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Optimization of Production Conditions of Cellulase Enzyme from Micro-Fungi *Aspergillus Fumigatus* for Agriculture Application

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Cellulase enzymes are belonging to the hydrolytic group of enzymes facilitates the sugar release and its bioconversion into different valuable industrial products. Isolated micro fungi from rice straw by dilution plating pouring method studied for playing a various role in industries as well as in agriculture application. Various micro-fungi show enzymatic degradation of lignocellulosic material. The present study optimized the growing conditions for cellulase enzymes production from *A.fumigatus*. Optimization of various growth conditions such as temperature, different pH level and nitrogen source were studied for the production of enzyme carboxymethyl cellulase during this study. The result showed that *A. fumigatus* produced highest cellulase activity (3.546 IU/ml) at pH 7.0 and temperature 30^{0}C with yeast extract and Fpase activity (0.653 IU/ml) through solid state fermentation. In future agriculture applications and in industries the cellulase enzyme production attains a crucial role to acquire biodegradable yield.

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1. INTRODUCTION

The practice of burning leftover rice straw is widespread throughout the Asia-pacific area. The negative result of burning rice straw on the environment, such as air pollution also contribute to changes in temperature, loss fertility of soil on agriculture land. Lignin, hemicelluloses and cellulose make up the majority of lignocellulosic biomass.

Cellulose is the most prevalent lignocellulosic biomass which accounts for 40-60% of its weight [1].Cellulose has a polyacetal form of cellobiose (4-0-D glucopyranosyl-D-glucose) [2]. According to [3] cellulose makes up the majority of plant biomass and is only present in the cell wall of plants and its strong influences on lignocellulosic biomass recalcitrance can be found. Therefore, the cellulose was digested by a bio-enzyme like cellulase to create glucose, which was then utilized in many businesses [4].The biopolymer of cellulose can be converted into reducing sugars by cellulase enzyme which have various biotechnological uses [5].Various microfungi, bacteria, actinomycetes generate this enzymes [6].

Micro-fungi, which can be easily exploited to produce commercial cellulases and are found in nature, as natural agents for cellulase degradation. For the synthesis of cellulase, *Trichoderma* and *Aspergillus* are thoroughly investigated [7]. *Aspergillus* species are present in almost all situation with high oxygen levels. The *Aspergillus* species has a number of qualities that make them exceptional organisms for use in agriculture and industries, including satisfactory fermentation proficiency, high levels of protein secretion, high sporulation capacity and ability to acclimate to various organic substrates [8]. In addition, they are involved in the synthesis of enzymes that aid in the breakdown of plant cell wall components like lipids, starch and protein. [9].

A perfect environment is needed for multiplication of micro fungi strain and increase the production of the cellulase enzyme. The yield of the enzyme often depends on a complex relation between numerous variables, including inoculums size, pH, temperature the presence of inducers, growth period, moisture and medium of cultivation [10-12].The optimal pH, solubility and amino acid content of the majority of cellulase investigated are comparable. The substrate's specificity and thermal stability can change. Therefore the objective of the current study is to investigate high level cellulase enzyme produced by *A. fumigatus* and optimize the parameter to hasten the production of cellulase.

2. MATERIALS AND METHODS

2.1 Sample Collection

Samples were collected in polythene from rice field of Rawali village near Muradnagar UP after harvesting of crop for study. Sample brought to laboratory in the Department of Biotechnology Shobhit deemed -to- be University, Meerut. For study we have using a Nylon net bag technique.

2.2 Isolation of Fungus

Isolated *A.fumigatus* fungus from buried rice straw by Dilution plating pouring method. The potato dextrose agar (Hi-media GMH09-India) was prepared according to instructions with pH 5.6 after that sterilized at 121^0C temperature and 15 lbs for 30 minutes and poured into petriplates,the plates were leave to solidify at room temperature. Weigh one gram rice straw (2-4 cm pieces) and taken into test tube with 9 ml distilled water and shaken at constant speed for 5 minutes. The rice straw suspension of 1µl from each dilution (upto10^{-3}) pours into petriplates and spread with the help of sterilized spreader. The plates were incubated fro 5-7 days at 28^0C.

2.3 Identification of Microfungi

Further fungus colony taken up from potato dextrose agar plate and prepared a microscopic slide with lactophenol and seen under microscope (Lieca EC4 at 400X) fungus identified as morphologically and structurally as describe by text books [13,5,14-16].Later it will also send to IARI, PUSA, New Delhi Plant Pathology lab for identification. Once confirmation done *A. fumigatus* was stored at 4^0C in refrigerator for further uses.

2.4 Cellulase Enzyme Production

Cellulase production was performed by using rice straw as the sole carbon source in a 500 ml an Erlenmeyer flask containing broth media. The

composition of the medium was in (g/l in distilled water yeast powder (2g/l), jaggery (5g/l) and urea (1g/l).

