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Original article

Enhanced cellulase enzyme production by *Aspergillus niger* using cellulase/iron oxide magnetic nano-composites

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ABSTRACT

Objectives: The cellulase enzyme is used for various industrial applications such as textile, paper, food and biofuel industries. Industrially, the fungal strain, *Aspergillus niger*, is widely used to produce cellulase enzymes. Emerging evidence has indicated the possible role of magnetic nanocomposites in enhancing cellulase production by *A. niger*. The cellulase/iron oxide nanocomposites are already reported to be an eco-friendly method for modulating microbial biochemical characteristics.
Methods: The present study was used to assess the efficacy of cellulase/iron oxide magnetic composites (CMNPS) for the enhanced production of cellulase enzyme. The fungal strain *Aspergillus niger* was isolated from the soil samples by using standard techniques. The fungi were then transfected with CMNPS and further evaluated for the production of cellulase enzyme.
Results: Synthesis of the cellulase/iron oxide magnetic composites was characterized by UV, FTIR, and XRD. The produced CMNPS was used as substrate and enzyme production. The cellulase enzyme production by *Aspergillus niger* was analyzed by CMC (0.82 IU/ml enzyme activity) and FPA assay (0.039 IU/ml enzyme activity). CMNPS was also analyzed by CMC (0.74 IU/ml enzyme activity) and FPA assay (0.039 IU/ml enzyme activity). Reuse of CMNPS is the ultimate application and ecofriendly approach for the synthesis of cellulase enzymes.
Conclusion: The study concludes that the cellulase/ iron oxide nanocomposites may be a useful tool for the enhanced production of cellulase enzyme from the soil fungus *A. niger*.

1. Introduction

Cellulases are a kind of enzyme that breaks down cellulose, the predominant component of the cell walls of the plant biomass (Garg et al., 2016). Cellulases are important industrial enzymes synthesized by microorganisms such as fungi and bacteria using cellulosic materials (EFSA Panel on Food Contact Materials et al., 2019). The action of the Cellulase enzyme mainly comes from three major enzymes that confirm the efficiency of this process. They are endoglucanases, exoglucanases, and β-glucosidase. B-1,4 linkages present in cellulose chains can be easily broken up by the cellulase enzymes. Enzymes are one of the key materials that are widely recognized for their diverse applications at the industrial level (Kuhad et al., 2011a). Cellulases enzymes are utilized for textile, biofuel, paper, food, and detergent industries (Li et al., 2018).

Cellulose is one of the richest biomass found on Earth. It is the prime product of photosynthesis in terrestrial environments and the richest renewable bioresource formed in the biosphere (Yamazawa et al., 2013). The cellulosic waste materials are classified as industrial, agricultural and municipal wastes (Omojasola and Jilani, 2008). The cellulosic waste materials are pretreated after usage. The common methods available for pretreatment are physical pretreatment, chemical pretreatment, and biological pretreatment methods. In the industrial sectors, widely used pretreatment methods are acid or alkaline treatment, the explosion of steam, wet oxidation, hot water and organic solvent pretreatment. Among

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them, acid pretreatment is the method of option in several industries processes (Kumar and Sharma, 2017).

Cellulase enzyme can be produced by various industrially important microorganisms, which includes fungi (*Aspergillus fumigatus, Fusarium solani, Trichoderma reesei and Sclerotium rolfsii)* as well as bacteria (*Clostridium thermocellum, Ruminococcus albus and Streptomyces species*) using different substrates (Cheese whey, Baggase and Rice straw) through submerged and solid-state fermentation (Mrudula and Murugammal, 2011). Filamentous fungi are the best choice for commercial enzyme production because the level of the enzymes produced by filamentous fungi is higher than those collected from yeast and bacteria. Almost all species of the genus *Aspergillus* is synthesizing cellulase (Mrudula and Murugammal, 2011). A large volume of cellulase enzyme is required for the industrial processes to break down cellulose. To minimize this demand, cellulase enzymes must be reused or recycled effectively.

In order to reuse the cellulase enzyme, the current study is planned to synthesize CMNPS, which may enable the reuse of cellulase. In recent years, "nanotechnology" has spread to almost all aspects of science and technology with several beneficial applications (Singh, 2017). In specific, magnetic nanoparticles based on magnetite (Fe3O4) for enzymes immobilization have gained high attention in different fields, which includes biomedical and environmental applications, due to their tiny size, large specific surface area, very little toxicity and ecofriendly nature (Joseph et al., 2020). The main advantages of using these magnetic composites are their easy and immediate separation from the mixture by creating an external magnetic field that may be appropriate for the reuse of cellulase enzyme (Sánchez-Ramírez et al., 2017).

