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Deep Eutectic Solvent Derived from Paracetamol for Efficient Enzymatic Biotransformation

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Abstract

Deep Eutectic Solvent (DES) has emerged as a promising green solvent for use in various chemical reactions, including enzymatic reactions. In this study, a paracetamol-based DES was synthesized and characterized for its physicochemical properties. Additionally, molecular simulation was performed to investigate the DES's effect on the *Candida rugosa* lipase activity. The characterization analyses confirmed that the paracetamol-based DES possessed functional groups similar to its original components but exhibited a lower melting point. The enzymatic activity of the lipase in the presence of the synthesized DES was significantly enhanced, leading to a high yield of free fatty acids (348.30 µmole). This study is the first to demonstrate the use of a paracetamol-based DES for enzymatic activity, providing evidence that DESs are an efficient replacement for conventional organic solvents in enzyme-catalyzed reactions. Molecular simulation results showed that the DES's active ingredient, pTSA, exhibited potent catalytic properties and protonation abilities, contributing to the observed lipase activity enhancement. Furthermore, the paracetamol molecule present in the DES was found to have a significant impact on the chemical environment surrounding lipase, leading to indirect effects on its activity. In conclusion, this study offers a promising strategy for utilizing greener solvents in enzymatic reactions, demonstrating the potential of DESs as a versatile tool for enhancing the catalytic activity of enzymes. The results of this study highlight the importance of exploring alternative green solvents and provide a foundation for future research in this area.

Keywords deep eutectic solvent, *Lipase Candida Rugosa,* biocatalysis, ionic liquids

1.0 INTRODUCTION

In recent years, the consumption of medicinal drugs has increased, triggering pharmaceutical industries to increase production. The presence of pharmaceutical compounds in aquatic systems has become a serious topic [1]. There are emerging contaminants in the environment through domestic, industrial waste, and hospital effluent [2].

Consequently, it tended to bioaccumulate in aquatic environments, posing a considerable risk to human health and the ecosystem due to the acute exposures through the food chain [3]. Considering the environmental impact of pharmaceutical residues, it is necessary to reduce pollution by developing a method to overcome this issue [4].

Paracetamol, also known as acetaminophen, is one of the most extensively used analgesic and antipyretic drugs around the world [2]. One of the most consumed drugs, with a production of more than 100,000 tonnes per year [5]. The widespread use and easy accumulation of paracetamol results in its being found in various environmental matrices such as soils, sediments, and drinking water [6]. Paracetamol will be used as a part of the deep eutectic solvent (DES) component in this study to reduce water contamination, as it has been considered an excellent hydrogen bond donor (HBD). Several non-conventional reactional media have been introduced as good solvents that focus on sustainability, such as ionic liquid (IL) and DES. However, DES has gained more attention due to its non-toxicity, low cost, and biodegradability over organic solvents and ionic liquids [7]. Novel deep eutectic solvents, a novel class of green solvents, have emerged as an excellent solvent urgently needed in biocatalytic activities [8]. Besides, DES can boost enzyme catalytic action and bioconversion efficiency significantly [9]. In eutectic solvents, a wide range of enzyme and chemo-enzymatic reactions can be carried out, including transesterification, epoxidation and forming carbon-carbon bonds, which are metabolized by lipase [10]. Nevertheless, it is also promising for substituting traditional solvents such as acetone, hexane, and methanol, which are volatile and harmful to the environment.

Owing to its ability to enhance substrate availability, modification and stability, DES has recently been introduced as a solvent in a variety of biocatalytic reaction [11]. DESs have been effectively employed as solvents in catalytic and biocatalytic organic processes, in biomass refining and materials science, as electrolytes for electrodeposition and electrochemistry [12, 13], and as solvents for extracting natural bioactive chemicals [8]. The pivotal role of lipase activity in various biochemical pathways has spurred research into strategies for maximizing its catalytic potential. Therefore, this study explores the intriguing prospect of utilizing DES to enhance lipase activity as catalysts and versatile reaction media. DESs, renowned for their unique physicochemical attributes, are investigated here to unravel their multifaceted influence on lipase catalysis.

