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Isolation and Identification of *Aspergillus protuberus* from Mahanandi Forest Sample and Investigation of Its Cellulase Production

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ABSTRACT

Newly isolated Aspergillus protuberus was used for cellulase production by submerged fermentation (SmF) in a laboratory scale was compared. The production of filter paperase (FPase), carboxymethyl cellulose (CMCase), β-glucosidase were monitored at alternate intervals for 10 days. Optimization of parameters such as inducers, carbon sources was performed for the maximal production of cellulase. Among inducers and carbon sources tested in the present study, cellulose as the best inducer and lactose found to be best carbon source for FPase and CMCase whereas β-glucosidase and protein content maltose and fructose were the best carbon sources of A. protuberus in SmF.

Key words: *Submerged fermentation, Filter paperase, Carboxymethyl cellulase, β-glucosidase, Aspergillus protuberus.*

1. INTRODUCTION

Cellulose is the most abundant and renewable natural biopolymer on earth available for bioconversion to value-added bioproducts. The bioconversion of cellulosic biomass is potentially sustainable approach to develop novel bioprocesses and products. Microbial conversion of cellulosic biomass into useful products is a complex process involving the combined action of at least three different enzymes, namely, endoglucanase, exoglucanase or filter paperase (FPase), and β-glucosidase [1]. All these three component of cellulase complex are the main factors that influence the application of enzyme-based bioconversion technology. Therefore, research has been directed to discover new microorganisms that have capability to produce cellulolytic enzymes with high specific activity and characteristics that favor the use in industrial applications [2]. Among the cellulolytic fungi, *Trichoderma* spp. and *Aspergillus* spp. have been widely studied for their ability to secrete high levels of cellulose-degrading enzymes [3]. *Trichoderma reesei* is the most efficient producer of endo and exoglucanases [4], but does not excrete a sufficient amount of β-glucosidase [5] for which *Aspergillus* strains are known to be good producers [6].

Microbial cellulases have become the important biocatalyst due to their complex nature and widespread applications. The potential applications of cellulase are in food, animal feed, textile, fuel, and chemical industries. Other avenues for its application include cotton processing, paper recycling and as animal feed additives [7]. The fungal cellulase is used for deinking of fiber surfaces in paper industries and to enhance the pulp drainage in textile industries [8]. There are many microbes capable of producing cellulase enzyme, but a few of them only produces significant quantities of enzyme [9]. Production of cellulases by the fungal isolates requires optimal conditions for their growth which leads to the release of extracellular enzymes. The growth conditions as well as extracellular enzyme production conditions is likely to vary among isolates. The major components of production medium like carbon and nitrogen sources and physical parameters such as temperature, pH, and incubation time were found to be critically affecting the cellulase production hence need to be optimized for every isolate [10,11].

The present study focuses on the optimization of various parameters for cellulase enzyme production using *Aspergillus protuberus*, a forest isolate under submerged fermentation (SmF). It would be beneficial to optimize the medium conditions for cellulase production under SmF when considering the ease in scaling up of the process for biotechnological applications.

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2. EXPERIMENTAL

2.1. Isolation of Fungi for Hyper Cellulase

Soil samples were collected from a forest located at Mahanandi, Nandyal, Kurnool, Andhra Pradesh into sterile polythene bags and transported to the laboratory for further enumeration experiments.

In each collected group, homogenized soil sample was prepared by thorough mixing of an equal quantity of soil. Single colonies were enumerated by subjecting to serial dilution, and appropriate dilutions were plated on Czapek Dox agar medium containing cellulose (0.5%) and streptomycin (500 mg/L), and plates were incubated at 30°C for 7 days.

2.2. Plate Screening for Cellulase Enzyme Activity

Isolated fungal strains were primarily tested for cellulase enzyme activity by culturing on Czapek Dox medium supplemented with 1% carboxymethylcellulose (CMC). The plates were incubated at 30°C ±2°C for 7 days. After incubation plates were stained with 1% Congo red reagent. Excess dye was washed gently with 0.1 M NaCl. The clear zone around the fungal colony was regarded positive for cellulase activity. The fungal species exhibiting hyper cellulase activity were selected for further experiments.