In this study Solid state fermentation (SSF) was used for the production of cellulase enzyme. Spore suspension were prepared with the same media for 4-5 days old culture of *A. fumigatus* and scratched with sterilized plastic loop under the aseptic conditions in laminar air flow. Then, 5ml of spore suspension were inoculated into the rice straw flasks media and gradually mixed. The flasks were then placed in static condition in incubator .The temperature of incubator was fixed at 28 ^{o}C. After selected time of incubation and growth flasks were taken out from incubator and adding 50 ml citrate buffer (50Mm,pH-4.8) in flask and filtered off with (Whatman filter paper No.1) and transferred into falcon tube for centrifugation (Eppendorf) at 12,000 rpm for 15 minutes to remove all cell debris. The supernatants were used to measure the cellulolytic activity by the standard test method [15].

2.5 Cellulase Assay

The extracellular carboxymethyl cellulase enzyme assay, as well as fpase-enzyme assay was performed. For Carboxymethyl cellulase assay among three tubes, 1st tube was for substrate blank containing 1.6 ml sodium citrate buffers, 0.4 ml carboxymethly cellulose and for enzyme blank 1.6 ml enzyme of desired fungus, 0.4 ml sodium citrate buffer while 3rd tube was for test sample, containing 1.6 ml fungus enzyme, 0.4 ml Carboxymethyl cellulose. Then all the test tube and control tubes were kept in water bath at 45°C for 15 min and cool under running tap water after that we have taken 1 ml sample from another same set of test tube and added 1 ml dinitrosalicylic acid (DNS) and were boiled for 5 min. The optical density (OD) of the mixture was checked by spectrophotometer (Eppendorf) at 540 nm wavelength.

2.6 Filter Paper Activity (FPase) Production

Filter paper assays were determined by standard methods [14].The filtrate of enzyme sample is collected in tube and added a whatman no.1 filter paper strip (1×60 cm, 50 mg) and 1 milliliter of 0.05M sodium citrate buffer of 5.0 pH.Incubate all the tubes at 50 ^{o}C into water bath for 1 hour and cool down the tubes. Reducing sugars released were estimated by dinitrosalicyclic acid (DNS) method [15].One unit of filter paper (FPU) activity was defined as the amount of enzyme required to liberate 1 µ mole reducing sugars from the filter paper per ml per minute under standard assay conditions [17].

3. OPTIMIZATION OF CULTURE CONDITIONS FOR CELLULOSE ENZYME PRODUCTION UNDER SOLID STATE FERMENTATION (SSF)

3.1 pH Effect on Cellulase Production

During this experiment different pH (5.0, 6.0, 7.0, 9.0) were tested for enhance production of cellulase and Fpase activity. pH of medium was adjusted with 0.1 HCL and 0.1 NaoH solutions. Initially all the flasks were incubating at 28 ^{o}C for 7 days in stationary stage. After 7 days of cultivation flasks were taken ,adding 50 ml citrate buffer (50Mm,pH-4.8) in flask and filtered off with (Whatman filter paper No.1) and transferred into falcon tube for centrifugation (Eppendorf) at 12,000 rpm for 15 minutes to remove all cell debris. The supernatants were used to measure the cellulolytic activity by the standard test method [15].The absorbance was measured by Bio spectrophotometer (Eppendorf AG-22331) at 540 nm.

The pH level will affect the yield of cellulase production which will later use for further study.

3.2 Temperature Effect on Cellulase Production

Incubation temperature influences various metabolic activities such as enzyme production. Therefore in this study different temperature (25°C, 30°C, 35°C, 40°C) were used for optimization of fugal strain cellulase production under solid state fermentation. All the flasks were incubated for 7 days. After 7 days of incubation period, adding 50 ml citrate buffer (50Mm,pH-4.8) in flask and filtered off with (Whatman filter paper No.1) and transferred into falcon tube for centrifugation (Eppendorf) at 12,000 rpm for 15 minutes to remove all cell debris. The supernatants were used to measure the cellulolytic activity by the standard test method [15]. The absorbance was measured by Biospectrophotometer (Eppendorf AG-22331) at 540 nm.

3.3 Nitrogen Source Effect on Cellulase Production

For cellulase production nitrogen is most important factor. During solid state fermentation medium of flasks supplemented with nitrogen source like urea, ammonium nitrate, yeast extract and diamonium phosphate (DAP) for hasten the cellulase production. After 7 days of incubation period, adding 50 ml citrate buffer (50Mm,pH-4.8) in flask and filtered off with (Whatman filter paper No.1) and transferred into falcon tube for centrifugation (Eppendorf) at 12,000 rpm for 15 minutes to remove all cell debris. The supernatants were used to measure the cellulolytic activity by the standard test method [15].The absorbance was measured by Biospectrophotometer (Eppendorf AG-22331) at 540 nm.

4. RESULTS AND DISCUSSION

4.1 Isolation of Microfungi

We have taken nylon bags (5*10) containing 20 gm of rice straw chopped into a 2-4 cm pieces were prepared and kept in pits. These nylon bags with rice straw were buried 15 cm from the soil surface. Nylon bags with rice straw taken out from pits at 15 day of incubation for isolation of fungus.

