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β-glucosidase from *Streptomyces griseus*: Ester hydrolysis and alkyl glucoside synthesis in the presence of Deep Eutectic Solvents

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ABSTRACT

Deep Eutectic Solvents (DES) are ionic liquid analogs that have attracted considerable attention as green solvents for biocatalytic transformations. The use of DES as part of a 'solvent engineering' approach to enhance enzyme stability holds great promise since they are biodegradable, relatively inexpensive and environmentally safe media for enzyme reactions. However, the behaviour of specific enzymes in such solvents is complex; some enzymes are inhibited in DES, while others appear to be activated.

Glucosidases are among the most widely used enzymes for commercial chemoenzymatic synthesis. In particular, their application in the synthesis of biodegradable alkyl glucosides by reverse hydrolysis is of great interest. Previous work in this laboratory identified *Streptomyces griseus* glucosidase (*Sgβgl*) as an interesting enzyme for biotechnological applications. In this study, we examined its behaviour in the presence of DES as a co-solvent using choline chloride as hydrogen bond donor and using glycerol, glucose and urea as hydrogen bond acceptors. We show that *Sgβgl* activity depends on both the nature of DES components, and their ratio in the eutectic mixture, as well as the water content of the reaction medium. A choline chloride/glycerol DES mixture at a level of 40% (v/v) caused activation of *Sgβgl* and increased its optimum temperature from 70 to 80 °C: it also led to a striking increase in its thermostability, doubling its half-life at 60 °C and almost tripling its half-life at 80 °C.

The synthesis of alkyl glucosides was explored using DES as a co-solvent. In the presence of DES, *Sgβgl* catalysed the formation of a range of alkyl glucosides. The presence of DES resulted in enhanced product yield, which was observed to increase with increasing temperature, up to 60 °C.

These studies show that the application of DES at relatively low % (v/v) levels can dramatically effect enzyme activity and stability. Specifically, enhanced thermostability can significantly increase the operating range for glucosidases for biocatalytic applications. Solvent engineering offers a simple and effective way to enhance glucosidase stability and will be useful as an alternative and/or adjunct to more complex methods such as immobilisation or protein engineering.

1. Introduction

Solvent waste in many industrial processes is a cause of concern [1]. In sustainable chemistry, the use of environmentally friendly solvents is a key objective [2]. Green chemistry focuses on the design of products and processes that minimise or eliminate the use, and generation of, hazardous substances [3]. In recent years, Deep Eutectic Solvents (DES) have been explored as environmentally friendly solvents for biotechnological and 'green' chemical applications [4,5]. DES have the advantage of being derived from natural compounds and are non-toxic and environmentally friendly. Furthermore, they are relatively inexpensive and biodegradable solvents [6]. Initially, DES were studied by Abbott and co-workers [7] who demonstrated that a eutectic mixture made from choline chloride and urea had a much lower melting point (12 °C) than choline chloride or urea alone, 302 °C and 133 °C, respectively. The structure of the DES components used in this study; hydrogen bond acceptors (HBA) and hydrogen bond donors (HBD), are shown in Fig. 1. Some areas for applications of these solvents are in biocatalysis [8,9], extraction [10,11], and synthetic chemistry [12,13]. Previous studies had shown that the presence of DES could have a stabilising, and potentially activating, effect on certain enzymes [14,15].

β-glucosidase catalyses the hydrolysis of glycosidic linkages [16], and plays a pivotal role in many biological and biotechnological processes [17–19]. However, like many enzymes, β-glucosidase has limitations

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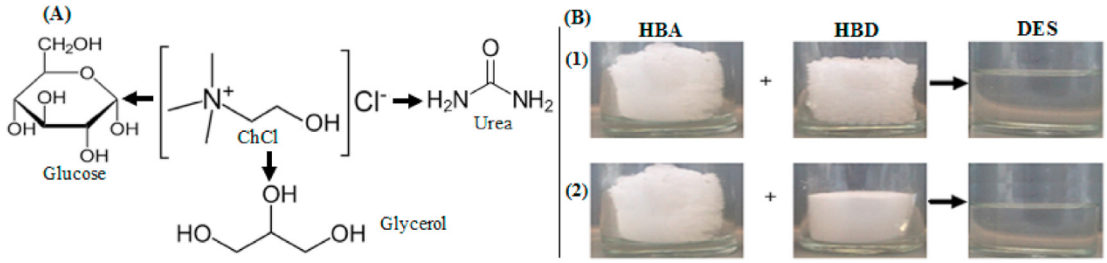


