

Encapsulation of fungal extracellular enzyme cocktail in cellulose nanoparticles: enhancement in enzyme stability

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We demonstrated the nano-immobilization of fungal enzymes through their encapsulation in cellulose nanoparticles (CNPs). An extracellular enzyme cocktail (a mixture of amylase, protease, lipase, and cellulose) was produced from *Aspergillus niger* and *Phanerochaete chrysosporium* through submerged fermentation. The process of encapsulation was carried out through a microemulsion nanoprecipitation method in the presence of a lipid, a surfactant, and a co-surfactant. The morphology of CNPs was determined by field-emission scanning electron microscopy and transmission electron microscopy; CNPs were less than 100 nm in diameter. Fourier transform infrared spectroscopy (FTIR) and energy dispersive spectroscopy demonstrated the successful encapsulation of the fungal enzyme cocktail and revealed C and O as its major components. FTIR peaks of CNPs with encapsulated enzymes occurred at 3421.80, 2828.91, 1649.29, 1450.24, and 1061.61 cm^{-1} as well as in the range of 1050–1150 cm^{-1} . Encapsulated enzymes showed excellent stability with a peak at -70.91 mV in zeta potential studies. Thermogravimetric analysis proved that the CNP-encapsulated enzymes had an initial weight loss at 250°C. The encapsulated fungal enzyme cocktail exhibited higher catalytic performance and stability than the free enzymes. The encapsulated fungal enzyme cocktail derived from *A. niger* at the concentration of 100 $\mu\text{g/mL}$, showed the highest amylase activity with a clear zone of 2.5 cm. Overall, the results of this research reveal the enhancement in the activity of fungal extracellular enzyme cocktail through nanoencapsulation.

Keywords: *Aspergillus niger*, Cellulose nanoparticle, Enzyme cocktail, Encapsulation, Nanocatalyst, *Phanerochaete chrysosporium*

Enzymes function as ubiquitous magicians in the biological world by catalyzing specific substrates into products with substantially different properties. Enzymes are proteins and biological macromolecules that act as biocatalysts. They are produced by living organisms and they play a crucial role in all important chemical interconversions¹. Enzymes accelerate the rate of specific biological/biochemical reactions by lowering the activation energy without altering the reaction equilibrium. Microbial enzymes have been gaining tremendous importance in recent years owing to the rapid evolution of enzyme technology²⁻⁵. Microbial enzymes are preferred over the animal and

plant enzymes, given their stability, economic feasibility, ease of product modification and optimization, high yield, consistency, frequent supply due to the absence of seasonal variations, rapid production with cheap culture media, high catalytic performance, and absence of concerns with social and political issues^{5,6}. Approximately, 60% of more than 260 enzymes that have been commercialized by the members of the Association of Manufacturers and Formulators of Enzyme Products (AMFEP) are produced from fungi as host organisms⁷. Fungal enzymes are produced extracellularly and are efficient, compatible, and stable for industrial processing. Furthermore, fungal enzymes are highly desirable for solid-based fermentation can penetrate hard substrates and accelerate the hydrolysis process^{8,9}.

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Fungal enzyme cocktail is an alternative strategy for integrating different fungal enzymes to optimize their applications as biocatalysts. In this research, a fungal extracellular enzyme cocktail served as a mixture of enzymes, including amylases, cellulases, lipases, and proteases, produced by fungi. Filamentous fungi naturally secrete an enzyme cocktail that comprises various hydrolases, oxidases, and esterases that are unsuitable for industrial applications^{10,11}. Individually, these enzymes may become unstable or inactive, owing to unfavorable pH and temperature. The activity of enzymes may also be decreased or inhibited by small molecules present in the process. On the other hand, the use of these enzymes in industrial processes under harsh and extreme conditions may improve the tendency of enzyme destabilization and decrease their industrial lifespan¹². Therefore, the technology of enzyme immobilization may serve as an effective method to circumvent these limitations by enhancing the catalytic properties of enzymes to increase operational stability and simplify downstream processing.

The combination of biotechnology and nanotechnology has encouraged the rapid development of immobilized enzymes using various nanomaterials¹³. With the advent of nanotechnology, nanomaterials with unique physicochemical characteristics have emerged as a novel and fascinating support materials for enzyme immobilization¹⁴. These nanomaterials have ideal properties to balance the key factors that influence the efficiency of biocatalysts, such as specific surface area, effective enzyme loading, and mass transfer resistance. Nanoparticle-based immobilization technique is preferred over conventional immobilization techniques, owing to the ease of synthesis of the nano-enzyme particle without using toxic reagents and surfactants, the possibility to modify particle size within effective operational confines, and achievement of well-defined core-shell and homogeneous nanoparticles with thick enzyme shells¹¹.

