequences.

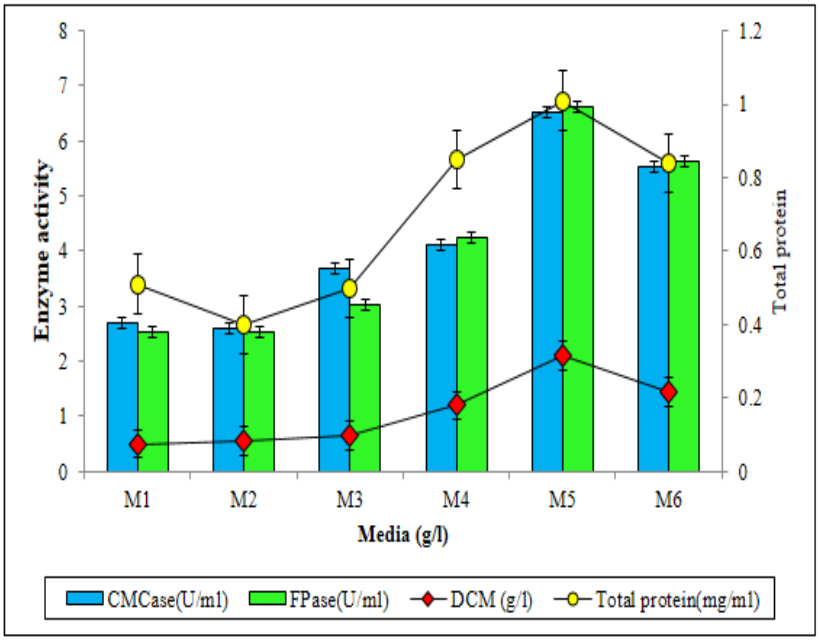


**Figure 2**. Effect of media on the cellulase production.

The time of fermentation greatly influences the product formation. In this investigation fermentation is carried out up to 120h (Figure 3A). The enzyme activity was increased with the rise in the fermentation time till 72hours. After this a gradual decrease occurs in the activity of the enzyme and drastic reduction was observed at 120h. The reason could be the exhaustion of nutrients or production of toxic substance may occur which retard the growth of organism and subsequently CMCase and FPase production (Malik et al. 2010). Our studies were contradictory to Karthikeyan et al. (2010) who investigated 120h was an optimum fermentation period for the production of cellulase. However, our studies were similar to Gori and Malana (2010) who reported 72h as a best incubation time for cellulase production.

A critical feature in fungal enzyme production is the optimal temperature that stimulates catalytic site optimum saturation, whereas higher temperature ultimately denatures the enzyme (Geoffry et al. 2018). The influence of varying the range of incubation temperature from 25-45°C was recorded (Figure 3B). The maximal CMCase and FPase activities was noted at 30°C further rise in temperature results decrease in the enzyme activity. Higher temperature during enzyme production inhibits the growth of fungi and cause denaturation of protein and consequently low enzyme yield (Yoon et al. 2014). The current results disagree with El-Hadi et al. (2014) who reported 37°C for cellulase production. The influence of varying media volume (25 - 200 ml) on the cellulase production was investigated (Figure 3C). The highest production of CMCase (6.52 U/ml) and FPase (6.62 U/ml) was achieved in 100ml. Further increase in volume caused the enzyme production to decrease. The reason lies in the fact that in the shake flask supply of oxygen is related to the volume of the medium when the volume of medium increase or decrease above the optimal level the growth of fungi and corresponding yield of the enzymes was reduced accordingly (Carlile et al. 2001).

The pH of medium has a strong influence on fungal growth, metabolism and enzymatic processes. It also affects the transport of different compounds through cell membrane. Effect of pH variations on the production of cellulase was evaluated (Figure 3D). The varying pH ranging from 3-10 was screened. The pH 5 proved to be best and gave maximal CMCase (7.42 U/ml) and FPase (7.65 U/ml) productivity. Any change more or less than the optimal state result in the less production of enzyme. The optimal pH is needed to retain three dimension shape of the enzyme active site. The variation in pH causes loss in shape of enzyme because of changes in ionic bonding of enzyme (Mmango-Kaseke et al. 2016). Akula and Golla (2018) reported pH 5 for optimal cellulase production which was comparable to our finding.