Fig. 1. DES components used in this study. (A), Chemical structures used for DES preparation: Choline chloride (ChCl) was used as a hydrogen bond acceptor (in the centre), with three hydrogen bond donors: Glucose (G), Urea (U) and Glycerol (Gly). (B), some typical DES prepared in this study (see methods for more details on preparation): (**1**), DES based on ChCl:U (1:1 M ratio); (**2**), DES based on ChCl:G (1:1 M ratio) HBA, hydrogen bond acceptor; HBD, hydrogen bond donor.

such as low operational stability and activity under certain reaction conditions. To improve its catalytic performance, various strategies such as mutagenesis [20] and immobilisation [21] have been explored. These strategies can result in some adverse effects on the enzyme, as well as being time consuming and expensive [18]. Recently, solvent engineering using DES has received increasing attention in biocatalysis, not only because it is environmentally friendly but because it can enhance enzyme performance and stability [22,23].

In this study, DES based on choline chloride as a H-bond acceptor, with H-bond donors, urea (U), glucose (G) and glycerol (Gly) were used to examine the effect of DES on the hydrolytic and synthetic activity of *Sgβgl*. For hydrolytic activity, *Sgβgl* behaviour was evaluated by following its hydrolysis of *p*-nitrophenyl β-D-glucopyranoside (*p*NPG) in the presence of DES. As well as examining effects on hydrolysis, the ability of *Sgβgl* to catalyse the synthesis of alkyl glucosides, via reverse hydrolysis, in the presence of DES (ChCl:Gly) as co-solvent was explored. Alkyl glucosides are highly effective carbohydrate-derived surfactants that are low in toxicity and are ecologically safe [24]. They have a diverse range of industrial applications including cosmetics, household detergents [25], agricultural products [26], pharmaceutical products [27–29] and applications in oil recovery [30].

2. Material and methods

2.1. Chemical and materials

Choline chloride, glucose, urea, glycerol, *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), potassium phosphate, acetonitrile, thin layer chromatography (TLC) plates, ethanol, sulphuric acid, butanol, pentanol, hexanol, heptanol, acetic acid, ethyl ether, orcinol reagent, hexyl glucoside, were purchased from Sigma-Aldrich (Ireland). All other chemical reagents used for *S. griseus* recombinant β-glucosidase expression and purification were described in our previous study [31].

2.2. Preparation of deep eutectic solvents

Choline chloride (ChCl) as H-bond acceptor and three H-bond donors (urea, glucose, glycerol), were used to prepare Deep Eutectic Solvents (Fig. 1). Briefly, choline chloride was mixed, separately, with urea, glucose and glycerol at molar ratios of 1:2, 1:1 or 2:1 in a beaker using a magnetic stirrer at 80 °C for 1–2 h until a clear liquid was formed. The resulting eutectic mixture was then cooled to room temperature and dried over P_2O_5, in a desiccator, at room temperature for at least two weeks prior to use [32].

2.3. Sgβgl activity assay using DES-containing aqueous solution

The *Sgβgl* utilised in this study was expressed and purified as described previously [31], and was used in lyophilised form. The *Sgβgl* activity was monitored by following the hydrolysis of *p*NPG using a 96-well-plate-based protocol. In brief, both enzyme and substrate were dissolved in DES-containing aqueous solution (50 mM potassium phosphate buffer, pH 7.0). The lyophilised *Sgβgl* was dissolved in DES/aqueous solution at a concentration of 0.5 mg mL^{-1} and enzyme activity measured by mixing 20 μL of enzyme with 120 μL DES/aqueous solution and 30 μL *p*NPG (7 mM) at 37 °C for 20 min. The reaction was terminated by adding 30 μL of 1 M Na_2CO_3 and the total reaction volume was 200 μL. The release of *p*-nitrophenol (*p*NP) product was measured at 405 nm using a microplate spectrophotometer (Bio-Tek PowerWave). All experiments were performed as three independent experimental runs, each carried out in triplicate. One unit (IU) of β-glucosidase activity was defined as the amount of enzyme required to release 1 μmol of *p*NP per minute under standard assay conditions [31].

