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Bioethanol Production from Lignocellulosic Biomass Using *Aspergillus niger* and *Aspergillus flavus* Hydrolysis Enzymes through Immobilized *S. cerevisiae*

Amira H. Alabdalall [1,2,*], Asma A. Almutari [1,2], Sumayh A. Aldakeel [3], Ahmed M. Albarrag [4], Lena A. Aldakheel [5], Maryam H. Alsoufi [1,2], Lulwah Y. Alfuraih [1,2] and Hesham M. Elkomy [1,2]

1 Department of Biology, College of Science, Imam Abdulrahman Bin Faisal University, P.O. Box 1982, Dammam 31441, Saudi Arabia
2 Basic and Applied Scientific Research Center (BASRC), Imam Abdulrahman Bin Faisal University, P.O. Box 1982, Dammam 31441, Saudi Arabia
3 The National Center for Genomic Technology (NCGT), Life Science and Environment Research Institute, King Abdulaziz City for Science and Technology (KACST), P.O. Box 6086, Riyadh 11461, Saudi Arabia
4 Department of Pathology, School of Medicine, King Saud University, P.O. Box 800, Riyadh 11461, Saudi Arabia
5 NEOM Community 1, Building 4758, P.O. Box 10, Gayal 49643, Saudi Arabia
* Correspondence: aalabdalall@iau.edu.sa

Abstract: Lignocellulose, the main component of a plant cell wall, is a potential renewable bioenergy source. It is composed of cellulose, hemicellulose, and lignin structures. Cellulose is a linear polysaccharide that is hydrolyzed chemically or enzymatically by cellulase. The addition of lignocellulosic biomass, such as wheat bran and coffee pulp, into the fermentation culture, induces the production of cellulases. Cellulose accounts for 20% of the enzyme market worldwide, demonstrating benefits in diverse applications, especially bioethanol and biogas generation. The aim is to evaluate the optimal condition for bioethanol production by previously isolated fungal species from different soil types in the eastern region of the Kingdom of Saudi Arabia. This study attempts to evaluate and optimize the culture conditions of lignocellulosic biomass under SSF using the highest cellulases-producer strains in the region: Aspergillus niger and Aspergillus flavus (GenBank Accession No. MT328516 and MT328429, respectively) to produce raw sugar that consequently is used in the next step of bioethanol production. This process has two parts: (1) hydrolyze lignocellulosic biomass to obtain raw sugar using *A. niger* and *A. flavus* that produce cellulase, and (2) produce bioethanol through the conversion of the raw sugar produced from the cellulolysis into ethanol using Saccharomyces cerevisiae. The optimal conditions under SSF were seven days of incubation, 5% glucose as a carbon source, 1% ammonium sulfate as a nitrogen source, and 80% moisture for both isolates. Biochemical characterization showed stability for the immobilized enzyme in all temperature ranges (from 20 °C to 70 °C), while the free enzyme exhibited its maximum at 20 °C of 1.14 IU/mL. CMCase production was the highest at pH 4.0 (1.26 IU/mL) for free enzyme and at pH 5.0 (2.09 IU/mL) for the immobilized form. The CMCase activity increased steadily with an increase in water level and attained a maximum of 80% moisture content. The maximum enzyme activity was with coffee pulp as a substrate of 7.37 IU/mL and 6.38 IU/mL for *A. niger* and *A. flavus* after seven days of incubation, respectively. The Carboxymethyl Cellulase (CMCase) activity in immobilized enzymes showed good storage stability under SSF for six weeks, maintaining 90% of its initial activity, while the free enzyme retained only 59% of its original activity. As a carbon source, glucose was the best inducer of CMCase activity with coffee pulp substrate (7.41 IU/mL and 6.33 IU/mL for *A. niger* and *A. flavus*, respectively). In both fungal strains, ammonium sulfate caused maximum CMCase activities with coffee pulp as substrate (7.62 IU/mL and 6.47 IU/mL for *A. niger* and *A. flavus*, respectively). Immobilized *S. cerevisiae* showed an increase in ethanol production compared to free cells. In the case of immobilized *S. cerevisiae* cells, the concentration of ethanol was increased steadily with increasing fermentation time and attained a maximum of 71.39 mg/mL (*A. niger*) and 11.73 mg/mL (*A. flavus*) after 72 h of fermentation.

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1. Introduction

Due to the high demand for fossil fuel, which is toxic to the environment, research into alternative fuel sources has become necessary. An example of these alternative sources is the use of lignocellulosic biomass mainly composed of polysaccharides to produce biofuels such as bioethanol and biogas. Bioethanol production, as a renewable biofuel, involves a conversion of the lignocellulose into fermentable sugar, followed by sugar fermentation to ethanol by certain microorganisms [1,2].

Lignocellulose, as the plant's main component of cell walls, is one of the most common renewable bioenergy sources in nature that has been the focus of research to meet future fuel demand [3,4]. Lignocellulose is composed of cellulose, hemicellulose, and lignin [5] (Figure 1). Cellulose is a linear polysaccharide of glucose residues with β-(1,4)-glycosidic linkages. Enzymes involved in cellulose hydrolysis are called cellulases or cellulases systems, which are a group of enzymes hydrolyzing the β-(1,4)-glycosidic bond of intact cellulose and other related cello-oligosaccharide. According to sequence analysis of amino acids and hydrophobic cluster analysis, cellulases are glycoside hydrolase families that catalyze the hydrolysis of cellulose to glucose units [6]. Cellulases systems contain three different types: exoglucanase (EC 3.2.1.91), β-glucosidase (EC 3.2.1.21), and endoglucanase (EC 3.2.1.4), all of which are to breakdown the hydrogen-bond of intact cellulose [7]. Carboxymethyl Cellulase (CMCase; known as endoglucanase or endo-β-1,4-glucanase) hydrolyzes carboxymethyl cellulose (CMC) randomly. Thus, the length of the polymer shortens, and the reduced-sugar concentration increases [8,9]. CMCase also converts cellodextrins, a byproduct of cellulolysis, into cellobiose (disaccharide) and glucose.

