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Full Length Research Article

Utilizing Agrowaste for Optimization and Production of Cellulase Enzyme from *Aspergillus Sp*

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Abstract

The current study is focused on the production of cellulase enzyme from *Aspergillus* sp by using agrowaste. The usage of agricultural wastes for enzyme production is considered an essential part in any approach to accomplish goals to reduce environmental pollution and disposal of waste. Optimization of some nutritional and environmental factors like pH, temperature (°C), incubation period (days), nutrient content (Carbon) (mg/g), nitrogen (mg/g), phosphorus (mg/g) and potassium (mg/g) were studied under submerged culture conditions for cellulolytic enzyme production. Cellulase production involves the use of two types of agricultural waste such as rice bran and sugarcane bagasse. The production of cellulase from *Aspergillus flavus, A. niger* and *A. terreus* by liquid state fermentation. *A. terreus* had the highest cellulase activity reached. Maximum celluloytic activity was observed in pH (8), temperature at (25 °C), Incubation period at (4), Nutrient content (carbon at 3), Nitrogen at (75), Phosphorus at (75) and Potassium at (25) respectively. Sugarcane bagasse showed maximum cellulase activity are presented respectively. From the current research, sugarcane bagasse was proven as a cheap and easily available source throughout the year for higher production of cellulase.

***Key words*:** Enzymes production, Cellulase, Sugarcane bagasse, Rice bran, *Aspergillus* sp.

Several agricultural wastes are highly abundant in celluloses and the effective cellulase enzymes do exist widely among microorganisms. Accordingly, the cellulose degradation using microbial cellulase to produce a low-cost substrate for ethanol production has attracted more attention [1]. Microbial cellulases find applications in many industries and constitute a significant share of the world's industrial enzyme market [2]. Enzyme production was evaluated by measuring enzyme activities in the crude [3]. Microbial cellulases find applications in many industries and constitute a significant share of the world's industrial enzyme market. In order to improve the cost function of the cellulase producing processes with enhanced yield and novel activities, superior bioprocesses are formulated these days. Designed to isolate and identify superior cellulose-degrading fungi from cellulosic waste and selection of different cellulosic waste like paper waste, cotton ginning, wheat Bran and sugarcane bagasse for cellulase production under solid state fermentation [2]. The complete hydrolysis of cellulose to fermentable sugar showing that the process is completed by the cellulase enzymes synergistically break down the homogenous polysaccharide. The complete hydrolysis of cellulose to fermentable sugar showing that the process is completed by the cellulase enzymes synergistically breakdown the homogenous polysaccharide [4-5]. Agro wastes such as crop residues, grasses, peanut husks, corn husks, coffee cherry husks, paddy, wheat, jowar straws, etc., contribute major sources of ligno cellulosic substances [6]. The production of cellulase by fungal strains from *Penicillium* sp and choosing the best organism that provides highest enzyme productivity, investigation of cellulase production under the optimum conditions, purification of cellulase, identification by high-performance liquid chromatography [7].

Filamentous fungi are mainly interesting in view of the fact that they secrete enzymes into the culture media and their enzymatic activity level is significant in comparison with bacteria and yeasts. For the production of xylanase from wastes, filamentous fungi including *Aspergillius* sp and *Trichoderma* sp, are extensively used [8]. *Paenibacillus* sp is strong xylanase producers using sugarcane bagasse and involved in the hydrolysis of hemicelluloses [9]. Fungi are important microorganisms that produce cellulase and *Aspergillus*, *Trichoderma* and *Penicillium* sp. are the ones that produce the highest amount of cellulase [10].

Determine the optimum conditions in producing cellulase enzyme under solid state fermentation using bagasse as substrate with *A. niger* ITB CC L74. A statistical approach

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such as RMS and factorial experiment design is used to involve a minimum number of experiments for a large number of factors where these methods have also been demonstrated to improve the cellulase production [11].

MATERIALS AND METHODS

The marine sediment soil samples were collected from Arichalmunai, Dhanushkodi, Ramnadu District, Tamil Nadu, India and also the substrates of *Oryza sativa* (Rice bran) and *Saccharum officinarum* (sugarcane bagasse) from local market Thanjavur Tamil Nadu India.