2.3.1. Effect of DES molar ratios and concentration on Sgβgl activity

DES (ChCl:Gly, ChCl:G, ChCl:U) prepared with three different ratios of ChCl to H-bond donors (2:1, 1:1, and 1:2), were initially evaluated for their effect on *Sgβgl* at a low DES inclusion level (10% v/v). Subsequently, when a 1:1 M ratio showed most activity, this ratio was used to evaluate the effect of DES concentration (10%–60% v/v) on *Sgβgl* activity. In all cases, a control reaction was performed without DES in 50 mM potassium phosphate buffer, pH 7.0.

2.3.2. Effect of DES on pH and temperature profiles of Sgβgl

The effect of DES-containing aqueous solutions, at an inclusion level of 40% (v/v), on pH and temperature profiles was evaluated for *Sgβgl*. For pH studies, enzyme activity was measured using buffers of different pH from 5 to 9 as per Bhoite and colleagues [33]. For temperature studies, the assay was carried out at temperatures ranging from 40 °C to 90 °C. Thermostability tests were performed at different temperatures (40 °C, 60 °C, and 80 °C) over a period of 4 h and residual activity measured. In this set of experiments, a comparison with a commercially available almond β-glucosidase (Sigma-Aldrich) was included to allow for a point of reference for these findings.

2.4. Synthetic activity of Sgβgl using DES as a co-solvent

To explore the ability of *Sgβgl* to synthesise alkyl glucosides using DES (ChCl:Gly), as a co-solvent, the synthesis of alkyl glucosides was initially carried out through screening various alcohols (butanol, pentanol, hexanol and heptanol) as glycosyl acceptors with glucose as glycosyl donor. Standard screening conditions were 100 mM glucose, 215 U/mL enzyme, 80% (v/v) alcohols, 20% (v/v) of DES (ChCl:Gly), at 50 °C, 220 rpm, for 6 h. After 6 h of reaction, samples were boiled for 5 min to terminate the biocatalytic reaction and cooled at room temperature before analysis.

2.4.1. TLC and UHPLC analysis

Alkyl glucoside products were analysed by thin-layer chromatography (TLC) or by Ultra-High Performance Liquid Chromatography (UHPLC). For TLC analysis, the reaction mixture was diluted (1:10) and applied to the TLC plate by capillary and developed in the following solvent system: butanol, acetic acid, ethyl ether, and water, 9:6:3:1 (v/v/

Table 1
Compositions of DES mixtures utilised in this study.

Hydrogen bond acceptor	Hydrogen bond donor		Molar ratio
	Type	Name	
Choline Chloride	Amide	Urea	2:1
			1:1
			1:2
	Alcohol	Glycerol	2:1
			1:1
			1:2
	Sugar	Glucose	2:1
			1:1
			1:2

v/v). The plates were sprayed with orcinol reagent (180 mg orcinol in 5 mL of distilled water, 75 mL neat ethanol, and 10 mL of H_2SO_4), and heated at 120 °C until spots were observed [20]. For UHPLC analysis, an Agilent UHPLC with a refractive index detector (RI) and Hi-Plex H, 7.7 × 300 mm column for carbohydrates was used. Samples were reconstituted in ultra-pure water (UPW), passed through a 0.22 μm syringe filter and eluted through a Hi-Plex H, 7.7 × 300 mm column, according to the manufacturer's specifications. A 100% UPW mobile phase was used at a flow rate of 0.6 mL/min, as per Gudmundsson and colleagues [34]. The column and detector were kept at a constant temperature of 35 °C and 60 °C, respectively. The quantitative analysis of alkyl glucosides was carried out by use of UHPLC peak area in comparison with peak area of commercially available standard, hexyl glucoside (Sigma-Aldrich) of known concentration [35].