2.3. Identification of Fungal Isolate

2.3.1. Microscopic method

Fungi were isolated as monocultures on Czapek Dox agar, and each pure culture was inoculated on the same medium and maintained at 4°C in a refrigerator. Cultural characteristics such as color and size of colonies during the growth were monitored.

A small amount of mycelia mat was taken with a sterile needle and placed on a clean glass slide and then stained with lactophenol cotton blue and covered by cover slip and microscopically analyzed for morphological characteristics of mycelia, conidia, and conidiophores/fruiting bodies.

2.3.2. Molecular method

2.3.2.1. Identification of the isolated fungi by sequencing amplified 28S rDNA gene

Ribosomal genes and spacers regions within the fungal genome have proven good candidates for amplification through polymerase chain reaction (PCR) because they are comprised highly conserved tracts with heterogeneous regions in between [12]. The gene coding for the 28S rDNA is amplified using the PCR, and the amplified product has been subjected to sequencing and the sequence obtained has been compared with the sequence obtained from the Nucleotide Database of National Centre for Biotechnology Information (NCBI).

2.3.2.2. Isolation of genomic DNA from the fungal isolates

The genomic DNA was isolated from MFS2 and quality was evaluated on 1.2% agarose gel, a single band of high-molecular-weight DNA was observed.

2.3.2.3. Amplification and sequencing of the 28S rDNA gene of the fungal chromosome

Amplification of consensus sequence of 28S ribosomal DNA was amplified using the universal primers (forward primer (DF): ACC CGC TGA ACT TAA GC and revere primer (DR): GGT CCG TGT TTC AAG ACG G) using BDT v3.1 cycle sequencing kit on ABI 3730xl genetic analyzer. The amplified product was purified and subjected to sequencing. The sequence so obtained was analyzed at gene bank data center using BLAST system at http://www.ncbi.nlm.nih.gov/ and the assembled sequence of 28S rDNA gene of the unknown fungi was determined as *A. protuberus* (GenBank accession number KX427028).

2.3.2.4. Pure culture and preparation of inoculum

A. protuberus isolated from the decaying forest litter soils was maintained on Czapek Dox medium, and spore suspension was prepared from 7 days grown old slants by adding adequate amount of sterile distilled water with Tween-20 (0.2%, v/v).

2.3.2.5. Effect of various inducers on cellulase production

To test the effect of different inducers on cellulase enzyme production and mycelia growth by *A. protuberus*, four inducers at a concentration of a 1% (w/v) of cellulose, cellobiose, sorbitol and carboxymethyl cellulose were used and incorporated into Czapek Dox medium which is supplemented with 0.5% cellulose. To examine cellulase activity, a time course was conducted, i.e., 2, 4, 6, 8, and 10 days of incubation at 30°C in an orbital shaker with 150 rpm. After incubation spent medium was filtered through Whatman No. 1 filter paper and supernatant was centrifuged at 10,000 rpm for 10 min at 4°C. The clear filtrate obtained was used as enzyme source. All samples were analyzed in triplicate and the mean values calculated.

2.3.2.6. Different carbon sources in fermentation media for cellulase production

This step consisted of evaluating four different carbon sources used on the enzyme production media at a concentration of 1% (w/v) of glucose, fructose, maltose, and lactose. The experiments were conducted under the same growing conditions from the previous experiment, changing the source of carbon for each test.

2.3.2.7. SmF

Tests were conducted in Erlenmeyer flasks of 250 mL capacity containing 50 mL of Czapek Dox medium

containing 0.5% cellulose. The flasks were autoclaved at 121°C for 15 min and thereafter cooled to room temperature and inoculated with 2×10^6 spores/flask and incubated in an orbital shaker at 150 rpm at ambient temperature (30°C±2°C). After incubation, the culture broths were aseptically passed through pre-weighed Whatman No. 1 filter paper to separate mycelial mat. The filter paper along with mycelial mat was dried and culture filtrate was used in enzyme assay experiments.

