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Adsorption of tannase from *Aspergillus ficuum* to carboxyl--functionalized multi-walled carbon nanotubes

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Abstract: The immobilization of cross-linked tannase onto carboxyl-functionalized multi-walled carbon nanotubes (MWCNT–COOH) was achieved *via* physical adsorption. Glutaraldehyde was used to cross-link the enzyme molecules. Spectroscopic and morphological characterizations of the enzyme-nanotubes composite were carried out, which authenticated the successful adsorption event. Enzyme composite is proven equal to, or even superior than free tannase, in terms of catalytic activities and stabilities, when measured under different thermal, pH and recycling conditions. Whilst both free and immobilized tannase preparations exhibited optimum catalysis at pH 5.0 and 35 °C, tannase-nanotubes composite possesses better thermal stability. The immobilized preparation retained 75 % of its initial catalytic activity following ten consecutive uses. The study demonstrated a facile method to produce catalytically efficient nanobiocatalyst composite for biotechnological applications.

Keywords: MWCNT; MWCNT–COOH; nanobiocatalyst; enzyme immobilization; enzymes stability and reusability.

INTRODUCTION

The initial hydrolysis of tannin is mediated by tannase (tannin acyl hydrolases, EC 3.1.1.20), which catalyzes the hydrolysis of ester and depside bonds found in tannins to release, *e.g.*, gallic acid, catechin and glucose.[1] The enzyme also fulfills a number of applications for different industrial sectors, such as food and beverages, cosmetics, as well as biological remediation. However, the role of tannase in the production of gallic acid is the most often highlighted. The compound is a feedstock in the production of antibacterial drugs, and in the synthesis

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of esters such as propyl gallate, an important additive and antioxidant in the food industry.[2]

Recently, developments in nanomaterials and their applications have opened up numerous opportunities in the area of nanobiocatalysis.[3] Carbon nanomaterials have been promoted as versatile supports for enzyme immobilization due to their small size, large surface area, mechanical and thermal stabilities.[4] Enzyme attachment to a nanoscale support material allows for a much higher enzyme loading, and catalytic stability was exceedingly better than those of free enzyme.[5] Among the various nanostructured materials, such as nanoparticles, nanofibers or carbon nanotubes (CNT), the latter is considered markedly promising for enzyme immobilization.[6]

Two main types of CNT namely, single-walled carbon nanotubes (SWCNT), and multi-walled carbon nanotubes (MWCNT) have been utilized as a support in enzyme immobilization.[3,7] Whilst SWCNT are attractive because of their higher surface area/high enzyme loading capacity, MWCNT are preferred alternative due to their better dispersibility, environmental and health safety. MWCNT have been frequently reported as a popular choice of starting nanomaterial for potential developments in recent years owing to their unique properties such as biocompatibility, high mechanical stability, good dispersibility, and high surface-to-volume ratio, ordered, nonporous structure, and large surface area.[8] Recently, a wide variety of enzymes such as ferredoxin-NAD(P)$^+$ reductase (NAhAa),[9] lipase,[10] L-ribose isomerase,[11] L-asparaginase,[8] peroxidase[12] and cellulase[13] have been successfully immobilized on carboxyl-functionalized MWCNT (MWCNT–COOH) for various applications. The nanobiocatalyst composites obtained are stable and active at a relatively high temperature, providing a unique combination of useful attributes such as low mass transfer resistance, as well as recycling of the catalyst.[13]

In this study we have investigated the attachment of tannase from *Aspergillus ficuum* to carboxyl-functionalized multi-walled carbon nanotubes (MWCNT–COOH) *via* facile adsorption, hence its immobilization. The efficiency of immobilized enzyme preparation, along with its stability and reusability were compared to its free counterpart.

