Volume 64, Issue 3, 2020
**Journal of Scientific Research**
Institute of Science,
Banaras Hindu University, Varanasi, India.

National Conference on Frontiers in Biotechnology & Bioengineering (NCFBB 2020), JNTU Hyderabad, India

# *Aspergillus niger* Based Production of Cellulase-A Study on Submerged and Solid State Fermentation

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***Abstract:*** **Cellulases are group of hydrolytic enzymes viz. FPase, carboxymethylcellulse (CMCase) that lead to release of sugars via bioconversion of lignocellulosic biomass into variety of value-added products. In the present study using wheat bran and groundnut shell as native lignocellulosic substrates, cellulase was produced through SmF and SSF. The source for cellulase production was *Aspergillus niger*. The extracts like peptone, tween and yeast extracts increased the productivity both in SmF and SSF. The enzyme concentration reached a maxima after an incubation period of 72 h for SSF and 96 h for SmF in both substrates. The activities of CMCase and FPase as observed in SSF were 24.4IU/g and 10.6 IU/g, 13.7IU/g and 8.8 IU/g for wheat bran and groundnut shell respectively, whereas in SmF they were found to be 6.32 IU/mL and 3.1 IU/mL, 3.12 and 1.3 IU/mL respectively. The production of extracellular cellulases in SSF was 4 fold higher than in SmF respectively. This data provides information on the cellulolytic potential of the fungal strain *Aspergillus niger*, a promising source for future commercial applications with economic feasibility.**

***Index Terms:*** ***Aspergillus niger*, Cellulase, Submerged fermentation, Solid-state fermentation.**

## I. INTRODUCTION

The rising energy demand and depletion of fossil fuels has provoked towards development of sustainable renewable energy source that are alternate to fossil fuels (Saranga, 2020, Panga,2017). Bioethanol is considered as one the most promising transportation fuel to reduce the emissions of greenhouse gases (GHG) (Yerra, 2020). The bioethanol can be produced from food crops, but this threatens the global foods chains. In this aspect, a great interest has been shown towards the bioethanol production from alternative feedstock such as food waste (Changbo Wang, 2020; Wei Han, 2020.). Lignocellulosic biomass is among the best source for bioethanol production (Uma Addepally,2019). In order to make lignocellulosic biomass an economically feasible for bioethanol production, an extensive investigation is required for conversion of lignocellulosic biomass into fermentable sugars by enzymatic hydrolysis. The selection of feedstock and microorganisms capability to breakdown the lignocellulosic polymers during cellulosic bioethanol production plays a vital role in the improvements of yields (Sai Praneeth Thota, 2016).

In this concern, a wide range of microorganisms were isolated and identified from the nature, for the production of enzymes that are capable for the conversion of cellulosic substrate into fermentable sugars (cellobiose and glucose). The enzymes involved in this process are called cellulases, which are a group of hydrolytic enzymes (carboxymethylcellulase (CMCase), filter paperase (FPase), and β-glucosidases) (K. Shruthi, 2019). A great interest has been shown in the cellulases production using filamentous fungi either in solid state fermentation (SSF) or submerged fermentation (SmF) for bioethanol production from lignocellulosic biomass (Norma N, 2010). SmF process is traditionally equipped in the industries for the production of enzymes because of its easy handling system and control of several environment conditions such as pH, temperature, agitation and aeration (G. Praveen Kumar Reddy, 2015). However, SSF grows on a solid (water free) substrate, which make more suitable filamentous fungi growth, is reported for higher productivity with lower energy consumption, lower sterilization requirement and less downstream process (Ankit Lodha, 2020).

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*DOI:* 10.37398/JSR.2020.640312
Selection and Peer-review under responsibility of the Program Chairs.

