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Culture-dependent isolation and optimization of cellulase-producing fungi from brewer's spent grains

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Abstract

The study involved isolating and optimizing cellulase-producing fungi from brewer's spent grain. Carboxylmethyl-cellulose (CMC) agar was used for the pour plate technique to screen the fungi. Based on the morphology of their colonies and other microscopic features, isolates were identified using lactophenol staining. The diameter and clear zones of colonies were evaluated to determine their hydrolysis efficiencies. The procedures used to optimize cellulase production were investigated using statistical Central Composite Design (CCD). Thirty-two microorganisms were isolated and screened for cellulolytic potential and only two (6.25%) demonstrated promising features. The prospective isolates were confirmed to be *Aspergillus nidulans* and *Aspergillus oryzae*. When prepared starch agar plates were flooded with iodine solution, *A. nidulans* and *A. oryzae* had hydrolysis efficiencies of 45.8% and 40%, respectively. *A. nidulans* had an optimal activity of 0.35UmL^{-1} at pH7 and 60°C and *A. oryzae* with optimal activity of 0.3UmL^{-1} at pH5 and 55°C. Moreover, *A. nidulans* had an optimal cellulase activity of 0.37UmL^{-1} at a temperature of 52.5°C and an incubation period of 30h, whereas *A. oryzae* gave up an optimal activity of 0.33UmL^{-1} at a temperature of 52°C and an incubation period of 26h, as a result of influence of temperature and incubation period. The two isolates were good cellulase producers, however, the study revealed that *A. nidulans* produced cellulase more effectively than *A. oryzae*.

Keywords: Cellulase; *Aspergillus nidulans; Aspergillus oryzae*; central composite design; optimization

1. Introduction

The bulk of plant biomass is made up of cellulose, which is the most prevalent naturally-occurring polysaccharide in the world. It is a hydroglucose linear polymer made up of units connected by -1, 4-glycosidic linkages. The cellulase enzyme produced by a number of bacterial and fungal species can be used to break down this substrate. The cellulose polymer, β-1, 4-glycosidic bonds are broken by the cellulase enzyme, which is extensively used in the industrial setting [1]. Due to their use in several industrial processes, the demand for enzymes like cellulase has steadily grown over time. Since the dawn of time, cellulose has been a cost-effective raw resource and prevalent natural polysaccharide for industrial uses. Cellulolytic materials have been employed as a source of heat energy, textile fiber, and soil fertilizer [2]. Due to its numerous applications in the food, animal feed, paper, textile, detergent, and leather sectors as well as the development of extraction, clarifying, and stabilization technologies, it has sparked interest. Additionally, it is employed in fiber modification, biomass fermentation, and medicinal applications. In order to boost production output, cellulase application in these industries necessitates the identification of stable enzymes and their generating microorganisms that can stay active at high temperatures and pH levels [3].

The majority of brewing waste is made up of brewer's spent grain (BSG), a byproduct of the brewing process. Regardless of the type of grain used, BSG is primarily a solid residue left over after the brewing process. It is frequently high in protein, lignin, cellulose, and hemicelluloses, making it a vital dietary additive that can take the place of low-fiber foods [4]. BSG is a good substrate for isolating cellulose-producing bacteria since it typically contains 20% or less of cellulose, lignin, and hemicelluloses [5]. It is also produced year-round from the brewing process without any potential pollutants, making it a sustainable bio-product that is friendly to the environment. As a result of floating polysaccharide substances such cellulose, hemicellulose, lignin, pectin, starch, metals, proteins, and tannins, fruit and vegetable juices become cloudy. This lowers the juice's quality and decreases consumer satisfaction, which has an impact on product demand. Osho *et al.* [6] reported synergistic effect in combining medium supplement for production of high extracellular cellulase by *Aspergillus flavus* using low cost and readily available plantain fruit stalks biomass as a source of carbon through submerged fermentation. Olive oil extraction and quality have been reported to be improved by the use of cellulases, either alone or in conjunction with hydrolytic pectinases [7]. The enzymatic treatment of olive oil during the extraction stage considerably increased its quality [8] and increased the ideal phenolic content and antioxidant