EXPERIMENTAL

Materials

Carboxyl-functionalized multi-walled carbon nanotubes (MWCNT–COOH, length of 1.5 μm×outer diameter of 9.5 nm, Cat. #755125), tannic acid, gallic acid, rhodanine, potassium hydroxide and tannase from *Aspergillus ficuum* (EC 3. 1. 1.20; ≥ 150 U g^{-1}) were purchased from Sigma–Aldrich. Citric acid and trisodium citrate dehydrate were procured from Merck. Ultrapure water (18.2 MΩ·cm resistivity at 25 °C) was used to prepare buffer and reagent solutions. All chemicals used are ACS reagent grade, and were used as received from the suppliers.

Immobilization of tannase onto MWCNT–COOH

The MWCNT–COOH (3 mg) was dispersed in 3.0 ml of 50 mM citrate buffer (pH 4.7) *via* sonication at 37 kHz frequency, and 0.8 W sonification power (Elmasonic P30H, Elma, Singen, Germany) for 30 min. To the suspension, 0.3 mg of tannase was added, followed by incubation at 25 °C for 60 min under constant shaking at 200 rpm. Subsequently, 0.18 ml glutaraldehyde (3 vol. %) was added to the mixture, followed by shaking at 200 rpm, 25 °C for 60 min. Afterwards, the suspension was centrifuged for 30 min (6153*g*), and the supernatant was decanted. The unbound proteins were removed by washing with citrate buffer (pH 4.7) for at least five times. Then, the recovered enzyme-nanotubes composite was lyophilized overnight, and stored at 4 °C prior to use.

The enzyme loading efficiency was estimated as reported by Ulu *et al.*:[8]

$$\text{Enzyme loading efficiency}(\%) = 100\frac{C_\text{i}V_\text{i} - C_\text{f}V_\text{f}}{C_\text{i}V_\text{i}} \quad (1)$$

where C_i is the initial enzyme protein concentration (mg ml^{-1}), V_i is the initial volume of enzyme solution (ml), C_f is the residual enzyme protein concentration in the total supernatant (mg ml^{-1}), and V_f is the total volume of total supernatant (ml). The enzyme protein concentrations in solutions were measured by the Bradford assay with Bovine Serum Albumin (BSA) as a standard protein.

Characterization of tannase–nanotubes composite

Fourier transform infrared spectroscopy (FTIR, Perkin Elmer, model: Spectrum™ 400) in the frequency range (450–4000 cm^{-1}) was employed to analyze the signature functional group signals of MWCNT–COOH and tannase-immobilized MWCNT–COOH composite from He–Ne laser that emits red light with a wavelength of 633 nm. Field emission scanning electron microscopy (FESEM, FEI, model: FEL Quanta™ 450 FEG, operating voltage 10 kV) and transmission electron microscope (TEM, Carl Zeiss, model: Libra® 120 FEG) operated at accelerating voltage of 120 kV were used to observe the fine morphologies of pristine MWCNT–COOH and tannase-nanotubes composite.

Tannase assay

Tannin hydrolysis was determined based on the formation of chromogen between gallic acid and rhodanine (2-thioxo-4-thiazolidinone), resulting in a colored product with strong absorption at λ_max 520 nm according to Sharma *et al.*[14] Tannic acid at a final concentration of 0.075 mM was used as the substrate, and different concentrations of gallic acid were applied in standard calibration. One unit (U) of tannase activity is defined as 1 μmol of gallic acid released per min at pH 4.7 and 30 °C.

Effects of pH and temperature on tannase activities

The optimum temperature for free- and immobilized tannase catalysis were determined by performing enzyme assay at different temperatures from 20 °C to 45 °C at pH 4.7. Another set of experiments was also carried out to examine the effects of pH on free and immobilized enzyme activities (at 30 °C) in 50 mM citrate buffer at different pH, *i.e.*, 3.5 to 5.5, and in 50 mM phosphate buffer at different pH, *i.e.*, 6.0 to 7.0. The ratio of enzyme activity-to-enzyme activity at optimal pH or temperature yields residual enzyme activity (%).