2.3.2.8. Enzyme assays

Each filtrate was monitored for FPase, carboxymethyl cellulase (CMCase), and β-glucosidase activity. Filter paper assay method [13] was employed to measure total cellulase activity of *A. protuberus* grown on SmF. The activity of cellulase was expressed in filter paper units. One unit of FPase activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar per minute. The activity of endoglucanase in the culture filtrate was quantified by CMCase method [14]. One unit of endoglucanase activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar per minute. β-glucosidase activity in the culture filtrate of *A. protuberus* was determined according to the method [15]. Activities of FPase, CMCase, and β-glucosidase, were measured on substrate - filter paper, CMCase and p-nitrophenyl β-glucosidase, respectively with appropriate enzyme control.

2.3.2.9. Protein determination

Aliquots of *A. protuberus* culture filtrates with appropriate dilution were used for estimation of soluble protein content according to the method Lowry *et al*. [16].

2.4. Statistical Analysis

Data presented are the averages of triplicates. Duncan's multiple range (DMR) test for all data was carried out by Megharaj *et al*. [17].

3. RESULTS AND DISCUSSION

3.1. Isolation and Screening of Cellulolytic Fungi

The soil sample collected from Mahanandi forest area was serially diluted in sterile distilled water and spread onto the Czapek Dox agar plates consists of 0.5% cellulose. The plates were incubated at 30°C for 7 days. A total of 11 primary fungal isolates were isolated and tested for cellulase activity by plate screening method. Isolated fungal colonies were cultured on CMC agar plates. The appearance of the clear zone around the colony after staining with 0.1% Congo red solution was strong evidence for the secretion of cellulase. Out of 11 isolates, only three fungal strains have exhibited considerable high activity zone around the colony and labeled them as MFS1, MFS2, and MFS3. MFS2 was exhibited high activity zone of 36 ± 1 mm (Figure 1). Therefore, it was selected for further experimentation.

3.2. Morphological Identification of the Fungal Isolates Obtained from the Soil Sample

The isolate *A. protuberus* (MFS2) was purified by repeated sub-culturing on the Czapek Dox agar medium at regular intervals and incubated at 30°C. The primary identification of fungal isolate was carried out based on the colony morphology, i.e., characteristics of mycelia, conidia, and conidiophores/fruiting bodies using light microscopy [18] and it were tentatively identified as *Aspergillus* sp. (Figure 2).

3.3. Molecular Characterization Based on 18S rDNA Sequence

DNA was isolated from the MFS2 strain and checked for its purity by taking absorbance ratio at A260/280 (A260/A280 ratio 1.8-2.0 to be pure), revealing that the DNA isolated from the sources was pure. The same DNA samples, when run on an Agarose gel also confirmed to be pure as the DNA band was single and distinct as showed in Figure 3a.

3.4. Sequencing of the 18S rDNA Gene

28S rDNA gene was amplified by PCR and subjected to Agarose gel electrophoresis along with the DNA

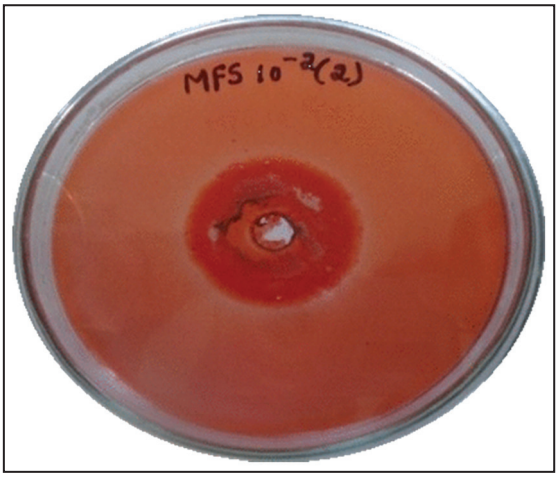

Figure 1: *Aspergillus protuberus* showing clear zone (hydrolyzed zone) around the colony on carboxymethyl cellulose agar plate after staining with 0.1% Congo red.