activity of olive oil. The identification and characterization of bacteria that produce cellulase can enhance the industrial manufacturing of materials made from organic materials as well as make biodegradation and bioremediation of organic wastes simpler, quicker, and safer. Thus, the purpose of this study was to isolate cellulase-producing fungi from brewer's spent grain using a culture-dependent technique, and statistically optimize the process conditions using central composite design of the Response Surface Methodology (RSM) tool.

2. Materials and Methods

2.1 Microorganisms

The microbes (*Aspergillus nidulans* and *A.orzya*) were isolated from brewers' spent grain that was collected from Brewery industry in Ogun State. Serial dilution was used for isolation, and Malt-extract Agar and Carboxyl-Methyl cellulose Agar were used for pour plating and sub-culturing. The pure colonies were kept on slants of malt-extract agar and Peptone water at 4°C prior use.

2.2 Sample collection, isolation and screening of cellulase-degrading fungi

Brewers' spent grain samples were collected in sterile polythene bags and appropriately labeled. These samples were placed in a refrigerator at 4°C to slow down microbial activity. Using 10mL of sterile distilled water in a 15mL test tube, 1g of brewer's spent grain was serially diluted. Each sample was cultured in one milliliter (ml) using malt-extract agar that had been produced by pour-plating in accordance with the manufacturer's instructions at 27°C, and was incubated for 48h. Pure cultures were obtained and preserved on a slant agar using sub-culturing techniques.

The organisms were subsequently sub-cultured on carboxymethyl-cellulose (CMC) agar plates in accordance with the methods demonstrated by Soeka & Ilyas [9]. Iodine solution was used as an indication to flood the fungal colony, which was then left for 30 to 60 min while it was checked for obvious zones of hydrolysis. The diameter of the colony and the clear zones were evaluated in order to assess the effectiveness of the fungi's hydrolysis [6]. It is stated that the hydrolysis efficiency is:

$$\text{Hydrolysis efficiency } (\%) = \frac{Z \times C}{C} \times 100 \qquad (1)$$

Where, Z is the zone of clearance and C is the colony diameter

2.3Cellulase production, extraction, and activity

The fermentation medium used to produce cellulase contained 1% (g/v) carboxymethyl cellulose and was made up of the following reagents: 2g KH_2PO_4, 1.4g $(NH_4)_2SO_4$, 0.3g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, and 1g peptone. Each of them was dissolved in 100mL of distilled water before being autoclaved at 121°C for 15min for sterilization. After being inoculated into the media, the fungi were then placed in the incubator shaker for 120h at a temperature of 27°C and an agitation of 120rpm [8]. The fermentation media were centrifuged at 4000rpm for 20min to separate the crude enzyme-containing supernatant from the substrate particles and fungus mycelia. As a basic enzyme for assaying, supernatant was used.

Cellulase activity was determined by adding 0.5mL of the supernatant to 0.5mL of 0.1M sodium acetate buffer. The mixture was then incubated at 30ºC for 30min, after which 3mL of 3,5-Dinitro salicylic acid was added to the mixture and heated in a boiling water bath for 10 min. The cellulase activity was analyzed and observed using a spectrophotometer at wave length of 540nm [9].

$$\text{Cellulase Activity} = \frac{(Gc \times Df) \times 1000}{(GmW \times IT)} \qquad (2)$$

Where, $Gc = Glucose\ content,$
$Df = Dilution\ factor,$
$GmW = Glucose\ molecular\ weigth\ and$
$IT = Incubation\ Time$

2.4 Optimization of cellulase enzyme

The crude cellulase enzyme was optimized using Central Composite Design to determine the effect of pH, temperature, incubation time, and the addition of different concentrations of Na^+ ions on cellulose production. The effect of pH against enzyme activity was carried out by reaction enzyme solution with a concentration of carboxyl-methyl cellulose of 1% in 0.05N of sodium acetate buffer at various pH ranges from 5.0 to 9.0. The effect of temperature was obtained by reacting enzyme solution in 1% CMC at various temperature ranges from 30-80ºC.

