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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 30⁰C for 72 hours in a shaking incubator with 160 rpm agitation speed. Later, the fermented broth was centrifuged for 15-20 min at 6000 x 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 (NH₄)₂SO₄; 0.3 urea; 2.0 KH₂PO₄; 0.3 CaCl₂; 0.3 MgSO₄.7H₂O; 0.75 tryptone; 0.005 FeSO₄.7H₂O; 0.0014 ZnSO₄; 0.0016 MnSO₄.3H₂O; 2 Tween-80 (Tao et al. 2010).
- M2: 1.0 KH₂PO₄; KCl 0.5; (NH₄)₂SO₄ 0.5; MgSO₄.7H₂O 0.2; L-aspartagine 0.5; CaCl₂ 0.1; yeast extract 0.5; 10 Wheat bran (Haq et al. 2005).
- M3: Urea 0.3; 1.4 (NH₄)₂SO₄; KH₂PO₄ 2.0; 0.4 CaCl₂·2H₂O; 0.3 MgSO₄·4H₂O; 1.0 peptone; 0.005 FeSO₄·7H₂O; 0.0016 MnSO₄·4H₂O; 0.0014 ZnSO₄·7H₂O; 0.002 CoCl₂·6H₂O; 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 (NH₄)₂SO₄; 2.0 KH₂PO₄; 0.3 CaCl₂; 0.0003 MgSO₄; 7H₂O; 0.005 FeSO₄·7H₂O; 0.0016 MnSO₄·4H₂O; 0.0014 ZnSO₄·7H₂O; 0.002 CoCl₂ (Singh et al. 2009).
- M6: KH₂PO₄ 2.0; MgSO₄·7H₂O 0.3; CaCl₂·2H₂O 0.3; (NH₄)₂SO₄ 1.4; FeSO₄·7H₂O 0.005; MnSO₄·4H₂O 0.0016; ZnSO₄·7H₂O 0.0014; CoCl₂·6H₂O 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): TCCTCGCTTATTGATATGC 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.
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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 NaNO₃ 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 ABT11 can be promising candidate for economic production of cellulases by using wheat straw as a substrate for large scale production.

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References

ABD-ELROUSOU, R.M.M.A. and BAKHIET, S.E.A. Optimization of factors influencing cellulase production by some indigenous isolated fungal species. Jordan Journal of Biological Sciences. 2018, 11(2), 31-36.

AKULA, S. and GOLLA, N. Optimization of cellulase production by Aspergillus niger isolated from forest soil. The open Biotechnology Journal. 2018, 12(1), 256-269. http://dx.doi.org/10.2174/1874070701812010256

BHAT, M. Cellulases and related enzymes in biotechnology. Biotechnology Advances. 2000, 18(5), 355-383. https://doi.org/10.1016/S0734-9750(00)00041-0

BRADFORD, M.M.A. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Analytical Biochemistry. 1976, 72(1-2), 248-254. https://doi.org/10.1006/abio.1976.9999

CARILE, M. J., et al. The Fungi. 2nd ed. London: Academic Press, 2001.

CHELLAPANDI, P. and JANI, H.M. Production of endoglucanase by the native strains of Streptomyces isolates in submerged fermentation. Brazilian Journal of Microbiology. 2008, 39(1), 122-127. https://doi.org/10.1590/S1519-673020080001000026

DA SILVA, V.C.T., et al. Effect of pH, Temperature, and Chemicals on the Endoglucanases and β-Glucosidases from the Thermophilic Fungus Myceliophthora heterothallica F. 2.1. 4. Obtained by Solid-State and Submerged Cultivation. Biochemistry Research International. 2016, 2016, 1-9. https://doi.org/10.1155/2016/9781216

DAMISA, D., AMEH, J. and EGBE, N. Cellulase production by native Aspergillus niger obtained from soil environments. Fermentation Technology and Bioengineering. 2011, 1, 62-70.

EL-HADI, A.A., et al. Optimization of cultural and nutritional conditions for carboxy methyl cellulase production by Aspergillus hortai, Journal of Radiation Research and Applied Sciences. 2014, 7(1), 23-28. https://doi.org/10.1016/j.jrras.2013.11.003

GAO, J., et al. Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal Aspergillus terreus M11 under solid-state cultivation of corn stover. BioResource Technology. 2008, 99(16), 7623-7629. https://doi.org/10.1016/j.biortech.2008.02.005

GEOFFRY, K. and ACHUR, R.N. Screening and production of lipase from fungal organisms. Biocatalyst and Agriculture Biotechnology. 2018, 14, 241-253 https://doi.org/10.1155/2016/1475258

GORI, M.I. and MALANA, M.A. Production of carboxymethyl cellulase from local isolate of Aspergillus species. Pakistan Journal of Life and Social Sciences. 2010, 8(1), 1-6.