Isolation [12]

One gram of marine sediment soil samples was diluted serially in distilled water and Potato Dextrose Agar medium (PDA) was prepared and sterilized in an autoclave at 121ºC for 15 minutes. The medium was incorporated with streptomycin sulphate solution (1:1) and poured into the petri plates. After solidification 0.1mL of serially diluted soil sample were inoculated into the medium. The inoculum was spread uniformly and kept undisturbed in dust free chamber at room temperature for a period of 3-5 days. The fungal colonies were counted. The pure cultures were maintained in the conventional potato dextrose agar medium.

Identification and photomicrography [13]

Morphological features of fungi were photographed using Nikon microscope. All the fungi were identified with the standard manual.

Solid substrate [14]

Agricultural solid waste is most cellulose abundant in nature. The agro solid waste like Rice bran and sugarcane bagasse dust are obtained from local market of Thanjavur. The present research work of cellulose production by solid state fermentation process using microorganism *Aspergillus flavus, Aspergillus niger* and *Aspergillus terreus*. Fermentation process and fungal spore suspensions were made using sterile water and were added to the sterile solid substrates. Flasks were incubated for 5 days in the B.O.D incubator at 27 °C.

Cellulase production [15]

The cultures that made a zone of hydrolysis around its colonies were inoculated in 250-ml Erlenmeyer conical flask containing 50ml of CMC medium containing the following (g/l): $(NH_4)_2SO_4$ 1.4 g; KH_2PO_4 2.0 g; urea 1.3 g; $CaCl_2$ 0.3 g; $MgSO_4.7H_2O$ 0.3g; $FeSO_4.7H_2O$ 0.005g; $MnSO_4.H_2O$ 0.0016 g; $ZnSO_4.7H_2O$ 0.0014 g; $CoCl_2$ 0.002 g; peptone 1.0 g; CMC–Na salt 10 g; and tween 80 (1%) 2.0ml. The pH of the medium was adjusted to 6.0. The inoculated flasks were incubated on a rotary incubator shaker at 150 rpm for 7 days at 28±1 °C. The mycelium of each isolate was collected by centrifugation at 4000–5000 rpm for 15 min at 4 °C. The cell-free supernatant was used as a crude enzyme for further determinations.

Effect of pH [16]

The optimum pH for enzyme (cellulase) activity was determined by running the assay activity between pH ranges of 4.0, 6.0 and 8.0. The pH was adjusted by addition of hydrochloric acid (0.1N) and 0.1N sodium hydroxide to achieve acidity and alkalinity respectively. The flasks were incubated at 37 °C for 48 h. Samples were taken at regular intervals and analyzed for cellulase activity.

Effect of temperature °C [16]

The optimal temperature for activity was determined by enzyme assay at different temperature ranges of 25, 30 and 35 °C for 48 h. Samples were taken at regular intervals and analyzed for cellulase activity.

Effect of incubation period [17]

To study the effect of incubation period, the fungal culture was inoculated in cellulase medium and incubated for different incubation time 2, 4 and 6 days at 28 °C on an incubator shaker at 150rpm under submerged conditions.

Effect of carbon sources [18]

The effect of different carbon sources such as rice bran, and sugarcane bagasse on protease production was investigated. Carbon source present in the production medium was replaced with 10.0g of each of the carbon source under study. Furthermore, for carbon source optimization, different concentrations of the best carbon source (25, 50 and 75/100ml) was used for cellulase production.

Effect of nitrogen source [18]

The influence of different nitrogen sources (5.0g) of yeast extract, peptone, meat extract, skim milk, soyabean meal, gelatin and ammonium nitrate was determined for protease production to optimize the best nitrogen source at different concentrations of 25, 50 and 75g/100ml) was used for produce cellulase.

Effect of phosphorous [18]

Aspergillius niger, Aspergillius flavus and *Aspergillius terreus* were grown in Erlenmeyer flasks (250 ml) containing 50ml of liquid medium of phosphorous were added individually to the basal medium in a 0.3% ratio. The flasks were sterilized at 121 °C for 20 min and inoculated with two mycelia disc (10mm) cut from 7-days fungal cultures grown on potato dextrose agar medium. The inoculated flasks were incubated at 30 °C for 6 days when cellulase activity was determined.

Effect of potassium [18]

Aspergillius niger, Aspergillius flavus and *Aspergillius terreus* were grown in Erlenmeyer flasks (250 ml) containing 50ml of liquid medium of potassium were added individually to the basal medium in a 0.3% ratio. The flasks were sterilized at 121 °C for 20 min and inoculated with two mycelia disc (10mm) cut from 7-day fungal cultures grown on potato dextrose agar medium. The inoculated flasks were incubated at 30 °C for 6 days when cellulase activity was determined.