Different carbon sources like sucrose, glucose, fructose, maltose, lactose, xylose were screened for the production of cellulase (Figure 4A). Lactose at 1% concentration gave the maximal production of CMCase and FPase (13.64 U/ml; 13.51 U/ml) respectively (Figure 4B). However, glucose inhibits the synthesis of cellulase. Many workers reported that lactose act as a strong inducer to produce cellulase (Akula and Golla 2016; EL-Hadi et al. 2014). The impact of using varying types of nitrogen sources including both organic and

inorganic i.e. ammonium sulphate, ammonium chloride, ammonium nitrate, meat extract, yeast extract, peptone, sodium nitrate and urea were evaluated (Figure 4C). Among all the above used nitrogen sources sodium nitrate gave highest activity of CMCase (13.95 U/ml) and FPase (13.82 U/ml). All other nitrogen sources exhibited less enzyme production (Figure 4D). So, different $NaNO_3$ concentrations (0.5 to 2%) were screened. The highest CMCase (14.86 IU/ml) and FPase (14.66 U/ml) activity was achieved at 1%. Our studies are comparable to Abd-Elrsoul and Bakhiet (2018) who also reported sodium nitrate as a best nitrogen source, but contrary to Pothiraj and Eyini (2007) who reported that optimal cellulase production can be achieved from organic nitrogen sources, such as a yeast extract and peptone.

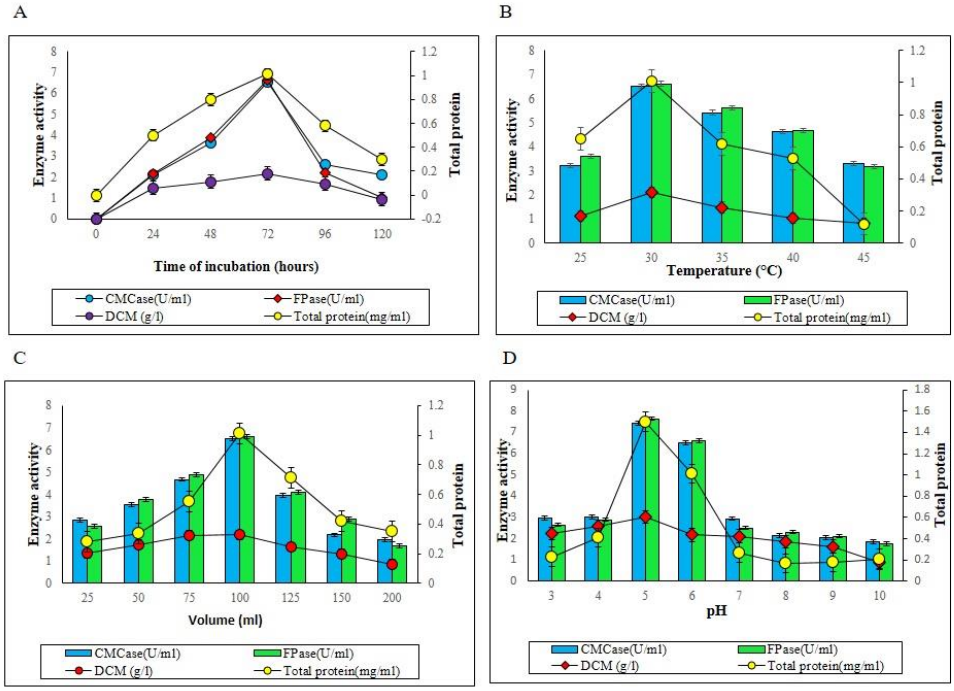


**Figure 3.** Influence of different parameters on cellulase production. A – time of incubation; B – temperature; C – volume; D – pH.