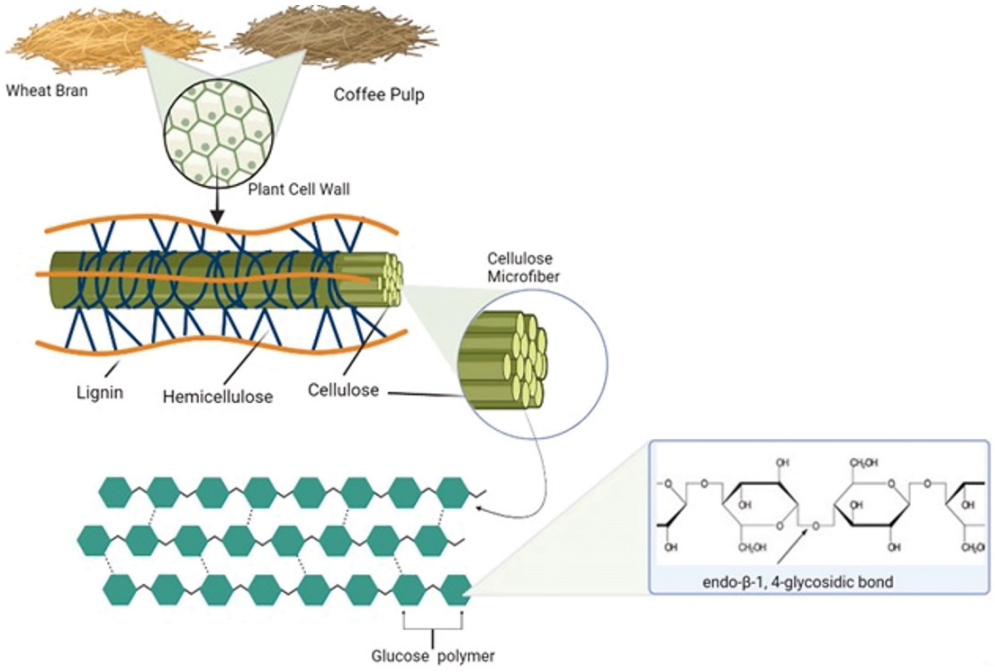

Figure 1. A brief diagrammatic structure and chemical composition of lignocellulose biomass. Wheat bran and coffee pulp cell walls contain a thick layer made of cellulose, hemicellulose, and lignin. This is an illustration of the β-(1,4)-glycosidic bond, which Carboxymethyl Cellulase (CMCase) would act upon.

Cellulases have shown promise in commercial applications, particularly in the food, chemical, detergent, cosmetics, pulp, and paper industries [10]. Cellulases are significant in the natural biodegradation processes as they break down cellulose into simple sugars. Yeast and bacteria could metabolize these simple sugars into ethanol [4]. Although some bacteria produce cellulases, the cellulases produced by fungi can synthesize and break down lignocellulosic materials fully with minimal pretreatment [11]. This suggests that these lignocellulolytic fungi are more productive to be used in the bioprocessing of cellulosic materials in various significant applications [12,13]. Fungi are recognized decomposers of organic substrate in general and cellulosic material specifically [13]. An example of a fungus that has been identified as containing all components of the cellulase system capable of degrading plant biomass polysaccharides is *Aspergillus* [14].

The agricultural lignocellulosic waste consists mainly of cellulose, such as corn cobs, which are an important source of bioethanol production via enzymatic hydrolysis and microbiological processes [15]. Enzymatic hydrolysis of organic lignocellulosic biomass, the second-generation bioethanol, is essential for the viability of bioethanol production [16]. The glucose produced from cellulose hydrolysis is utilized in bioethanol processes [17]. The use of lignocellulosic biomass in bioethanol production is cost-effective compared to the use of crop starch, the first-generation bioethanol [18]. A report from 2021 states that about 109 million cubic meters of ethanol are produced worldwide using first-generation bioethanol [5].

During bioethanol production from lignocellulosic, cellulases play a critical part in cellulose digestion. Decreased costs of cellulase production, increased cellulase activity, and higher sugar yields are three crucial factors in lowering bioethanol production costs from cellulosic materials [19]. The commonly employed microorganism in bioethanol production is *Saccharomyces cerevisiae* due to its durability and other favorable physiological features [20].

S. cerevisiae is a common organism used for sugar fermentation [21]. For enhanced production of bioethanol, immobilization by adhesion of cells on solid substances or trapping of cells in a matrix-like as calcium alginate pellets and K-carrageenan has been used [22–24]. This approach proved inexpensive, non-toxic to cells, and simple to achieve. Immobilizing cellulase onto modified chitin, chitosan, and calcium alginate produced good results, such as reusability and improved enzyme stability [25,26].

Submerged Fermentation (SmF) is the most widely utilized system for the large-scale production of enzymes. SmF is typically carried out in a free-flowing fluid where soluble materials are dissolved. Unlike the SmF liquid medium, filamentous fungi, such as *Aspergillus*, produce large amounts of cellulase in the solid medium, Solid State Fermentation (SSF). However, the ease of monitoring SmF conditions and controlling its downstream processes makes it a more appealing approach [27]. Ideally, *Aspergillus* would be grown in SmF for the synthesis of cellulase; nevertheless, cellulase production in SmF is influenced by several parameters, including pH value, temperatures, substrate concentration, inducer, and composition of media [7]. Medium and culture conditions are among the main parameters affecting fungal lignocellulolytic activity. Therefore, the two fundamental disadvantages of SmF medium are the long fermentation time accompanied by the low cellulose production. To improve enzyme production and overcome these medium limitations, various synthetic and natural carbon sources could be used within the SmF medium [28]. In terms of cellulase production, the application of appropriate fermentation strategies is determined based on the efficiency of a fungus [7]. One of those strategies is the application of SSF, the most prominent technique used in the industry for increasing enzyme output.

SSF has recently gained popularity as a viable approach for recycling nutrient-dense waste like lignocelluloses. SSF is performed on solid substrates in the absence of free water, which serves as both a solid support and a source of nutrients. SSF permits the bioconversion of agricultural leftovers into value-added products and the effective recycling of lignocellulosic materials with decreased energy use [29]. SSF also promotes high enzyme

production at minimal cost, the use of agricultural waste as a substrate, and a wide range of additional enzyme activities compared with SmF [30,31].

The study aims to contribute to achieving the Saudi 2030 vision by finding alternative renewable and sustainable energy sources. This study attempts to evaluate and optimize the culture conditions of lignocellulosic biomass under SSF using the two highest cellulases-producer strains: *Aspergillus niger* and *Aspergillus flavus* (GenBank Accession No. MT328516 and MT328429, respectively) to produce raw sugar that consequently is used in the next step of bioethanol production. This process has two parts: (1) use *A. niger* and *A. flavus* that produce cellulase which can hydrolyze lignocellulosic biomass to obtain raw sugar, and (2) convert the raw sugar produced from the cellulolysis into ethanol using *S. cerevisiae*.