The advancement in nanoscience has led to the development of cellulose-based nanomaterials. Cellulose nanoparticles (CNPs) are the preferred choice for enzyme immobilization, given their large surface area, ease of functionalization, chemical accessibility, and absence of any internal diffusion¹⁵⁻¹⁷. The rapid growth in nanotechnology over the years has facilitated cellulose extraction at a nanoscale level with minimal changes in cellulose hierarchical structure to allow for the creation of a novel template of cellulose-based

nanomaterial¹⁸. CNPs are non-poisonous, inexpensive, have low density and high accessibility, specific strength, and modulus. Hence, CNPs may be used in several fields such as nanocomposites, tissue engineering framework, filtration media, cosmetics, medicines, food, and industries¹⁹.

Enzyme immobilization by encapsulation is an entrapment method, wherein the enzymes are enclosed within a confined spherical semipermeable membrane. Encapsulation is a reproducible process that is performed without the need for sophisticated equipment²⁰. The multi-enzyme system could be developed by trapping more than one enzyme inside the membrane. Enzyme encapsulation increases the surface area between the enzyme and the polymeric material and provides integrity of enzyme structure and activity²¹. Enzyme encapsulation offers significant advantages to the permeability of matrices, as the encapsulated enzymes are resistant to leakage during the transportation of low molecular weight compounds. Furthermore, different sizes of enzymes may be easily accommodated inside the material with flexible porosity to provide an ideal microenvironment that is more suitable for controlled enzyme release or specific biocatalysis in drug delivery system through the chemical modification of matrix.

In this study, a fungal enzyme cocktail was extracted and subsequently encapsulated in CNPs to achieve the following properties; (i) enhanced enzyme stability, (ii) a slow and gradual release of enzymes, (iii) encapsulation of multiple enzymes in a single system. These properties enable the enzymes to be active in a wide range of environments. The activities of free and encapsulated enzymes were compared with a well-diffusion method. The CNP-encapsulated fungal enzymes were also characterized by Fourier transform infrared spectroscopy (FTIR), field-emission scanning electron microscopy (FESEM), transmission electron microscopy (TEM), energy dispersive spectroscopy (EDS), thermogravimetric analysis (TGA), and zeta potential measurement.

Materials and Methods

Organisms

The selected fungi (*Aspergillus niger* and *Phanerochaete chrysosporium*) were chosen from the School of Bioprocess Engineering, Universiti Malaysia Perlis, Arau, Malaysia.

Agar Plate Assay- Screening for Amylase Production

Nutrient agar medium composed of peptone (5.0 g/L), meat extract (3.0 g/L), agar-agar (20.0 g/L), sodium chloride (5.0 g/L), glucose (10.0 g/L), and soluble starch (2.0 g/L) was prepared in distilled water by adjusting the pH to 6.0, followed by autoclaving. A total of 20 mL of starch nutrient agar medium was poured into a sterile petri dish and allowed to solidify. A pin-point inoculum of the fungal colony was removed from working cell culture and inoculated at the center of the starch nutrient agar plate. The plate was incubated for 3 days at ambient temperature. Iodine solution was poured onto the plate after the incubation period to observe the formation of clearance zones around fungal colonies. The circular clear zones around colonies were yellow in color after reaction with iodine solution, indicative of the hydrolysis of starch in amylase assay plate²². The positive or negative results for enzyme production were recorded.

Screening for Cellulase Production

Czapekdox agar medium composed of sucrose (30.0 g/L), sodium nitrate (3.0 g/L), agar-agar (13.0 g/L), dipotassium hydrogen phosphate (1.0 g/L), magnesium sulfate (0.5 g/L), potassium chloride (0.5 g/L), iron (III) sulfate (0.01 g/L), and cellulose (10.0 g/L) was prepared in distilled water and its pH was adjusted to 7.0. The medium was autoclaved, and 20 mL of the autoclaved medium was poured into a sterile petri dish. The medium was allowed to cool, and a pin-point inoculum of fungal colony was removed from working cell culture and inoculated at the center of the solidified Czapekdox agar plate. The plate was incubated for 3 days at ambient temperature²² and the formation of a clear zone around colonies were observed. The positive or negative results for enzyme production were recorded.