The utilization of these lignocellulosic biomass waste as a substrate in SSF and SmF for cellulase production reduces the production cost due to its large quantity accruing and high nutritional value. Wheat and ground-nut shell are the major crop cultivated in all parts of the world and India is the second largest producers of these crops globally. The residues of these crops are generally considered as agricultural waste. Groundnut shell contains abundant natural cellulose which can be utilized as a feedstock for cellulase enzymatic fermentation by cellulolytic fungi (*Aspergillus niger*). The groundnut shells are the leftover byproduct which are obtained after the separation of their seeds from its pod (Ashish Vya, 2016). Groundnut shells is high in lignin content, which slower the degradation rate of the shell under natural environment conditions. A wheat kernel part consists of three principle fractions namely germ, endosperm and bran. Wheat bran is a rich source of fiber, mineral, vitamins and phenolic compounds. Worldwide around 690 million tonnes of wheat is produced annual. The wheat contains 25:75 ratio of bran and milled wheat, resulting in accumulation of around 150 million tonnes of wheat bran. So, these agronomic by products (wastes) can be transformed into a valuable bio-product such as biofuels through biochemical treatment. (Bharti Amit Kumar, 2018; Pham Anh Duc, 2019).

In the current study, the quantification of extracellular cellulases by *A.niger* in SmF and SSF were studied for commercial cellulase using as a substrate which is wheat bran and Groundnut shell.

## II. MATERIALS AND METHODS

### *A. Microorganism Preparation*

The locally isolated *Aspergillus niger* fungal culture was used as an inoculum for both submerged fermentation (SmF) and Solid state fermentation (SSF) process. The strains were cultured on slants of potato dextrose agar (PDA) at 30$^{o}$C for 7 days and stored at 4$^{o}$C, these cultures were sub cultured for every two weeks on seed media (PDA) with potato dextrose-20g/L, glucose-15g/L and agar-20g/L. Fresh Potato dextrose slants were prepared and fungal strains were streaked. The formation of spores after 8-10days, 10ml of sterile distilled water was added to the slant and disrupted the spores by gentle pipetting so that the spores get suspended in the water.

### *B. Inducer Substrate Preparation*

The lignocellulosic substrates which is Groundnut shell (GS) and wheat bran (WB) were used in the study, harvested from local agricultural field sources located at Sangareddy District, Telangana State, India. The sun dried GS and WB substrates were grounded into small particles size about 2mm. these dried grounded particles was used as a substrate for SmF and SSF experiments.

### *C. Cultivation of Enzymatic Hydrolysis*

The cultivation of enzymatic hydrolysis were carried out in 250ml Erlenmeyer flask with 100ml as a working volume. The nutrient media was used in the study for the growth and the cellulolytic enzyme production. The initial pH of media was adjusted to 4.8 prior to the fermentation process. The incubation of the media was carried out for seven days. All the experiments were conducted in triplicate and the average results were represented in the current study.

#### *1) Nutrient Medium*

The composition of the nutrient medium used for SSF and SmF experiments and during cellulolytic enzyme production were prepared as per the Mandels (1976) process. The composition of the media is as shown in table1.

**Table.1** Composition of Nutrient medium

| **Nutrients** | **Composition (g/L)** |
|---|---|
| Urea | 0.3 |
| Ammonium sulphate | 1.4 |
| Potassium dihydrogen phosphate | 2.0 |
| Calcium chloride | 0.3 |
| Magnesium sulphate | 0.3 |
| Yeast extract | 0.2 |
| Peptone | 0.75 |
| Ferrus sulphate | 0.05 |
| Cobalt chloride | 0.02 |
| Manganese sulphate | 0.016 |
| Zinc sulphate | 0.014 |
| Tween 80 | 0.1% |

#### *2) Pre culture for Submerged Fermentation (SmF)*

In SmF process, the pre-culture was initiated for hydrolysis in 250mL Erlenmeyer flask consists of 4% of substrate (GS and WB) and 90 ml culture medium. The media was sterilized at 121$^{o}$C, 15mins at 15psi. The sterilized media was inoculated with 10% (v/v) of $10^{6}$spore suspension. The incubation of media was carried out for 7days in a controlled shaking incubator (Bio-technics India), at 28°C and 100rpm. The samples were collected every 24h for the quantification of enzyme.