2.5. Purification and product identification

Butyl glucoside was purified by butanol partitioning [36]. The product was extracted into an immiscible butanol layer, while the unreacted sugar and enzyme remained in the lower aqueous layer following shaking of the reaction medium with an equal volume of butanol. The product was subsequently subjected to rotary evaporation at 47 °C, to remove butanol. The purified product was then separated by UHPLC and the purified butyl glucoside analysed by commercial LC-MS (TCD, Dublin) to confirm the molecular mass of this product.

3. Results and discussion

3.1. Deep eutectic solvents used in this study and their preparation

Table 1 shows the DES prepared in this study. Glycerol-based DES were transparent mobile liquids, while urea-based DES were transparent viscous liquids. Glucose-based DES were found to be highly viscous. Chemical structures and images of the DES prepared in this study are shown in Fig. 1.

3.2. Sgβgl activity in DES-containing aqueous solutions

3.2.1. Effect of DES molar ratios and concentrations on Sgβgl activity

Previous studies showed that deep eutectic solvents, formed from choline chloride and various hydrogen bond donors, were of interest due to their ability to solvate a wide range of compounds [7,23] and increase enzyme activity and stability [37]. Therefore, it was important to understand how *Sgβgl* was affected by different DES compositions. In this study, different DES mixtures (ChCl:Gly, ChCl:G, ChCl:U), with various ratios of ChCl to hydrogen bond donor (2:1, 1:1, 1:2), were synthesised. These were first examined using *p*NPG as substrate at a low level of DES inclusion (10% v/v) in the assay mix. In this case the DES is acting as a co-solvent rather than a reaction medium. This level of DES inclusion was reported to cause 55% activation of a lipase from *Candida rugosa* in a recent study [6]. However, for *Sgβgl* no such activation was observed for 10% (v/v) DES (Fig. 2A). It was also reported that the molar ratio of DES components had a significant effect on enzyme activity [6]. Here, different DES preparations, based on their molar ratios of H-bond acceptor and H-bond donor, had markedly different effects on the activity of *Sgβgl* (Fig. 2A). DES preparations where the ratio of ChCl to hydrogen donors was 1:1 showed most activity, closely followed by those at a ratio of 2:1. Those with a lower amount of ChCl (1:2), showed a substantially lower enzyme activity. The DES components with the most positive effect on *Sgβgl* were those containing choline chloride and glycerol. In all cases the activity in DES was lower than the control although for ChCl:Gly (1:1) this was a loss in activity of only 5–10% (see Fig. 2A).