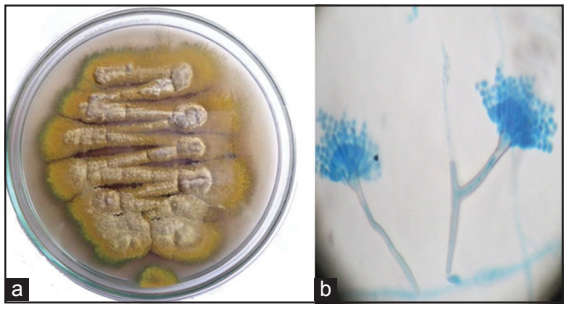

Figure 2: (a) Growth of *Aspergillus protuberus* on Czapek Dox agar medium, (b) lactophenol cotton blue staining of *A. protuberus* under light microscope.

marker (Figure 3b). The consensus sequence of 700 bp of D2 region of LSU gene was generated from forward and reverse sequence data using aligner software. The 28S rDNA gene sequence was used to carry out BLAST analysis at NCBI gene bank database. Based on maximum identity score, fifteen sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 5 software (Figure 4).

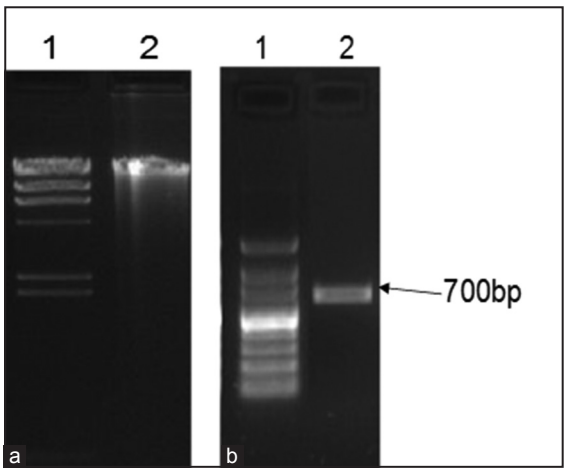

Figure 3: (a) Agarose gel electrophoresis showing the purity of isolated genomic DNA. Lane 1: Hind III marker, Lane: 2: MFS2 genomic DNA, (b) 1.2% agarose gel showing single 700 bp of partial 28S rDNA amplicon band. Lane 1: 1Kb DNA ladder, Lane 2: D2 region of 28S rDNA amplicon.

3.5. Effect of Various Inducers on Cellulase Production

In the present study, *A. protuberus* was grown on the Czapek Dox medium which is supplemented either with one of the inducers and investigated its effect on the production of cellulase. According to results in Figure 5, cellulose was found to be the best inducer which showed FPase activity of 7.05 FPU/ml on the 6th day of incubation with significant difference with enzyme activity followed by CMC (1.913 FPU/ml), cellobiose (1.792 FPU/ml) and sorbitol (1.683 FPU/ml) on the 4th and 6th day of incubation, respectively. Thus, cellulose was the most effective inducer for FPase production followed by CMC.

Cellulose was the most effective inducer for the production of CMCase, as reflected by recovery of 3.658 U/ml on the 1st day of incubation and considered as its maximum production followed by cellobiose (0.596 U/ml), CMC (0.539 U/ml), and sorbitol (0.4958 U/ml) on the 2nd day of incubation, respectively (Figure 6). Thus, cellulose was the most effective inducer for the production of CMCase among all inducers tested.