2. Materials and Methods

The two highest cellulases-producer strains, *A. niger* MT328516, and *A. flavus* MT328429 were identified by partial 18S rDNA sequencing from a previous study by Almutairi [32] at Basic and Applied Scientific Research Center (BASRC), Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia. Pure cultures of these two fungi were revived on potato dextrose agar slants and stored at 4 °C.

Fungal spores of isolates were obtained by growing the culture on potato dextrose agar at 30 °C for 5 days. The culture was harvested using sterile distilled water containing 0.1% (*w/v*) Tween 80. The spore concentration was estimated by direct microscopic counting using a hemocytometer (LW Scientific, Lawrenceville, GA, USA). At 4.3×10^6 spore/mL for *A. niger* and 5×10^7 spore/mL for *A. flavus*, the spore suspension was used as inoculums (2.5 mL) for subsequent SSF and SmF culturing.

2.1. Biochemical Characterization of Cellulase under SmF

As previously identified in one of our group studies (under publication), *A. niger* MT328516 is the highest cellulases-producer strain at 1.07 ± 0.05 IU/mL/min CMCase production under SmF. Our identified optimal culture conditions of *A. niger* under SmF were 5 days incubation at 30 °C (pH 5.0) with lactose as a carbon source and yeast extraction as a nitrogen source. Under these SmF conditions, CMCase production by *A. niger* was performed to characterize and evaluate the improvement of cellulase immobilization on enzyme activity and stability.

The optimization of cellulase crude enzyme was performed in two forms: the free and the immobilized form. The crude enzyme was immobilized as follows: 2% sodium alginate (*w/v*) solution was prepared by dissolving 20 g in 1000 mL of distilled water and sterilized in an autoclave at 121 °C for 15 min. Then, the crude enzyme was mixed with 100 mL of 2% sodium alginate solution. The final mixture was dropped using a syringe into a sterile solution of 0.6% calcium chloride-forming beads. The beads were left for 2 h in the solution to get the final hardened form of a 2 mm average bead diameter. The final beads were washed five times with distilled water to remove the excess calcium chloride [26].

Characterization of stabilities for both cellulase forms. To evaluate temperature stability, free and immobilized cellulase was incubated at different temperatures from 20 °C to 70 °C (with an interval of 10 °C) for 30 min before the addition of CMC substrate. The residual activities were measured following the standard procedure of CMCase assay stated below [33,34]. For the determination of stability at varying pH levels, 100 µL of free and immobilized cellulase was mixed with 450 µL buffers of varying pH values from 3.0 to 9.0 (with an interval of 1 pH). The buffers used were citrate phosphate buffer (pH 3.0 to 7.0) and Tris buffer (7.0 to 9.0). Each mix was incubated at room temperature for 30 min, and the residual activities were measured under the standard CMCase assay [33,34]. For storage stability, both free and immobilized enzymes were stored at 4 °C, and enzyme activities were measured every week over 6 weeks using the CMCase assay [34,35].

2.2. Carboxymethyl Cellulase (CMCase) Production Assay and the Analytical Method

The CMCase (endoglucanase) activity was assayed using the standard procedure of CMCase assay by Ghose, 1987 [34,36]. Carboxymethyl cellulose (CMC) was used, as a substrate; therefore, CMCase activity was determined by the amount of reduced sugar (glucose). In brief, a mixture containing 0.1 ml enzyme solution and 0.9 ml 0.5% CMC (diluted in 50 mM of sodium acetate buffer; pH 5) was prepared and vortexed for 1 min. The mixture then was incubated for 30 min at 50 °C in a water bath. The reaction was stopped by adding 2 mL of 98% DNS reagent (Thermo Fisher Scientific, Auburn, AL, USA). The mixture was then boiled for 15 min at 100 °C in a water bath and cooled in cold water. The optical density was measured at 540 nm using UV-Spectrophotometer (Mettler Toledo, Columbus, OH, USA). An appropriate blank of 0.1 mL distilled water instead of crude enzyme extract was also run along with the test. Using the glucose standard curve, enzyme activity was determined by the International Unit (IU), which is defined as the amount of enzyme produced by one μ mole of glucose per minute under these specified conditions. The calibration curve was prepared using a standard glucose solution in the 0–0.1 mg/mL glucose range. Results of the standard curve were drawn using the equation of Y = [11.007x − 0.0286] mg/mL to make the standard curve, as shown in (Figure S1).

1. $Y = [11.007x - 0.0286]$ mg/mL
2. $Y = [11.007x - 0.0286] \times 1000/180]$ μmol/mL [34].

Where Y = the amount of glucose equivalent liberated from CMC hydrolysis; X = Optical density measured at 540 nm.

2.3. Characterization of SSF Culture Condition for CMCase Production

Wheat bran, coffee pulp, orange peel, and sea algae are four selected lignocellulosic substrates based on local sustainable availabilities. All selected substrates were obtained locally from random stores in Al-Khobar city. They were washed, dried at 60 °C, then chopped into small pieces. The pieces were ground into smaller particles using a grain mill machine (SX 100, Retsch, Newtown, PA, USA). Finally, the milled materials were sized and separated using a 20-mesh sieve. The four lignocellulosic substrates were used to produce CMCase enzyme from *A. niger* and *A. flavus* inoculums under SSF.

Of each lignocellulosic substrate, 10 g were transferred in 250 mL Erlenmeyer flask and mixed in 10 mL stock mineral salt solution (0.05 g K_2HPO_4, 0.02 g $MgSO_4 \cdot 7H_2O$, 0.1 g NH_4NO_3, 0.01 g $CaCl_2 \cdot 2H_2O$ and 1 mL of 1% $FeCl_3$). All flasks then were autoclaved at 121 °C for 15 min and incubated [37]. After 5 days of incubation at 30 °C, the culture was extracted by adding 100 mL distilled water. Then, filtered using (Whatman No.1 filter paper) and centrifuged at 10,000 rpm for 15 min. The supernatants were used to assay CMCase activity.

2.3.1. Optimization of SSF Culture Condition for CMCase Production

Based on their higher induction of cellulase activities under SSF by *A. niger* and *A. flavus*, wheat bran and coffee pulp substrates were selected subsequently for these optimization studies of culture conditions.

Incubation Period. The optimum incubation time for CMCase production by *A. niger* and *A. flavus* under SSF using wheat bran and coffee pulp as a substrate was determined by incubating the inoculum flasks at 30 °C for different intervals (3, 5, 7, 10, and 15 days). The culture was extracted at the end of each incubation period, and the enzyme activity was measured.