Overall, the present study aims to isolate the soil *Aspergillus niger* strain and the enhancement of cellulase production by using metal nanocomposites, especially derived from iron oxide nanoparticles.

2. Materials and methods

2.1. Isolation of microbes from a soil sample

The soil sample was collected from the garden area located at Thiruvanaikovil, Tiruchirappalli, Tamil Nadu. The collected sample was kept in a sterile container and transferred to the laboratory. In the laboratory it was serially diluted, plated and fungus was isolated as per standard protocols described by Jin et al. (2019).

2.1.1. Identification and characterization of the isolated fungus

The isolated fungus was identified and characterized through their morphological and microscopic observation. Smears of the isolated fungi were prepared in Lactophenol cotton blue and examined with a microscope (McClenny, 2005).

2.2. Screening of Aspergillus niger for cellulase activity

Primary qualitative analysis was carried out using Congo red dye for the confirmation of cellulolytic microorganisms. The isolated fungus was inoculated in the CMC agar plate and incubated for 24 hrs at 25 °C. After successful incubation, the culture plate was flooded with Congo red (1%) and allowed to stand for 15 min at room temperature. After 15 min, the material was counterstained with NaCl (1 M) solution and left undisturbed for another 15 min. Finally, the tested plates which showed a clear zone around the line of growth confirm cellulose hydrolysis (Ahmad et al., 2020).

2.3. Synthesis of CMNPS

The CMNPS was prepared using the co-precipitation method. Ferric chloride (Three equivalent), Ferrous sulphate (Two equivalent), and 5μM of commercial *A. niger* cellulase were added and stirred for 15 min. Once the mixing is complete, about 50 ml of NaCl (0.2 M) was added to it and stirring was continued for another 1 h. The ammonium hydroxide 200 ml (w/v) was added at a slow rate with continuous stirring for 15 min. The black colour precipitate formed was washed with 5% ammonium hydroxide solution and the precipitate was then dried in the presence of nitrogen flow (Hassan et al., 2019).

2.4. Characterization of synthesized CMNPS

The structure and composition of the CMNPS were confirmed by UV, FTIR and XRD analysis. UV–visible spectroscopic analysis was performed on Hitachi double beam equipment (Model Lambda 35), in the 200–1100 nm range. In the FTIR spectrum (Spectrum RX 1-One), all spectral transmittance was acquired over the mid-infrared region (4000–400 cm^{-1}) using 32 scanning at a resolution of 8 cm^{-1}. For quantitative analysis of functional groups, single-beam attenuated total reflectance (ATR) spectra were collected from each sample and recorded. As a background, an air spectrum was used. Spectra were recorded repeatedly and average calculated before being used for model optimization (Rohman et al., 2014).

In XRD the crystalline nature and average size of the particles of the synthesized CMNPS were analyzed by X-ray diffraction (XRD) at 25 °C with a D8 Advanced X-ray diffractometer (Gonio model) using CuKα (λ = 1.54060 Å) radiations as an X-ray source and nickel (Ni) as a filter (Yu et al., 2013).

2.5. Production of cellulase enzyme

The Czapek dox media was used for the production of the cellulase enzyme. The media was dispensed into a 250 ml conical flask using a measuring cylinder and 1.0 g of each of the CMNPS were added into the Erlenmeyer flask and labelled properly. CMC was used as a control and sterilized in Autoclave at 121 °C for 15 min (Ghose, 1987).

2.6. Cellulase enzyme assay

The sample was collected and centrifuged at 2800 g for 10 min. The supernatant collected from centrifugation contains the enzyme. The supernatant was analyzed for the Endo-β-1,4- Glucanase assay and filter paper assay (FPA) (Ferrari et al., 2014).