2.0 EXPERIMENTAL

2.1 DES preparation

The chemicals used to prepare the DES, namely paracetamol (also known as acetaminophen) and p-toluenesulfonic acid (pTSA) were purchased from Sigma-Aldrich. All solid chemicals were dried overnight in a vacuum oven (Memmert VO500, ThermoFisher, America) at 60ºC. Next, via magnetic stirring, paracetamol and pTSA were mixed according to the given molar ratio (i.e., 1:3) at 70ºC. The resulting homogeneous mixtures were transferred to a well-sealed and dark (covered with aluminum foil) bottle.

2.2 Physicochemical characterization

Fourier Transform-Infrared Spectroscopy (FTIR) analysis was conducted according to the previous studies [14, 15] to confirm the presence of functional groups from the initial ingredients in the synthesized DES using a Perkin Elmer 1600 FTIR spectrometer. The sample was measured in the range of 400-4000 cm^{-1} for five scans [16, 17]. ^{1}H Nuclear Magnetic Resonance (NMR) Liquid state Bruker NMR Avance II (400 MHz) was also used to validate the chemical structure of DES. Deuterium oxide (D2O) was used as a solvent for the sample preparation. The melting point (Tm) and freezing point (Tc) of the synthesized DES were determined using a DSC STARe System Mettler TOLEDO V9.10 (Mettler Toledo). The sample was cooled from 313 to 203 K at a 1 K/min cooling rate for two cycles under a nitrogen gas atmosphere. The sample was weighed in less of 10 mg and sealed in an aluminium crucible with a lid. The sample was heated from -70°C to 25 °C at 10°C/min and cooled at - 70°C to 25°C in 5°C/min.

2.3 Method of quantum calculation

The starting structures of the paracetamol and pTSA were built and optimized in the ground state using the hf/3-21g level of theory in the *Gaussian 09* quantum chemical suite [18, 19] and Gauss View software [20]. To extract the

frontier molecular orbital, further energy analysis at the same level of theory was conducted on the optimized geometries using a similar procedure as reported previously [21, 22].

2.4 Lipase activity assay

The lipase activity assay was conducted according to the modified method of Lowry assay [23] . 30 µl of *Candida rugosa* lipase (Sigma Aldrich, USA) was dissolved in 970 µl of 0.1 M sodium phosphate buffer at pH 7. For the case of DES-treated lipase, the lipase was treated with 100 µl of DES before the experiment. The solution was incubated in a bench orbital shaker at 37°C for 5 min. Next, 2.5 ml of emulsion system (6 ml of olive oil and 4 ml of isooctane) and 0.02 ml of calcium chloride were added to the solution. The solution was vortexed for 10 min at 150 rpm and incubated for 30 min. The reaction was terminated by adding 1 ml of 6N HCl and 5 ml of isooctane. Next, the solution was vortexed for 1 min and was heated in a water bath at 95°C for 5 min. Then, 5 ml of the upper layer was transferred into the centrifuge tube, followed by the addition of copper reagent (1 ml). The absorbance of the free fatty acid formed from the lipase catalytic activity was measured at 715 nm using a spectrophotometer. The amount of free fatty acid was estimated from the standard curve of oleic acid (145 µmole – 1250 µmole).

3.0 Results and Discussion

3.1 DES Formation

Figure 1 shows that the formation of DES paracetamol: pTSA (1:3) begins from a non-covalent interaction between paracetamol as HBD and pTSA as hydrogen bond acceptor (HBA). The Mulliken atomic charges shown in Figure 1 indicate that 15(O), 14(O) oxygen atoms in pTSA (HBA) are negative with magnitudes of -0.6501 and -0.6505 a.u, respectively. Similarly, the Mulliken atomic charges shown for paracetamol indicate that 12(H), 14(H) hydrogen atoms are positive with magnitudes of 0.3881 and 0.3571 a.u, respectively. Comparing these Mulliken atomic charges, non-covalent interaction between 15(O) - 12(H) or 14(O)- 14(H) is highly possible after mixture paracetamol and PTSA to form DES particularly at the molar ratio of 1:3. The as formed homogenous liquid arise due to the hydrogen bonding interaction between 15(O) - 12(H) or 14(O)- 14(H). This is in agreement with the work done in the previous studies [24, 25].