Carbon Source. To evaluate the impact of carbon sources on enzyme activity under SSF, wheat bran and coffee pulp were supplemented with different soluble sugar (glucose, fructose, maltose, and sucrose) at a concentration of 5% (*w/w*). After autoclaving, the flasks were inoculated with 2.5 mL inoculum suspension of *A. niger* and *A. flavus*. The enzyme was extracted after 7 days of incubation at 30 °C and its activity was measured.

Nitrogen Source. The effect of nitrogen sources was carried out by supplementing the substrates of wheat bran and coffee pulp with inorganic (ammonium sulfate and sodium nitrite) and organic (peptone and yeast extract) nitrogen sources at a concentration of 1% (*w*/*w*). After autoclaving, the flasks were inoculated with 2.5 mL inoculum suspension of *A. niger* and *A. flavus*. After 7 days of incubation at 30 °C, the enzyme was extracted, and its activity was measured.

Moisture Content. Different percentages of water content, including 50%, 60%, 70%, 80%, and 90%), were studied for their effect on enzymatic activity. All the liquid added into the flask and the original moisture content of the wheat bran (5%) and coffee pulp (6%) was considered in calculating the percentage of water in the medium. After autoclaving, the flasks were inoculated with 2.5 mL inoculum suspension of *A. niger* and *A. flavus*. After 7 days of incubation at 30 °C, the enzyme was extracted, and its activity was measured.

2.3.2. Measuring Cellulase Activity through CMCase Production

All measurements were carried out by adding 100 mL distilled water, filtering using Whatman No.1 filter paper, then centrifuging at 10,000 rpm for 15 min. The supernatants were employed to assay enzyme activity using the standard procedure of CMCase assay mentioned above [34].

2.4. Bioethanol Production Using S. cerevisiae

The resultant optimal conditions for the highest CMCase production by *A. niger* and *A. flavus* under SSF using wheat bran and coffee pulp as substrates were used for the subsequent bioethanol production. The reduced glucose sugar, as the product of lignocellulolytic biomass digestions, was used for fermentation to ethanol by *S. cerevisiae* in free and Na-alginate immobilized cells.

S. cerevisiae, or baker's yeast, was acquired locally and cultivated on nutrient agar. A 1 g of dried *S. cerevisiae* sample was added to 10 mL sterile distilled water in an Erlenmeyer flask, then shaken to ensure even distribution of the cells. A 1 mL of these suspensions was immediately transferred into a 9 mL sterile distilled water blank. Each sample was transferred immediately through successive 9 mL of sterile distilled water until it reached the required final dilution. Each suspension was shaken by hand for a few seconds then inoculate 0.1 mL of the cell suspension on a nutrient agar medium (Sigma-Aldrich, Saint Louis, MO, USA). All plates were incubated at 30 °C for 48 h. Colonies were purified by sub-culturing on nutrient agar medium, and the pure cultures were maintained on agar slants at 4 °C [38].

To prepare *S. cerevisiae* inoculum, 100 mL of nutrient broth medium (Sigma-Aldrich, Saint Louis, MO, USA) was inoculated with pure colonies of *S. cerevisiae* from an agar slant with the aid of an inoculating loop. This was incubated on a shaker at 150 rpm at 28–30 °C. After 48 h of incubation, the cell concentration was estimated by direct microscopic counting using a hemocytometer (LW Scientific, Lawrenceville, GA, USA) [38]. At 4×10^7 cells/mL for *S. cerevisiae*, the cell suspension was used as inoculums for bioethanol production.

2.4.1. Immobilization of *S. cerevisiae* Cells

The solution of sodium alginate 2% (*w*/*v*) was prepared by dissolving 2 g of sodium alginate powder in 100 mL distilled water and then autoclaving at 121 °C for 15 min. 10 mL of the prepared *S. cerevisiae* inoculum was centrifuged at 10,000 rpm for 15 min; then, the supernatant was discarded to obtain the free cells. Some *S. cerevisiae* cells were then mixed with 100 mL of 2% sodium alginate solution. The final mixture was dropped using a syringe injector into a 500 mL solution of 0.6% calcium chloride forming beads. The beads were left for 2 h in the solution to get the final hardened form of a 2 mm average bead diameter. Finally, beads were removed and washed five times with distilled water to remove the excess calcium chloride [26].

2.4.2. Fermentation of Targeted Sugar (Glucose) for Bioethanol Production

The fermentation broth is comprised of (*w/v*), 2 g peptone, 2 g yeast extract, and the product of enzymatic hydrolysis of a substrate (wheat bran and coffee pulp) as the fermenting sugar. The broth (90 mL) was filled into a 250 mL sealable flask and sterilized in an autoclave at 121 °C for 15 min. After autoclaving, the flasks were inoculated with 10 mL of the *S. cerevisiae* inoculum of free or immobilized cells at 4×10^7 cells/mL and incubated at 30 °C for 24 to 72 h. The flasks were removed at 24 h intervals to determine the amount of ethanol produced in the medium [38].

2.4.3. Bioethanol Production Assay and Analytical Methods

The fermented broth was assayed for ethanol concentration using the potassium dichromate method [39]. In brief, produced bioethanol was determined by mixing: 10 mL of each sample, 5 mL of potassium dichromate solution (Loba Chemical Pvt. Ltd., Mumbai, India), and 10 mL of 2 N sulfuric acid. The mixture was shaken gently for 1 min and allowed to stand for 120 min at room temperature as an incubation period. After incubation, the absorbance was read at 578 nm versus blank.

The blank was composed of the same volume of the sample, but after removal of ethanol by heating at 60 °C for 5 min, then cooled [39]. The calibration curve was prepared using a standard ethanol solution in the 1.6–12.8 mg/mL ethanol range. Results of the standard curve were drawn using the equation of Y = [0.0219x + 0.03] mg/mL to make the standard curve (Figure S2).

2.5. Statistical Analysis

All experiments were carried out in three replications, statistically evaluated by excel and SPSS, and results have been presented as mean ± standard error (SE).

3. Results and Discussion

3.1. Biochemical Characterization of Cellulase under SmF

For the characterization of enzyme immobilization activity, the CMCase production by *A. niger* under SmF optimal culture conditions of five days incubation at 30 °C (pH 5.0) with lactose as a carbon source and yeast extraction as a nitrogen source was used. Both forms of the free and immobilized enzyme (in sodium alginate gel 2%) were utilized to improve the cellulase stability and activity as the following:

3.1.1. Thermal Stability

Thermal stability is a very important factor when considering the industrial application of enzymes. To study the temperature stability of CMCase activity, both the free and immobilized crude enzyme were incubated at different temperatures (20, 30, 40, 50, 60, and 70 °C) for 30 min. Results show the effects of the temperatures on free and immobilized enzyme stability (Table 1 and Figure S3).