The effect of incubation time was studied by reacting enzyme solution in 1% CMC at different incubation time ranging from 24h to 96h.

3. Results and Discussion

3.1 Morphological and microscopic characteristics of A. nidulans and A. oryzae

Thirty-two (32) microbes were isolated and screened for cellulolytic potential and only two (6.25%) demonstrated promising features. The potential isolates were identified based on their morphological and cultural properties, both fungal samples were each placed on clean slide and stained with lactophenol blue, then viewed under the microscope at X40 objective magnification to observe the microscopic characteristics of the fungi. Colonies on potato dextrose agar were dark green with orange to yellow in areas with closed, globose ascocarp structure. The growth rate of the *Aspergillus* species was slow in comparison with other clinically ones. Conidial chain which appeared as long

fluffy strands on the surface of the substrate was an extended long filament vesicle. The asci or spore-bearing cells were produced within the ascocarp. The isolates were identified as *Aspergillus nidulans* and *A. oryzae* (Fig. 3.1). There is need for basic understanding of the selection and physiology of the cellulase producing microbes, and optimization of the parameter conditions for a thriving and efficient cellulolytic enzyme system [10]. The cellulolytic enzyme system has an affinity for cellulose-degrading materials with many potentials. Other agricultural wastes used for cellulase production include groundnut shell biomass [11]. *A. nidulans* and *A. oryzae* isolated from brewer's spent grain were selected as possible cellulase producers on agar plates with *A. nidulans* showing optimal enzyme activity of $0.37UmL^{-1}$ whereas *A. oryzae* activity of $0.33UmL^{-1}$ at a temperature of 52°C and an incubation period of 26 and 30h, respectively. However, Osho *et al.* [6] reported that untreated plantain fruit stalk biomass at 2% (w/v) had an optimum cellulase activity of 4.45U/mL at 72h incubation period due to random collision between substrates and enzyme active sites as they occur more frequently [12, 13].

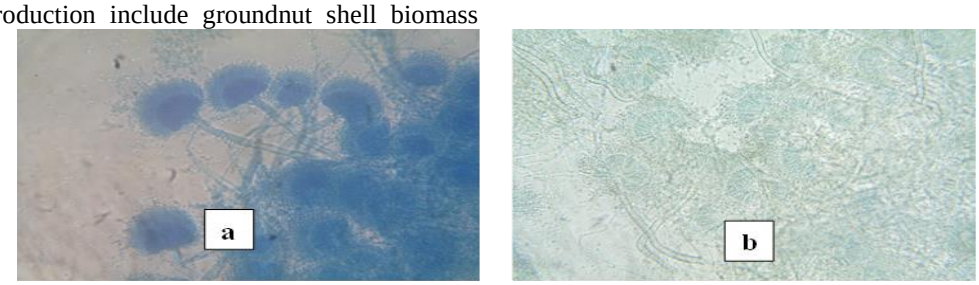

Figure 3.1**:** Microscopic diagrams of: **(a)** *Aspergillus nidulans* and **(b)** *A. oryzae* (x40)