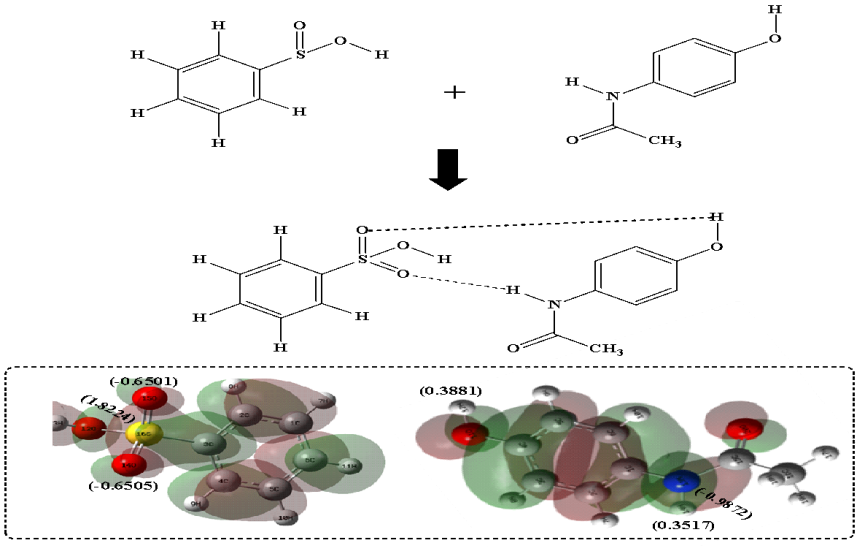

Figure 1: Combination of paracetamol and pTSA to form a paracetamol-based DES.

Figure 2 further supports this interpretation by visualizing these H-bonds, where green dashed lines represent the intermolecular hydrogen bonding between the two components. The strong electrostatic interactions

inferred from the Mulliken charges are critical in promoting the miscibility and homogeneity of the paracetamol:pTSA (1:3) mixture, ultimately forming a clear, stable eutectic system. This finding is consistent with previous reports on hydrogen bond-driven DES formation mechanisms [24, 25].

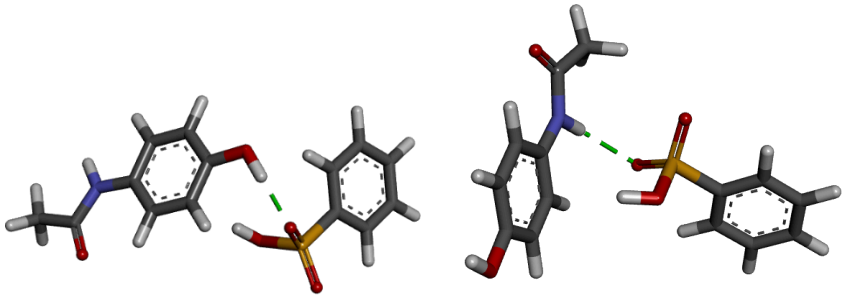

Figure 2: The H-bond between paracetamol and pTSA to form a paracetamol-based DES.

3.2 FTIR analysis

The IR spectrum for the synthesized paracetamol-based DES spectrum was compared with its components (i.e., paracetamol and pTSA) (Figure 3). The details of functional groups were provided in Tables 1, 2 and 3. The IR result of the paracetamol-based DES showed a broad absorption band at 3265.16 cm^{-1}, which corresponds to O-H bond. C-H aromatic stretching was detected at 3032.96 cm^{-1}, while C=C aromatic bands appeared at 1600.48 cm^{-1} and 1453.90 cm^{-1}, and carbonyl peaks appeared at 1675.72 cm^{-1} and S =O stretching band at 1030.63 cm^{-1} (Table 1).

Table 1: The IR data of DES paracetamol: pTSA.

Functional Group	Wavenumber, cm^{-1}
O-H broad	3265.16
C-H aromatic	3032. 96
C=C aromatic	1600.48 and 1453.90
C=O	1675.72
S=O	1030.63

The individual component of paracetamol showed a sharp, broad peak indicating an O-H bond at 3160.78 cm^{-1}. Meanwhile, a spike for secondary N-H absorption band was found at 3325.31 cm^{-1}, aromatic vibration at 1611.55 and 1440.50 cm^{-1}, and absorption band for C=O amide at 1654.71 cm^{-1} (Table 2).

Table 2: The IR data of paracetamol.

Functional Group	Wavenumber, cm^{-1}
O-H	3160.78
N-H secondary	3325.31
C-H aromatic	3109.81
C=C aromatic	1611.55 and 1440.50
C=O amide	1654.71

pTSA showed an absorption peak at 3418.00 cm^{-1} for the O-H bond, absorptions at 1601.55 cm^{-1} and 1498.87 cm^{-1} for the C=C aromatic stretching, and 1034.64 cm^{-1} for the S=O amide (Table 3).

