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Effect of deep eutectic solvents on the biocatalytic properties of β-glucosidase@ZnOFe nano-biocatalyst

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ABSTRACT

In this work, we investigate the use of aqueous deep eutectic solvents (DESs) consisting of different hydrogen bond acceptors (choline chloride, ammonium salts or betaine) and hydrogen bond donors (amines, sugars and alcohols), as co-solvents in biocatalytic reactions catalyzed by a nano-biocatalyst with β-glucosidase (BGL) activity. For this purpose, β-glucosidase from *Thermotoga maritima* was immobilized on the surface of green zinc oxide-iron oxide nanoparticles (BGL@ZnOFe) derived from an aqueous olive leaf extract. The covalent bonding of β-glucosidase on the surface of nanoparticles was demonstrated by X-ray photoelectron spectroscopy. The effect of a wide range of DES solutions on the biocatalytic features of BGL@ZnOFe was thoroughly investigated. The concentration of DES as well as the specific combination of hydrogen bond acceptors and hydrogen bond donors had a significant impact on the hydrolytic activity of immobilized β-glucosidase. Most choline chloride and betaine-based DESs enhanced the enzymatic activity and thermal stability of BGL@ZnOFe up to 50%. The enzymatic activity of the nano-biocatalyst towards the hydrolysis of bioactive phenolic compounds depends on the nature of the DES used. Choline chloride:butylene glycol-based medium had beneficial effect on a transglycosylation reaction, such as the synthesis of salidroside, increasing the total turnover number of the biocatalytic process by 55%. This work indicates that the use of several choline chloride and betaine-based DESs can be successfully employed as green co-solvents in various hydrolytic and synthetic biocatalytic processes.

1. Introduction

Biocatalytic processes exploiting enzymes to produce target molecules with high purity have gained significant ground over the years. Enzymes are implemented in several biotechnology sectors as they meet the growing demand for safe and sustainable industrial processes due to their natural origin, biocompatibility, biodegradability, and their ability to function in ambient reaction conditions which leads to the elimination of wastes and noxious reagents (Chapman et al., 2018). Especially, hydrolases are widely used due to their broad substrate specificity, wide availability, and easy handling (Hanefeld et al., 2022; Wu et al., 2021). Nevertheless, the instability of biocatalysts under specific conditions (temperature, pH, organic solvents) is a matter of concern. To circumvent this is-

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sue, different strategies have been proposed including immobilization, protein engineering and the exploitation of neoteric solvents (Gkantzou et al., 2021; Singh et al., 2013; Woodley, 2018).

One representative type of neoteric solvents are Deep Eutectic Solvents (DESs). DESs are liquid solvents at room temperature that are prepared by mixing two or more components which act either as Hydrogen Bond Acceptors (HBA), such as a quaternary ammonium salt, or Hydrogen Bond Donors (HBD), such as alcohol, organic acids etc. (Smith et al., 2014). Concerning their properties, DESs outperform volatile organic and petrochemical solvents. While the latter are mostly explosive, flammable, unstable, volatile, and toxic, DESs present an adequate solubility for a broad range of compounds, biocompatibility, biodegradability, low to no toxicity, low volatility, they are incombustible, environmentally friendly and, they can be either hydrophilic or hydrophobic depending on their components (Sekharan et al., 2022). In addition, these solvents are prepared by the simple mixing of the HBAs and HBDs whereas no reaction takes place, thus assuring 100% atom economy. Moreover, the formation of DESs is held at room or moderate temperature and ambient pressure, hence promoting energy efficiency (Panić et al., 2021). Therefore, DESs apply to the principles of green chemistry, and are considered as green solvents (Anastas and Eghbali, 2010). Owing to these benign traits, DESs are employed in many biocatalytic processes (Chanquia et al., 2020; Hessel et al., 2022; Perna et al., 2020), being suitable for the function of numerous enzymes (hydrolases, oxidoreductases) by acting either as co-solvents or replacing non-conventional media in which many enzymatic reactions are proceeded (Bittner et al., 2022; de María and Hollmann, 2015; Tan and Dou, 2020). For instance, the use of certain DESs as co-solvents has led to an increase in the hydrolytic or synthetic activity, and stability of β-glucosidases (Ma et al., 2020; Uhoraningoga et al., 2021; Xu et al., 2018), whereas in the case of laccase, pre-incubation in aqueous-DES solutions resulted in the enhancement of the enzyme's thermostability (Delorme et al., 2020). Additionally, DESs' components can also function as substrates in a synthetic reaction catalyzed by lipase-, offering solvent-free conditions and thus, a more sustainable reaction system (Hümmer et al., 2018).

Moreover, immobilization of enzymes on nanosized materials is an intriguing approach due to the enhanced enzyme stability, easy recovery and reusability, and limited purification steps needed for the final products (Gkantzou et al., 2021). Green synthesis of nanoparticles is gaining ground over conventional chemical techniques as a more environmentally friendly and cost-effective alternative (El-Seedi et al., 2019; Shah et al., 2015). By-products of the agro-industrial sector replace chemical catalysts as reducing agents for the production of metallic nanoparticles, eliminating the needs for derivatization and protection/deprotection steps, minimizing energy supplies and toxic wastes, enhancing the recycle of processed natural sources and hence, promoting the paradigm of bioeconomy (Aswathi et al., 2022). Renewable feedstocks such as olive oil production biowastes can act as reductants and stabilizers owing to their bioreactive compounds yielding nanoparticles with a functional surface for enzyme coupling (Fotiadou et al., 2021; Shah et al., 2015). In this framework, the employment of agricultural by-products for the one-pot synthesis of nanomaterials complies precisely with the principles of green chemistry (Anastas and Eghbali, 2010). Meanwhile, the valorisation of olive sector biowastes is strongly encouraged since their accumulation has adverse environmental effects. Green zinc oxide, iron oxide and silver oxide nanoparticles from different natural sources have already been synthesized and employed as green immobilization carriers (Dutt and Upadhyay, 2018; Fotiadou et al., 2021).

β-Glucosidases (BGLs) derived from micro and macro-organisms constitute a ubiquitous enzyme family. BGLs catalyze the cleavage of β-1-4 glycosidic bonds between two glucose residues (such as cellobiose) or glucose-substituted compounds (such as phenolic glucosides), while playing a pivotal role in biological systems involving in physicochemical reactions. The exploitation of BGLs on biotechnological processes has been proved an advantageous strategy regarding the production of biofuels, flavour enhancement, edible extracts' clarification, and pharmaceuticals synthesis (Singh et al., 2016). More specifically, by using hydrolytic enzymes as β-glycosidases, food industries can improve the organoleptic properties of their products (juice, wine) or enrich them with nutritional aglycones (*o*-diphenols, coumarins, flavonoids, isoflavones), which mostly demonstrate enhanced bioactivities and aroma (Fernández-Pacheco et al., 2021; Monteiro et al., 2019); Several aglycone derivatives are of high pharmaceutical interest too (Cheng and Zhang, 2017; Ma et al., 2020). BGLs can also catalyze transglycosylation reactions (Ahmed et al., 2017; Xia et al., 2022); Especially immobilized β-glucosidases are ideal candidates for targeted enzymatic glycosylation of natural compounds due to the decreased enzyme inhibition by the substrates (Geronimo et al., 2019). To date, diverse BGLs have been explored to produce valuable compounds benefiting from the inclusion of DESs as co-solvents (Cheng and Zhang, 2017; Uhoraningoga et al., 2021).