2.6.1. CMC (Carboxymethylcellulose) assay

The Citrate buffer (0.1 M) was taken and about 1 ml of cell-free supernatant was added to the tube; it was then incubated at 50 °C for 30 min followed by the addition of 3 ml of 3, 5-dinitro salicylic acid (DNS). The tubes were incubated at 100 °C in a boiling water bath for 15 min and the optical density of the solution was determined spectrophotometrically at 540 nm. The glucose was used as a standard for final calculations (Yan and Chai, 2021).

$$\text{CMC} = \frac{\text{Glucose released}(\mu\text{mol})}{\text{Concentration}}$$

2.6.2. FPA assay (filter paper assay)

Sodium citrate, filter paper strip, and 1 ml of culture supernatant were added to a test tube, and the tube was incubated at 50 °C at 60 min. After the incubation period, about 3 ml of DNS was added to a test tube and further maintained at 100 °C in a boil-

ing water bath (15 min). The optical density of the solution was read at 540 nm using a spectrophotometer (Yu et al., 2016).

$$\text{FPA} = \frac{\text{Glucose released}(\mu\text{mol})}{\text{Concentration}}$$

3. Results

3.1. Screening of Aspergillus niger for cellulase activity

The preliminary qualitative analysis was conducted by using Congo red dye for cellulolytic microorganisms. Fig. 1 shows isolated fungus produced the cellulase enzyme. 2 cm of clear zone appeared. The plates that showed a zone of clearance around the line of growth indicated cellulose hydrolysis

3.2. Characterization of synthesized CMNPS

The CMNPS is separated using a magnet (Fig. 2) from the medium and the CMNPS is reused for the next industrial process. The Czapek dox media was used for the production of the cellulase enzyme. The cellulase enzyme assay and FPA were used to analyze enzyme production.

3.2.1. UV–VIS spectroscopy

The UV/visible absorption spectra for CMNPS are shown in Fig. 3. The magnetite composites show a surface Plasmon resonance (SPR) band at 400 nm. The SPR band of the magnetite composites shows a redshift and broadening of the peak in the spectrum.

3.2.2. Fourier transform infrared spectrometer (FTIR)

The FTIR spectrum of the CMNPS is shown in Fig. 4. The transmittance band at 3409 cm^{-1} corresponds to the OH stretching vibration of cellulose. The band at 2922 cm^{-1} denotes the C–H asymmetric and symmetric tensile vibration in the pyranoid ring and the peak at 1634.01 cm^{-1} is formed due to the OH bending vibration. The CH_2 symmetric scissoring in the pyranoid ring, C–O anti-symmetric bridge stretching, the crystal absorption peak of cellulose, C–O–C pyranoid ring skeletal vibration, and the n-glycosidic linkages are attributed to the absorption peaks at 1420.27, 1111.85, 1033.87, and 1057.53, respectively. The peak at 1634.01 cm^{-1} formed from the bending mode of the absorbed water. In the CMNPS, a new peak develops at 447 cm^{-1}, which

Fig. 2. Demonstration of the attraction potential of Cellulase/Iron oxide magnetic composites (CMNPS) towards a magnet kept aside.

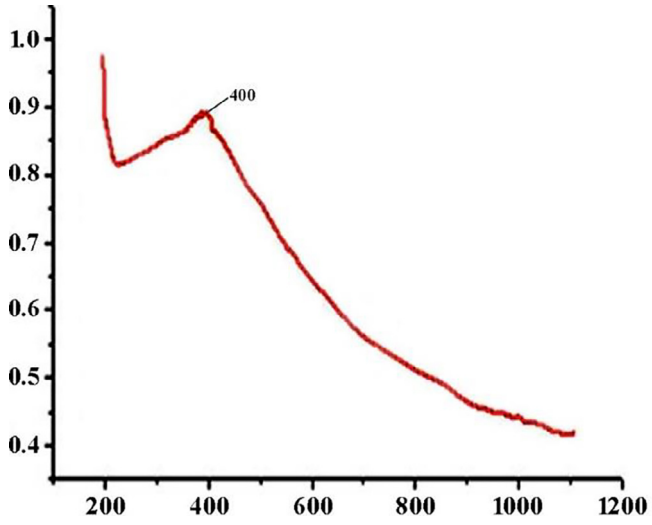

Fig. 3. UV –Visible Spectroscopic analysis of Cellulase/Iron oxide magnetic composites (CMNPS) from 200 to 1200 nm.