Thermal stability of free and immobilized tannase

Thermal stability of the free- and immobilized tannase was investigated by incubating the preparations at different temperatures (30, 40, 50, 60 and 70 °C) and pH 4.7 (50 mM citrate buffer) for 60 min in the absence of enzyme substrate. After 60 min, the residual tannase

activities were determined. The relative activities of the free and immobilized tannase without incubation were considered to be 100 %.

Reusability of immobilized tannase

The reusability of the immobilized preparation was evaluated according to the standard assay conditions. After each cycle, the immobilized tannase was recovered by centrifugation (6153*g*, 30 min). The pellet was washed thrice with assay buffer before the subsequent cycle. For the consecutive successive cycles, the immobilized preparation was resuspended in a fresh substrate solution, and the reaction was carried out as described earlier. The activity of the immobilized preparation in the first cycle was considered to be 100 %.

Statistical analysis

All experiments were performed in triplicates. The data obtained were expressed as mean ± standard deviation. Microsoft Office Excel (Microsoft Corporation, USA) was used to calculate the means and standard deviations, and graph plotting.

RESULTS AND DISCUSSION

The enzyme loading efficiency

In this study, 0.1 mg of MWCNT–COOH was found efficient for the immobilization of 0.1 mg tannase *via* adsorption mechanism with the protein loading efficiency of 81 % under the experimental conditions used.

Fourier transform infrared spectroscopy (FTIR)

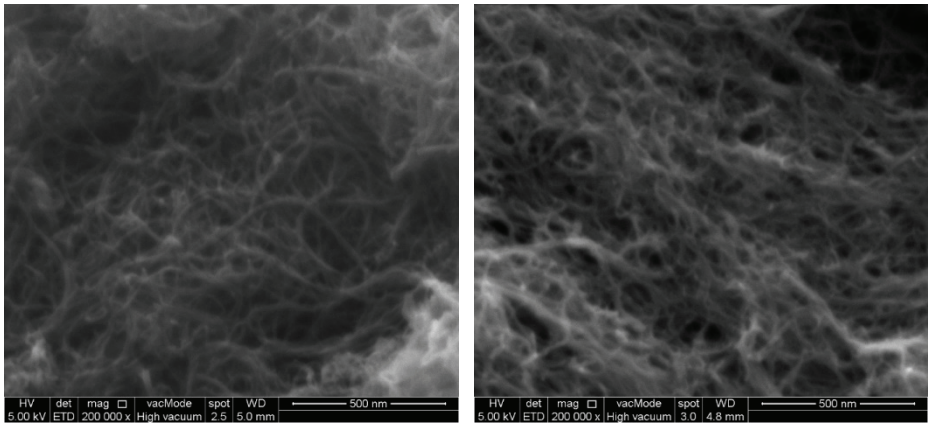
From Table I, for MWCNT–COOH, the signal peaks at 1740 and 2922 cm^{-1} are due to C=O and O–H stretching of carboxylic groups (O=C–OH) from the side wall of the graphite structure.[15] For MWCNT–COOH–tannase composite, signal peaks at and 1543 cm^{-1} are attributed to the amine C–N stretching and amine N–H bending lend support that tannase enzyme was successfully attached to the MWCNT–COOH carrier,[10] presumably *via* non-specific interactions involving hydrogen bonding and van der Waals forces between the amino acids of the enzyme and MWCNT–COOH carrier. Stable non-specific, multipoint interactions between tannase molecules and MWCNT–COOH are hypothesized to minimize enzyme conformational changes hence preventing the distortion of its active site.