Statistical analysis

Analysis was performed on the collected data using the statistical analysis software Microsoft Office Excel 2007.

RESULTS AND DISCUSSION

Cellulase production

Aspergillius niger, Aspergillius flavus and *Aspergillius terreus* was initially grown on the surface of PDA slants at 30 °C for 7 days. The fermentation process was carried out in 250-ml conical flasks. Separate flask was used for two substrates. Each flask was filled with 5 g of solid substrates followed by the addition of 3 ml of water. Then the flasks were plugged with cotton and autoclaved at 121 °C for 15 min at 15 psi. Under aseptic conditions fungal spores were transferred from culture slants to the solid substrate and mixed thoroughly. Then the flasks were kept in incubator maintained at 30 °C. The extra-cellular enzyme was extracted by soaking the fermented solid

material with 50 ml of sterile water over night at 40 °C and filtering through muslin cloth. Two filtrates were centrifuged at 10,000 rpm for 30 min and temperature has been maintained 40 °C. The supernatant was analyzed for the amount of cellulase produced. Optimization of incubation period influencing cellulase yield. An experiment with different incubation periods was executed in 250ml conical flasks at 30 °C. All experiments were carried out in triplicate and the mean values are calculated.

Optimization conditions for production of cellulase

Optimization of the conditions of the culture for producing cellulase by selecting the best environmental conditions and increase the yield of the cellulase production.

The results are given in (Fig 1), where pH 8 was found to be optimal for the largest production of cellulase by *Aspergillius terreus* (18.2 IU/ml) and pH 4 was found to be optimal for the lowest production of cellulase by *Aspergillius niger* (10.2 IU/ml) in sugarcane bagasse. In rice bran, pH 4 was found to be optimal for the lowest production of cellulase by *Aspergillius niger* (1.52) and pH 8 was found to be optimal for the highest production of cellulase by *Aspergillius terreus* (4.00 IU/ml). However, sugarcane bagasse showed higher cellulase production activity as compared to rice bran.

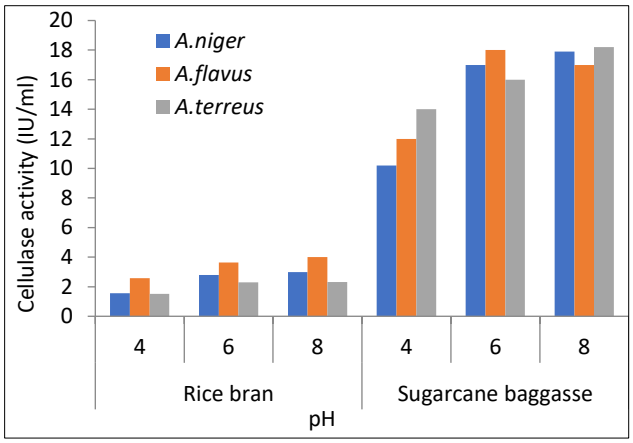

Fig 1 Effect of pH on cellulase production using different agro waste

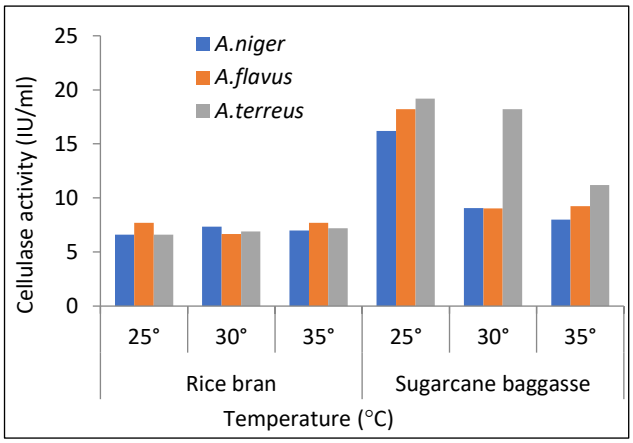

Fig 2 Effect of temperature (°C) on cellulase production using different agro waste

Results showed that, in sugarcane bagasse, cellulase production increased as incubation and temperature increased, maximum at *A. terreus* (19.2 IU/ml) at 25 °C and minimum at *A. niger* (8.00 IU/ml) at 35 °C. They showed that *A. flavus* had a maximum cellulase activity temperature of 25 °C at (7.69 IU/ml) and a minimum cellulase activity temperature of *A. niger* 25°C at (6.60 IU/ml) for rice bran (Fig 2). These two different substrates function well in cold temperatures because high temperatures can alter membrane composition, promote protein catabolism and inhibited the growth of fungi, there was a reduction in enzyme synthesis as the temperature increases.