4.2 Identification and Screening of Micro Fungi

Primarily many fungi, molds, yeast were isolated from rice straw. At the starting isolated fungi identification done by prepared microscopic slide by using lactophenol and morphological and structurally features(size of spore,conidia,color of mycelium)seen under microscope. But *A.fumigatus* selected as cellulase producers by performing cellulase activity test on CMC media plate (carboxy methyl cellulase) agar. *A. fumigatus* selected on the basis of it produces a clear zone on CMC media plate when it is treated with 1% congo red dye and wash with 1M Nacl solution.

4.3 pH Effects Cellulase Production

All the three replicate were grow in media using various pH value of 5.0, 6.0, 7.0 and 9.0.Higher CMase activity were obtained in medium of pH-7.0 (2.672 IU/ml) and Fpase activity obtain were (0.130 IU/ml) as shown in Table 1.The finding of study is disagree with [17] who reported that maximum CMase and FPase enzyme activity was observed in pH-6.5.The present study were linen with the finding [18] says that maximum cellulase production achieved at pH 7.0 by *Aspergillus* species.

4.4 Temperature Effects Cellulase Production

Our study of *A.fumigatus* at different temperatures value 25^{0}C,30^{0}C,35^{0}C,40^{0}C showed the highest CMase(3.546 IU/ml) and FPase enzyme activity(0.653 IU/ml) at 30 ^{0}C which is shown in Table 2.The present research disagreed with [19] who says the optimum temperature for cellulase production by *A.flavus* and *A.fumigatus* at 40^{0}C-50^{0}C. The result of our study quite resemble to [17] was found maximum cellulase production by *A.fumigatus* at 32^{0}C.

4.5 Nitrogen Effects Cellulase Production

Nitrogen source affected the enzyme production. The result are similar to [17,11] who reported that yeast extract was optimum nitrogen source for cellulase production similarly our result shows Optimum nitrogen source is yeast extract which enhance the CMase activity (2.681IU/ml) and Fpase(0.242 IU/ml) activity during degradation process, indicated in Table 3.

Fig. 1. Nylon bag with rice straw kept into soil

Fig. 2. Pure culture of *A. fumigatus* on PDA plate

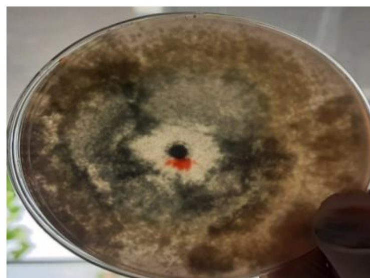

Fig. 3. Screening of *A. fumigatus* on CMC plate

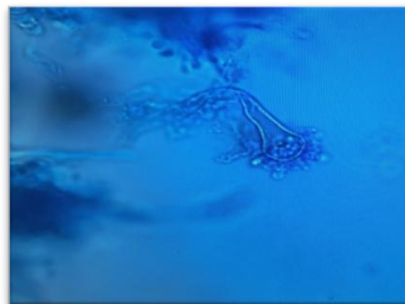

Fig. 4. Slide of *A. fumigatus* under microscope at 400 X

Table 1. Optimization of pH

pH	CMase(IU/ml) Mean ± SD	Fpase(IU/ml) Mean ± SD
5.0	1.084±0.89	0.050±0.07
6.0	1.943±1.51	0.105±0.07
7.0	2.672±2.30	0.130±0.13
9.0	0.993±0.81	0.080±0.06

Values present a Mean ± SD of three replication under solid state fermentation

Table 2. Optimization of temperature

Temperature ^{0}C	CMase(IU/ml) Mean ± SD	Fpase(IU/ml) Mean ± SD
25	2.010±1.70	0.119±0.06
30	3.546±1.93	0.653±0.53
35	1.645±1.39	0.114±0.06
40	0.952±0.78	0.089±0.04

Values present a Mean ± SD of three replication under solid state fermentation

Table 3. Optimization of nitrogen

Nitrogen source 2 %(w/v)	CMase(IU/ml) Mean ± SD	Fpase(IU/ml) Mean ± SD
Ammonium nitrate	1.434±1.20	0.171±0.11
Urea	2.195±1.86	0.217±0.15
Yeast extract	2.681±2.29	0.242±0.18
Di ammonium phosphate	1.462±1.23	0.204±0.14

Values present a Mean ± SD of three replication under solid state fermentation

5. CONCLUSION

In world lignocellulosic material degradation is a major problem. India and China are producing 90% of rice amongst Asian countries. As a result burning of rice straw is done by farmers. The cellulase enzymes play a vital role in degradation of lignocellulosic materials as well as used as alternative energy resources. Present study result showed that isolated micro-fungi was confirmed as *A.fumigatus* has cellulolytic enzyme activity and used for degradation of lignocellulosic material . So the optimization of media parameters is important for fermentation. The growth of fungus depends on media pH and effect the stability of product. The present study shows highest cellulase and Fpase acivity by *A. Fumigatus* at pH 7.0, Temperature 30^0C and best nitrogen source is yeast extract.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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