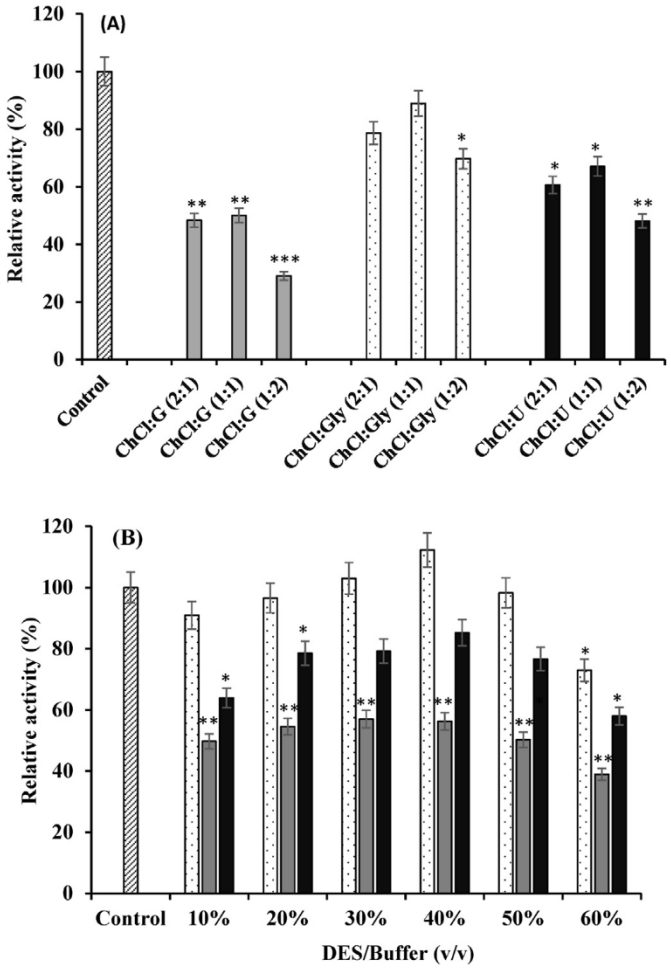


Fig. 2. **(A)** Effect of DES at different molar ratios on *Sgβgl* activity. Screening for the effect of DES: ChCl:G, (▩); ChCl:Gly, (□); and ChCl:U, (■) made from different molar ratios of hydrogen bond donor to hydrogen bond acceptor (2:1, 1:1, 1:2) at an inclusion level of 10% (v/v) in 50 mM potassium phosphate buffer, pH 7.0. The activity assay was carried out at 37 °C, for 20 min using 7.0 mM *p*NPG as substrate and 0.5 mg mL^{-1} enzyme. Activity (%) was estimated relative to a DES-free system (50 mM potassium phosphate buffer, pH 7.0) as a control (▨). Each histogram represents the mean of triplicate experiments; error bars represent the SD. * *p*-value ≤ 0.05, ** *p*-value ≤ 0.01, and *** *p*-value ≤ 0.001 represent significant, very significant, and extremely significant difference respectively, based on two-tailed *t*-test. The abbreviations ChCl represent Choline Chloride; G, glucose; Gly, glycerol; and U, urea. **(B)** Effect of DES inclusion level on *Sgβgl* activity on ester hydrolysis. Enzyme activity in 1:1 M ratio DESs: ChCl:G, (▩); ChCl:Gly, (■); and ChCl:U, (□) at different concentrations (10%–60% v/v) was carried out at 37 °C, for 20 min using 7.0 mM *p*NPG as substrate and 0.5 mg mL^{-1} enzyme (see Section 2.3). Activity (%) was estimated relative to DES-free system (50 mM potassium phosphate buffer, pH 7.0) as a control (■). Each histogram represents the mean of triplicate experiments; error bars represent the SD. * *p*-value ≤ 0.05, ** *p*-value ≤ 0.01, and *** *p*-value ≤ 0.001 represent significant, very significant, and extremely significant difference respectively, based on two-tailed *t*-test.

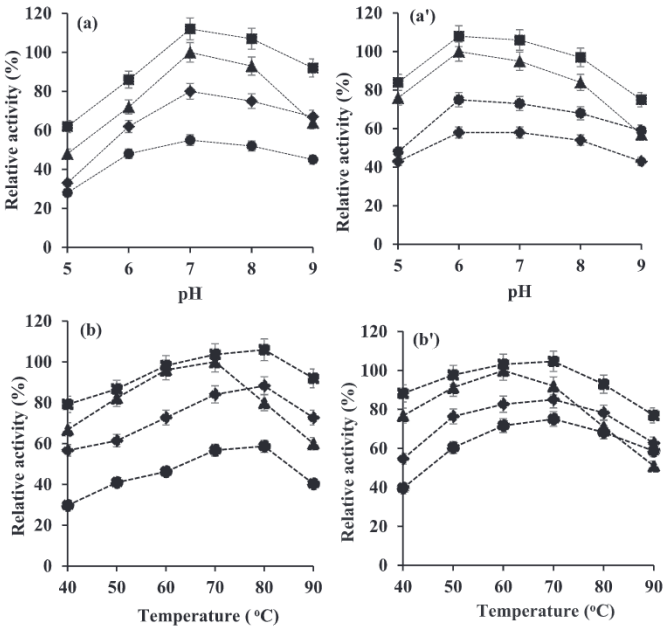


Fig. 3. The effect of DES (ChCl:Gly, (---■---); ChCl:U, (---◆---); ChCl:G, (---●---) at a 1:1 M ratio on pH and temperature optimum of *Sgβgl* (a, b) and commercial almond *βgl* (a', b'). DES at a concentration of 40% v/v in 50 mM potassium phosphate, pH 7.0 was used. Assays were carried out at 37 °C for 20 min using 7.0 mM *p*NPG as substrate and 0.5 mg mL^{-1} enzyme. A control (—▲—) using DES-free buffer (50 mM potassium phosphate buffer, pH 7.0) was included. The data represent the mean of three independent experiments; error bars represent standard deviation.