TABLE I. Signal assignments for: a) pristine MWCNT–COOH and b) MWCNT–COO–tannase composite

Sample	Frequency, cm-1	Functional group
MWCNT–COOH	1740	C=O stretching
	2922	O–H stretching
MWCNT–COOH-tannase	1168	Amine C–N stretching
composite	1543	Amine N–H bending

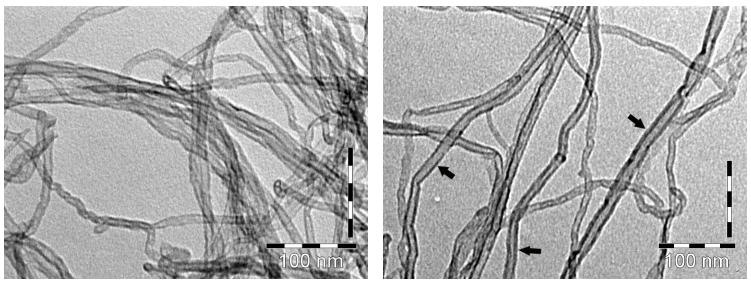
Morphologies of MWCNT–COOH and tannase-immobilized MWCNT–COOH

Figs. 1 and 2 show the FESEM and TEM micrographs of the MWCNT–COOH and tannase-immobilized MWCNT–COOH, respectively. As shown in Fig. 1a, the MWCNT–COOH sample exhibits a distinct, smoother strands of tubes. On the other hand, for tannase-immobilized MWCNT–COOH sample (Fig. 1b), the morphology is in contrast to pristine MWCNT–COOH, which indicates successful deposition of the enzyme molecules onto the carbon nanotubes. The observed increase in thickness dimension of the strands of the enzyme–nanotubes composite sample (Fig. 2b) compared to MWCNT–COOH sample (Fig. 2a) is ascribed to the adsorption of tannase onto the outermost wall of MWCNT–COOH. These morphological characteristics bear strong resemblance to the images reported in the previous study of lipase enzyme immobilization on MWCNT–COOH.[15]



(a) (b)

Fig. 1. FESEM micrographs of the MWCNT–COOH: a) before and b) after tannase immobilization.



(a) (b)

Fig. 2. TEM micrographs of the MWCNT–COOH: a) before and b) after tannase immobilization. The increased thickness of the carbon nanotubes is due to deposition of enzyme molecules onto outermost wall *via* physical adsorption.

Optimum pH and temperature for free and immobilized tannase

Fig. 3 shows the effect of pH on the relative activities of free and immobilized tannase. The enzyme activities were highest at pH 5.0 for both free and immobilized preparation, hence, 5.0 is the optimum pH value. Immobilized tannase showed no observable alteration in its optimum pH and shared a similar pH-activity profile with its free counterpart. The finding is in general agreement to the previous study, *i.e.*, identical optimum pH at 5.0 for both free and immobilized tannase obtained from *Aspergillus ficuum*.[16,17] In contrast, Li *et al.*[18] reported that the optimal pH value for immobilized tannase (pH 4.5) was lower than that of the free tannase (pH 5.5) when sourced from *A. niger*.

The effects of temperature (20–45 °C) on the free and immobilized enzyme activities were studied in 50 mM citrate buffer (pH 4.7, Fig. 4). Both free and immobilized preparations showed identical optimum temperature at 35 °C. Although

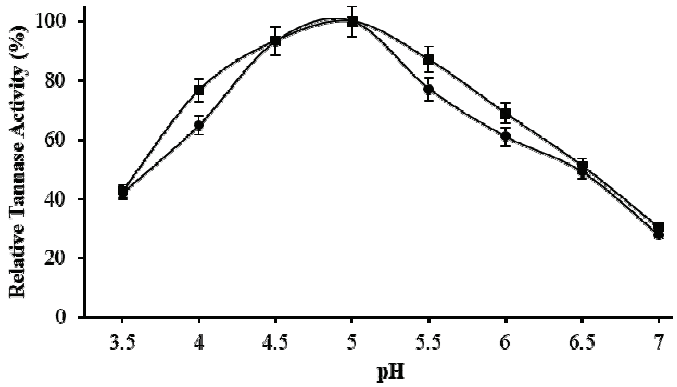


Fig. 3. Effects of pH on the enzyme activities. Square marker refers to the immobilized enzyme preparation while circle marker to free tannase.