Maximum titer (0.121 U/ml) of β-glucosidase was recorded on the 6th day of incubation when *A. protuberus* was induced with CMC and considered it as maximum production. Cellobiose, cellulose and sorbitol act as poor inducers for the production of β-glucosidase as reflected by the recovery of 0.021, 0.0195, and 0.0193 U/ml on the 4th and 10th days

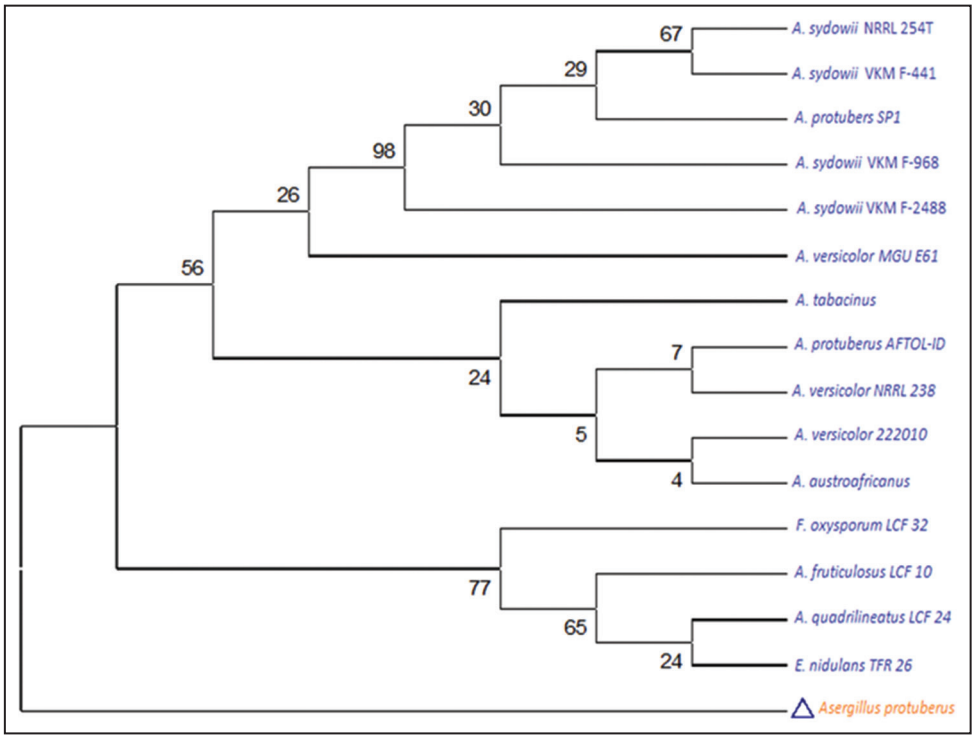

Figure 4: Phylogenetic tree showing the relationship of *Aspergillus protuberus* with other related fungal species retrieved from GenBank based on their sequence homologies of 18S rDNA.

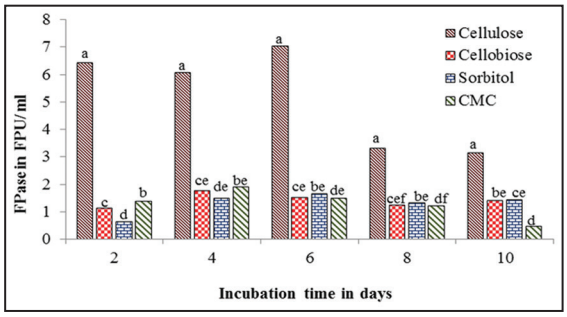

Figure 5: Effect of different inducers on the production of filter paperase by *Aspergillus protuberus* in submerged fermentation. Means, in each column, followed by the same letter are not significantly different ($p \leq 0.05$) from each other according to Duncan's multiple range test.