Aspergillus terreus, *A. flavus*, and *A. niger* were all capable of producing cellulase during the various tested incubation durations, as shown by the results in (Fig 3). *A. flavus* produced the lowest amount of cellulase activity on day two of the incubation time (10.9 IU/ml), while *A. terreus* produced the highest on day four of the incubation period (21 IU/ml) in sugarcane bagasse. In the rice bran substrate, the maximum amount of cellulase production was showed after six days of incubation in *A. terreus* (1.63 IU/ml), and the minimum level of cellulase production was showed after two days of incubation in *A. flavus* (0.23 IU/ml).

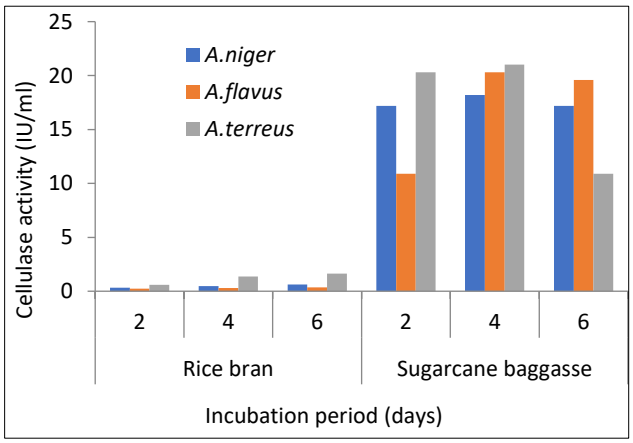

Fig 3 Effect of incubation period on cellulase production using different agro waste

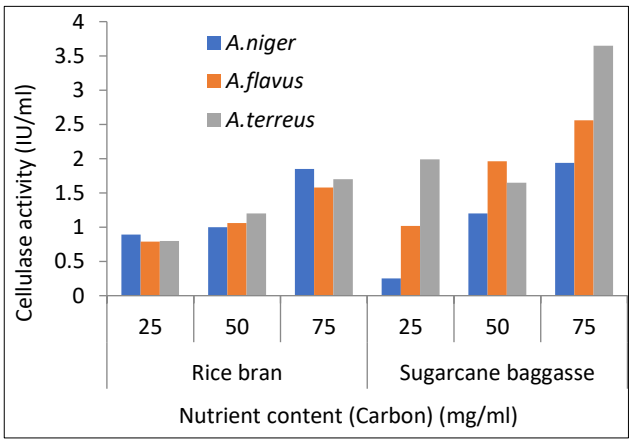

Fig 4 Effect of carbon source on cellulase production using different agro waste

Different agricultural wastes such as rice bran and sugarcane bagasse were tested for the production of enzymes. The carbon source was used at concentrations of 25, 50, and 75 mg/ml. According to the carbon source, the sugarcane bagasse produced the largest amount of cellulase (3.65 IU/ml) at 75 in *Aspergillus terreus* and the lowest amount (1.02 IU/ml) at 25 in *Aspergillus niger*. The rice bran substrate had comparatively less enzyme production. The maximum cellulase activity was (1.85 IU/ml) at 75 in *Aspergillus niger* and the lowest cellulase activity (0.79 IU/ml) at 25 in *Aspergillus flavus* (Fig 4).

The results of this study showed that various sources have different effects on enzyme activity. Sugarcane bagasse extract had the highest level of enzyme activity among the tested organic nitrogen sources. *Aspergillus niger* had the maximum cellulase activity (2.97 IU/ml) at 75 and *Aspergillus flavus* had the lowest cellulase activity in sugarcane bagasse (0.95 IU/ml) at 75. The maximum cellulase activities were obtained with nitrogen source *Aspergillus niger* (2.00 IU/ml) at 75 in rice bran and minimum cellulase activities were obtained with (0.67 IU/ml) at 25 in *A. flavus*. It was reported that good cellulase yield can be obtained with sugarcane bagasse as the nitrogen source (Fig 5).