The activity of free CMCase exhibited a maximum of 1.14 ± 0.01 IU/mL at 20 °C, thermal stability then gradually decreased with an increase in temperature. The immobilized enzyme was stable in all temperature ranges (from 20 °C to 70 °C). At 70 °C, the free enzyme retained around 61% activity, while the immobilized enzyme retained up to 92% of its original activity. This proved that the immobilized enzyme was more thermostable than the free enzyme. These results agree with the findings of [40], who reported that the immobilized cellulase on chitosan showed a significant increase in thermal stability compared to the free one.

Table 1. The thermal stability of free and immobilized CMCase produced by *A. niger* under SmF.

Temperature	Enzyme Activity (IU/mL)		*p*-Value
	Free Enzyme	Immobilized Enzyme	
Control	1.15 ± 0.08	1.95 ± 0.09	0.003 *
20 °C	1.14 ± 0.01	1.95 ± 0.02	<0.001 *
30 °C	1.13 ± 0.01	1.92 ± 0.02	<0.001 *
40 °C	1.10 ± 0.01	1.90 ± 0.01	<0.001 *
50 °C	1.09 ± 0.01	1.88 ± 0.01	<0.001 *
60 °C	0.77 ± 0.01	1.84 ± 0.01	<0.001 *
70 °C	0.71 ± 0.01	1.81 ± 0.03	<0.001 *
p-value	<0.001	0.154	

* Each value is a mean of three replicates (±SE).

Although Akkaya et al. [41] have reported that optimal temperatures for immobilized enzymes may be higher, lower, or the same as for the native enzyme; immobilization of cellulase enhanced heat stability. The results for the immobilized form showed significantly better heat stability than those of the native enzyme at temperatures of over 75 °C [42]. This enhanced thermostability is attributed to the covalent binding of cellulase with the copolymer [43].

3.1.2. pH Stability

The pH stability of free and immobilized enzymes was determined by incubating the enzyme at different pH values (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0) for 30 min. The free enzyme displayed good activity at acidic pH values of 3.0 to 5.0, with maximum activity at pH 4.0 (1.26 IU/mL). The maximal activity of the immobilized enzyme was recorded at pH 5.0 (2.09 ± 0.05 IU/mL). At neutral pH (7.0), the immobilized enzyme retained up to 96% of its initial activity, while the free enzyme retained 80% of its original activity (Table 2). At pH 9.0, the immobilized enzyme retained up to 93% of its original activity, whereas the free enzyme retained around 67% of its activity. Higher pH values reduced activity in free cellulase.

Table 2. The pH stability of free and immobilized CMCase produced by *A. niger* under SmF.

pH Value	Enzyme Activity (IU/mL)		*p*-Value
	Free Enzyme	Immobilized Enzyme	
Control	1.15 ± 0.08	1.95 ± 0.09	0.003 *
pH 3	1.07 ± 0.01	1.88 ± 0.01	<0.001 *
pH 4	1.26 ± 0.00	1.92 ± 0.03	<0.001 *
pH 5	1.20 ± 0.00	2.09 ± 0.05	<0.001 *
pH 6	1.05 ± 0.00	1.90 ± 0.03	<0.001 *
pH 7	0.92 ± 0.01	1.88 ± 0.01	<0.001 *
pH 8	0.86 ± 0.02	1.85 ± 0.02	<0.001 *
pH 9	0.78 ± 0.00	1.83 ± 0.01	<0.001 *
p-value	<0.001 *	0.010 *	

* Each value is a mean of three replicates (±SE).

The present results were similar to results by [34], who reported that the immobilized cellulase remained nearly stable at a pH range of 7.0 to 8.0, while the free enzyme showed an acute decrease (68%) at pH 9.0. Esway et al. [44] reported similar results; they found that the stability of free and immobilized pectinase showed that the immobilization process strongly protected the enzyme from severe alkaline pHs. Both free and immobilized cellulase proved stable only at acidic pHs [42].

3.1.3. Storage Stability

Another important aspect of measurement when assessing enzyme efficiency was storage stability. The results show the changes in enzyme activity as a function of storage time at 4 °C over the six weeks (Table 3 and Figure S4). After four weeks, the free enzyme lost about 27% of its initial activity, while the immobilized enzyme showed good storage stability and lost only 6% of its activity. After storage for six weeks, the immobilized enzyme's residual activity was 90% of its initial activity, while the free enzyme retained 59% of its original activity.

Table 3. The storage stability of free and immobilized CMCase produced by *A. niger* under SmF.

Time (Week)	Enzyme Activity (IU/mL)		*p*-Value
	Free Enzyme	Immobilized Enzyme	
Control	1.15 ± 0.08	1.95 ± 0.09	0.003 *
1	1.02 ± 0.02	2.00 ± 0.06	<0.001 *
2	0.98 ± 0.00	2.00 ± 0.07	<0.001 *
3	0.91 ± 0.01	1.91 ± 0.01	<0.001 *
4	0.84 ± 0.01	1.84 ± 0.01	<0.001 *
5	0.76 ± 0.01	1.79 ± 0.01	<0.001 *
6	0.68 ± 0.01	1.76 ± 0.01	<0.001 *
p-value	<0.001	0.019	

* Each value is a mean of three replicates (±SE).

These results agreed well with the results by [34], who reported that the immobilized cellulase has better storage stability than the free one, in which the residual activity of the immobilized enzyme is up to 80% of its original activity after six weeks, while the free enzyme retained 71% of its activity. The results finding of [40] reported that the free enzyme lost about 79% of its activity after 28 days of storage, while the immobilized cellulase lost only 16% of its initial activity. This is most likely due to a decrease in the dissociation of peptide subunits and the rate of enzyme denaturation [45].

3.2. Characterization of SSF Culture Condition of the Lignocellulolytic Substrates for CMCase Production

SSF is mostly useful for the production of enzymes by fungi. Cellulase production was influenced by the type of substrate utilized in fermentation. Therefore, various lignocellulolytic biomass were used as substrates in SSF to produce CMCase by selected *Aspergillus* isolates (wheat bran, coffee pulp, orange peel, and sea algae). The results show the effect of substrate nature on CMCase activity produced by *A. niger* and *A. flavus* (Table 4 and Figures S5 and S6). The results indicate that the optimum substrate for inducing CMCase was coffee pulp, followed by wheat bran, then orange peel, and the lowest one was sea algae in both isolates. The CMCase activities were 5.09 ± 0.15 IU/mL and 4.82 ± 0.18 IU/mL for *A. niger* and *A. flavus* using coffee pulp as a substrate, respectively. Wheat bran as a substrate induced CMCase activities for isolates, 4.76 ± 0.07 IU/mL for *A. niger and* 3.93 ± 0.33 IU/mL for *A. flavus*.