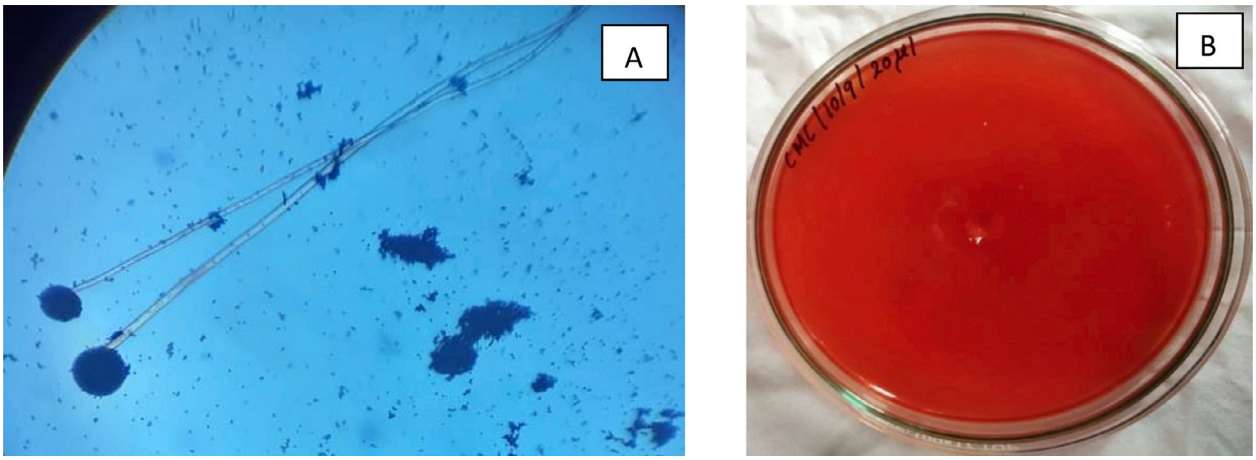

Fig. 1. Identification of *Aspergillus niger* from the soil sample, cultured using standard protocols (A) and Screening of *Aspergillus niger* colony using the cellulose hydrolysis assay (B).

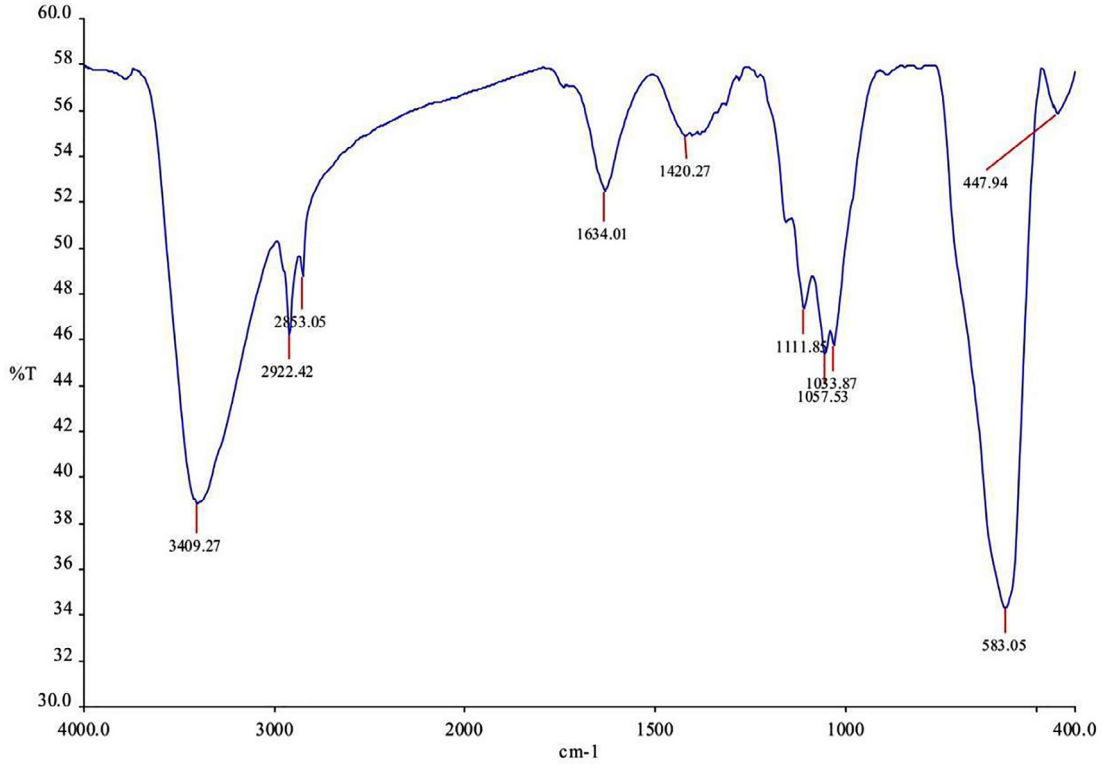

Fig. 4. Fourier transform infrared Spectroscopic analysis of Cellulase/Iron oxide magnetic composites (CMNPS) and the indicative peaks are labelled appropriately.

can be attributed to the vibration of γ-Iron oxide due to the overlapping bands around 583.05 cm^{-1} in cellulose and the CMNPS.