Table 3: The IR data of pTSA.

Functional Group	Wavenumber, cm^{-1}
O-H broad	3418.00
C=C aromatic	1601.55 and 1498.87
S=O	1034.64

The DES shared several similar functional groups with pTSA and paracetamol, namely O-H bond and C=C aromatic. However, upon the formation of the DES, the wavenumber for each identical functional group was shifted, signifying subtle yet significant alterations in these functional groups. The O-H peak became broader and less intense. In addition, the C=O amide functional group identified in paracetamol was not observed in the DES spectrum. The N-H peaks might appear in the spectrum but may overlap with strong O-H peaks. An S=O functional group was observed in the DES spectrum, which was contributed by pTSA.

A pivotal factor governing these spectral changes is the establishment of hydrogen bonding interactions after DES formation. These interactions, entailing a dynamic interplay between hydrogen bond donors (HBD) and acceptors (HBA), intricately shape the observed infrared bands by broadening and shifting the bands (26). Specifically, the broadening of the O-H peak within the DES paracetamol: pTSA spectrum indicates robust hydrogen bonding between the HBD and HBA entities, underscoring the intimate nature of their association. A similar trend was observed in previous studies on the hydrogen bonding interactions within DESs [14, 27].

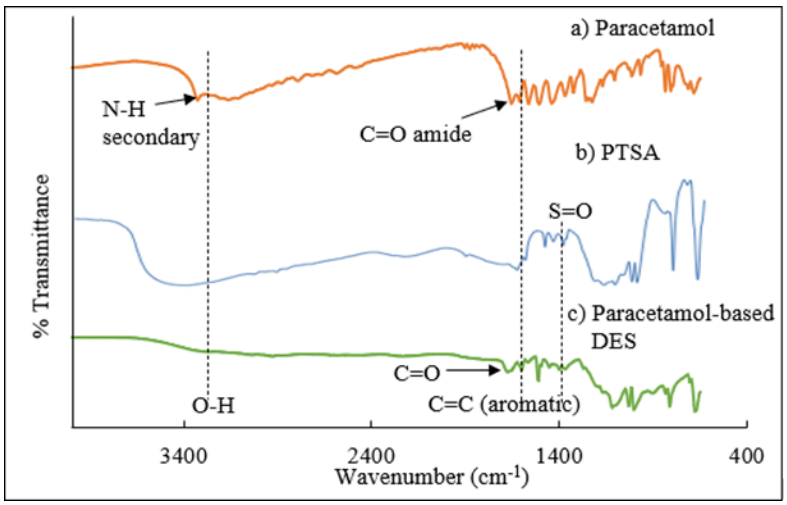

Figure 3: FTIR spectra a) Paracetamol, b) pTSA, and c) Paracetamol-based DES.

3.3 Melting and freezing point

According to Figure 4, a concave melting curve (black) occurred at 5°C and a cooling curve (red) occurred at -30°C. Based on its concave melting curve characterized at peak maxima -3.17°C, DES is classified as a eutectic impurity. The exothermic peak has Tonset at -31.17 °C and the cooling enthalpy (ΔHf) is 6.7074 J/g while the endothermic peak has Tonset at -7.65 °C and the melting enthalpy (ΔHf) is 7.6449 J/g.