The increase in enzyme production under SSF could be due to the limited availability of resources in solid-state substrates compared to liquid-state putting pressure on fungi to evolve more efficient pathways to a breakdown of the substrate [46,47]. The selection of a suitable inducing substrate was essential to achieving high cellulase production [48].

Dutt and Kumar [49] reported that rice straw was regarded as the best substrate for the induction of cellulase because it provided enough nutrients and remained loose in moist circumstances, allowing for good aeration and a vast surface area for fungi to utilize for growth and metabolism. Utilizing wheat bran and sugarcane bagasse as a substrate for the production of cellulase has previously been reported [48,50]. The amount of lignin in the substrates was often inversely related to the digestibility of lignocellulosic material [51].

Table 4. Production of CMCase under SSF by *A. niger* and *A. flavus* using lignocellulolytic biomass as substrates.

The Substrate	CMCase Activity (IU/mL)		*p*-Value
	A. niger	*A. flavus*	
Coffee pulp	5.09 ± 0.15	4.82 ± 0.18	0.311 *
Wheat bran	4.76 ± 0.07	3.93 ± 0.33	0.071 *
Orange peel	3.97 ± 0.02	3.62 ± 0.06	0.0070 *
Sea algae	1.92 ± 0.11	1.63 ± 0.22	0.301 *
p-value	<0.001	<0.001	

* Each value is a mean of three replicates (±SE).

3.3. Optimization of SSF Culture Condition for CMCase Production

Wheat bran and coffee pulp as a substrate was used for optimization studies of culture conditions based on their higher induction of cellulase activities under SSF by *A. niger* and *A. flavus*.

3.3.1. The Effect of the Incubation Period

The fermentation duration highly influences enzyme production. For optimization of the incubation period, the inoculated flasks of fermentation media were incubated at different periods ranging from 3, 5, 7, 10, and 15 days. Table 5 and Figure S7 show the effect of the incubation period on enzyme production by *A. niger* and *A. flavus*. The highest CMCase activities were after seven days of incubation for both fungal strains. The enzyme activity using wheat bran substrate was 7.1 ± 0.36 IU/mL and 5.75 ± 0.06 IU/mL for *A. niger* and *A. flavus* on the seventh day of incubation, respectively. The maximum enzyme activity was with coffee pulp as a substrate of 7.37 ± 0.4 IU/mL and 6.38 ± 0.23 IU/mL for *A. niger* and *A. flavus* after seven days of incubation, respectively.

Table 5. Optimization of the incubation period, carbon source, nitrogen source, and moisture content for CMCase production under SSF by *A. niger and A. flavus* using wheat bran and coffee pulp as a substrate.

Variation in Conditions	CMCase Activity (IU/mL/min)					
	Wheat Bran			Coffee Pulp		
	A. niger	*A. flavus*	*p*-Value	*A. niger*	*A. flavus*	*p*-Value
Incubation time						
3 days	2.5 ± 0.49	1.6 ± 0.37	0.220 *	2.49 ± 0.31	2.07 ± 0.24	0.348 *
5 days	4.76 ± 0.07	3.93 ± 0.33	0.071 *	5.09 ± 0.15	4.82 ± 0.18	0.311 *
7 days	7.1 ± 0.36	5.75 ± 0.06	0.021 *	7.37 ± 0.4	6.38 ± 0.23	0.100 *
10 days	4.72 ± 0.26	3.55 ± 0.19	0.023 *	4.44 ± 0.2	3.43 ± 0.25	0.033 *
15 days	3.08 ± 0.18	1.87 ± 0.23	0.015 *	3.35 ± 0.26	2.52 ± 0.11	0.45 *
p-value	<0.001	<0.001		<0.001	<0.001	
Carbon source						
Glucose	7.12 ± 0.28	6.19 ± 0.19	0.049 *	7.41 ± 0.11	6.33 ± 0.25	0.017 *
Fructose	6.42 ± 0.19	5.05 ± 0.26	0.014 *	6.65 ± 0.39	5.14 ± 0.32	0.040 *
Maltose	5.38 ± 0.26	4.99 ± 0.11	0.236 *	6.23 ± 0.24	5.29 ± 0.05	0.019 *
Sucrose	3.46 ± 0.17	3.1 ± 0.22	0.266 *	3.7 ± 0.15	3.23 ± 0.25	0.186 *
p-value	<0.001	<0.001		<0.001	<0.001	
Nitrogen source						
Peptone	5.54 ± 0.12	4.38 ± 0.16	0.005	6.08 ± 0.36	4.53 ± 0.16	0.017 *
Ammonium sulfate	7.11 ± 0.1	5.94 ± 0.16	0.004	7.62 ± 0.2	6.47 ± 0.15	0.010 *
Sodium nitrate	4.21 ± 0.23	2.84 ± 0.19	0.011	4.46 ± 0.18	3.08 ± 0.25	0.011 *
Yeast extract	6.09 ± 0.2	5.01 ± 0.3	0.039	6.36 ± 0.3	5.59 ± 0.09	0.068 *
p-value	<0.001	<0.001		<0.001	<0.001	
Moisture content						
50%	4.18 ± 0.12	2.74 ± 0.26	0.007 *	4.37 ± 0.38	3.46 ± 0.17	0.094 *
60%	5.31 ± 0.28	4.18 ± 0.16	0.024 *	5.87 ± 0.06	4.78 ± 0.08	0.000 *
70%	6.52 ± 0.17	5.07 ± 0.18	0.004 *	6.73 ± 0.1	5.24 ± 0.25	0.005 *
80%	7.24 ± 0.05	6.12 ± 0.08	0.000 *	7.84 ± 0.05	6.69 ± 0.13	0.001 *
90%	6.45 ± 0.06	4.38 ± 0.32	0.003 *	6.85 ± 0.16	4.86 ± 0.52	0.022 *
p-value	<0.001	<0.001		<0.001	<0.001	

* Each value is a mean of three replicates (±SE).

These results are in agreement with the findings of [52], who found that maximum cellulase activity by *T.viride* and *T.reesei* using empty fruit bunch as substrate in SSF was after seven days of incubation. As a result of time courses of cellulase production by *A. niger* under SSF using textile waste as a substrate, the incubation period was reduced (from nine days to six days) with a 25.8% increase in total cellulase activity [53].

On longer incubation, the activities of the enzyme were decreased for both fungal strains. Increased periods cause decreased enzyme production, which may result from cell death, denaturation, or pH changes [54].