DES concentration can effect enzyme activity and stability [15]. These initial studies, with 10% (v/v) DES, indicated that the 1:1 mixture of ChCl and a hydrogen bond donor retained the greatest activity in comparison to a control. The effect of DES on *Sgβgl* activity over a range of inclusion levels was then explored using this ratio (1:1). DES mixtures (ChCh:Gly; ChCl:G; ChCl:U) in aqueous solution at different concentrations (10%–60% v/v) were used to observe the effect on ester hydrolysis (Fig. 2B).

Typically, DES inclusion of 40% (v/v) was found to give the greatest activity with glycerol-based DES being the most favoured solvent. For DES levels of 50% (v/v) and above a *decrease* in activity was observed. While urea-based DES showed some decline in activity, glucose-based DES significantly inhibited enzyme activity (see Fig. 2B) possibly due to the sensitivity of this enzyme to glucose inhibition [38]. A similar glycerol-based DES at 40% (v/v) has been reported to show the greatest activity for *A. niger* β-glucosidase [15], and for a 6-O-rhamnosyl β-glucosidase [39]. This study is consistent with the observation that glycerol has a generally stabilising effect on enzymes and that choline chloride activates some enzymes [14]. The favourable effect of glycerol on enzyme activity is probably due to its hydroxyl groups H-bonding with surface residues on the enzyme [36]. In general, glycerol-based DES have been shown to increase activity and stability of different enzymes such as β-glucosidase [15], lipase [36], and protease [40]. The level of 40% (v/v) inclusion was adopted for exploring alkyl glucoside synthesis experiments (described in Section 3.3).

3.2.2. *Effect of DES on pH and temperature profiles*

Since relatively little data are available in the literature concerning the behaviour of β-glucosides in DES, the response of *Sgβgl* and commercial almond *βgl* to DES solvents in terms of pH and temperature was explored to provide baseline data on these enzymes' performance in such solvents. The inclusion of the commercial enzyme allows for a reference for future studies. The presence of ChCl-based DES at a level of 40% (v/v) gave rise to an unchanged pH optimum indicating that the DES was not influencing the catalytic residues' state of ionisation. However, a striking effect on thermal activity profiles was observed for all of the DES mixtures examined. Thus, 40% (v/v) DES showed a considerable effect on the optimum temperature for both *Sgβgl* and commercial almond *βgl* (see Fig. 3.b,b'). The control (DES-free buffer) showed maximum activity at 70 °C for *Sgβgl*, but maximum activity was obtained at 80 °C in the presence of DES. Similarly, for commercial almond *βgl*, the control showed maximum activity at 60 °C, but with DES this was increased to 70 °C. The comparable response for both *Sgβgl* and commercial almond *βgl*, along with the recent findings that the optimum temperature of *A. niger* β-glucosidase was increased by 10% in DES [15], suggest that this stabilisation may be a common property for glucosidases. Mohammad and co-workers [41] explain that the formation of a strong ionic supramolecular net around the enzyme, and the shielding effect of hydrogen bonding in DES may be responsible for maintaining the integrity of the enzyme at elevated temperatures.

Having shown that the optimum temperature was increased in the presence of 40% (v/v) DES, it was of interest to examine the effect of DES on thermostability.

3.2.3. *Thermostability*

A highly significant (p-value $\leq$ 0.01) increase in thermostability, particularly for temperatures over 60 °C, was observed (see Fig. 4). This was observed as an increase in half-life of *Sgβgl* of 2-fold at 60 °C and approximately 3-fold at 80 °C when DES based on ChCh:Gly was used. Similar results were also seen for the commercial almond *βgl*. Interestingly, the presence of the DES, even those shown to inhibit *Sgβgl*, still provided enhanced thermostability over control albeit with lower activity.