Screening for Lipase Production

Culture medium composed of peptone (10.0 g/L), sodium chloride (5.0 g/L), agar-agar (20.0 g/L), and calcium chloride dihydrate (0.10 g/L) was prepared in water, autoclaved, and subjected to pH adjustment (pH of 6.0). A solution of 1.0% (v/v) Tween-20 was separately filter-sterilized and mixed with the autoclaved culture medium. A total of 20 mL of Tween-20 agar medium was poured into a sterile petri dish and allowed to solidify. A pin-point inoculum of the fungal colony was removed from the working cell culture and inoculated at the center of Tween-20 agar

plate, followed by the incubation of the plate for 3 days at ambient temperature²². Positive or negative results for lipase enzyme production were observed and recorded. Positive results were indicated by the formation of a clearance zone around a colony because of the complete degradation of calcium salt of fatty acid or through the formation of a visible precipitate owing to the deposition of calcium salt crystals produced from the hydrolysis of fatty acids by lipases²³.

Screening for Protease Production

Nutrient agar medium composed of peptone (5.0 g/L), meat extract (3.0 g/L), agar-agar (20.0 g/L), sodium chloride (5.0 g/L), and glucose (10.0 g/L) was autoclaved after pH adjustment to 6.0. A solution of 5.0% (v/v) gelatin (8.0% w/v) was autoclaved and mixed well with autoclaved nutrient agar medium, and 20 mL of the gelatin nutrient agar medium was measured and poured into a sterile petri dish. The plate was allowed to cool, and a pin-point inoculum of the fungal colony was inoculated at the center of the gelatin nutrient agar plate. The plate was incubated for 3 days at ambient temperature and the formation of clearance zones around fungal colonies was observed following the complete degradation of gelatin²⁴. A saturated solution of ammonium sulfate was poured onto the plate after incubation, resulting in a more opaque agar plate and increased visibility of a clear zone around the colony. The positive or negative results for enzyme production were recorded.

Enzyme Cocktail Production

Enzyme cocktail production from *A. Niger* and *P. Chrysosporium* was conducted with submerged fermentation (SmF) using the prepared inoculum in the form of spore suspension. Spore suspensions were prepared by scraping conidiospores from working cell cultures into sterile distilled water. Spore suspension comprising approximately 10^7 spores/mL, as determined with a hemocytometer, was used as inoculum for SmF.

Submerged Fermentation

The nutrient medium used for growth and enzyme production contained the following components (w/v): 0.14% (NH₄)₂SO₄, 0.20% KH₂PO₄, 0.03% CaCl₂, 0.02% MgSO₄·7H₂O, 0.50% peptone, 0.20% yeast extract, 0.03% urea, 0.10% Tween-20, and 0.10% of salt solution (5 mg/L FeSO₄·7H₂O, 1.6 mg/L MnSO₄·H₂O, 1.4 mg/L ZnSO₄·7H₂O, and 2.0 mg/L CoCl₂). The medium was supplemented with 2.0%

glucose, 2.0% cellulose, 2.0% gelatin, 2.0% olive oil, and 2.0% starch and its pH were adjusted to 6.0, followed by sterilization at 120°C for 20 min. The medium was inoculated with 1 mL spore suspension (10^7 spores) and the inoculated flasks were incubated at 37°C for 72 h in an incubator shaker operating at 150 rpm. The culture medium was centrifuged and filtered before being tested.

Enzyme Assays by Well-diffusion Method

The enzyme cocktail was subjected to enzyme activity assay for amylase, cellulase, protease, and lipases using a well-diffusion method and an enzyme assay plate with a specific substrate. The aforementioned media were prepared in different Petri dishes. After solidification, agar was punched with a 6 mm diameter well at the center. The crude fungal enzyme cocktail was filled in the well and the plates were incubated for 3 days at ambient temperature. The results were measured as stated above.