In this work, the effect of a wide range of DES solutions on the activity and stability of immobilized β-glucosidase was investigated. More specifically, β-glucosidase from the bacterium *Thermotoga maritima* was immobilized on the surface of zinc oxide-iron oxide nanoparticles (BGL@ZnOFe), synthesized via a straightforward green route, to evaluate the efficiency of the nano-biocatalyst in biocatalytic reactions of interest, employing green solvents. For this purpose, different types of DESs were prepared with various HBAs and HBDs. Furthermore, the impact of specific DESs on the hydrolytic and transglycosylation activity of BGL@ZnOFe towards the production of valuable phenolic compounds was evaluated. The aim of this work was to broaden the applicability of neoteric solvents in reactions catalyzed by the immobilized enzyme, investigating the effect of aqueous DESs on the biochemical characteristics of the immobilized BGL.

2. Materials and methods

2.1. Materials

β-glucosidase from *Thermotoga maritima* was purchased from Megazyme (E-BGOSTM). Zinc acetate dihydrate (Merck), ferrous (II) sulfate heptahydrate (Fluka), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, Merck), 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES, Sigma), *p*-nitrophenol (*p*-NP, Sigma), *p*-nitrophenyl β-D-glucopyranoside, (*p*-NPG, Sigma), citric acid monohydrate (Riedel de Haen), sodium phosphate dibasic dihydrate (Sigma), N-hydroxysuccinimide (NHS, Sigma), choline chloride (ChCl, Sigma), ethylammonium chloride (EAC, Merck), betaine (Bet, ACROS Organics), choline dihydrogen phos-

phate (Chol DHP, IOLITEC), urea (U, Fluka), glycerol (Gly, 0.5% max water, Fisher Scientific), ethylene glycol (EG, AppliChem), propylene glycol (PG, ACROS Organics), butylene glycol (BG, TCI), D(+)-glucose (Glc, Sigma), D(−)-fructose (Fru, Sigma), esculin sesquihydrate (Fluka), D(−)-salicin (Sigma), potassium bromide (Acros), tyrosol (Alfa Aesar), D(+)-cellobiose (Alfa Aesar) and acetic acid (Sigma) were of analytical grade. Acetonitrile, methanol, and water were for liquid chromatography and purchased from Fisher Scientific. Deuterium dimethyl sulfoxide-d_6 (DMSO-d_6, ≥ 99.9%), for NMR experiments, was acquired from Deutero. Buffers and aqueous solutions were prepared in double-distilled water (ddH_2O).

2.2. Synthesis of DESs

A hydrogen bond acceptor and a hydrogen bond donor were mixed in predetermined molar ratios and different DESs were prepared as described by Delorme and coworkers (2020). All DESs are listed in Table 1. In brief, for each DES, specific quantities of a HBD and a HBA were weighed and mixed in a glass vial, incubated at 80–100 °C for a total time of 1–2 h in an oven (BINDER GmbH, Tuttlingen, Germany) and agitated per 10 min until a transparent liquid was obtained. After the preparation, all DESs were stored at 30 °C. Aqueous solutions of DESs were prepared by adding a certain volume of DES in citrate-phosphate buffer (100 mM, pH 6.5). The final concentrations of DESs in the aqueous solutions were 5, 10, 25 and 50% (v/v).

2.3. Covalent immobilization of β-glucosidase from Thermotoga maritima on ZnOFe nanoparticles

β-glucosidase from *Thermotoga maritima* was immobilized on the surface of green hybrid zinc oxide-iron oxide (ZnOFe) nanoparticles from an olive leaf extract. The synthesis of ZnOFe nanoparticles from aqueous olive leaf extract was previously reported by Fotiadou et al. (2021). BGL was immobilized via covalent linkages using EDS/NHS coupling reaction. In brief, 4 mg mL^{-1} ZnOFe were dispersed in HEPES buffer (50 mM, pH 7.5) in an ultrasonic bath for 20 min (Elma Schmidbauer GmbH, Singen, Germany). In the next step, 1 mL of aqueous solution of EDC/NHS (1:2 M ratio) was added in the solution with the dispersed nanoparticles and the final mixture was incubated at 30 °C for 1 h under stirring in an oven (Eppendord SE, Hamburg, Germany). Activated nanoparticles were rinsed thrice with HEPES buffer (50 mM, pH 7.5) and separated by centrifugation (Ortoslresa, Madrid, Spain) at 12,000 rpm, 7 °C for 10 min. ZnOFe were re-dispersed in citrate-phosphate buffer (50 mM, pH 6.5) and then, 5 μL of β-glucosidase from *Thermotoga maritima* (bottle concentration 460 U/mL) were added. The immobilization process was carried out at 30 °C for 1 h under stirring. Finally, the nano-biocatalyst was recovered by applying a magnet (grade N42), rinsed thrice, and placed into a speed-vacuum concentrator (Kisker Biotech GmbH & Co. KG, Steinfurt, Germany). The unbound enzyme was estimated by Bradford's method and the immobilization yield was calculated according to Fotiadou et al. (2021).

2.4. Characterization of β-glucosidase on the surface of ZnOFe nanoparticles

X-ray photoelectron spectroscopy (XPS) was performed with a ESCA SSX-100 spectrometer (Surface Science Instruments, Mountain View, CA., U.S.A.), equipped with a monochromatic Al Kα X-ray source (hν = 1486.6 eV). The measurement chamber pressure was maintained at 1×10^{-9} mbar during data acquisition; the photoelectron take-off angle was 37° with respect to the surface normal. The diameter of the analysed area was 1000 μm; the energy resolution was 1.26 eV (or 1.67 eV for a broad survey scan). Samples were dispersed in chloroform, sonicated and stirred for 30 min, and drop-casted on a thin gold film deposited on mica. All measurements were carried out on freshly prepared samples, and three different spots were measured on each sample to check for homogeneity. Spectral analysis with the help of the least-squares curve-fitting program WinSpec (LISE, University of Namur, Belgium) included a Shirley background subtraction and fitting with a minimum number of peaks consistent with the expected composition of the probed volume, taking into account the experimental resolution. Peak profiles were taken as a convolution of Gaussian and Lorentz-

Table 1
Composition, abbreviation and molar ratios of the deep eutectic solvents studied.