HAQ, I., et al. Sugar cane bagasse pretreatment: an attempt to enhance the production potential of cellulases by Humicola insolens TAS-13. Biokemistri. 2006, 18(2), 83-88. https://doi.org/10.4314/biokem.v18i2.56396

HAQ, I., et al. Cotton saccharifying activity of cellulases produced by co-culture of Aspergillus niger and Trichoderma viride. Research Journal of Agriculture and Biology Sciences. 2005, 1(3), 241-245.

HUSSAIN, A., et al. Cellulolytic enzymatic activity of soft rot filamentous fungi Paecilomyces variotii. Advances in BioResearch. 2012, 3(3), 10-17.

IRFAN, M., et al. UV mutagenesis of Aspergillus niger for enzyme production in submerged fermentation. Pakistan Journal of Biochemistry and Molecular Biology. 2011, 44(4), 137-140.

KARNCHANATAT, A., et al. A novel thermostable endoglucanase from the wood-decaying fungus Daldinia schscho1zii. Enzyme and Microbial Technology. 2008, 42(5), 404-413. https://doi.org/10.1016/j.enzmitec.2007.11.009
KARTHIKEYAN, N. and PALANI, P.S.M. Screening, identifying of *Penicillium* K-P strain and its cellulase producing conditions. *Journal of Ecobitechology*. 2010, 2(10), 4-7.

MMANGO-KASEKE, Z., et al. Optimization of cellulase and xylanase production by *micrococcus* species under submerged fermentation. *Sustainability*. 2016, 8(11), 1168(1-15)  https://doi.org/10.3390/su8111168

KIM, D., et al. Identification and molecular modeling of a family 5 endocellulase from *Thermus caldophilus* GK24, a cellulolytic strain of *Thermus thermophilus*. *International Journal of Molecular Sciences*. 2006, 7(12), 571-589.  https://doi.org/10.3390/i7120571

KUMAR S., et al. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*. 2018, 35(6), 1547-1549.  https://doi.org/10.1093/molbev/msy096

LYND, L.R., et al. Microbial cellulose utilization: fundamentals and biotechnology. *Microbiology and Molecular Biology Reviews*. 2002, 66(3), 506-577.  https://doi.org/10.1128/MMBR.66.3.506-577.2002

MALIK, S.K., et al. Optimization of process parameters for the biosynthesis of cellulases by *Trichoderma viride*. *Pakistan Journal Botany*. 2010, 42(6), 4243-4251.

MILLER, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*. 1959, 31(3), 426-428.  https://doi.org/10.1021/ac60147a030

NISAR, K., et al. Hyper production of carboxy methyl cellulase by *Thermomyces dupontii* utilizing physical and chemical mutagenesis. *Revista Mexicana de Ingenieria Quimica*. 2020, 19(2), 617-625.  https://doi.org/10.24275/rmiq/Bio823

OLIVEIRA, P., et al. Cocoa shell for the production of endoglucanase by *Penicillium roqueforti* ATCC 10110 in solid state fermentation and biochemical properties. *Revista Mexicana de Ingenieria Quimica*. 2019, 18(3), 777-787.  https://doi.org/10.24275/um/itz/dcbi/revmexingquim/2019v18n3/Oliveira

PADMAVATHI, T., et al. Optimization of the medium for the production of cellulases by *Aspergillus terreus* and *Mucor plumbeus*. *European Journal of Experimental Biology*. 2012, 2(4), 1161-1170.

POTHIRAJ, C. and EYINI, M. Enzyme activities and substrate degradation by fungal isolates on cassava waste during solid state fermentation. *Mycobiology*. 2007, 35(4), 196-204.

REDDY, P.L.N., et al. Screening, identification and isolation of cellulolytic fungi from soils of Chittoor district, India. *International Journal of Current Microbiology and Applied Sciences*. 2014, 3(7), 761-77.

SINGH, A., et al. Production of cellulases by *Aspergillus heteromorphus* from wheat straw under submerged fermentation. *International Journal of Civil and Environmental Engineering*. 2009, 1(1), 23.26.

TAO, Y.M., et al. Purification and properties of endoglucanase from a sugar cane bagasse hydrolyzing strain, *Aspergillus glaucus* XC9. *Journal of Agricultural and Food Chemistry*. 2010, 58(10), 6126-6130.  https://doi.org/10.1021/jf1003896

YOON, L.W., et al. Fungal solid-state fermentation and various methods of enhancement in cellulase production. *Biomass and Bioenergy*. 2014, 2014(67), 319–338.  http://dx.doi.org/10.1016/j.biombioe.2014.05.013

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