Encapsulation of Fungal Extracellular Enzyme Cocktail in CNPs

The prepared cellulose from cotton wool was subjected to dissolution to prepare CNPs as previously described²⁵ and used for the encapsulation of fungal enzyme cocktail. The preparation of CNPs was conducted using microemulsion nanoprecipitation method both in the presence and absence of enzyme solutions. Various concentrations (10, 20, 50, and 100 $\mu\text{L}/\text{mL}$) of fungal enzyme cocktail solution were added into mixtures containing 1.0% (*v/v*) Tween-20 (surfactant), 1.2% (*v/v*) olive oil (lipid), and 20.0 mL of absolute ethanol (co-surfactant). The oil/surfactant/co-surfactant mixture was continuously stirred for 1 h at 900 rpm to achieve complete homogenization. A microemulsion solution was successfully formed upon homogenization of the mixture. A total of 1 mL of cellulose solution (0.001% *w/v*) was drop-wise added into the microemulsion under sonication. The formation of CNPs was indicated by the cloudy dispersion observed immediately in the microemulsion solution. The solution was centrifuged at $4500 \times g$ for 20 min for the separation of CNPs from the unreacted solution. The suspended CNPs were washed with absolute ethanol and centrifuged at $4500 \times g$ for another 20 min. The suspended CNPs were washed again with absolute ethanol to remove the unincorporated components before the final centrifugation process at a higher speed of 10000 rpm for 5 min. The suspended CNPs were dried and stored.

Characterization of CNPs

Fourier Transform Infrared Spectroscopy

FTIR (Vertex 80V, Bruker, Germany) analysis was used to identify the functional group for microcrystalline cellulose (MCC) in the range of 500 to 4000 cm^{-1} . Absorption by different stretching modes of functional groups of molecules was reported between 1500 and 4000 cm^{-1} and below 1500 cm^{-1} of wave number significant for deformation, bending, and ring vibrations that are frequently used as the fingerprint regions of the spectrum.

Morphological Analysis

The surface morphology of the samples was analyzed with FESEM (Hitachi, S-4300 SE, Japan), and TEM (JEOL, Jem-2100F, Japan). Characterization of the particles based on shape, size, and size distribution was performed with FESEM at an acceleration voltage of 15 kV. FESEM equipped with an EDS analyzer was used for the identification of the elemental composition of samples at an acceleration voltage of 15 kV. For TEM analysis, the samples were negatively stained with uranyl acetate solution and placed on a copper grid substrate before observation at 200 kV acceleration voltage²⁶.

Thermogravimetric Analysis

Thermal stability of the sample was evaluated by the progressive increase in the temperature in TGA (TG 209 F3 Tarsus, Netzsch, Germany). Approximately 6.3 mg of samples were positioned in an alumina pan, which was subsequently heated from 30°C until 600°C at a rate of 10°C/min.

Enzymatic Activity of Encapsulated Fungal Enzymes

A total of 100 μL of sterile distilled water was added to various concentrations (0, 10, 20, 50, and 100 $\mu\text{L}/\text{mL}$) of dried encapsulated fungal enzyme cocktail with gentle mixing. An amylase assay plate was punched with five wells of 6 mm diameter and filled with different concentrations of encapsulated fungal enzyme cocktail solution. The same process was also carried out for the free enzyme of fungal enzyme cocktail at various concentrations (0, 10, 20, 50, and 100 μL) using sterile distilled water (100 μL working volume). Both agar plates with encapsulated and free enzymes were incubated at 50°C for 1 day. Iodine solution was poured onto the plate after the incubation period to observe the formation of clearance zones around fungal colonies. The circular clear zones around colonies were yellow in color after reaction with

iodine solution, indicative of the hydrolysis of starch in the amylase assay plate²². The developed yellow zones on amylase assay plates were measured and the diameter of clear zones was compared between the encapsulated and free enzymes.

Results and Discussion

The test fungal confirmation was carried out with microscopic observations at 100X magnification for *A. niger* and 40X magnification for *P. chrysosporium*. The hyphae, spore, and fruiting body of *A. niger* and *P. Chrysosporium* were clearly visible, and the genus of the fungi was confirmed by comparing the structure with other reports²⁷⁻²⁹. Agar plate assay was used for the primary screening of enzyme cocktail-producing fungi and the ability of *A. niger* and *P. Chrysosporium* to produce the enzyme cocktail was screened with the pinpoint inoculum method in amylase, cellulase, protease, and lipase assay plates. The results of the screening are shown in (Fig. 1). Both fungi showed positive results in all enzyme assay plates, as evident from the formation of clear zones around colonies. In this research, a special focus was given to the encapsulated cocktail enzymes and we evaluated the independent enzyme activities. Unlike in the unencapsulated system, after encapsulation, the enzyme release is delayed until an appropriate substrate is available. The CNPs do not dissolve quickly and require some time before they dissolve completely in the cellular milieu. This mechanism allows enzymes to have higher stability while being highly active.