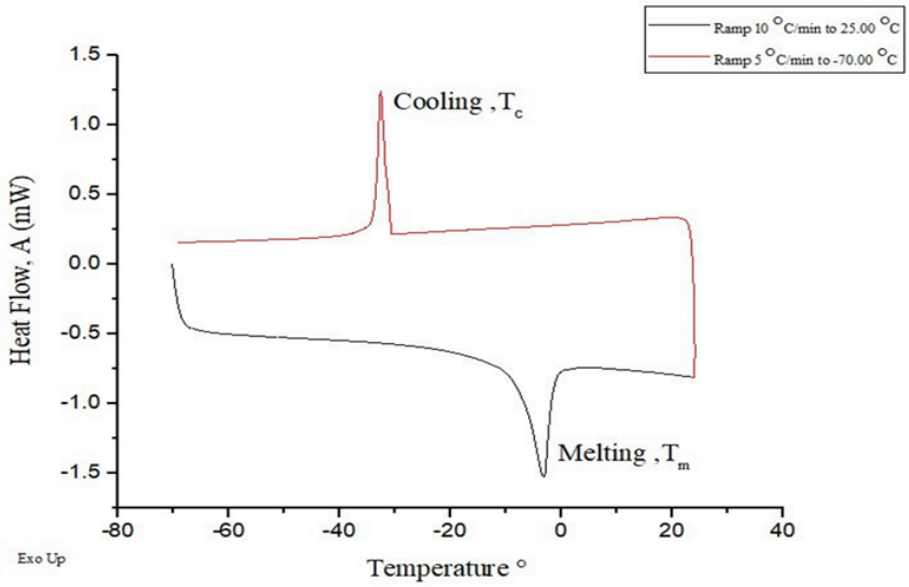

Figure 4: DSC thermogram of paracetamol-based DES.

Remarkably, upon the formation of the DES, a profound alteration in melting and freezing points becomes evident when juxtaposed with the individual components, as tabulated in Table 4. Following the formation of the DES, it significantly reduced the melting point (-32.58°C) and freezing point (-3.17°C) compared to its individual components (Table 4). This is due to the formation of the hydrogen bond that weakens the individual components' internal molecular interactions, hence lowering the Tc and Tm [28]. This consequential impact on phase transitions adds to the intricate tapestry of the DES's physicochemical behavior.

Table 4: The melting and freezing point of DES and individual components.

Compounds	Melting point /°C	Freezing point /°C	Reference
DES	-32.58	-3.17	This study
Paracetamol: pTSA			
Paracetamol	168.00 to 172.00	-	[29]
pTSA	105 to 107	-	[30]

3.4 Lipase activity assay

The lipase activity of untreated and DES-treated lipase was determined using a colorimetric technique. The free fatty acid level was measured at a wavelength of 715 nm. The absorbance of DES-treated lipase and untreated lipase obtained was used to estimate the amount of free fatty acid from the prepared standard curve of oleic acid. The DES-treated lipase showed higher absorbance, and therefore, higher free fatty acids than the untreated lipase. The amount of free fatty acids implies lipase catalytic activity. Figure 5 visually illustrates the absorbance values of DES-treated and untreated lipase. Strikingly, DES-treated lipase exhibited markedly higher absorbance than untreated lipase, suggesting elevated free fatty acid concentrations. The quantified amounts were 348.30 µmole of free fatty acid for DES-treated lipase and 333.90 µmole for untreated lipase. The noticeable surge in absorbance and the subsequent elevation in free fatty acid content upon DES treatment substantiate the remarkable enhancement of lipase catalytic activity. This enhancement not only reaffirms findings from a previous study that showcased similar improvements using diverse ChCl-based DESs but also amplifies the potential of DESs as adaptable media for enhancing lipase activity [8]. The observed increase in enzymatic activity can be attributed to the unique physicochemical properties of DESs, such as their ability to form hydrogen bonds, alter enzyme microenvironments, and improve substrate solubility [31]. Moreover, the ability of DESs to stabilize enzyme conformations while simultaneously reducing diffusional limitations may contribute to the observed enhancement in lipase activity. This is particularly relevant given that DESs can act as both reaction media and co-solvents, mitigating substrate inhibition

effects and facilitating a more efficient catalytic process [32, 33]. The results reinforce the growing body of evidence that DESs provide an alternative to conventional organic solvents, offering a greener and more tunable platform for enzymatic bioconversions.

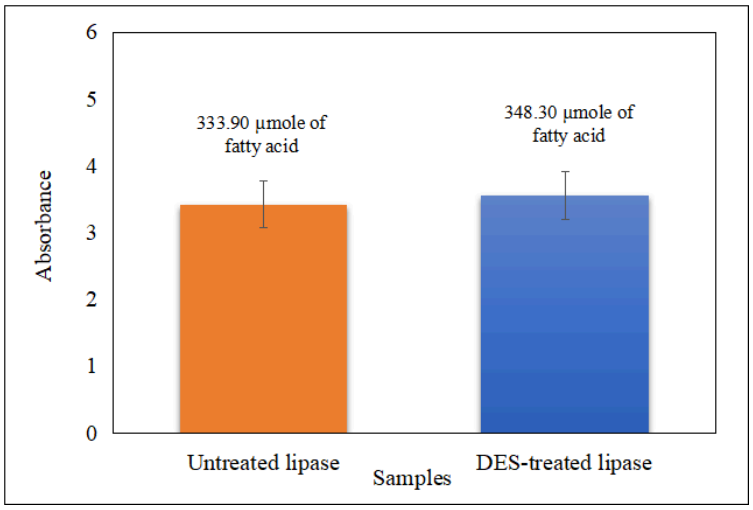

Figure 5: Free fatty acid formed from the esterification process catalyzed by untreated and DES-treated lipase.