**Fig. 1 schematic view of Submerged fermentation**

*3) Pre-culture for Solid state fermentation (SSF)*

In SSF process, the moisture content in the substrate was adjusted using distilled water to maintain 1:2 (Solid: liquid) ratio and adjusted the pH to 4.8. The experiments was carried out in 250ml Erlenmeyer flasks contain 5.0 g dry substrates (particle size 2.0 mm). These flaks were subjected for sterilization using autoclave at 121$^{o}$C for 15 min. Under room temperature, culture inoculation was performed with 24 h grown active culture of *Aspergillus niger* on PD broth followed by the incubation. The fermentation experiments was carried out at 28°C in an incubation chamber with controlled humidity (70% Moisture content). The extraction of enzyme was carried by adding 1:7 ratio of citrate buffer (pH 4.8) for solid state cultures for 1 hr in on rotary shaker at 28 $^{o}$ C *(Namita Bansal*, 2011). The fermented product was passed through a metallic sieve and the solid residue was further pressed to release leftover liquid. The obtained liquid product was centrifuged at 10,000 rpm for 10 min under 4$^{o}$C to remove the solid and the clear supernatant was analyzed for cellulase enzyme. Crude enzyme was preserved at 4°C for further analysis. (Namitha, 2012).

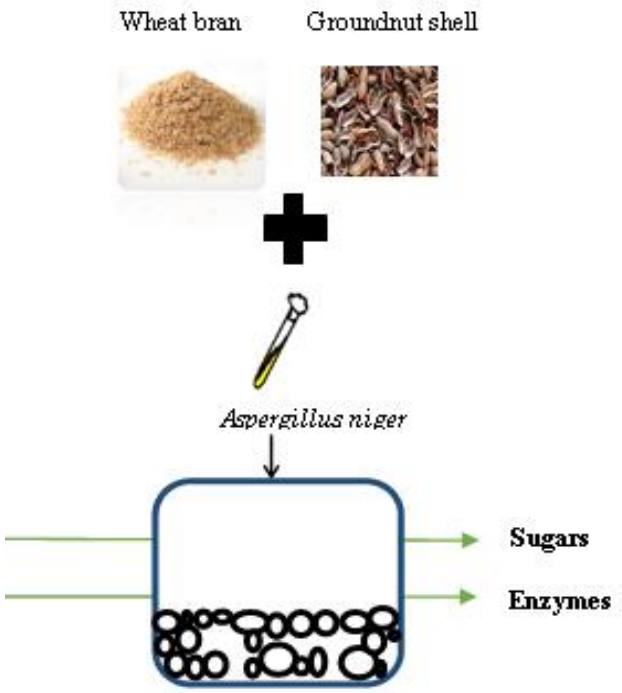


**Fig. 2 Solid state fermentation (SmF) process.**

*D. Analytical Methods*

*1) Determination of Enzyme Activities:*

The supernatants containing the extracellular enzyme released by the *Aspergillus niger* was assayed by filter paper activity (FPA) and expressed in terms of filter paper units (FPU) (Ghose TK, 1987, Miller GL, 1959). The cellulase activity of exo-β-1,4-β-glucanaseactivity and endo-β-1,4-glucanase were analyzedin the form of reducing sugars by DNS method. The quantification of exo-β-1,4-β-glucanase were measured using enzyme solution (0.5 mL) and citrate buffer (0.05 M, pH 4.8, 1 mL) and 50mg Whatman No. 1 filter paper (1×6 cm strip) and incubated at 50ºC for 60mins. For endo-β-1,4-glucanase Carboxymethyl cellulose (CMC) -0.5 mL, enzyme solution (0.5 mL) and citrate buffer (0.05 M, pH 4.8, 0.5 mL) and incubated at 50ºC for 30mins. The productivity of enzyme were expressed in terms of international units per gram dry substrate (IU/g) for SSF and international units per milliliters (IU/mL) for SmF which is defined as one unit of enzyme activity is equivalent to one micro mol of reducing sugars released by the enzyme in 1 min.

*E. Statistical Analysis*

All the assays were carried out in triplicates and results are expressed as arithmetic mean ± standard deviation.