The calculated hydrolysis efficiency of the fungal isolates was 40% to 45.83% for *A. nidulans* and *A. oryzae* respectively. The zones of inhibition exhibited by the screened fungi showed differences in their ability to hydrolyze cellulose, *A. nidulans* and *A. oryzae* had zones of inhibition of 35mm and 21mm, respectively. The screening of cellulase producers using this method had been extensively reported [6, 8]. The production of cellulase by *A. nidulans* and *A. oryzae* was studied with submerged fermentation using 2% carboxymethyl cellulose as carbon source to enhance the production, which is an inducible enzyme that was synthesized during both fungi's growth [14]. The structural complexity of pure cellulose and the difficulty of insoluble substrates lead to the widespread use of carboxylmethyl cellulose for cellulase studies [15]. Carboxylmethyl cellulose is a soluble cellulose derivative with a high degree of polymerization, the isolates were found to have CMCase activity [6]. With regard to cellulase enzyme adsorption, enzymatic hydrolysis rate, and the bioenergetics of microbial cellulose utilization, quantification description of cellulose hydrolysis was addressed. Producing cellulolytic enzymes, hydrolyzing biomass, and fermenting the resulting sugars into desirable products all take place in one phase during the development of organisms, which is referred to as consolidated bioprocessing [14]

3.2 Optimization of cellulase activity

The Central Composite Design method was used for statistical analysis to optimize the parameter conditions. Temperature, incubation time, and pH are among the variables examined to determine the optimum conditions for cellulase activity. Temperature and incubation time were the most significant factors in cellulase activity of optimization of *Aspergillus nidulans.* At 60°C and a 24-h incubation period, the cellulase activity of *Aspergillus oryzae* was at its highest level. *A. nidulans* and *A. oryzae* each had ten experimental runs with different settings, and the outcomes are displayed in Tables 3.1 and 3.2 below, respectively.

Table 3.1: Experimental design and responses (cellulase activity of *Aspergillus nidulans*) of different runs

STD	Run	Factor pH	Factor Temperature (°C)	Factor Incubation Period (Hrs.)	Cellulase Activity (UmL^{-1})
4	1	7	60.00	72	0.221
8	2	7	47.50	72	0.133
3	3	7	35.00	120	0.165
9	4	7	47.50	72	0.199
6	5	7	47.50	72	0.251
5	6	7	47.50	72	0.156
10	7	7	47.50	72	0.173
7	8	7	47.50	24	0.274
2	9	9	47.50	72	0.211
1	10	5	47.50	72	0.104

Table 3.2: Experimental design and responses (Cellulase activity of *Aspergillus oryzae*) of different runs

STD	Run	Factor pH	Factor Temperature (°C)	Factor Incubation Period (Hr)	Response Cellulase Activity (UmL^{-1})
4	1	7	60.00	72	0.126
8	2	7	47.50	72	0.187
3	3	7	35.00	120	0.079
9	4	7	47.50	72	0.163
6	5	7	47.50	72	0.098
5	6	7	47.50	72	0.096
10	7	7	47.50	72	0.075
7	8	7	47.50	24	0.237
2	9	9	47.50	72	0.175
1	10	5	47.50	72	0.198

2.3 Effects of pH and temperature; and temperature and incubation period on cellulase activity

A. nidulans had optimum cellulase activity of 0.35 UmL^{-1} at pH7 and 60°C as shown in Fig. 3.2a. As temperature and pH declined cellulase activity began to steadily decrease. In the optimization process of *Aspergillus oryzae*, a decrease in pH and an increase in temperature resulted in an increase in the cellulase activity, as shown in Fig. 3.2b. The optimum cellulase activity of $0.3UmL^{-1}$ was attained at a temperature of 55°C and pH5. *A. nidulans* optimal cellulase activity of $0.37UmL^{-1}$ was detected at a temperature of 52.5°C and an incubation time of 30h, as illustrated in Fig. 3.2c. As the temperature was lowered and the incubation time increased, a steady decrease in cellulase activity was noted. *Aspergillus oryzae* optimal cellulase activity was observed to be $0.33UmL^{-1}$ at a temperature of 52°C and an incubation duration of 26h, as shown in Fig. 3.2d. This observation was as a result of the interaction between temperature and incubation period. As the incubation time and temperature increased, the cellulase activity decreased. This study showed that increasing the amount of growth increased the yield of cellulase from *Aspergillus nidulans* and *A. oryzae*, which was in accordance with a previous study by Sachslehner *et al.* [16] that found that the amount of mannanase, cellulase, and xylannases increased with an increase in the concentration of mycelia.