These findings echo previous reports where a β-glucosidase from *Aspergillus niger* [15] and β-glucosidase from *Thermus thermophiles* [42] increased their activity and stability in the presence of ionic liquids. The enhancement of enzyme activity and stability in the presence of DES based on choline chloride was also reported for other types of enzymes such as *Penicillium expansum* lipase [32], *Candida rugosa* lipase [6], *Candida Antarctica* lipase [43], and *Bacillus licheniformis* protease [40]. Some plausible explanations for this stability suggest that DES can prevent enzyme unfolding and aggregation at high temperatures [44] or that the interaction between the salt and H-bond donors may lessen the negative impact of these anions on the enzyme [45]. Bell and colleagues

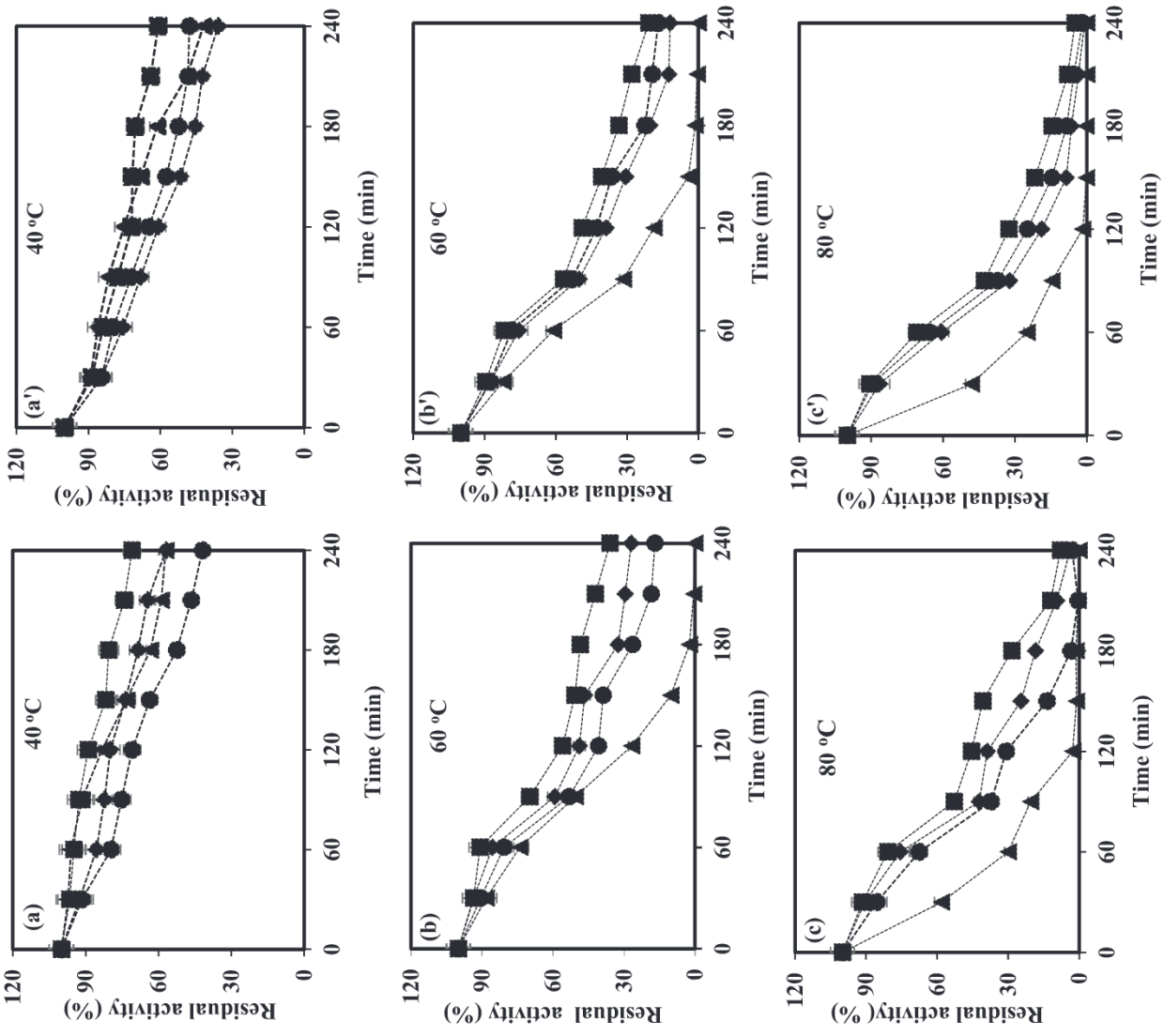


Fig. 4. Thermal stability of *Sg*βgl (a, b, and c) and commercial almond *β*gl (a', b', and c') at different temperatures (40 °C, 60 °C, and 80 °C) using 40% DESs such ChCl:Gly (—■—); ChCl:U (—◆—); ChCl:G(■0) in aqueous solution. Relative residual activity (%) was calculated against the initial rate in the DES-free system as control (■1). Assays were carried out using 7.0 mM *p*NPG substrate and 0.5 mg mL^{-1} enzyme. The data represent the mean of three independent experiments; error bars represent the standard deviation.