3.2.3. X-ray diffraction (XRD)

The XRD curve of the CMNPS is shown in Fig. 5. The diffraction peaks for cellulose at 15.20° and 22.70° are assigned to native cellulose. Moreover, the characteristic peaks at 30.20°, 35.50°, 43.20°, 53.70°, 57.20°, and 62.70° planes of Iron oxide (JCPDS card no. 39–1346), also appear for the resultant composites, suggesting that Iron oxide has been successfully prepared in the cellulose matrix. The CMNPS size range between 2 and 18 nm.

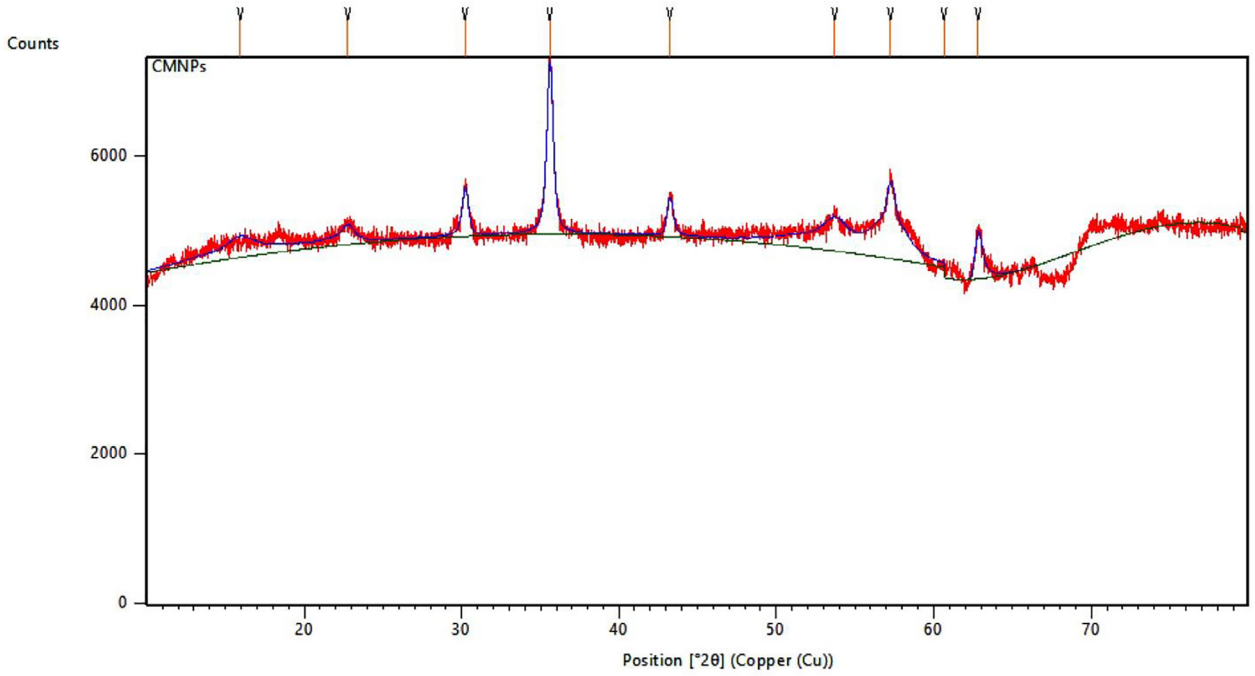

Fig. 5. X-Ray Diffraction analysis of Cellulase/Iron oxide magnetic composites (CMNPS).