3.3.2. The Effect of Carbon Source

Soluble sugars as a carbon source influence fungal strains' growth, hence enhancing cellulase production. Different carbohydrates (glucose, fructose, maltose, and sucrose) at a concentration of 5% (*w/w*) were added to the fermentation medium for the optimization of carbon sources. The results show glucose to be the best inducer of CMCase activity with coffee pulp substrate (*A. niger*, 7.41 ± 0.11 IU/mL and *A. flavus*, 6.33 ± 0.25 IU/mL) and wheat bran substrate (*A. niger*, 7.12 ± 0.28 IU/mL and *A. flavus*, 6.19 ± 0.19 IU/mL) followed by fructose, with wheat bran as substrate (*A. niger*, 6.42 ± 0.19 IU/mL and *A. flavus*, 5.05 ± 0.26 IU/mL) and coffee pulp as substrate (*A. niger*, 6.65 ± 0.39 IU/mL and *A. flavus*, 5.14 ± 0.32 IU/mL) (Table 5 and Figure S8).

Both the SmF and SSF systems have shown an increase in the production of an enzyme with additional carbon sources [55]. The present study's results agree with the findings of [56], indicating that glucose was the best inducer of cellulase production with rice bran substrate by *Aspergillus flavus*. In several fungal species, carbon sources (methylcellulose, hydroxyethylcellulose, glucose) operate as an effective inducer of cellulase secretion [57]. This is in contrast to [49], who reported that the CMCase activities by *A. niger* and *A. flavus* were minimal with glucose and lactose compared to cellobiose.

3.3.3. The Effect of Nitrogen Source

To determine the impact of the nitrogen source on cellulase activity, the fermentation medium was supplemented with inorganic (ammonium sulfate and sodium nitrite) and organic (peptone and yeast extract) nitrogen sources at a concentration of 1% (*w/w*). The results indicate that in both fungal strains, ammonium sulfate caused maximum CMCase activities with coffee pulp as substrate (*A. niger*, 7.62 ± 0.2 IU/mL and *A. flavus*, 6.47 ± 0.15 IU/mL) and wheat bran as substrate (*A. niger*, 7.11 ± 0.1 IU/mL and *A. flavus*, 5.94 ± 0.16 IU/mL) followed by yeast extract, with coffee pulp as substrate (*A. niger*, 6.36 ± 0.3 IU/mL and *A. flavus*, 5.59 ± 0.09 IU/mL) and wheat bran as substrate (*A. niger*, 6.09 ± 0.2 IU/mL and *A. flavus*, 5.01 ± 0.3 IU/mL) (Table 5 and Figure S9). Sodium nitrate as a nitrogen source caused minimum CMCase activities in both fungal strains.

These results agreed with the results of [49] who found that *A. niger* and *A. flavus* showed the highest production of CMCase utilizing ammonium sulfate as an inorganic nitrogen source. Kocher et al. [58] reported that the best nitrogen source of *T. harzianum* MTCC 8230, when grown on rice straw, was ammonium sulfate as a nitrogen source.

Gokhle et al. [59] observed that the best inorganic nitrogen sources for the production of CMCase and xylanase activity by the *A. niger* NCIM1207 strain were ammonium sulfate and diammonium phosphate. In SSF using rice bran as substrate, yeast extract proved to be the most effective nitrogen source for the production of cellulase by *A. flavus* [56].

3.3.4. The Effect of Moisture Content

In SSF, the moisture content of the fermentation medium plays an important role in microbial growth hence, it is influencing enzyme production. To detect the effect of moisture content on CMCase activity, different moisture levels (50%, 60%, 70%, 80%, and 90%) were examined in both substrates (wheat bran and coffee pulp). The results show that the CMCase activity increased steadily with an increase in the level of water and attained a maximum of 80% of moisture content (Table 5 and Figure S10). Moreover, when

coffee pulp was used as the substrate, the enzyme activities were 7.84 ± 0.05 IU/mL and 6.69 ± 0.13 IU/mL for *A. niger and A. flavus*, respectively. The decrease in moisture content resulted in a decrease in enzyme activity of both fungal strains and attained a minimum at 50% of water content with wheat bran as a substrate, the enzyme activities were 4.18 ± 0.12 IU/mL and 2.74 ± 0.26 IU/mL for *A. niger and A. flavus*, respectively.

These results agreed with the results of [60] who reported that the best activity of cellulase was in straw with 80% of water content by *Aspergillus niger* ITBCC L74. At 50% of moisture content in SSF, cellulase production by the mutant fungal strain, *Aspergillus* sp. SU14-M15 was significantly higher than other moisture levels [61].

The amount of water in the solid substrate has an impact on microbial growth. The nutrient diffusion and solubility of the solid substance will be reduced if the water concentration is low, resulting in limited nutrients for the growth of microorganisms and decreased enzyme production [11]. However, if the moisture content was high, the porosity and gas volume will decrease but contamination will increase, hence the enzyme production was reduced [62].

3.4. Bioethanol Production Using S. cerevisiae

The resulting SSF optimal culture conditions for both strains achieving the highest CMCase production were: seven days incubation period, glucose as a carbon source, Ammonium sulfate as a nitrogen source, and 80% moisture content. The products of wheat bran and coffee pulp waste biomass digestion by *A. niger* and *A. flavus* under SSF were used for fermentation to ethanol by *S. cerevisiae* as free or Na-alginate immobilized cells.

The two forms of *S. cerevisiae* were used to ferment the targeted sugar (glucose) as products of enzymatic hydrolysis of substrates wheat bran and coffee pulp by *A. niger* and *A. flavus*. Ethanol production yields were determined every 24 h over three days using the potassium dichromate method. The results of ethanol production using *S. cerevisiae* as free, and Na-alginate immobilized cells are presented in (Table 6).

Table 6. The ethanol concentration (mg/mL) is produced by fermenting the products of enzymatic hydrolysis of wheat bran and coffee pulp by *A. niger* and *A. flavus* using *S. cerevisiae* as free or Na-alginate immobilized cells.