## III. RESULTS AND DISCUSSION

*A. Submerged Fermentation (SmF):*

Fig: 3, 4 shows the effect of fermentation period on the cellulolytic enzymes production with GN and WB in submerged fermentation (SmF).In the present study, the fermentation experiments were conducted for an incubation of 7 days. The cellulolytic enzymes production started from day 2 with wheat bran yielded FPase: 0.26IU/Ml and CMCase 0.32IU/mL whereas with groundnut shell yielded FPase: 0.13, and CMCase yielded as 0.32 IU/mL. The cellulase activity (CMCase, FPase) increased constantly and reached maximum at 96 hours (5 days) of incubation period. The findings were in accordance with Praveen, 2015. who studied the cellulolytic enzymes production in SmF with wheat bran in batch vials (250ml Erlenmeyer flasks) for 7days and obtained a significant increase in cellulase production the average cellulase production was FPase, CMCase were 2.632 IU/mL and 2.478 IU/mL which was higher than the present study. From the present investigation the highest cellulase productivity was obtained with substrates (wheat bran and Groundnut shell). The highest FPase and CMCase of wheat bran and Groundnut shell is 3.1, 6.32 IU/mL and 1.3, 3.12 IU/mL, insist that the submerged fermentation might be applied to achieve high cellulase production with wheat bran and groundnut shell substrates.

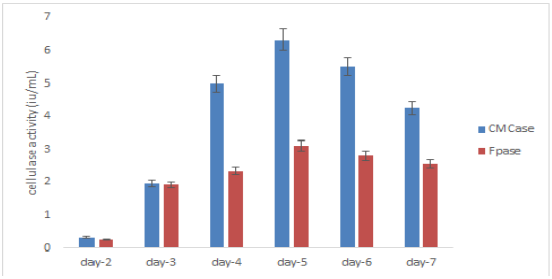


**Figure 3.** Cellulase enzyme production with wheat bran by *Aspergillus niger* in SmF.

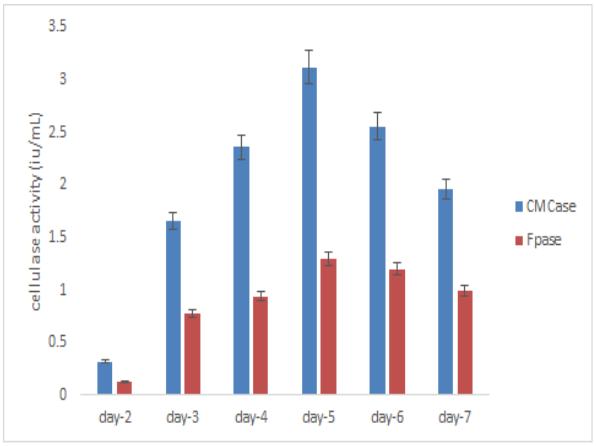


**Figure 4.** Cellulase enzyme production with groundnut shell by *Aspergillus niger* in SmF.

*B. Solid State Fermentation (SSF)*

The Solid state fermentation (SSF) is similar to microbial process where the microorganisms cultivate under its favorable conditions which is closer to their natural habitat which results in the improved production of extracellular enzymes and other enzymes than that of submerged fermentation (Sato K, 1990). *Aspergillus niger* was cultivated in SSF with readily available and inexpensive lignocelluloses. The production of FPase and CMCase activities on Wheat bran and groundnut shell as a substrate was carried out for 7 days (Fig5 and 6). At the initial stage of fermentation the FPase and CMCase yields were lower or in undetectable range at the incubation of day-1and increased from day-2 in both wheat bran yield of FPase: 2.26 IU/g and CMCase: 3.32IU/g and groundnut shell yielded FPase: 0.26IU/g and CMCase 0.32IU/g. Production of the cellulases improved and reach to maximum activity on 4$^{th}$ day in both wheat bran and groundnut shell with CMCase 24.4 IU/g, FPase 10.6IU/g and CMCase 13.7 IU/g, FPase 8.8 IU/g respectively.After incubation period of 4$^{th}$ day, in wheat bran and groundnut shell, a decline phase of cellulolytic enzyme activity was observed.