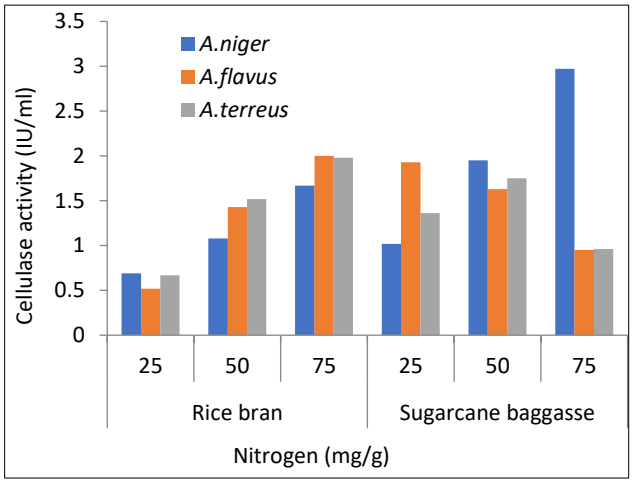

Fig 5 Effect of nitrogen source on cellulase production using different agro waste

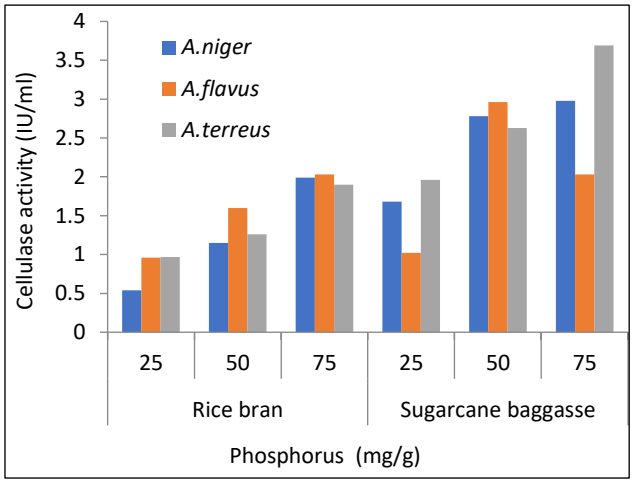

Fig 6 Effect of phosphorus source on cellulase production using different agro waste

The results of this study showed that phosphorus sources have different effects on enzyme activity. Sugarcane bagasse extract had the highest level of enzyme activity among the tested organic phosphorus. *A. terreus* had the maximum cellulase activity (3.69 IU/ml) at 75 and *A. flavus* had the lowest cellulase production in sugarcane bagasse (1.20 IU/ml) at 25. The maximum cellulase activities were obtained with phosphorus *A. flavus* (2.03 IU/ml) at 75 in rice bran and minimum cellulase activities were obtained with (0.96 IU/ml) at 25 in *A. niger*. It was reported that good cellulase yield can be obtained with sugarcane bagasse as the phosphorus (Fig 6).

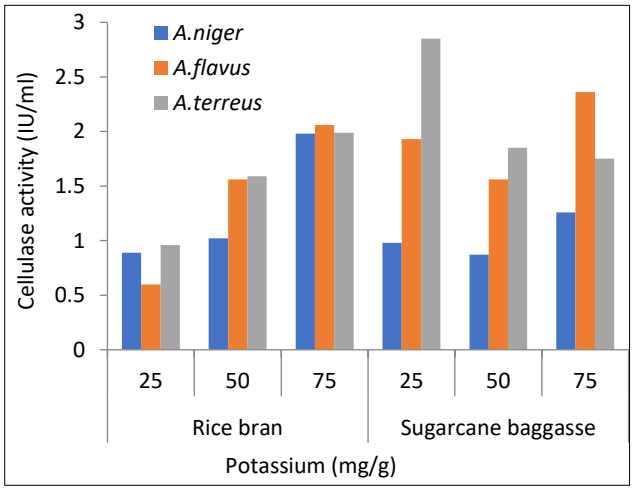

Fig 7 Effect of nitrogen source on cellulase production using different agro waste

It is important to investigate about potassium effect during fermentation process. According to the potassium results, the sugarcane bagasse produced the largest amount of cellulase activity (2.85 IU/ml) at 25 in *A. terreus* and the lowest amount (0.87 IU/ml) at 50 in *A. niger*. The rice bran substrate had comparatively less enzyme production. The maximum cellulase activity was (2.06 IU/ml) at 75 in *A. niger* and the lowest cellulase activity (0.60 IU/ml) at 25 in *A. flavus* (Fig 7).