Hydrogen Bond Acceptor (HBA)	Hydrogen Bond Donor (HBD)	Abbreviation	Molar Ratio
Choline Chloride	Urea	ChCl:U	1:2
Choline Chloride	Glycerol	ChCl:Gly	1:2
Choline Chloride	Ethylene Glycol	ChCl:EG	1:2
Choline Chloride	Propylene Glycol	ChCl:PG	1:2
Choline Chloride	Butylene Glycol	ChCl:BG	1:2
Choline Chloride	Butylene Glycol	ChCl:BG	1:4
Choline Chloride	Glucose, H_2O	ChCl:Glc:H_2O	5:2:5
Choline Chloride	Fructose, H_2O	ChCl:Fru:H_2O	5:2:5
Choline Chloride	Urea, Glycerol	ChCl:U:Gly	1:1:1
Choline Chloride	Urea, Ethylene Glycol	ChCl:U:EG	1:1:1
Choline Chloride	Glycerol, Ethylene Glycol	ChCl:Gly:EG	1:1:1
Ethylammonium Chloride	Urea	EAC:U	1:1.5
Ethylammonium Chloride	Glycerol	EAC:Gly	1:1.5
Ethylammonium Chloride	Ethylene Glycol	EAC:EG	1:1.5
Betaine	Ethylene Glycol	Bet:EG	1:3
Betaine	Glycerol	Bet:Gly	1:2
Betaine	Glycerol	Bet:Gly	1:3
Choline Dihydrogen Phosphate	Ethylene Glycol	Chol DHP:EG	1:2
Choline Dihydrogen Phosphate	Glycerol	Chol DHP:Gly	1:3

ian functions; binding energies were referenced to C1*s* photoemission peak centered at 284.8 eV and are accurate to ±0.1 eV when deduced from the fitting procedure.

2.5. Determination of the enzymatic activity

The activity of β-glucosidase was assayed spectrophotometrically. The hydrolysis of *p*-nitrophenyl β-D-glucopyranoside (*p*-NPG) to *p*-nitrophenol (*p*-NP) was selected as a standard reaction. For the determination of the enzymatic activity of the BGL@ZnOFe, 20 μg of the nano-biocatalyst were added in 1 mL citrate-phosphate solution (100 mM, pH 6.5) or in aqueous DES solutions of different concentrations (% v/v) containing 2 mM of *p*-NPG. The reactions were carried out at 50 °C for 5 min. The linear increase in absorbance due to the release of a colour product (*p*-NP) was monitored at 410 nm using a UV–Vis spectrophotometer equipped with a Peltier temperature controller (Agilent, Santa Clara, CA., USA) over time. *p*-NP concentration was estimated based on a standard curve. One unit of enzymatic activity (U) was defined as the amount of enzyme that liberated 1 μmol of *p*-NP per minute of reaction at 50 °C. Blank samples without enzyme were also prepared and no coloured product was detected. Enzymatic activity was expressed as U per mg of the nano-biocatalyst (BGL@ZnOFe) or U per mg of the immobilized enzyme. Experiments were performed in triplicate.

2.6. Kinetic analysis

The kinetic analysis was performed to investigate the effect of specific DESs on the apparent kinetic constants (K_m, V_{max}, k_{cat}) of BGL@ZnOFe at a concentration of 10% (v/v). The enzymatic activity was measured as described in subsection 2.5, monitoring the initial reaction rates. *p*-PNG concentration ranged from 0.1 to 10 mM. K_m, V_{max} and k_{cat} were estimated from nonlinear regression fitting of the experimental data on the concentrations studied. Experiments were performed in triplicate.

2.7. Stability test for β-glucosidase immobilized on the surface of ZnOFe nanoparticles in DES aqueous solutions

BGL@ZnOFe was incubated in DES aqueous solutions (0, 5, 10, 25, 50% v/v) at 50 °C to evaluate the thermostability of the green nano-biocatalyst. In brief, 20 μg mL^{-1} BGL@ZnOFe were added to different DES aqueous solutions, and the mixtures were incubated at 50 °C and stirred at 700 rpm for 24 h. After that, the nano-biocatalyst was separated by applying a magnet (grade N42) and rinsed thrice with citrate-phosphate buffer (100 mM, pH 6.5). The enzymatic activity was measured in buffer as described in subsection 2.5. Experiments were performed in triplicate. The relative activity was estimated according to the following equation (1):

$$\textbf{Relative activity} = (\text{Activity after incubation in x\% (v/v) DES solution / Activity after incubation in DES-free system}) * 100 \quad (1)$$

2.8. Hydrolysis of phenolic glucosides in DES aqueous solutions

The hydrolysis of phenolic glucosides was investigated in citrate-phosphate buffer (100 mM, pH 6.5) or DES aqueous solutions (10, 25, 50% v/v) using the nano-biocatalyst BGL@ZnOFe. In brief, 50 μg mL^{-1} BGL@ZnOFe were added in an aqueous solution containing 2 mM of esculin or 4 mM of salicin and incubated at 50 °C under stirring, for 1 or 2 h, respectively. Aliquots were withdrawn at predetermined time intervals to monitor the reaction rates (μM of hydrolysed substrate min^{-1}). Each sample was diluted (1:1) with a mixture of ACN/water (50:50% v/v) prior to High Performance Liquid Chromatography (HPLC) analysis. Blank samples were also prepared and tested. Experiments were performed in triplicate.

2.9. Synthesis of salidroside in DES aqueous solutions

The enzymatic synthesis of salidroside was carried out in citrate-phosphate buffer (100 mM, pH 6.5) or DES aqueous solution (10% v/v of ChCl:BG). In brief, 2 mg mL^{-1} BGL@ZnOFe were added in aqueous solutions containing 0.15 M tyrosol and 0.45 M cellobiose. The mixtures were incubated at 50 °C and stirred at 900 rpm for 24 h in a thermoshaker (IKA-Werke GmbH & Co. KG, Staufen, Germany). The nano-biocatalyst was separated from the reaction mixture by applying a magnet (grade N42). Further, the samples were lyophilized to remove water and analysed using ^{1}H NMR spectroscopy. Blank samples were also prepared and tested. Experiments were performed in triplicate.

2.10. Qualitative and quantitative analysis

The progress of hydrolysis reactions of the glycosylated compounds was monitored by measuring the concentration of the remaining unreacted substrate by HPLC (Shimadzu, Tokyo, Japan) equipped with a μBondapak C18 reversed-phase column (particle size 10 μm, length 300 mm, diameter 3.9 mm) and a diode array UV detector. The column temperature was set at 27 °C and the flow rate and injection volume were 1 mL min^{-1} and 20 μL, respectively. The mobile phase consisted of acetonitrile (A) and water (B, with 0.1% acetic acid) with initial conditions of 10–90% B, a gradient elution of 100-0% at 0–25 min and reset at the initial conditions. Salicin and salilyl alcohol were detected at 270 nm, and esculin and esculetin at 340 nm using a photodiode array (PDA) detector. Conversion yields were determined according to equation (2).

$$\textit{Conversion yield (\%)} = 100 - (\textit{Total Area of substrate at } t_{(reaction)} \textit{ x } 100 \textit{ / Total Area of substrate at } t_0) \quad (2)$$

The characterization of salidroside was carried out using ^{1}H–^{13}C HSQC-HMBC NMR spectroscopy. Reaction samples were dissolved in 600 μL DMSO-d_6 in 5 mm NMR tubes. NMR spectra were recorded on a Bruker 500 MHz AV spectrometer (Bruker Biospin, Rheinstetten, Germany), equipped with a broadband inverse probe, at 25 °C. The software TopSpin 3.2 was used to control the NMR system. The pulse sequences for ^{1}H–^{13}C HSQC, ^{1}H–^{13}C HMBC were standard Bruker library sequences, acquired with 2K data points over a