Validation of Enzyme Production by Well-diffusion Method

An enzyme assay was conducted using the well-diffusion method for the screening of crude enzymes produced by fungi to ensure the presence of selected enzymes such as amylase, cellulase, protease, and lipase. The overall results are shown in (Fig. 2). The crude fungal enzyme cocktail showed positive results toward amylase, protease, and lipase assay plates but exhibited negative results for cellulase plates. Thus, only three enzymes were predominantly present inside the test fungal enzyme cocktail. The diameter of clear zones around the wells was measured and denoted as x. The range of diameter of clear zones was classified as follows: +++, >3 cm; ++, 3 cm < x < 2 cm; +, 2 cm < x < 0 cm; -, undetected. Among the positive results, the amylase assay plate showed the largest diameter of clear zones. Hence, the amylase plate was chosen for the validation of the encapsulated enzymes.

Characterization of Encapsulated CNPs

Fourier Transform Infrared Spectroscopy

The FTIR spectra of MCC, CNPs, and CNP-encapsulated fungal enzyme cocktail were compared in the range of wave numbers between 500 and 4,000 cm^{-1} . The OH stretching, C-H stretching, carbonyl ring, C-O stretching, and pyranose ring were observed at 2828.91–2928.91, 1649.50, 1050–1150, and 1061.61 cm^{-1} , respectively (Table 1 & Fig. 3). The encapsulated enzymes showed similar peaks with MCC and CNPs, indicative of the successful encapsulation of the enzymes inside CNPs. The spectra obtained were overlaid and a huge difference was observed at the wave number between 1400 and

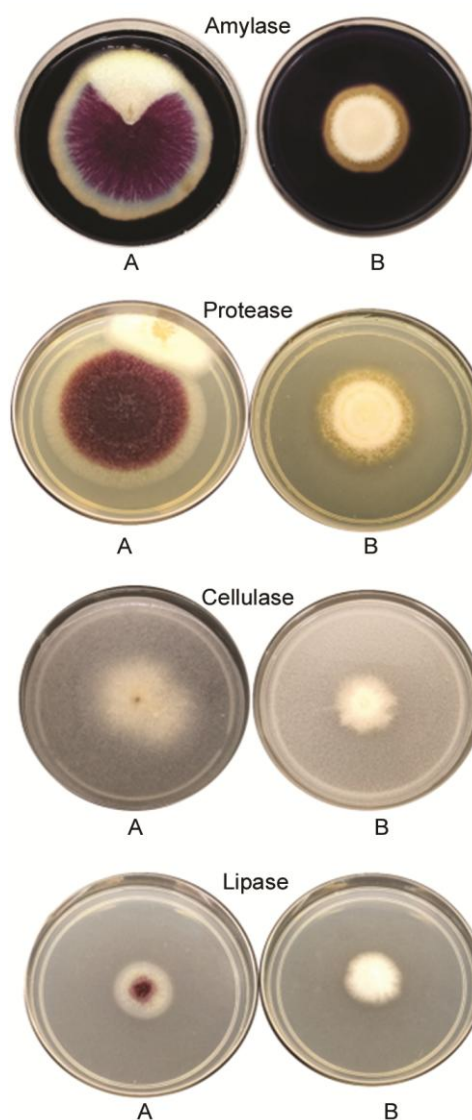


Fig. 1 — Screening fungi for enzyme production. (A) *A. niger*; and; (B) *P. chrysosporium*. The strains were screened for different enzymes using the pin-point inoculum