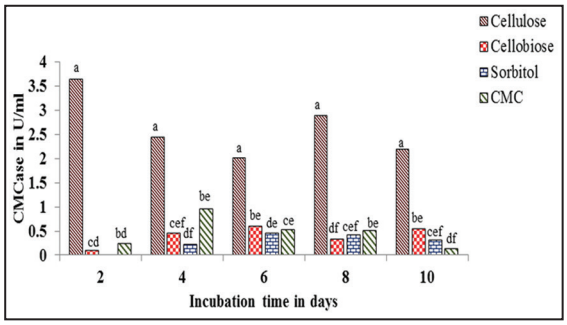

Figure 6: Effect of different inducers on production of carboxymethyl cellulase by *Aspergillus protuberus* in submerged fermentation. Means, in each column, followed by the same letter are not significantly different ($p \leq 0.05$) from each other according to Duncan's multiple range test.

of incubation, respectively (Figure 7). Thus, CMC was the most effective inducer for the production of β-glucosidase among all inducers tested.

The secretion of protein content into liquid medium under shaking conditions for 10 days was measured (Figure 8). Secretion of extracellular protein content was obtained maximum (6.68 mg/ml) when *A. protuberus* was induced with cellulose on the 10th day of incubation. Cellobiose, sorbitol, and CMC act as poor inducers for the production of extracellular protein as reflected by the recovery of 4.63, 4.06, and 5.45 mg/ml on 2nd, 6th, and 10th day of incubations, respectively. Thus, cellulose was found to be the best inducer for the production of protein among all inducers tested.

3.6. Effect of Various Carbon Sources on Cellulase Production

It is apparent from literature that cellulase production depends on nature of carbon sources. Different fermentable sugars have been shown to either induce or inhibit cellulase production depending on individual species. To decipher the suitable sugar

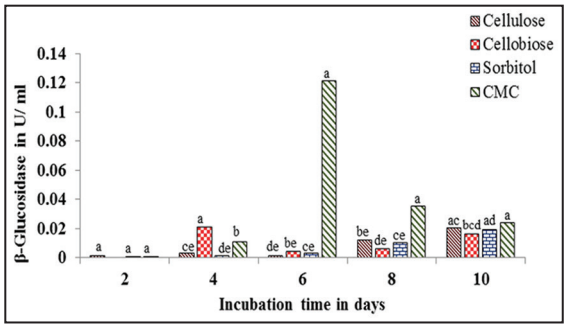

Figure 7: Effect of different inducers on production of β-glucosidase by *Aspergillus protuberus* in submerged fermentation. Means, in each column, followed by the same letter are not significantly different ($p \leq 0.05$) from each other according to Duncan's multiple range test.

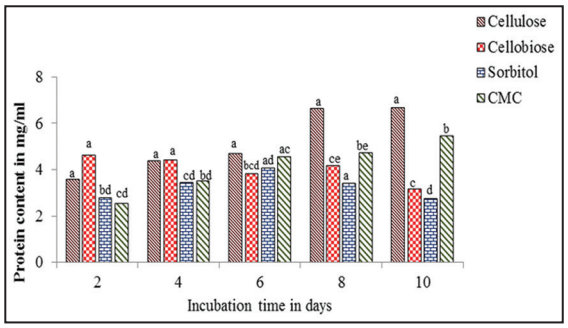

Figure 8: Effect of different inducers on production of extra cellular proteins by *Aspergillus protuberus* in submerged fermentation. Means, in each column, followed by the same letter are not significantly different ($p \leq 0.05$) from each other according to Duncan's multiple range test.

for the *A. protuberus*, glucose, fructose, lactose and maltose were tested. *A. protuberus* utilized all carbon sources for growth and cellulase production. However, lactose and fructose exhibited extensive role to enzyme yield and production. Among different carbon sources, lactose was the best carbon source followed by fructose for cellulase production.

Maximum FPase activity was recorded on the 2nd day of incubation irrespective of carbon sources used, and activity was gradually decreased along with the incubation time increases (Figure 9). Lactose was the best carbon source for the production of FPase activity of 2.17 FPU/ml on the 2nd day of incubation with a significant difference with enzyme activity followed by fructose (1.93U/ml), maltose (1.213 FPU/ml) and glucose (1.19 U/ml), respectively. Thus, lactose was the best carbon source for FPase production followed by fructose.