The treated bagasse with 2% NaOH (w/v) was analyzed for its cellulose, hemicellulose, and lignin [19]. The content of cellulose, hemicellulose, and lignin after pretreatment is 57.76 ± 0.49%, 12.44 ± 0.35%, and 21.34 ± 0.18% respectively. This is compatible with the results of the research that has been done [20]. The endophytic fungi with cellulase production would have transparent circles around the colony. Furthermore, the higher the ratio of transparent circle diameter to colony diameter, the more cellulase activity is produced [21]. *Penicillium* has the ability to produce more cellulose-degrading enzymes and has certain advantages in terms of enzyme system degradation performance and strain [22] growth rate indicating great potential for industrial applications.

The pH of the growth medium plays an important role in enzyme secretion by fungi. Six different pH values ranged between 3.0 and 8.0 were applied. Cellulase activity production was low when *P. oxalicum* R4 was cultured in a medium with an initial pH of 3.0 [23]. The cellulase production was optimum (23.97 U/ml) by *P. decumbens* at an optimal pH of 4.0^{7}. The production of exoglucanase (1.76 & 2.16 U/ml), endoglucanase (1.25 & 1.94U/ml), and β-glucosidase (1.44 & 1.71U/ml) by *Aspergillus niger* and *Trichoderma* sp. was found between 6-7 and 5-6 pH [24].

Maximum enzyme activity was recorded at 25 °C [25]. A *Trichoderma* strain in a recent paper preferred a temperature of 35 °C for maximum growth [26]. The cellulase activity of *Acinetobacter* sp. KKU44 was determined when grown in LB medium at 37 °C, 150 rpm for 72 h. The bacterial culture of 36 h. showed the maximum of cellulase activity at 83, 93, and 46 U/ml when determined at 50 °C, 60°C and 70 °C, respectively [1]. At 30 °C, *B. megaterium* had amylase activity of 0.47 mg/ml. When the temperature reached 40 °C, there was an increase in amylase activity, which was followed by a sharp decrease at 50 °C (0.39 mg/ml) [27].

The cellulase activity was measured at regular intervals. However, the maximum yield of exoglucanase (1.64 U/ml) and endoglucanase (1.84 U/ml) activity was obtained after 4 days [24]. However, the maximum glucosidase activity (1.61 U/ml) was observed after 3-5 days of incubation. Incubation periods. *P. decumbens* gave the highest amount of cellulase activity

(21.99 U/ml) on the sixth day of the incubation period [7]. However, after fermentation for 3 days, the highest activity could be obtained. This is due to nutrient consumption and the production of other components in the fermentation medium [28]. Various concentrations of carbon sources were used to replace 1% sugar which was the original concentration in growth media, with 2 to 5%. The results of a 24-hour incubation period revealed that a 5% carbon source produced the most cellulase when compared to other % carbon sources [29]. We compared *Aspergillus niger* cellulase production (filter paper activity, endoglucanase, and glucanase) on three different carbon sources. Glucose containing media gave the highest mycelia weight of 1.294 mg/flask. The culture containing cellulose showed the highest cellulose enzyme activity (filter paper activity, endoglucanase, and beta-glucanase). The waste cellulosic material can be used as a low-cost carbon source for commercial cellulose production [30].

The peptone was used as the nitrogen source in the *Aspergillus niger* growth medium, the cellulase yield reached its peak (0.79 U mol1) [31]. This data supports earlier results [32]. Poptone is one of the best nitrogen sources for cellulase production by *Aspergillus niger* 1433 [33]. Furthermore, that maximum cellulase activity can be achieved by using yeast extract or peptome as organic nitrogen sources [34]. Cellulase production by *Pycnoporus coccineus* gave its maximum activity when the growth medium nitrogen source was organic [35].

CONCLUSION

The present study reached the conclusion that the incubation period is the most significant element for microorganism development as well as the quantity of cellulose enzyme production. The work was to isolate and identify a high-cellulase producer from soil fungi. The production of enzymes uses two different agricultural byproducts, rice bran and sugarcane bagasse. The fungi *Aspergillus flavus*, *Aspergillus niger* and *Aspergillus terreus* were able to produce cellulase. Sugarcane bagasse had the optimal pH, temperature, incubation periods, and nutrient content (carbon, nitrogen, phosphorus, and potassium) for its highest cellulase production. The soil pathogen *Aspergillus terreus* is capable of producing a significant yield of cellulase from wheat bran and sugarcane bagasse containing cellulose.

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