14 ppm spectral width. ^{1}H and ^{13}C NMR assignment of chemical shifts (δ, ppm) of salidroside: ^{1}H NMR (500 MHz, DMSO-d_6, 25 °C): δ = 2.63 (t, 2H, J = 7.1 Hz, 7′c-H), 3.09 (t, 1H, J = 9.1 Hz, 6c-aH), 3.16 (d,1H, J = 9.1 Hz, 6c-bH), 3.42 (t,1H, J = 9.1 Hz, 4c-H), 3.49 (d,1H, J = 3.8 Hz, 3c-H), 3.55 (q, 2H, J = 6.1 Hz, J = 14.3 Hz, 8′c-H), 3.60 (dd, 1H, J = 5.3 Hz, J = 10.5 Hz, 5c-H), 3.7 (d,1H, J = 11.5 Hz, 2c-H), 4.39 (s,1H,OH), 4.47 (s,1H,OH), 4.65 (s, 1H,OH), 4.9 (s,1H,OH), 5.33 (d,1H, J = 3.13 Hz 1c-H), 6.83 (d, 2H, J = 8.45 Hz, 3′c-H, 5′c-H), 7.15 (d, 2H, J = 8.45 Hz, 2′c-H, 6′c-H), 9.06 (s, 1H, 4′c-OH)ppm; ^{13}C NMR (500 MHz, DMSO-d_6, 25 °C): δ = 38.8(C7′), 62.4(C4), 70.3(C8′), 71.7(C6), 72.7(C2), 77.2(C3), 81.2(C5), 108.0(C1), 115.8(C3′,C5′),130.8(C2′,C6′), 131.4 (C1′), 155.7(C4′) ppm; Synthesis of salidroside was expressed as Total Turnover Number according to equitation (3).

Total Turnover Number (TTN) = moles of salidroside produced per moles of immobilized enzyme in the reaction mixture (24 h of continuous process) (3)

2.11. Statistical analysis

All the experiments were performed in triplicate. Data were expressed as means ± standard deviation (SD). The SD is indicated as error bars in the plots. The results were analysed by IBM SPSS statistics (version 28.0.1.0, IBM Corporation, NY, USA). Enzyme kinetics were estimated from non-linear regression fitting of the experimental data applying the Michaelis-Menten model.

3. Results

3.1. Preparation and characterization of β-glucosidase immobilized on the surface of ZnOFe nanoparticles

In the present work, β-glucosidase from the bacterium *Thermotoga maritima* was immobilized for the first time on the surface of green hybrid magnetic nanoparticles (zinc oxide-iron oxide) derived from an aqueous olive leaf extract via covalent bonding through EDC/NHS coupling chemistry. The immobilization yield of BGL on the surface of zinc oxide-iron oxide nanoparticles was 94% and the enzyme loading was about 16.27 μg mg^{-1} support. Moreover, the enzyme activity was estimated to 85.5 U mg^{-1} nano-biocatalyst and the enzyme specific activity to 5.3 U μg^{-1} immobilized BGL, using the hydrolysis of *p*-NPG as a standard enzyme reaction. The immobilization yield of BGL was higher or comparable with previous reported works for immobilized β-glucosidase on different nanoscaffolds (Chatzikonstantinou et al., 2019). The enzyme activity of BGL@ZnOFe was about 2-fold higher in comparison to β-glucosidase from *Thermotoga maritima* that was immobilized on the surface of magnetic nanoparticles functionalized with chitin (Alnadari et al., 2020). These differences could be attributed to the different immobilization methods or the type of the functionalized nanosupports used. Apart from the fact that the hybrid magnetic nanoparticles were efficient immobilization support matrix for BGL, ZnOFe-NPs were also prepared by a one-pot, eco-friendly and cost-effective method compared to relevant studies.

X-Ray Photoelectron Spectroscopy was used to clarify the type of interactions between the enzyme and the green nanoparticles (ZnOFe). The elemental composition of zinc oxide-iron oxide nanoparticles has been analysed in our previous work (Fotiadou et al., 2021). There we showed that because of the specific synthesis protocol, these nanoparticles were capped by stabilizers presenting C–C, C–H, C–O, C–O–C and C(O)O functionalities and are, thus, a functional nanomaterial. It is important to note that no peak ascribable to amide bonds (O = C–N) was observed. After the immobilization of β-glycosidase, the C1*s* core level spectrum shown in Fig. 1a presents a new component at a binding energy (BE) of 288.2 eV, which testifies to the existence of amide bonds and confirms both the successful immobilization of β-glycosidase and the type of bonding between the enzyme and the functional surface of the ZnOFe nanoparticles. The covalent bond between enzyme and nanoparticles is corrobated by the N1*s* core level spectrum, displayed in Fig. 1b, where the peak centered at a BE of 400.3 eV is attributed to C–NH_2 groups of the enzyme, while the component at 401.1 eV is assigned to amide bonds (O = C–NH) derived from the covalent linkages. The component at even higher BE (402.8 eV) is ascribed to NH_3^+ groups of β-glycosidase (Chatzikonstantinou et al., 2020; Kumamoto et al., 2021).

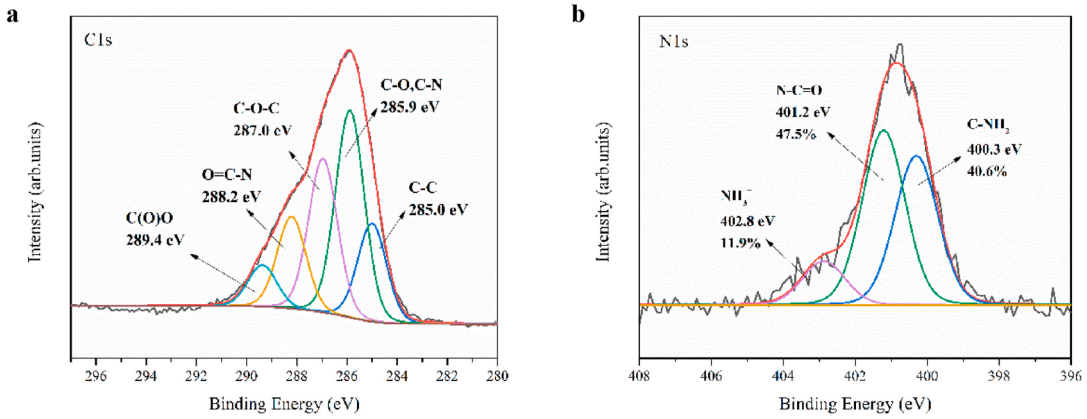

Fig. 1. XPS spectrum of (a) the C1*s* and (b) the N1*s* core level regions of β-glucosidase immobilized on the surface of zinc oxide-iron oxide nanoparticles (BGL@ZnOFe).