As data shown in Figure 10, maximum production of CMCase was recorded on the 2nd day of incubation in all carbon sources studied except lactose. Lactose

was the most effective carbon source for CMCase, as reflected by recovery of 1.37 U/ml on 8th day of incubation and considered as its maximum production followed by fructose (0.87 U/ml) maltose (0.787 U/ml) and glucose (0.2 U/ml) on the 2nd day of incubation, respectively. Thus, lactose was the most effective carbon source for the production of CMCase among all carbon sources tested.

Unlike FPase and CMCase enzyme activities, β-glucosidase activity was increased when incubation time increased and reached its maximum activity on the 10th day of incubation in case of all carbon sources studied except fructose (Figure 11). Maximum titers (1.419 U/ml) of β-glucosidase was recorded on the 10th day of incubation when *A. protuberus* was grown on maltose as sole source of carbon and considered it as maximum production. Fructose, glucose and lactose act as poor carbon source for the production of β-glucosidase as reflected by the recovery of 0.99, 0.28, and 0.12 U/ml on 8th and 10th day of incubation, respectively. Thus maltose was the most effective carbon source for the production of β-glucosidase among all carbon sources tested.

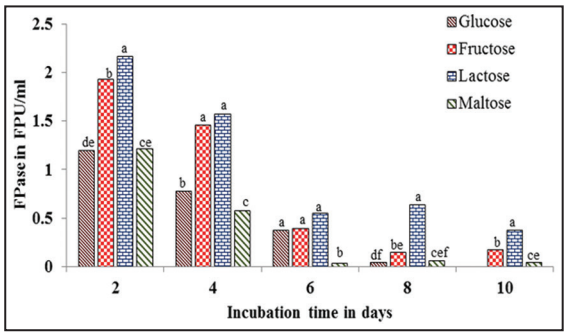

Figure 9: Effect of different carbon sources on production of filter paperase by *Aspergillus protuberus* in submerged fermentation. Means, in each column, followed by the same letter are not significantly different (p≤0.05) from each other according to Duncan's multiple range test.

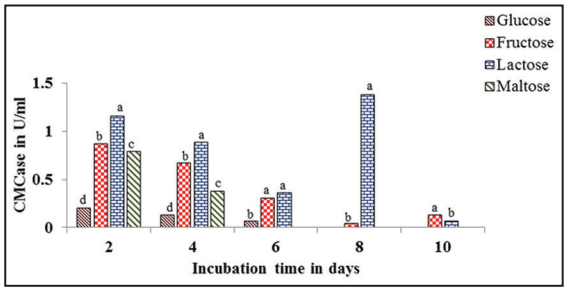

Figure 10: Effect of different carbon sources on production of carboxymethyl cellulase by *Aspergillus protuberus* in submerged fermentation. Means, in each column, followed by the same letter are not significantly different (p≤0.05) from each other according to Duncan's multiple range test.

The secretion of protein content into liquid medium under shaking conditions for 10 days was measured (Figure 12). Secretion of extracellular protein was maximum (6.34 mg/ml) when *A. protuberus* was cultivated on fructose as a sole source of carbon on the 2nd day of incubation and considered it as maximum. Lactose, maltose, and glucose act as poor carbon source for the production of extracellular protein as reflected by the recovery of 3.775, 2.33, and 2.11 mg/ml on 8th and 10th days of incubations, respectively. Thus, fructose was the best carbon source for the production of extracellular proteins among all carbon sources tested.

A diverse spectrum of cellulolytic organisms have been isolated and identified over the years and this list is still continues to grow rapidly. In this investigation, we have navigated the cellulolytic fungi from forest litter samples and subjected to optimization of inducers and carbon sources for cellulase production under SmF. In general, SmF was used to produce commercially important enzymes in which the microorganisms are cultivated in a nutrient rich aqueous medium. The main advantages of the submerged processes are ease in controlling the physicochemical process, greater efficiency of nutrient absorption, and excretion of