3.5 Interaction of lipase with DESs

Delving into the mechanistic aspects, the unique properties of DESs, such as their solvent capacity and ability to facilitate enzyme-substrate interactions, likely underpin the observed enhancement. Furthermore, the presence of pTSA within DESs introduces a captivating dimension that can function as a catalyst or a source of protons. The potent organic acid's interaction with the lipase's serine (SER) catalytic side chain via protonation offers a compelling mechanism. By giving its proton to the hydroxyl group (-OH) of serine (SER), one of the catalytic side chains in Lipase,[34] the pTSA can interact with lipase through serine (SER) activity, producing a protonated SER molecule (Figure 6). The effects of protonation extend to the total lipase activity and SER characteristics. The hydroxyl group's increased acidity and increased reactivity towards nucleophiles can be inferred from the protonation. The serine molecule's shape may change due to protonation, which may affect its capacity to engage in interactions such as hydrogen bonds or other interactions. Conversely, including paracetamol in DESs introduces the intriguing concept of indirectly modifying the chemical microenvironment surrounding the lipase enzyme, thereby potentially influencing its catalytic activity.

Acknowledging potential limitations, our study underscores the necessity of exploring unaccounted variables, which could significantly impact observed outcomes. These future research avenues hold immense potential in refining our comprehension of the intricate interplay governing DES-mediated enhancement of lipase activity.

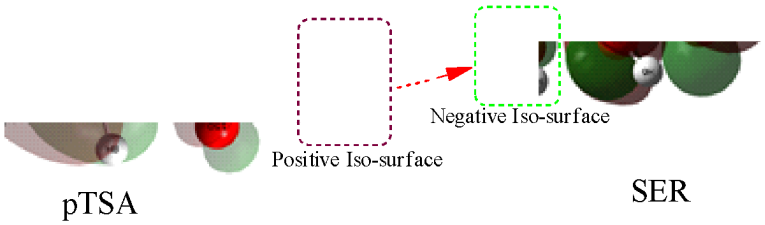

Figure 6: Frontier molecular orbital of LUMO level of pTSA and SER functionality in Lipase.

4.0 Conclusion

In conclusion, the use of a paracetamol-based deep eutectic solvent (DES) is an effective approach for improving the catalytic activity of lipase. The results presented compelling evidence that the DES effectively improved the enzymatic activity of lipase, resulting in a significant increase in its catalytic efficiency. Notably, the DES's active ingredient, pTSA, demonstrated potent catalytic properties and protonation abilities, contributing to the observed enhancement in lipase activity. Furthermore, the paracetamol molecule present in the DES was found to have a significant impact on the chemical environment surrounding lipase, leading to indirect effects on its activity. These findings highlight the potential of DESs as a versatile and promising tool for improving the performance of lipase and other enzymes in various biotransformation applications, where enzymatic catalysis facilitates the conversion of substrates into valuable bioproducts with enhanced efficiency and selectivity. Overall, this study provides valuable insights into the intricate interplay between DESs and enzymatic activity, paving the way for future research to explore this exciting avenue further.