3.2. Screening of the activity of β-glucosidase immobilized on ZnOFe nanoparticles in the presence of different DESs

To fully deploy green solvents such as DESs in biocatalysis for developing more sustainable processes, it is imperative to investigate how a series of different DESs affect the catalytic performance of an enzyme of interest (Huang et al., 2014). Hence, the activity of BGL@ZnOFe was first screened in 19 different aqueous DES solutions at a predetermined volume ratio (10% v/v) in order to explore the impact of DESs as co-solvents on the catalytic performance of the immobilized β-glucosidase. The hydrolysis of *p*-NPG was used as the model reaction, and the activity of the enzyme in DES-free solution was defined as 100%. Table 1 summarizes the composition and molar ratios of all prepared DESs. As shown in Fig. 2, the catalytic activity of BGL@ZnOFe was significantly affected by the hydrogen bond donor, the hydrogen bond acceptor, and in specific cases, by the molar ratio of the two components. Most choline chloride and betaine-based DESs enhanced the catalytic activity of immobilized β-glucosidase, whereas ethylammonium chloride and choline DHP-based DESs afflicted dramatically the enzyme performance. It is possible that EAC and Chol DHP ions cause enzyme denaturation or affect the physicochemical properties of the reaction media, thus hindering the substrate-enzyme interactions. ChCl and Bet-based DESs had a marked impact on the enzymatic activity of the nano-biocatalyst; for instance, the inclusion of 10% (v/v) ChCl:BG, ChCl:PG or Bet:EG significantly improved the catalytic performance of BGL@ZnOFe, leading to an enhancement between 17 and 50% as for the hydrolysis of *p*-NPG. Recent studies involving β-glucosidases have also reported enhanced enzymatic activities using ChCl-based DESs as co-solvents (Ma et al., 2020; Xu et al., 2018); however, the origin of the enzyme seems to play a role too, since the activity of β-glucosidase from *Streptomyces griseus* has been found to decrease when 10% (v/v) ChCl based-DES/buffer solutions were used (Uhoraningoga et al., 2021).

Furthermore, the activity of immobilized BGL was also found to be influenced by the molar ratio of the DES components in the cases of ChCl:BG and Bet:Gly. More specifically, in the case of Bet:Gly, the activity of BGL@ZnOFe was lower when a higher amount of glycerol (HBD) was employed for the preparation of the DES, whereas in the case of ChCl:BG, an increase in activity was observed when a higher amount of BG (HBD) was used. Uhoraningoga and coworkers (2021) have also reported that the molar ratio of ChCl-based DES constituents significantly affected the relative activity of β-glucosidase from *Streptomyces griseus*.

Also evident from Fig. 2 is the fact that the BGL@ZnOFe activity was markedly influenced by the hydrogen bond donor in the case of ChCl-based DESs. Immobilized β-glucosidase exhibited a higher relative activity in ChCl-based DESs where polyols were used as HBDs in contrast to DESs comprised of amines or sugars. It has been proposed that due to the hydroxyl groups of polyols, a stronger hydrogen bond network can be formed (Xu et al., 2018). Sugar-based DESs exhibit instead a significant viscosity that limits mass transfer in enzyme catalyzed reactions (Sun et al., 2020), and particularly glucose-based DESs have been reported to inhibit the catalytic activity of β-glucosidase from *Streptomyces griseus* (Uhoraningoga et al., 2021). Hence, we saw that the catalytic performance of immobilized β-glucosidase in DES aqueous solutions depends on different parameters such as the viscosity and polarity of the medium, the molar ratio of the DES components, and the HBD/HBA combination, similarly to what Huang and coworkers (Huang et al., 2014) found for a lipase.

The concentration of the DES in the reaction system is also an important parameter that affects the enzyme activity. Hence, the relative activity of BGL@ZnOFe in the presence of different concentrations of selected DESs was examined. As shown in Fig. 3a and b, ChCl or Bet-based DESs substantially improved enzyme activity at concentrations up to 10% (v/v) which is similar to that recently observed for β-glucosidase from *Streptomyces griseus* (Uhoraningoga et al., 2021). At higher DES concentrations, the activity of immobilized β-glucosidase rapidly decreased. The inhibitory effect at higher DES concentrations could be attributed to the viscosity of DES-containing solutions increasing the substrate's diffusion limitations. Recent research has presented a similar pattern indicating that

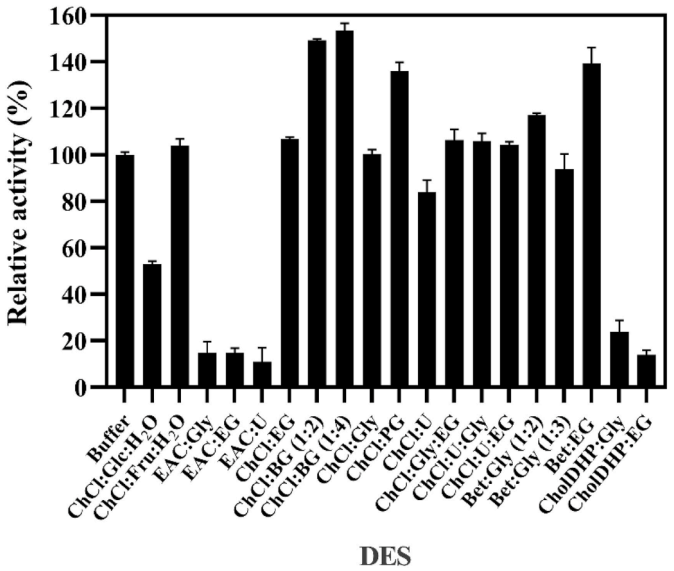

Fig. 2. Relative activity of β-glucosidase immobilized on ZnOFe nanoparticles in citrate-phosphate buffer (100 mM, pH 6.5) containing different DESs (10% v/v). The relative activities (%) were expressed as the percentages of the initial reaction rates obtained in the aqueous DES solutions relative to the DES-free system.

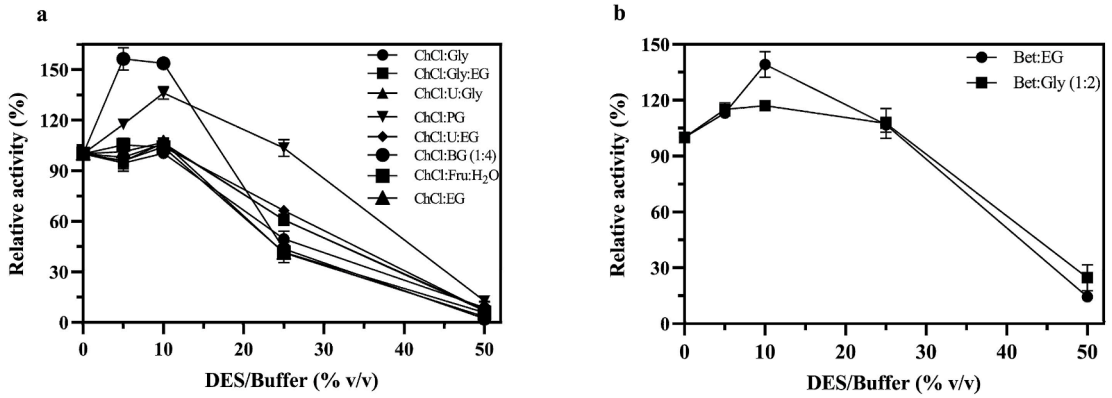

Fig. 3. Relative activity of β-glucosidase immobilized on ZnOFe nanoparticles in citrate-phosphate buffer (100 mM, pH 6.5) containing different concentrations of selected a) ChCl-based DESs and b) Bet-based DESs.

water is essential for hydrolytic reactions by improving the solution viscosity (Ma et al., 2020). It was also proposed that the active site of BGL could be blocked by the DES's components, which hinders the substrate binding (Uhoraningoga et al., 2021). It is important to note that DESs dissociate at concentrations below 50% (v/v) (Sapir and Harries, 2020). However, certain studies have ascribed the enhancement of the enzyme activity to the existence of DES "clusters" at low DES concentrations (Huang et al., 2014; Weiz et al., 2016), as also recently stated for the function of a different β-glucosidase (Xu et al., 2018). Individual DESs' components were not responsible for the enzymes' activation, therefore the researchers presumed that DES "clusters" were present in the mixtures affecting the biocatalytic characteristics of the enzymes. It seems that the effect of aqueous DES-based media on BGL activity is a multifactorial issue that depends on the enzyme, the nature of DESs and their concentration, and also on the interactions that are formed between the enzyme and HBA/HBD complex, DES "clusters" or the individual components of DESs in the reaction mixture.