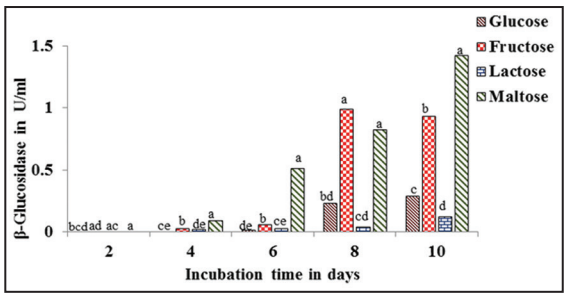

Figure 11: Effect of different carbon sources on production of β-glucosidase by *Aspergillus protuberus* in submerged fermentation. Means, in each column, followed by the same letter are not significantly different (p≤0.05) from each other according to Duncan's multiple range test.

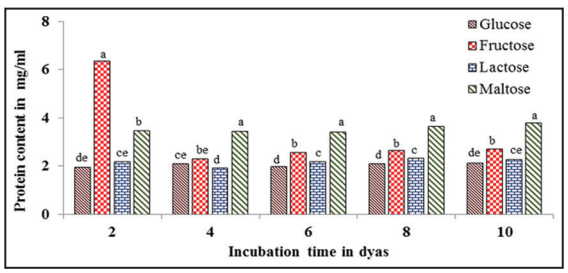

Figure 12: Effect of different carbon sources on production of extracellular protein by *Aspergillus protuberus* in submerged fermentation. Means, in each column, followed by the same letter are not significantly different (p≤0.05) from each other according to Duncan's multiple range test.

metabolites through the cells, leading to lower process times and, consequently, productivity gains. Moreover in SmF; modern methods of control are more easily adapted to fermentation [19]. From an economic viewpoint, the solid state fermentation (SSF) method has its own advantages over the traditional SmF method for enzyme production. There are also technical problems associated with the large-scale implementation of SSF because of uncontrolled heat and decline in available oxygen results in the cessation of mesophilic aerobic microbial activity leads to the consequential cessation of enzyme production [20,21].

In the present study, impact of different inducers on the growth and total cellulase enzyme production by *A. protuberus* under SmF was studied and observed that the strain was exhibited all three enzyme activities. Different components of cellulolytic enzymes reached peak production at different time intervals. In fungi, the production of cellulolytic enzymes is subject to transcriptional regulation by available inducers. The cellulase genes are repressed in the presence of glucose. Earlier it has been reported that endoglucanase was induced by CMC but repressed by glucose [22]. In this present study, we recorded the similar results with very less activities of cellulase in the presence of sorbitol, while cellulose proved to be a strong inducer of cellulose.

Cellulase production was found to be dependent upon the nature of the carbon source used in the culture media. To evaluate the carbohydrates to cause induction or repression of cellulase, the organism was grown on monosaccharide and disaccharides. In the present study, evaluated four carbon sources induction the peak productions of FPase and CMCase on the 2^{nd} day of incubation and in case of β-glucosidase production except fructose remaining carbon sources induced the peak levels on the 10^{th} day of incubation whereas fructose induces on the 8^{th} day of incubation. In case of extracellular protein content glucose and maltose induced on a 10^{th} day whereas fructose and lactose on 2^{nd} and 8^{th} day of incubation, respectively. This study substantiates the work of Kathiresan and Manivannan [10] and Devanathan *et al*. [23] who demonstrated lactose as best inducer of *Aspergillus* sp. Nochure *et al.* [24] identified fructose as the best inducer of cellulase in *Clostridium thermocellum*. In another study, dextrin was found to enhance the production of cellulase by *Trichoderma* sp. in SSF (2006).

4. CONCLUSION

To the best of our knowledge, this is the first report on cellulase production from *A. protuberus* under SmF. Therefore, an attempt was made in this study to enhance the cellulase production by optimizing the inducers as well as carbon sources. We found that among the inducers and carbon sources evaluated in the present study glucose is the best inducer and lactose is found to be the best carbon source for the production of FPase and CMCase whereas β-glucosidase and protein content CMC and fructose was found to be the best inducers and carbon sources, respectively.

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