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References

1. Fatima, S., Asif, N., Ahmad, R., & Fatma, T. (2020). Toxicity of NSAID drug (paracetamol) to nontarget organism-*Nostoc muscorum*. *Environmental Science and Pollution Research International, 27*(28), 35208–35216.
2. Spaltro, A., Pila, M. N., Colasurdo, D. D., Noseda Grau, E., Roman, G., Simonetti, S., & Ruiz, D. L. (2021). Removal of paracetamol from aqueous solution by activated carbon and silica. Experimental and computational study. *Journal of Contaminant Hydrology, 236*, 103739.
3. Courtier, A., Cadiere, A., & Roig, B. (2019). Human pharmaceuticals: Why and how to reduce their presence in the environment. *Current Opinion in Green and Sustainable Chemistry, 15*, 77–82.
4. Pollap, A., Baran, K., Kuszewska, N., & Kochana, J. (2020). Electrochemical sensing of ciprofloxacin and paracetamol in environmental water using titanium sol-based sensor. *Journal of Electroanalytical Chemistry, 878*.
5. Mane, S. N., Gadalkar, S. M., & Rathod, V. K. (2018). Intensification of paracetamol (acetaminophen) synthesis from hydroquinone using ultrasound. *Ultrasonics Sonochemistry, 49*, 106–110.
6. Zur, J., Wojcieszynska, D., Hupert-Kocurek, K., Marchlewicz, A., & Guzik, U. (2018). Paracetamol – toxicity and microbial utilization. *Pseudomonas moorei* KB4 as a case study for exploring degradation pathway. *Chemosphere, 206*, 192–202.
7. Gotor-Fernandez, V., & Paul, C. E. (2019). Deep eutectic solvents for redox biocatalysis. *Journal of Biotechnology, 293*, 24–35.

8. Tan, J. N., & Dou, Y. (2020). Deep eutectic solvents for biocatalytic transformations: Focused lipase-catalyzed organic reactions. *Applied Microbiology and Biotechnology, 104*(4), 1481–1496.
9. Mao, S., Wang, X., Zhang, Z., Wang, S., Li, K., Lu, F., & Qin, H. (2020). 15α-hydroxylation of D-ethylgonendione by *Penicillium raistrickii* in deep eutectic solvents DESs containing system. *Biochemical Engineering Journal, 164*.
10. Patzold, M., Siebenhaller, S., Kara, S., Liese, A., Syldatk, C., & Holtmann, D. (2019). Deep eutectic solvents as efficient solvents in biocatalysis. *Trends in Biotechnology, 37*(9), 943–959.
11. Sun, H., Xin, R., Qu, D., & Yao, F. (2020). Mechanism of deep eutectic solvents enhancing catalytic function of cytochrome P450 enzymes in biosynthesis and organic synthesis. *Journal of Biotechnology, 323*, 264–273.
12. Halilu, A., Hadj-Kali, M. K., Hashim, M. A., Yusoff, R., & Aroua, M. K. (2022). Bifunctional ionic deep eutectic electrolytes for CO_2 electroreduction. *ACS Omega, 7*(42), 37764–37773.
13. Halilu, A., Hadj-Kali, M. K., Hashim, M. A., Ali, E. M., & Bhargava, S. K. (2022). Electroreduction of CO_2 and quantification in new transition-metal-based deep eutectic solvents using single-atom Ag electrocatalyst. *ACS Omega, 7*(16), 14102–14112.
14. Zainal-Abidin, M. H., Eng, J. J., Khairuzi, K., Kristianto, S., Wan Mahmood, W. M. A., Al-Fakih, A. M., Matmin, J., Abdul Wahab, R., Abdullah, F., Mohamad, M. F., Hashim, S. E., Teo, H. L., & Syed Yaacob, S. N. (2024). Effectiveness of ammonium-based deep eutectic solvents in extracting polyphenol from *Chlorella vulgaris*. *Algal Research, 79*, 103436.
15. Zainal-Abidin, M. H., Sonny, K., Nuresyanah, E. S., Juan, M., Roswanira, A. W., Wan Mahmood, A. W. M., & Widodo, W. T. (2025). Green extraction of phenolics using deep eutectic solvents: A promising neoteric method. *Natural Product Research, 39*(7), 1955–1967.
16. Zainal-Abidin, M. H., Hayyan, M., Ngoh, G. C., & Wong, W. F. (2019). From nanoengineering to nanomedicine: A facile route to enhance biocompatibility of graphene as a potential nano-carrier for targeted drug delivery using natural deep eutectic solvents. *Chemical Engineering Science, 195*, 95–106.
17. Zainal-Abidin, M. H., Hayyan, M., Ngoh, G. C., Wong, W. F., & Looi, C. Y. (2020). Potentiating the anti-cancer profile of tamoxifen-loaded graphene using deep eutectic solvents as functionalizing agents. *Applied Nanoscience, 10*, 293–304.
18. Frisch, M., Trucks, G., Schlegel, H., Scuseria, G., Robb, M., Cheeseman, J., et al. (2009). *Gaussian 09 Rev. D.01*. Wallingford, CT.
19. Peppernick, S. J. (2009). United atoms to superatoms and the isoelectronic principle. *The Pennsylvania State University*.
20. Dennington, R., Keith, T., & Millam, J. (2009). *GaussView (Version 5)* [Software].
21. Halilu, A., Hayyan, M., Aroua, M. K., Yusoff, R., & Hizaddin, H. F. (2019). In situ electrosynthesis of peroxydicarbonate anion in ionic liquid media using carbon dioxide/superoxide system. *ACS Applied Materials & Interfaces, 11*(29), 25928–25939.
22. Halilu, A., Hayyan, M., Aroua, M. K., Yusoff, R., & Hizaddin, H. F. (2021). Mechanistic insights into carbon dioxide utilization by superoxide ion generated electrochemically in ionic liquid electrolyte. *Physical Chemistry Chemical Physics, 23*(2), 1114–1126.
23. Deepachandi, B., Weerasinghe, S., Andrahennadi, T. P., Karunaweera, N. D., Wickramarachchi, N., Soysa, P., & Siriwardana, Y. (2020). Quantification of soluble or insoluble fractions of *Leishmania* parasite proteins in microvolume applications: A simplification to standard Lowry assay. *International Journal of Analytical Chemistry, 2020*(1), 6129132.
24. García, G., Atilhan, M., & Aparicio, S. (2015). The impact of charges in force field parameterization for molecular dynamics simulations of deep eutectic solvents. *Journal of Molecular Liquids, 211*, 506–514.
25. Wagle, D. V., Deakyne, C. A., & Baker, G. A. (2016). Quantum chemical insight into the interactions and thermodynamics present in choline chloride based deep eutectic solvents. *The Journal of Physical Chemistry B, 120*(27), 6739–6746.
26. Rodriguez Rodriguez, N., Machiels, L., & Binnemans, K. (2019). p-Toluenesulfonic acid-based deep-eutectic solvents for solubilizing metal oxides. *ACS Sustainable Chemistry & Engineering, 7*(4), 3940–3948.
27. Zainal-Abidin, M. H., Hayyan, M., Hayyan, A., & Jayakumar, N. S. (2017). New horizons in the extraction of bioactive compounds using deep eutectic solvents: A review. *Analytica Chimica Acta, 979*, 1–23.