3.3. Kinetic studies of β-glucosidase immobilized on ZnOFe nanoparticles in the presence of different DESs

To better assess the effect of DESs on the BGL@ZnOFe activity, different DESs were exploited as co-solvents to determine enzyme kinetics using the Michaelis-Menten kinetic model. From the Lineweaver-Burk plots, the apparent Michaelis-Menten constant (K_m) and the apparent maximum reaction rates (V_{max}) were determined. The results are presented in Table 2. As mentioned above, EAC and Chol DHP-based DESs noticeably decreased the enzymatic activity. When EAC:Gly and Chol DHP:Gly were used as co-solvents (10% v/v), the K_m values substantially increased, and the V_{max} values were lowered. These results indicate that the enzyme performance was negatively affected when EAC and Chol DHP-based DESs were used as co-solvents, possibly due to a decreased enzyme affinity or to a destabilization of enzyme-substrate interactions (Juneidi et al., 2017). In the cases of the addition of ChCl and Bet-based DESs, K_m, V_{max} values and turnover numbers (k_{cat}) increased. K_m values reveal a lower enzyme affinity although the V_{max} values and turnover numbers are higher than those of the DES-free system. A possible explanation is that DESs which contain polyols as HBDs may affect the catalytic mechanism of β-glucosidase, causing higher reaction rates (Xu et al., 2018). The same effect has also been reported for other hydrolytic enzymes in DES-containing solutions (Huang et al., 2014). Overall, ChCl:BG demonstrated the most noticeable effect on BGL@ZnOFe performance. k_{cat}/K_m value was slightly higher than that of the DES-free system, testifying to an improved catalytic efficiency of the nano-biocatalyst in the DES-containing solution.

Table 2
Apparent kinetic parameters of the activity of β-glucosidase immobilized on ZnOFe nanoparticles regarding the hydrolysis of *p*-NPG in selected DES aqueous solutions.

	K_m (mM)	V_{max} (μmol L^{-1} min^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
DES-free system	0.48 ± 0.02	41.36 ± 0.47	$6.60 \times 10^3 \pm 0.07 \times 10^3$	$13.73 \times 10^3 \pm 0.01 \times 10^3$
ChCl:BG (1:4)	0.94 ± 0.05	81.59 ± 0.36	$13.01 \times 10^3 \pm 0.06 \times 10^3$	$14.52 \times 10^3 \pm 0.01 \times 10^3$
ChCl:Gly	2.14 ± 0.25	79.19 ± 2.26	$12.63 \times 10^3 \pm 0.36 \times 10^3$	$5.90 \times 10^3 \pm 0.20 \times 10^3$
ChCl:U:Gly	2.36 ± 0.20	80.77 ± 2.11	$12.88 \times 10^3 \pm 0.34 \times 10^3$	$5.47 \times 10^3 \pm 0.15 \times 10^3$
ChCl:Fru:H_2O	2.33 ± 0.06	69.12 ± 1.04	$11.02 \times 10^3 \pm 0.17 \times 10^3$	$4.73 \times 10^3 \pm 0.04 \times 10^3$
ChCl:U	1.32 ± 0.41	64.99 ± 4.39	$10.37 \times 10^3 \pm 0.70 \times 10^3$	$7.83 \times 10^3 \pm 0.33 \times 10^3$
ChCl:Gly	2.14 ± 0.25	79.20 ± 2.26	$12.63 \times 10^3 \pm 0.36 \times 10^3$	$5.90 \times 10^3 \pm 0.20 \times 10^3$
EAC:Gly	4.81 ± 0.12	14.49 ± 0.35	$2.31 \times 10^3 \pm 0.06 \times 10^3$	$0.48 \times 10^3 \pm 0.12 \times 10^3$
Bet:Gly (1:2)	1.32 ± 0.04	81.81 ± 0.17	$13.05 \times 10^3 \pm 0.03 \times 10^3$	$9.85 \times 10^3 \pm 0.04 \times 10^3$
Chol DHP:Gly	1.18 ± 0.17	13.01 ± 0.49	$2.07 \times 10^3 \pm 0.08 \times 10^3$	$1.76 \times 10^3 \pm 0.13 \times 10^3$

3.4. Stability of β-glucosidase immobilized on ZnOFe nanoparticles in the presence of different DESs

Enzyme stability is an important feature for biocatalytic related applications. Therefore, BGL@ZnOFe was pre-treated with a series of different aqueous DES solutions and incubated at 50 °C for 24 h in order to determine the stability of the immobilized β-glucosidase in the presence of DESs. Next, the nano-biocatalyst was rinsed and assayed in a fresh reaction mixture using *p*-NPG as the substrate to assess its residual activity. The residual activity of BGL@ZnOFe incubated in the DES-free system was defined as 100%. As shown in Fig. 4, the HBD had a major impact on the stability of the nano-biocatalyst. The incubation of BGL@ZnOFe in a sugar-based DES (ChCl:Fru:H_2O) had a marked deactivating effect possibly due to the denaturation of the enzyme by the DES's components (Uhoraningoga et al., 2021). Instead, ChCl-based DESs with Gly:EG and PG as HBDs preserved or enhanced the enzymatic activity of BGL@ZnOFe. The prominent role of polyols as HBDs has been highlighted before (Xu et al., 2018). Uhoraningoga and cowokers (2021) have also reported an enhancement on the stability of β-glucosidase using aqueous ChCl-based DES solutions, particularly with glycerol as HBD. Among Bet-based DESs, the one with EG as HBD demonstrated a stronger stabilizing effect. In addition, it seems that the pre-treatment of the nano-biocatalyst with a higher concentration of DESs (50% v/v) led to destabilization. Overall, ChCl:PG (5, 10% v/v) and Bet:EG (10% v/v) provided a conspicuous thermostability to the nano-biocatalyst, underlining the positive impact of the aqueous DES solutions on the stability of BGL@ZnOFe after the pre-treatment under specific conditions. Concerning the stability of the immobilized enzyme, the advantage of this study compared to previous related works is that the nanobiocatalyst can be separated from the reaction mixture by applying magnetic force and re-utilized (Ma et al., 2020; Xu et al., 2018). Hence, it is more cost-effective approach.