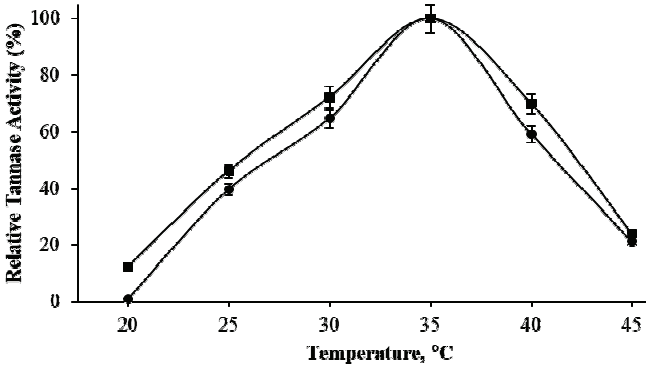


Fig. 4. Effects of temperature on the enzyme activities. Square marker refers to immobilized enzyme preparation while circle marker to free tannase.

temperature profiles of immobilized enzyme exhibited similar trend to those of free enzyme, significantly higher relative activities were observed for immobilized preparation over the temperature range studied. Similar observation was reported where free and immobilized *A. ficuum* tannase exhibited the same optimal temperature after immobilization onto pristine MWCNT.[16] It has been reported that immobilization may not necessarily alter the optimum temperature of tannase catalysis.[18,19]

Thermal stability of free and immobilized tannase

Fig. 5 shows the effect of temperature on the stability of the free and immobilized tannase preparations. The immobilized tannase preserved over 60 % of its initial activity while its free counterpart lost half its initial activity after incubation for one hour at 60 °C. It is clear that immobilization provides a more thermally-stable enzyme preparation compared to free tannase. Similar results were observed for tannase from *A. tubingensis* when immobilized onto carboxyl-functionalized super-paramagnetic ferroferric oxide particles,[19] and also tannase from *A. ficuum* immobilized onto pristine MWCNT nanocomposites.[16] The immobilization had put a restriction on the enzyme molecule conformational mobility, thus reducing the likelihoods of thermal-mediated deactivation of the enzyme.[20]

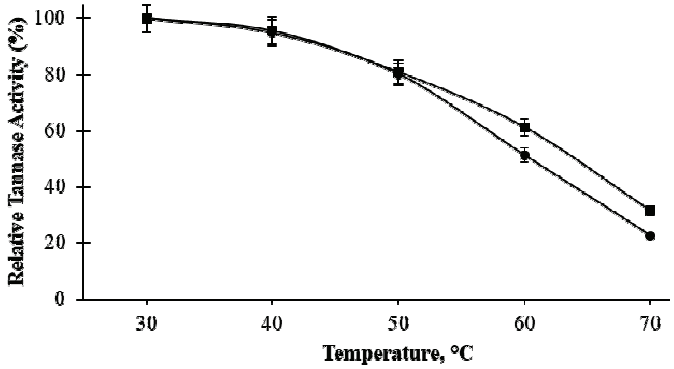


Fig. 5. Thermal stabilities of the free- and immobilized tannase preparations. Square marker refers to immobilized enzyme preparation while circle marker to free tannase.

Reusability of the immobilized tannase

The tannase-immobilized MWCNT–COOH composites retained residual activity above 75 % following ten batches of consecutive reactions (Fig. 6), whereas tannase immobilized onto pristine MWCNT recorded significantly lower residual activity at 34 % after ten cycles.[16] In other study, L-asparaginase-immobilized MWCNT–COOH doped calcium-alginate beads retained only 37 % of its initial activity after ten successive catalytic cycles.[8] From the results, it can be concluded that tannase is far more stable when immobilized onto the MWCNT–

–COOH surface compared to the single use, free enzyme, which opens up a wide possibilities for practical applications of tannase in the future.