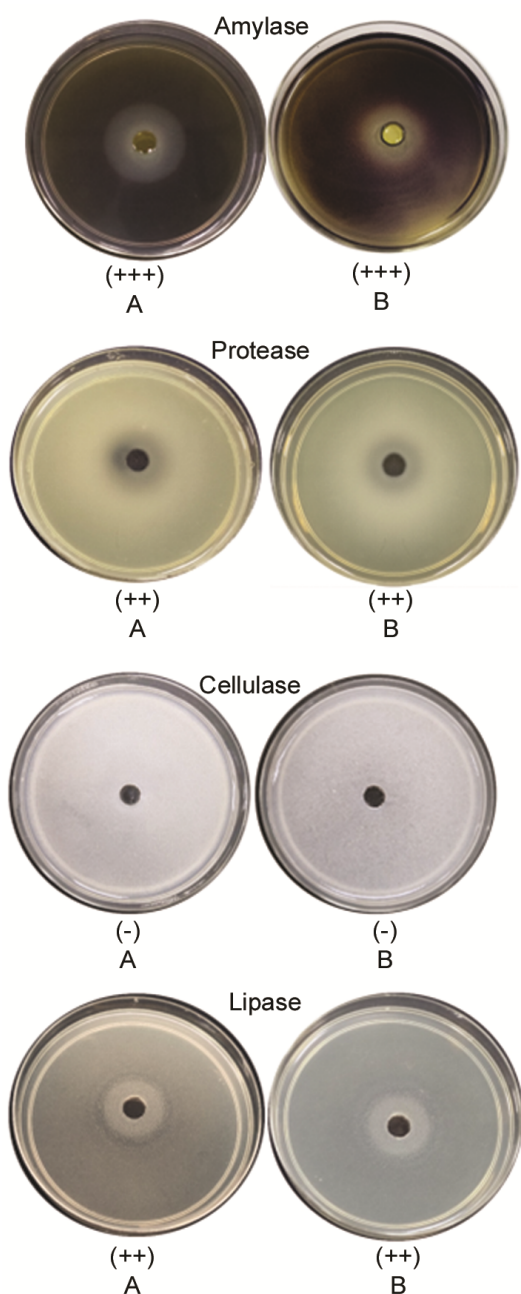


Fig. 2 — Fungal extracellular enzyme cocktail produced by (A) *A. niger*; and (B) *P. chrysosporium* were examined by the well-diffusion assay

1600 cm^{-1} with an enhanced sharp peak profile (indicated by the red star), confirming the encapsulation of enzymes.

Morphological and Compositional Analysis

The surface morphology of the CNPs prepared at 1.0% (w/v) concentration was examined using FESEM with accelerated electrons. Figure 4 displays the FESEM results of the encapsulated enzymes. The rod-like shape of CNP was evident that increased the surface area for the encapsulated enzymes. However, CNPs at 1.0% (w/v) concentration had a particle size of around 2 μm , which is outside the nanoscale range. Thus, a lower concentration (0.001% w/v) of cellulose solution was used to encapsulate the fungal enzyme cocktail to provide higher surface area and achieve nano-sized particles. FETEM was used to determine the morphology of the encapsulated enzymes under high magnification that revealed a particle size of 50 nm. FETEM analysis of the encapsulated enzyme is shown in (Fig. 4). Under high magnification, the shape of the particle was different from that observed in the FESEM image. The sphere-shaped CNP had a particle size within the nanometer range. From the morphological analysis, we observed that the particles were intact without any distortion. Even though there are some differences in shape between the images, they are uniform.

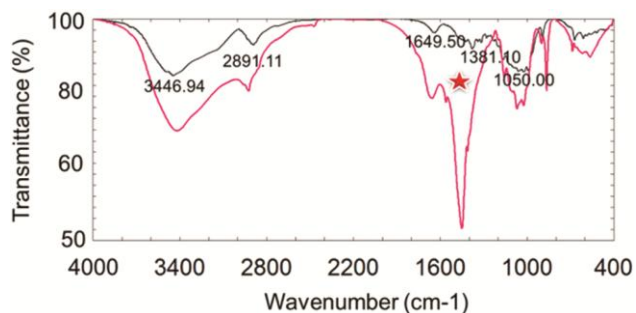


Fig. 3 — FTIR analysis with microcrystalline cellulose (blackline) and CNP-encapsulated fungal enzyme cocktail (Redline). The red star indicates the appearance of a new peak after encapsulation

Table 1 — Comparison of FTIR peaks between MCC, CNPs, and encapsulated enzymes

Description	MCC	CNPs	Encapsulated enzymes
O–H stretching	3446.94 cm^{-1}	3431.28 cm^{-1}	3421.80 cm^{-1}
C–H stretching	2891.11 cm^{-1}	2928.91 cm^{-1}	2828.91 cm^{-1}
C=O carbonyl ring stretching	1649.50 cm^{-1}	1634.70 cm^{-1}	1649.29 cm^{-1}
Cellulosic material	1380.10 cm^{-1}	1450.24 cm^{-1}	1450.24 cm^{-1}
C–O stretching	1050–1150 cm^{-1}	1050–1150 cm^{-1}	1050–1150 cm^{-1}
C–O–C pyranose ring stretching	1061.19 cm^{-1}	1061.61 cm^{-1}	1061.61 cm^{-1}