3.5. Phenolic glucoside hydrolysis by β-glucosidase immobilized on ZnOFe nanoparticles using DESs as co-solvents

The positive effects of specific DESs on the activity and stability of the immobilized enzyme emphasize the advantage of using them as additives in various BGL@ZnOFe catalyzed reactions of interest. Furthermore, it is well-established that DESs can increase the solubility of certain organic compounds in aqueous solutions (Cheng and Zhang, 2017). To evaluate these effects, the enzymatic hydrolysis of phenolic glucosides (esculin, salicin) using DESs as co-solvents was thoroughly explored. The aglycone derivatives of the aforementioned compounds have been reported to exhibit significant biological activities. More specifically, esculetin acts as lipoxygenase inhibitor, as well as anti-inflammatory and anti-tumor agent, and salicyl alcohol can be used as an intermediate for the synthesis of perfumes and pharmaceuticals, for example for acne treatment (Liang et al., 2017; Muñiz-Mouro et al., 2017). The hydrolysis reactions were monitored by HPLC. The impact of elevated concentrations of certain DESs on the degree of hydrolysis and the reaction rates was also evaluated. As shown in Fig. 5, the hydrolysis yields of esculin in DES aqueous solutions were nearly 100% except for the case of EAC:EG. The same phenomenon was observed as for the hydrolysis of *p*-NPG. Hence, DESs with EAC as HBA possibly denaturate β-glucosidase or hinder the substrate's access to the active site of β-glucosidase. In the case of salicin hydrolysis, the addition of a series of DESs as co-solvents resulted in decreased hydrolysis yields as compared to the DES-free solution. EAC:EG demonstrated a similar outcome as mentioned above. The differences between the hydrolysis yields of salicin when different DESs are used as co-solvents (10% (v/v)) were more pronounced; ChCl:PG and Bet:EG led to reaction yields up to 85%. Conversely, the reaction rates of phenolic glucosides hydrolysis were reduced when adding DESs as co-solvents, while the inclusion of Chol DHP:Gly at a concentration of 10% (v/v) increased the hydrolysis rate of esculin up to 1.4-fold (Table 3).

Our study revealed that the addition of DESs affected differently the hydrolysis yields and rates of salicin and esculin. These results demonstrate that the outcome of a reaction depends not only on the enzyme function in DES-containing solutions but also on the interactions between DESs and solutes. Hence, physicochemical parameters such as the polarity and the viscosity of the co-solvents can significantly affect the advance of a specific reaction by changing the medium properties, the enzyme-substrate interactions, and the substrate's solubilization. It is interesting to note that certain DESs' compounds such as ChCl can act as hydrotropes enhancing the sol-

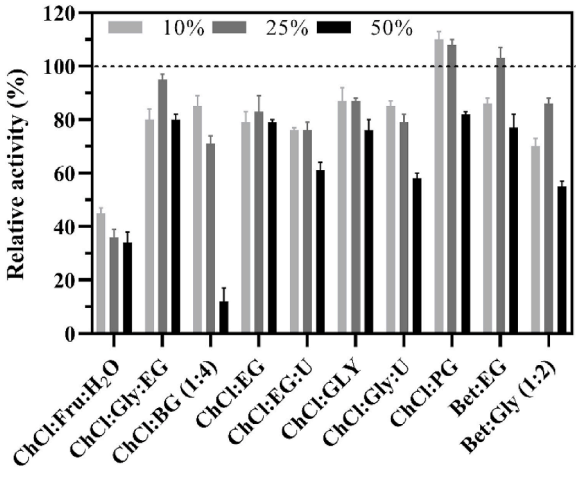

Fig. 4. Relative activity of β-glucosidase immobilized on ZnOFe nanoparticles, pre-treated with elevated concentrations of different aqueous DES solutions at 50 °C for 24 h. The residual activity of BGL@ZnOFe incubated in the DES-free system was defined as 100%.

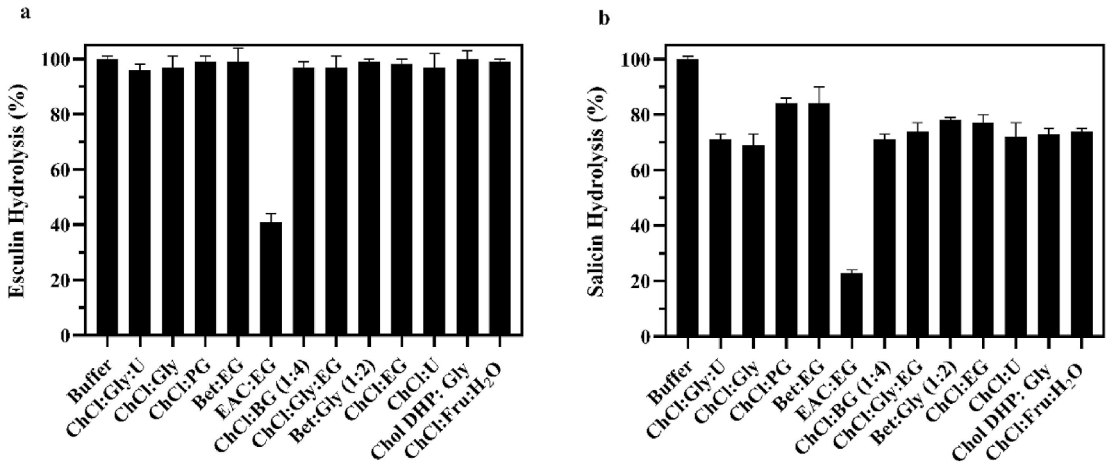

Fig. 5. Effect of different DESs (10% v/v) on the hydrolysis yields of a) esculin and b) salicin catalyzed by β-glucosidase immobilized on ZnOFe nanoparticles.

Table 3
Reaction rates (μM min^{-1}) of phenolic glucoside hydrolysis by β-glucosidase immobilized on ZnOFe nanoparticles.

Reaction mixture	Esculin hydrolysis rate (μM min^{-1})	Salicin hydrolysis rate (μM min^{-1})
Buffer	21.7	35.5
EAC:EG	0.8	8.0
Chol DHP:Gly	30.1	17.7
ChCl:U	13.2	12.4
ChCl:PG	17.9	21.2
ChCl:Gly	16.3	13.5
ChCl:Gly:U	14.0	13.6
ChCl:Fru:H_2O	16.8	15.4
ChCl:EG	15.8	14.6
ChCl:BG (1:4)	15.9	15.2
Bet:Gly (1:2)	17.5	21.8
Bet:EG	18.5	28.0
ChCl:Gly:EG	15.5	24.1

ubility of moderate/poorly water-soluble compounds (Sintra et al., 2021) such as esculin, and thus, facilitating its biocatalytic transformation.