3.3. Enzyme production

3.3.1. Cellulase assay

In a 250 ml conical flask, *Aspergillus niger* was inoculated into Czapek dox media and incubated for 1–12 days at 25 °C. At three-day intervals, the cellulase activity was assessed (Fig. 6). However, after 9 days, the maximal yield of endoglucanase activity in the CMNPS (0.82 mg/mL) was attained. After 3–6 days, however, minimum β-glucosidase activity (0.74 mg/mL) was seen. The largest amount of glucose was observed on the sixth day, which was used to identify the best incubation period for *Aspergillus sp*. enzyme production. Throughout the incubation period, *A. niger* was the most active cellulolytic species. The isolate *A. niger* required 4 and 6 days of incubation to obtain maximal cellulase activity, which was adequate for commercial use.

3.3.2. Filter paper assay (FPA)

In a 250 ml conical flask, *Aspergillus niger* was inoculated into Czapek dox media and incubated at 25 °C for 1–12 days. At three-day intervals, the filter paper assay was measured (Fig. 7). However, after 9 days, the maximal yield of FPA activity in the CMNPS (0.81 mg/mL) was attained. After 3–6 days, however, the Minimum FPA activity (0.79 mg/mL) activity was visible. The largest amount of glucose was observed on the sixth day, which was used to identify the best incubation period for *Aspergillus sp*. enzyme production. Throughout the incubation period, *A. niger* was the most active cellulolytic species. The isolate *A. niger* required 4 and 6 days of incubation to obtain maximal cellulase activity, which was adequate for commercial use.

4. Discussion

Microbial organisms are important agents in genetic engineering, especially in industrial microbiology (Vitorino and Bessa, 2017). They are widely utilized for the production of various biologically important molecules including enzymes, proteins, or other bioactive compounds (Abdel-Aziz et al., 2017; Ditu and Gheorghe, 2017). Fungal enzymes are widely used in industries and the most prominent among them is the cellulase enzyme (Singh et al., 2021b). This enzyme is of great significance due to its ability in cleaving the beta 1–4 glycosidic linkages in cellulose. It is highly utilized in the textile industry, pharmaceutical production, biofuel production and so on (Ejaz et al., 2021; Kuhad et al., 2011b). The commonly utilized microorganisms for cellulase production are *Cellulomonas, Clostridium, Trichoderma and Thermomonospora*. Apart from these, *Aspergillus* sp. is also emerging as an important producer of cellulase enzymes (Li et al., 2020).

Recent reports have indicated the use of metal nanocomposites for the production and enhancement of cellulase production (Rotaru et al., 2018). A study by Khalilzadeh et al. (2020) indicated the possible use of green synthesized cellulose nanocrystals using iron oxide for various purposes. Among the various models, iron oxide-based cellulose nanocomposites are promising in biotechnological applications (Yadav et al., 2015).

The results indicated the potential of CMNPS in enhancing the cellulase enzyme production by the *Aspergillus niger* in the Czapek dox medium. Previously it has been found that the iron oxide/ grapheme oxide nanocomposite modified by chitosan has been shown to increase the production of cellulase in *Trichoderma reesei* (Asar et al., 2020). Similarly, Han et al. (2018) has reported a similar increase in the production of cellulase and the production of sugars during the application of grapheme/ iron oxide nanocomposite. Reports have also indicated that the cellulase produced by the nanocomposite mediated method have better reusability and storability. Apart from using the magnetic nanocomposites, there are alternative methods like solid-state or submerged fermentation methods based on different substrates; however compared to the methods described the results of the present study has a significantly higher yield of cellulase and its activity (Mrudula and Murugammal, 2011; Singh et al., 2021a). In this study, the highest amount of cellulase enzyme produced using the CMNPS as substrate was 0.800 mg/ml of cellulase enzyme production. And the magnetic nanoparticle is reused and the enzyme is produced in a high amount (Islam and Roy, 2018). The use of molasses as substrate and the highest enzyme activity is 0.90 μmol ml^{-1} min. In this study, the CMNPS produces the highest amount of cellulase enzyme activity at 0.148 μmol ml^{-1} min.