EDS analysis based on FESEM was conducted to identify the chemical elements of CNPs, including C, O, Na, and K content. Sodium (Na) was detected in CNPs owing to the dissolution of cellulose in the presence of a strong alkaline sodium hydroxide (NaOH)/urea solution during the synthesis of CNPs. Hence, Na content increased, and Na showed the second-highest peak for CNP as compared with MCC (Fig. 4). The elemental analysis of MCC and CNPs is shown in (Table 2), which shows the presence of Na at an increased level of 36.93%.

Stability Analysis

The thermal behaviour of the encapsulated enzymes is important to withstand the high temperature of operation conditions. TGA pattern of the encapsulated enzymes is shown in (Fig. 5A). As indicated before, the initial weight loss of the encapsulated enzymes observed from 50°C to 100°C, was owing to the evaporation of moisture from the surface of cellulose^{30,31}. The thermal decomposition of the encapsulated enzyme was initiated at 250°C, indicative of its higher thermal stability than that of free enzymes.

Zeta potential analysis was carried out to determine the stability of the sample particles by measuring their surface charge in solution (colloids)^{32,33}. The zeta potential for the encapsulated enzymes was -70.91 mV (Fig. 5B). As shown in (Table 3), the encapsulated enzymes had excellent stability.

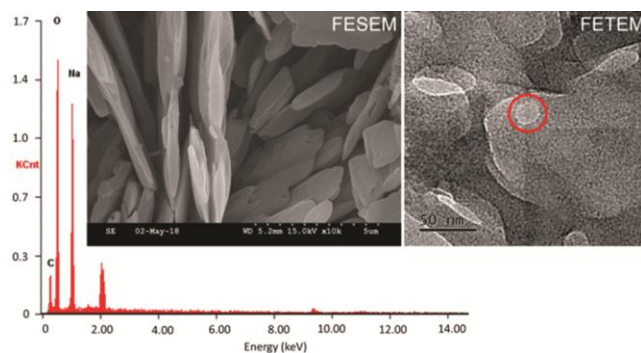


Fig. 4 — Morphology and EDS analyses of CNPs. FESEM and FETEM images are shown as insets

Table 2 — EDS analysis on FESEM images of MCC and CNPs

ELEMENTS	MCC	CNPs		
	Amount present, Wt %	Amount present, Wt %	Increase, %	Decrease, %
C	42.53	17.12	-	59.75
O	57.47	45.95	-	20.05
Na	-	36.93	36.93	-
Totals	100	100		

The Activity of Encapsulated Fungal Enzymes

The most important aspect in the encapsulation of enzyme is the retention of its activity upon immobilization. The encapsulation process has to be mild enough to retain most of the activity of the encapsulated molecule. For enzymes encapsulated in CNPs, the catalytic activity was determined by measuring the diameter of the clear zone as mentioned above. The amylase assay plate was selected for the comparison of activity between the encapsulated and free enzymes, as amylase showed the clearest and largest zones in the previous screening tests. Thus, it is easier to observe the difference in the enzyme activity between both enzymes. Autoclaved distilled water was filled in the central well as control. The formation of clear zones on the amylase assay plate was attributed to the enzymes filled into the wells.

According to the results shown in (Fig. 6A), the encapsulated enzymes exhibited higher enzyme activity along with the formation of larger clear zones than free enzymes. Thus, CNP is an excellent support material for the stabilization of the encapsulated enzymes against high temperature, as the free enzymes showed some degree of denaturation under similar conditions. Thus, the enzyme activity of the encapsulated enzymes was greater than that of the free enzymes owing to better stability. Previous studies have proven that immobilization by

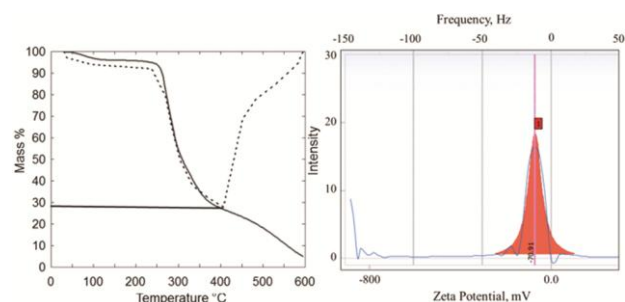


Fig. 5 — Stability assays (A) TGA analysis. The drop-down peak is shown by the broken line. Indicated the bottom-line temperature and; (B) Zeta potential analysis