Moreover, as shown in Fig. 6, at higher DES concentrations the hydrolysis yields of the glycosylated phenolic compounds were significantly decreased. Mass transfer limitations due to higher levels of viscosity or lack of essential water molecules hindered the pro-

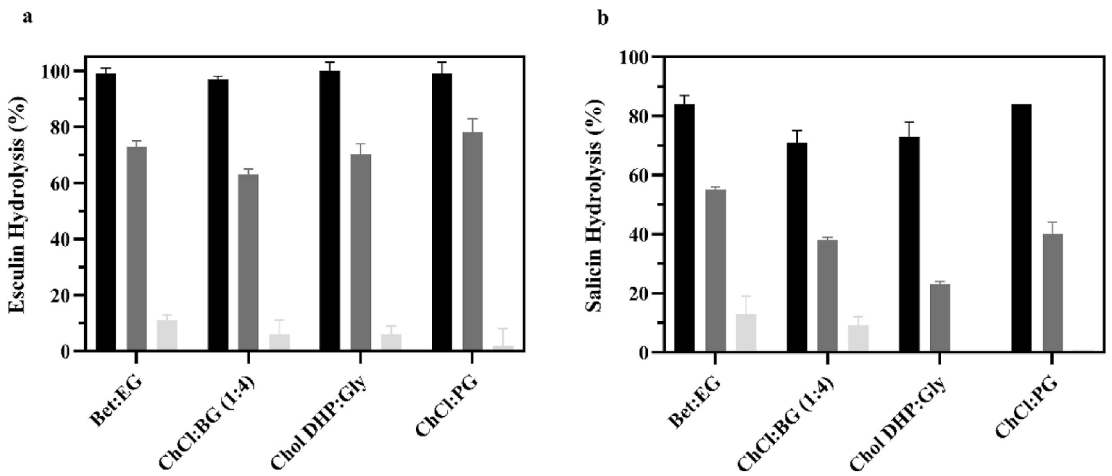

Fig. 6. Effect of DES concentration on the hydrolysis yields of a) esculin and b) salicin catalyzed by β-glucosidase immobilized on ZnOFe nanoparticles. Black bars represent 10%, grey bars 25% and light grey bars 50% (v/v) of the DES concentration.

cessing of reactions catalyzed by the immobilized BGL (Ma et al., 2020). The hydrolysis of hesperidin in DES aqueous solutions was also reported to be affected by the concentration of DESs in a similar manner (Weiz et al., 2016).

3.6. Salidroside synthesis using DESs as co-solvents

Considering the significant effect of DESs on the hydrolytic activity of β-glucosidase, the role of DES on the transglycosylation activity of BGL@ZnOFe was also explored. For this purpose, aryl glucoside synthesis was chosen as a model reaction. Salidroside is a phenolic glucoside of utmost importance due to its biological activities (Fig. 7) (Wang et al., 2019). Therefore, ChCl:BG (at a concentration of 10% v/v) was tested as co-solvent in the glycosylation of tyrosol; cellobiose was exploited as the glycosyl donor. The total turnover number was defined as the total moles of salidroside produced per moles of immobilized enzyme added in the reaction mixture and was used to express the aryl glucoside synthesis (24 h of continuous process). Salidroside was successfully synthesized using BGL@ZnOFe as biocatalyst. More specifically, the addition of ChCl:BG enhanced salidroside synthesis from 60.1×10^2 TTN (DES-free reaction mixture) to 93.4×10^2 TTN (inclusion of 10% v/v, ChCl:BG). An increase up to 55.5% was observed, revealing the importance of using DESs as co-solvents in synthetic reactions of interest involving an immobilized β-glucosidase. It is important to point out that salidroside synthesis was enhanced by employing lower and reusable enzyme quantity in contrast to previous works (Potocká et al., 2015). The use of immobilized enzyme with magnetic capabilities enables the easy recovery of the nanobiocatalyst, whereas in the case of non-immobilized enzyme, separation steps are needed for the recovery of the product (Delavault et al., 2022; Tong et al., 2004). Moreover, in previous works ionic liquids and organic solvents were explored as co-solvents for the enzymatic preparation of salidroside (Bi et al., 2012; Tong et al., 2004); However, most of these solvents are considered harmful. More specifically, dioxane as co-solvent exhibited a favourable effect on the production yield of salidroside, but its use is of major concern. Our study highlighted the implementation of green solvents in order to improve the synthetic performance of the immobilized β-glucosidase as a more benign approach. Another research group confirmed that using DESs as co-solvents, the butyl glucoside synthesis was noticeably enhanced (Uhoraningoga et al., 2021). Therefore, DESs present an advantageous effect on the glycosylation activity of β-glucosidases, triggering the need for further studies.

4. Conclusion

Enzymatic processes meet the demands of green chemistry for the preparation of bioactive compounds by being more environmentally friendly and cost-effective. Numerous studies have evaluated the effect of DESs on the catalytic features of different enzymes. Considering that immobilized enzymes are more stable, reusable, and apply to the needs of industries, the biocatalytic properties of an immobilized β-glucosidase were explored in the presence of different aqueous DES solutions. This is the first report that investigates the function of an enzyme immobilized on the surface of green nanoparticles in aqueous DES solutions. Hence, a completely green approach is presented for the development of a new nanobiocatalytic system, the function of the immobilized enzyme in aqueous DES solutions, and the production of bioactive compounds in comparison to recent literature. In this framework, β-glucosidase was successfully immobilized on the surface of green zinc oxide-iron oxide nanoparticles and applied to reaction systems with neoteric solvents. Choline chloride-based DESs and betaine-based DESs significantly enhanced the activity, stability, and catalytic efficiency of the nano-biocatalyst. Choline chloride:propylene glycol and betaine:ethylene glycol are ideal candidates for hydrolytic reactions of interest due to the enhancement of the nano-biocatalyst's stability. In addition, the incorporation of choline chloride:butylene glycol had a favourable effect on the production of salidroside resulting in a higher total turnover number in contrast to the DES-free system.

Furthermore, it is important to mention that the combination of experimental data with computational techniques can give deeper insights into the enzyme catalysis in DESs and in this way may bring the use of DESs as media for biocatalytic reactions, one step closer to industrial applications.

CrediT author statement

Renia Fotiadou: Conceptualization; Investigation; Visualization; Methodology; Formal analysis; Data curation; Validation; Roles/Writing - original draft; Writing - review & editing. Myrto G. Bellou: Investigation; Visualization; Methodology; Formal analysis; Data curation; Validation; Roles/Writing - original draft; Writing - review & editing. Konstantinos Spyrou: Investigation; Data curation; Validation; Writing - original draft; Writing - review & editing. Feng Yan: Investigation. Petra Rudolf: Validation; Writing - review & editing. Dimitrios Gournis: Writing - review & editing; Supervision. Haralambos Stamatis: Conceptualization; Visualization; Methodology; Data curation; Validation; Roles/Writing - original draft; Writing - review & editing; Resources; Project administration; Funding acquisition; Supervision. All authors have read and agreed to the published version of the manuscript.

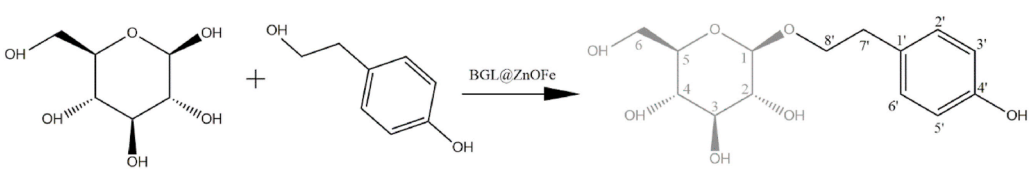

Fig. 7. Illustration of enzymatic synthesis of salidroside catalyzed by β-glucosidase immobilized on ZnOFe nanoparticles.

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.

Data availability

No data was used for the research described in the article.

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