At pH values of 5 and temperatures of 60°C, cellulase activity increased, which is consistent with the results of Soeka *et al.* [9], who found that cellulase activity could be influenced by pH change and depended on the fungal strains.

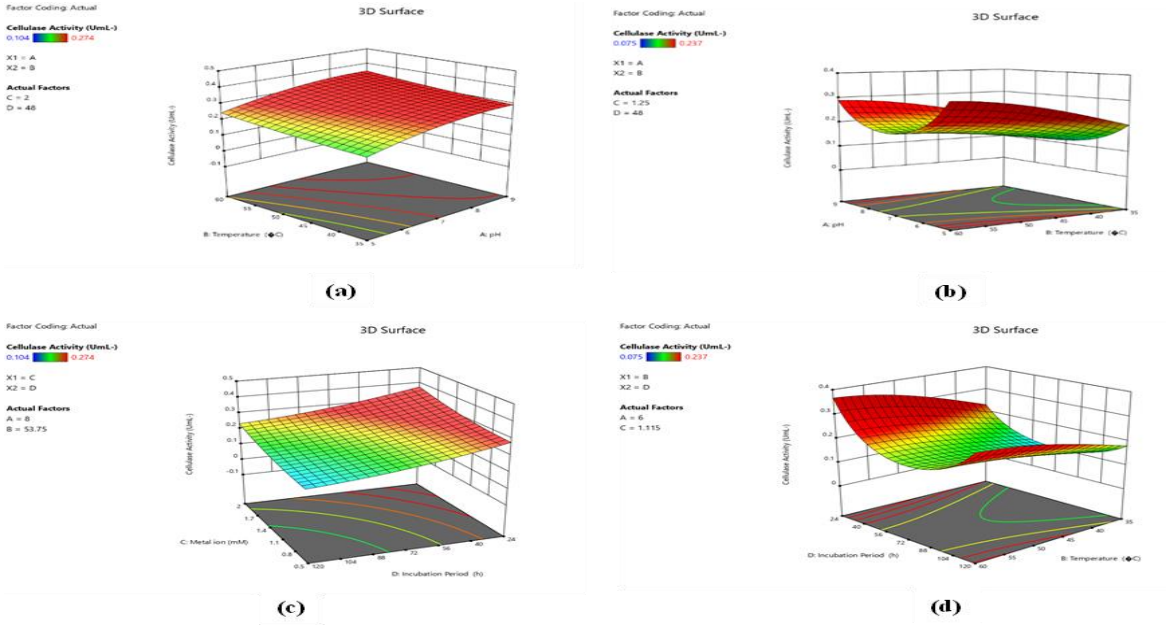

Figure 3.2**:** 3D Response Surface Graphs showing the effects of pH and Temperature interaction**(a)** - *Aspergillus nidulans;* **(b)**- *A. oryzae)* and Temperature and Incubation period**(c)** - *A.nidulans;***(d)**- *A.oryzae*) on Cellulase Activity

4. Conclusion

Cellulase complex enzymes derived from fungal sources provide an opportunity for achieving tremendous benefits in biomass utilization. Literature studies have shown that an assemblage of fungal strains from genus *Aspergillus* had the potential ability to produce cellulose. Two strains isolated from the brewer's spent grain used in the study showed positive indicators as cellulase producers. The quantitative analysis showed that the clearing zones were formed around the colony of *Aspergillus nidulan* and *A. oryzae* with a cellulosic index of 45.83% and 40%, respectively. The optimum cellulase activity for both isolates were obtained with the aid of the central composite design and shown to reach optimal activity at 48h at temperatures of 50-60℃ and pH5. Fungal-derived cellulase complex enzymes provide the potential for achieving remarkable benefits in biomass utilization. A variety of fungal strains from the *Aspergillus* genus have the capability to manufacture cellulose.

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