Aspergillus species are more effective in terms of cellulase activity compared to *Alternaria, Rhizopus*, and *Penicillium*, which had moderate cellulase activity, while *Trichoderma* and *Fusarium* had low cellulase activity (Famurewa and Olutiola, 1991; Zhang et al., 2017). Besides, the Aspergillus isolates have been found to have endoglucanase activity and cotton degrading abilities (an indicator of the polygalacturonase activity), which is in corroboration with

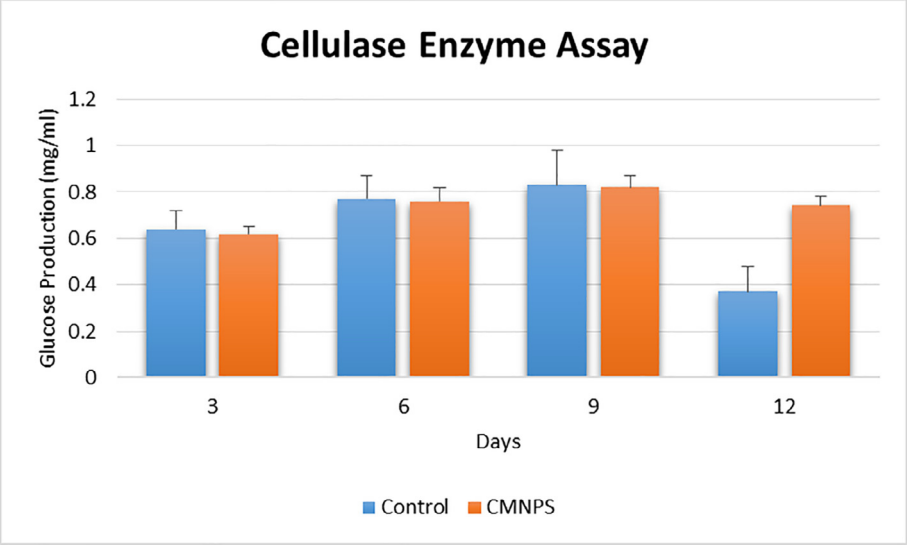

Fig. 6. Changes in the activity of cellulase enzyme in control group and Cellulase/Iron oxide magnetic composites (CMNPS) group for 12 days.

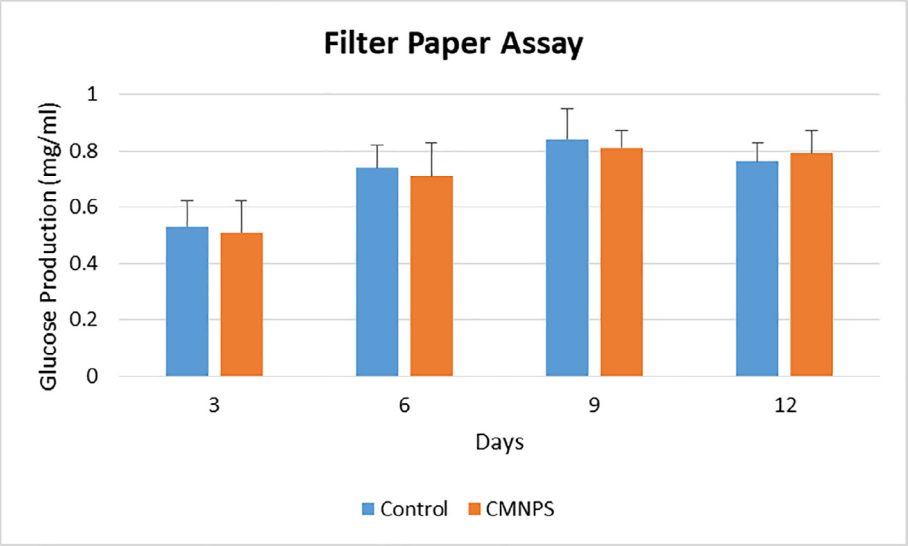

Fig. 7. Changes in the cellulase activity as indicated by the Filter paper assay by the formation of glucose (mg/mL) over different time periods.

the previous reports of Anuradha et al. (2010) and El Bergadi et al. (2016). Overall, the study indicated the possible use of cellulase/iron oxide nanocomposites in the production and enhancement of cellulase activity from *A. niger* strains. It may be useful for the industrial production of the enzyme as an easier and cheaper method.

5. Conclusion

Microorganisms are widely employed for the production of industrially important molecules such as enzymes. The present study utilized the soil fungus, *Aspergillus niger,* for the production of cellulase enzyme and its enhancement by using the cellulase/iron oxide nanocomposite. Overall the study concludes that the substrate, CMNPS, is easily separated and reused 1–5 times and also found to enhance the production of cellulase enzyme. Hence, the study suggests the use of CMNPS or similar nanocomposites for the modulation of cellulase enzyme production in *Aspergillus* sp. and which can bring revolutionary changes in the industrial applications of the fungi.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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