	Wheat Bran			**Coffee Pulp**		
Incubation Period (Hour)	**Free *S. cerevisiae***	**Immobilized *S. cerevisiae***	***p*-Value**	**Free *S. cerevisiae***	**Immobilized *S. cerevisiae***	***p*-Value**
			A. niger			
24 h	4.04 ± 0.61	12.08 ± 0.67	0.001 *	15.26 ± 0.72	16.0 ± 0.20	0.378 *
48 h	52.19 ± 1.2	64.34 ± 1.89	0.006 *	41.26 ± 2.46	58.21 ± 4.19	0.025 *
72 h	57.41 ± 5.71	68.91 ± 0.3	0.115 *	61.94 ± 2.48	71.39 ± 3.59	0.096 *
p-value	<0.001	<0.001		<0.001	<0.001	
			A. flavus			
24 h	0 ± 0	0.84 ± 0.14	0.004 *	1.26 ± 0.54	2.4 ± 1.06	0.392 *
48 h	4.8 ± 0.46	6.21 ± 1.85	0.500 *	6.06 ± 0.17	8.99 ± 1.36	0.099 *
72 h	5.65 ± 0.57	7.81 ± 0.76	0.085 *	7.58 ± 0.1	11.73 ± 1.33	0.036 *
p-value	<0.001	0.012		<0.001	0.005	

* Each value is a mean of three replicates (±SE).

3.4.1. Fermentation of the Targeted Produced Glucose by *A. niger*

In the case of immobilized *S. cerevisiae* cells, the concentration of ethanol was increased steadily with increasing fermentation time and attained a maximum of 68.91 ± 0.3 mg/mL and 71.39 ± 3.59 mg/mL for sugar product from hydrolysate of wheat bran and coffee pulp, respectively, after 72 h of fermentation (Figure S11). In free cell form, the amount of ethanol was a little less when compared to immobilized *S. cerevisiae*; it achieved the highest concentration of 57.41 ± 5.71 mg/mL and 61.94 ± 2.48 mg/mL for sugar produced from hydrolysate of wheat bran and coffee pulp, respectively, after 72 h of incubation.

3.4.2. Fermentation of the Targeted Produced Glucose by *A. flavus*

In immobilized *S. cerevisiae* cells, the concentration of ethanol was increased with an increase in fermentation time and attained the highest 7.81 ± 0.76 mg/mL and 11.73 ± 1.33 mg/mL for products of hydrolysate of wheat bran and coffee pulp, respectively, after 72 h of fermentation (Figure S12). In the case of free *S. cerevisiae* cells, the results show that the lowest production of ethanol achieved after 72 h of incubation was 5.65 ± 0.57 mg/mL and 7.58 ± 0.1 mg/mL for products of hydrolysate of wheat bran and coffee pulp, respectively.

These results agreed with the results of [63], who found that the concentration of ethanol obtained by fermentation of sago and sweet sorghum substrates achieved maximum yield at the end of fermentation which was 72 h of incubation. Itelima et al. [15] found the optimal ethanol concentration in corn cobs of 1.87% (*v/v*) was on the first day and gradually increased to 10.08% (*v/v*) after seven days of fermentation.

The immobilization of *S. cerevisiae* protects cells from damage during the fermentation period, hence increasing their activity and ability in fermentation. In a study undertaken by [64], the yield of ethanol was 10.19 g/L in the case of immobilized cells and 9.65 g/L in free cells when the glucose was fermented using *S. cerevisiae* for 72 h. Patel and Desai [38] observed that the ethanol production by fermented hydrolysis of sawdust with cellulase using *S. cerevisiae* was increased with an increase in incubation time and attained the highest concentration after 120 h of fermentation. Because the immobilization technique for *S. cerevisiae* cells in fermentation processes reduces the contamination risks [65], makes it easier to separate the cell mass from the bulk liquid [65], maintains cell activity stability [66], allows for biocatalyst recycling [67], and protects the *S. cerevisiae* from inhibitors [66], it has been regarded as a promising option for increasing ethanol production. According to various studies, the immobilization of *S. cerevisiae* enhanced ethanol production compared to the free cells [68–71].

4. Conclusions

The two isolates, *Aspergillus niger*, and *Aspergillus flavus* were selected as promising fungal strains that can produce biomass-dissolving enzymes in order to obtain raw sugar to be used in the next step of bioethanol production. Both strains show good results in cellulase production and its utilization in bioethanol production. Thus, for commercial application, the study recommends: (1) the necessity of purifying the enzyme produced from the two isolates on a large scale; (2) specifying all chemical compositions of biomass samples to calculate the yields of cellulose to glucose and glucose to ethanol conversion. The study contributes to achieving the Saudi 2030 vision by suggesting alternative renewable and sustainable energy sources. It suggests bioethanol production on a large scale using the cellulase enzyme produced from *Aspergillus* isolates through *S. cerevisiae*.

This study concludes that *A. niger* MT328516 and *A. flavus* MT328429 represented their high ability to produce cellulase enzymes capable of analyzing biomass rich in cellulosic compounds with high efficiency. It also determines that producing bioethanol using immobilized *S. cerevisiae* cells gave a higher CMCase production, production continuity, and bioethanol stability compared to free cells. The optimum culture conditions for the two isolates to achieve the highest CMCase production under SSF are seven days incubation period, glucose as a carbon source, Ammonium sulfate as a nitrogen source, and 80% moisture content.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/en16020823/s1, Figure S1: Standard curve of glucose; Figure S2: Standard curve of ethanol; Figure S3: The Thermal Stability of Free and Immobilized CMC-ase Produced by *A. niger* MT328516 under SmF; Figure S4: The Storage Stability of Free and Immobilized CMC-ase Produced by *A. niger* MT328516 under SmF; Figure S5: Production of CMC-ase Under Solid State Fermentation (SSF) by *A. niger* MT328516 and *A. flavus* MT328429 Using Cellulolytic Wastes as a Substrate; Figure S6: Sea Algae from Arabian Gulf Coast in Al-Khobar City. (a) Before processing, (b) Under an optical microscope; Figur S7: The Effect of Incubation Period on CMC-ase Production under SSF by *A. niger* MT328516 and *A. flavus* MT328429; Figure S8: The Effect of Carbon Source on

CMC-ase Production under SSF by *A. niger* MT328516 and *A. flavus* MT328429; Figure S9: The Effect of Nitrogen Source on CMC-ase Production under SSF by *A. niger* MT328516 and *A. flavus* MT328429; Figure S10: The Effect of Moisture Content on CMC-ase Production under SSF by *A. niger* MT328516 and *A. flavus* MT328429; Figure S11: The Ethanol Concentration (mg/mL) Produced by Fermented the Products of enzymatic hydrolysis of Wheat Bran and Coffee Pulp by *A. niger* MT328516 Using *S.cerevisiae* as Free or Alginate Immobilized Cells; Figure S12: The Ethanol Concentration (mg/mL) Produced by Fermented the Products of enzymatic hydrolysis of Wheat Bran and Coffee Pulp by *A. flavus* MT328429 Using *S.cerevisiae* as Free or Alginate Immobilized Cells.

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