Since tannase adsorption onto the MWCNT–COOH is exclusively due to non-specific interactions such as hydrogen bonding and van der Waals, it is highly likely that enzyme leakage, over the combined course of applications and washing steps, is responsible for the observed minor losses of activities during consecutive uses of the immobilized enzyme preparation (Fig. 6). Such occurrence is not uncommon, and almost certain to ensue in the case of physical adsorption of an enzyme to a solid support compared to when an enzyme is covalently coupled to the support. Nonetheless, the observed loss is rather negligible for each cycle relative to its precursor.

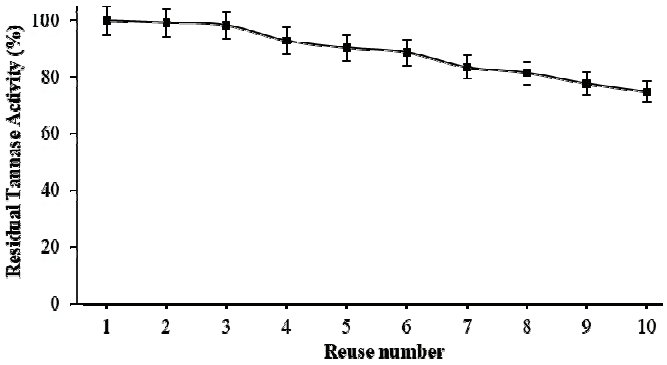


Fig. 6. Reusability of tannase-immobilized MWCNT–COOH preparation.

CONCLUSIONS

This study shows that tannase from *Aspergillus ficuum* was successfully immobilized onto carboxyl-functionalized MWCNT *via* the physical absorption method. The immobilized preparation exhibits identical optimum pH and temperature with its free counterpart with the exception of better thermostability. The facile immobilization also allows for catalytic reusability up to ten consecutive cycles with minor reduction in activity. Potentially wide applications for nano-biocatalyst, with such attributes, are envisaged.

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ИЗВОД

АДСОРПЦИЈА ТАНАЗЕ ИЗ *Aspergillus ficuum* НА ВИШЕСЛОЈНЕ УГЉЕНИЧНЕ НАНОЦЕВИ СА КАРБОКСИЛНИМ ГРУПАМА

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Имобилизација умрежене таназе на мултићелијске угљеничне наноцеви са карбоксилним групама (MWCNT–COOH) је постигнута физичком адсорпцијом. Глутаралдехид је коришћен за умрежавање молекула ензима. Спектроскопска и морфолошка карактеризација композитног материјала ензима и наноцеви је потврдила успешну адсорпцију. Ензимски композит се показао једнаким или бољим од слободне таназе у смислу каталитичке активности и стабилности, под различитим температурним, pH и условима рециклирања. Иако слободна и имобилизована таназа испољавају оптималне каталитичке особине на pH 5,0 и 35 °C, композит таназе и наноцеви има већу термалну стабилности. Имобилизовани препарат задржава 75 % почетне каталитичке активности након десет узастопних примена. У раду је приказан једноставан метод за производњу каталитички ефикасног нанобиокатализатора за биотехнолошку употребу.