Among both solid support, wheat bran resulted to be the best solid support matrix than the groundnut shell in production of cellulase enzymes in SSF and the outcomes of the results are supported by (M. Subhosh Chandra, 2007). The higher production of the cellulolytic enzymes was observed in wheat bran since it is known for presence of abundant source of nutrients, in the form of loose texture in moist conditions, and with a large surface area (Smith,1996). However, enzyme activity can be further improved through reduction/degradation of hemicelluloses and lignin by various pretreatment techniques (Ortega, 2000).

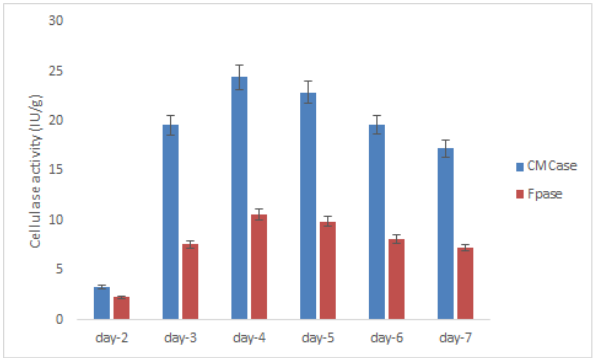


**Figure 5.** Cellulase enzyme production with Wheat bran by *Aspergillus niger* in SSF

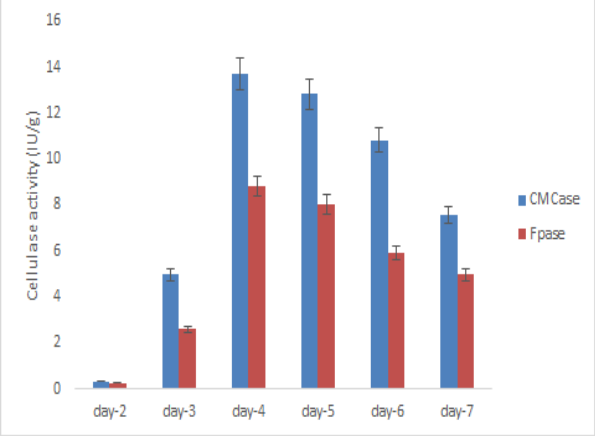


**Figure .6** Cellulase enzyme production with Groundnut shell by *Aspergillus niger* in SSF.

The comparative study between using submerged and solid state fermentation. From the experimental study, the result clearly indicates that both substrates has a very good potential for the cellulolytic enzyme production. It was also observed that SmF and SSF fermentation process were also suitable for the cellulase production using wheat bran and groundnut shell. In the comparative study with the wheat bran and groundnut shell as a substrate in SSF fermentation a higher yields of cellulase activities was observed in both the substrates.

From the experimental investigation solid-state medium showed maximum cellulase activity than submerged fermentation process. During the literature study, a high number of studies were represent on the cellulase production in submerged fermentation but in case of solid state fermentation very few studies were conducted. Hence, the present experimental investigation was focused on the comparative efficiency of both SSF and SmF fermentation process were carried out. The outcome of the study suggest that SSF has a good commercial importance due to its higher efficiency than SmF. But in order to attract industrial prospective, further investigation is required to improve the cellulase enzyme activity yields which can be improved by addition of pretreatment step. On the other hand, SSF require lower energy and sterility demands compared with SmF with higher stability of products and several of microorganisms.

The present investigation studies indicated that the wheat bran and groundnut shell are suitable for cellulases production in both the fermentation process (SSF and SmF). SSF showed for highest production, possibilities for effective utilization of the cellulose into value added product through biotechnological method. Such processes would not only help in reducing the cost of production but also pave the way in effective solid-state fermentation management.

## CONCLUSION

From this study both wheat bran and groundnut shell was found to be effective for the production ofcellulolytic enzymes activity form *Aspergillus niger* in both the fermentation process (SSF and SmF). Solid state fermentation (SSF) than submerged fermentation (SmF) with both the substrates. Higher cellulolytic enzyme production in SSF was observed in wheat bran as a best solid matrix than groundnut shell.

## ACKNOWLEDGEMENT

The author would like to thank JNTUH-TEQIP-III for their financial support to carry out the research.

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