Table 3 — Stability behavior of colloid suspension corresponding to zeta potential value

Zeta potential (mV)	Stability behaviour of the colloids
0 to ± 5	Rapid coagulation/flocculation
± 10 to ± 30	Incipient instability
± 30 to ± 40	Moderate stability
± 40 to ± 60	Good stability
$> \pm 61$	Excellent stability

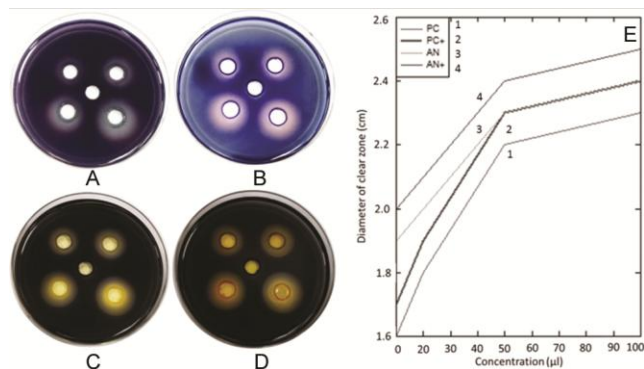


Fig. 6 — Activity for (A) free enzyme from *P. chrysosporium*; (B) CNP-encapsulated enzyme from *P. chrysosporium*; (C) free enzyme from *A. niger*; and (D) CNP-encapsulated enzyme from *A. niger*; and (E) Graph of the diameter of clear zones versus different concentrations of enzymes from *A. niger* and *P. chrysosporium*

encapsulation results in increased stability of the encapsulated enzymes, owing to their protection from the negative effects of the reaction conditions (*i.e.*, temperature and pH)^{34,35}. Based on the results obtained in our study, the higher concentrations of enzymes produced larger diameters of clearance zones. The enzymatic activity increased with an increase in enzyme concentration owing to the availability of more active sites, resulting in the continuous hydrolysis of the substrate to form larger clearance zones. The result obtained from the comparison of activity between the encapsulated enzymes and free enzymes was used to plot a graph (Figs. 6B-6E). Based on (Fig. 1), the activity of the encapsulated enzymes was greater than that of the free enzymes and the fungal enzyme cocktail secreted by *A. niger* was superior to that obtained from *P. chrysosporium*.

Conclusion

Fungi thrive well under extreme and inhospitable environment, owing to their efficient enzyme system. In this research, fungal extracellular enzyme cocktails produced by *A. niger* and *P. Chrysosporium* were extracted and subsequently encapsulated within CNPs. Although we obtained positive results during the screening of the ability of both fungi for enzyme production, we reported positive results only for amylase, protease, and lipase assay plates after SmF. The encapsulation of the fungal enzyme cocktail within CNPs was carried out using nanoprecipitation in a microemulsion method. Fungal extracellular enzyme cocktail, a mixture of amylase, protease, lipase, and cellulase, was produced from *A. niger* and

P. chrysosporium through SmF. The morphology of CNPs was determined by FESEM and TEM; CNPs had a 20 nm spherical shape and exhibited a high surface area. Furthermore, FTIR and EDS analyses revealed the successful encapsulation of the fungal enzyme cocktail within CNPs. FTIR peaks of the CNP-encapsulated enzymes were observed at 3421.80, 2828.91, 1649.29, 1450.24, 1061.61, and 1050–1150 cm^{-1} . CNP-encapsulated enzymes showed excellent stability with a peak at -70.91 mV in zeta potential studies. Thermogravimetric analysis proved that the CNP-encapsulated enzymes exhibited high thermal stability. Hence, the encapsulated fungal enzyme cocktail showed higher catalytic performance and stability than the free enzymes. The encapsulated fungal enzyme cocktail produced by *A. niger* showed maximum activity at 100 $\mu\text{g}/\text{mL}$ concentration with a 2.5 cm clear zone in the presence of the substrate amylase. Overall, the results of this research revealed the increase in the activity of fungal extracellular enzyme cocktail following encapsulation in CNPs.

Acknowledgments

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