28. Kamarudin, A. F., Hizaddin, H. F., El-Blidi, L., Ali, E., Hashim, M. A., & Hadj-Kali, M. K. (2020). Performance of p-toluenesulfonic acid-based deep eutectic solvent in denitrogenation: Computational screening and experimental validation. *Molecules, 25*(21).
29. Duranovic, M., Obeid, S., Madzarevic, M., Cvijic, S., & Ibric, S. (2021). Paracetamol extended release FDM 3D printlets: Evaluation of formulation variables on printability and drug release. *International Journal of Pharmaceutics, 592*, 120053.
30. Feng, C., Zhu, J., Hou, Y., Qin, C., Chen, W., Nong, Y., Liao, Z., Liang, C., Bian, H., & Yao, S. (2022). Effect of temperature on simultaneous separation and extraction of hemicellulose using p-toluenesulfonic acid treatment at atmospheric pressure. *Bioresource Technology, 348*, 126793.
31. Zainal-Abidin, M. H., Hayyan, M., Ngoh, G. C., Wong, W. F., & Looi, C. Y. (2019). Emerging frontiers of deep eutectic solvents in drug discovery and drug delivery systems. *Journal of Controlled Release, 316*, 168–195.
32. Kovács, A., Yusupov, M., Cornet, I., Billen, P., & Neyts, E. C. (2022). Effect of natural deep eutectic solvents of non-eutectic compositions on enzyme stability. *Journal of Molecular Liquids, 366*, 120180.
33. Guajardo, N., Müller, C. R., Schrebler, R., Carlesi, C., & Domínguez de María, P. (2016). Deep eutectic solvents for organocatalysis, biotransformations, and multistep organocatalyst/enzyme combinations. *ChemCatChem, 8*(6), 1020–1027.
34. Dodson, G. G., Lawson, D. M., & Winkler, F. K. (1992). Structural and evolutionary relationships in lipase mechanism and activation. *Faraday Discussions, 93*, 95–105.