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REFERENCES

1. A. S. Ristinmaa, T. Coleman, L. Cesar, A. Langborg Weinmann, S. Mazurkewich, G. Brändén, M. Hasani, J. Larsbrink, *J. Biol. Chem.* **298** (2022) (https://dx.doi.org/10.1016/j.jbc.2022.101758)
2. C. S. de Lima, B. T. A. Koelher, E. G. P. da Silva, A. Góes-Neto, R. P. Rezende, A. P. T. Uetanabaro, A. M. da Costa, *Fungal Biology* **126** (2022) 471 (https://dx.doi.org/10.1016/j.funbio.2022.04.001)
3. M. d. R. G. Dias, G. P. C. da Silva, A. de Pauloveloso, N. Krieger, C. Pilissão, *Chirality* **34** (2022) 1008 (https://dx.doi.org/10.1002/chir.23454)
4. M. Markiton, S. Boncel, D. Janas, A. Chrobok, *ACS Sustain. Chem. Eng.* **5** (2017) 1685 (https://dx.doi.org/10.1021/acssuschemeng.6b02433)
5. Z. L. Li, L. Cheng, L. W. Zhang, W. Liu, W. Q. Ma, L. Liu, *Process Saf. Environ. Prot.* **107** (2017) 463 (https://dx.doi.org/10.1016/j.psep.2017.02.021)
6. N. Singh, B. S. Dhanya, M. L. Verma, *Mater. Sci. Energy Technol.* **3** (2020) 808 (https://dx.doi.org/10.1016/j.mset.2020.09.006)
7. Y. Yamada, K. Obuchi, N. Kikuchi, A. A. Almarasy, A. Fujimori, *Langmuir* **38** (2022) 5692 (https://dx.doi.org/10.1021/acs.langmuir.2c00283)
8. A. Ulu, M. Karaman, F. Yapıcı, M. Naz, S. Sayın, E. İ. Saygılı, B. Ateş, *Catal. Lett.* **150** (2020) 1679 (https://dx.doi.org/10.1007/s10562-019-03069-y)
9. S. Zhao, P. Feng, Z. Yu, T. Zhou, T. Gao, M. M. Redina, P. Liu, X. Li, *Chemosphere* **291** (2022) 132934 (https://dx.doi.org/10.1016/j.chemosphere.2021.132934)
10. M. K. Ghide, K. Li, J. Wang, S. A. Abdulmalek, Y. Yan, *Food Chem.* **390** (2022) 133171 (https://dx.doi.org/10.1016/j.foodchem.2022.133171)
11. A. Singh, S. K. Rai, M. Manisha, S. K. Yadav, *Mol. Catal.* **511** (2021) 111723 (https://dx.doi.org/10.1016/j.mcat.2021.111723)

12. L. Y. Jun, N. M. Mubarak, L. S. Yon, C. H. Bing, M. Khalid, P. Jagadish, E. C. Abdullah, *Sci. Rep.* **9** (2019) 2215 (https://dx.doi.org/10.1038/s41598-019-39621-4)
13. R. Ahmad, S. K. Khare, *Bioresour. Technol.* **252** (2018) 72 (https://dx.doi.org/10.1016/j.biortech.2017.12.082)
14. S. Sharma, T. K. Bhat, R. K. Dawra, *Anal. Biochem.* **279** (2000) 85 (https://dx.doi.org/10.1006/abio.1999.4405)
15. M. Mohammadi, M. Ashjari, M. Garmroodi, M. Yousefi, A. A. Karkhane, *RSC Advances* **6** (2016) 72275 (https://dx.doi.org/10.1039/C6RA14142K)
16. C.-B. Ong, M. S. M. Annuar, *Preparative Biochemistry & Biotechnology* **48** (2018) 181 (https://dx.doi.org/10.1080/10826068.2018.1425707)
17. J. S. de Lima, M. P. Cabrera, C. M. de Souza Motta, A. Converti, L. B. Carvalho, Jr., *Food Res. Int.* **107** (2018) 470 (https://dx.doi.org/10.1016/j.foodres.2018.02.066)
18. R. Li, G. Fu, C. Liu, D. J. McClements, Y. Wan, S. Wang, T. Liu, *Int. J. Biol. Macromol.* **114** (2018) 1134 (https://dx.doi.org/j.ijbiomac.2018.03.077)
19. C. Wu, C. Xu, H. Ni, Q. Yang, H. Cai, A. Xiao, *Bioresour. Technol.* **205** (2016) 67 (https://dx.doi.org/10.1016/j.biortech.2016.01.032)
20. A. Xiao, C. Xu, Y. Lin, H. Ni, Y. Zhu, H. Cai, *Electron. J. Biotechnol.* **19** (2016) 1 (https://dx.doi.org/10.1016/j.ejbt.2015.10.001).