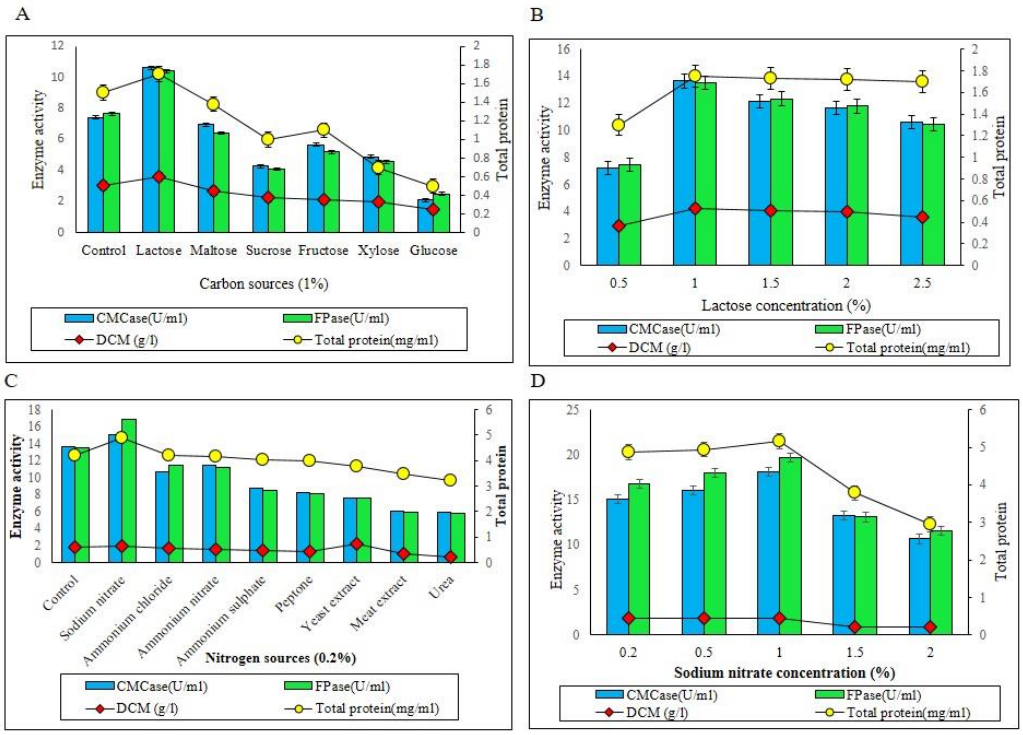


**Figure 4.** Influence of nutritional factors on cellulase production. A – carbon sources; B – concentration of lactose; C – nitrogen sources; D – sodium nitrate concentration.

## 4. Conclusions

The indigenously isolated *Aspergillus niger* ABT11 after optimization of cultural conditions exhibited the highest cellulase producing potential. The result indicates *Aspergillus niger* ABT1I can be promising candidate for economic production of cellulases by using wheat straw as a substrate for large scale production.

**Authors' Contributions:** ABDULLAH, R.: conception and design, acquisition of data, analysis and interpretation of data, and drafting the article; AKHTAR, A.: acquisition of data; NISAR, K.: acquisition of data, and drafting the article; KALEEM, A.: conception and design, acquisition of data, analysis and interpretation of data, and drafting the article; IQTEDAR, M.: conception and design, acquisition of data, analysis and interpretation of data and drafting the article; IFTIKHAR, T.: drafting the article; SALEEM, F.: conception and design, acquisition of data, analysis and interpretation of data and drafting the article; ASLAM, F.: conception and design, acquisition of data, analysis and interpretation of data and drafting the article. All authors have read and approved the final version of the manuscript.

**Conflicts of Interest:** The authors declare no conflicts of interest.

**Ethics Approval:** Not applicable.

**Acknowledgments:** Not applicable.

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**Received:** 16 April 2020| **Accepted:** 18 October 2020| **Published:** 12 May 2021