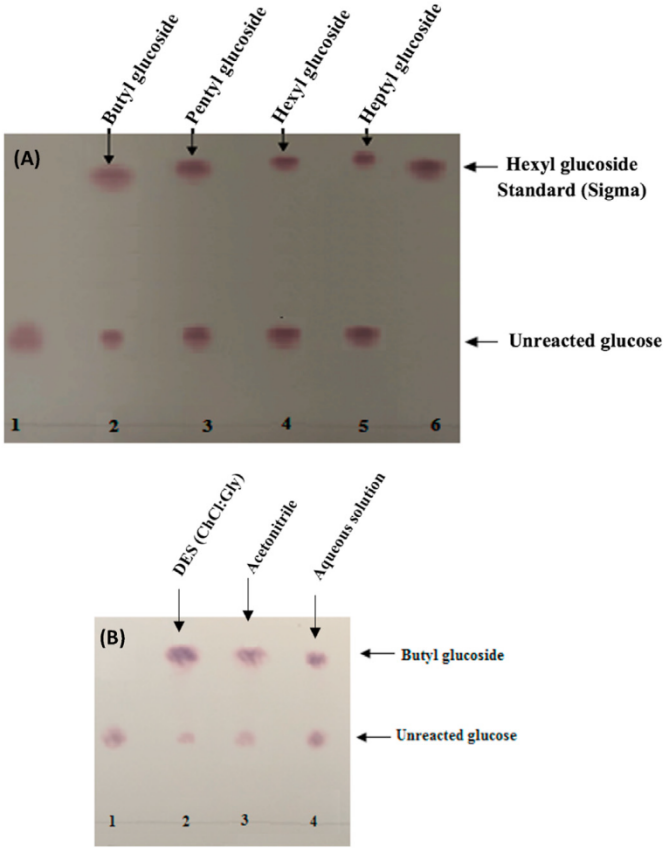


Fig. 5. **(A)** TLC analysis of alkyl glucoside synthesis using DES (ChCl:Gly) as co-solvent. The reaction conditions were: 100 mM glucose, 215 U/mL enzyme, 80% (v/v) alcohols, and 20% (v/v) DES solution (ChCl:Gly, 40% v/v in 50 mM potassium phosphate, pH 7.0) as co-solvent at 50 °C for 6 h. Lane 1, control; lane 2, butanol; lane 3, pentanol; lane 4, hexanol; lane 5, heptanol; and lane 6, hexyl glucoside standard (Sigma Aldrich). The control was without enzyme and showed no product formation. Spot density of products was determined using *ImageJ* densitometry software [57]. **(B)** TLC analysis of the effect of DES on butyl glucoside synthesis. The synthesis was catalysed by 215 U/mL *Sgβgl* using 100 mM glucose, 80% (v/v) butanol, 20% (v/v) DES (ChCl:Gly) at 50 °C, 220 rpm, for 6 h. Lane 1, control; lane 2, 20% (v/v) DES (ChCl:Gly) as a co-solvent; lane 3, 20% acetonitrile a co-solvent; lane 4, 20% (v/v) aqueous solution (potassium phosphate buffer, pH 7.0). The control was run under the same conditions using 20% (v/v) DES (ChCl:Gly), but without enzyme.

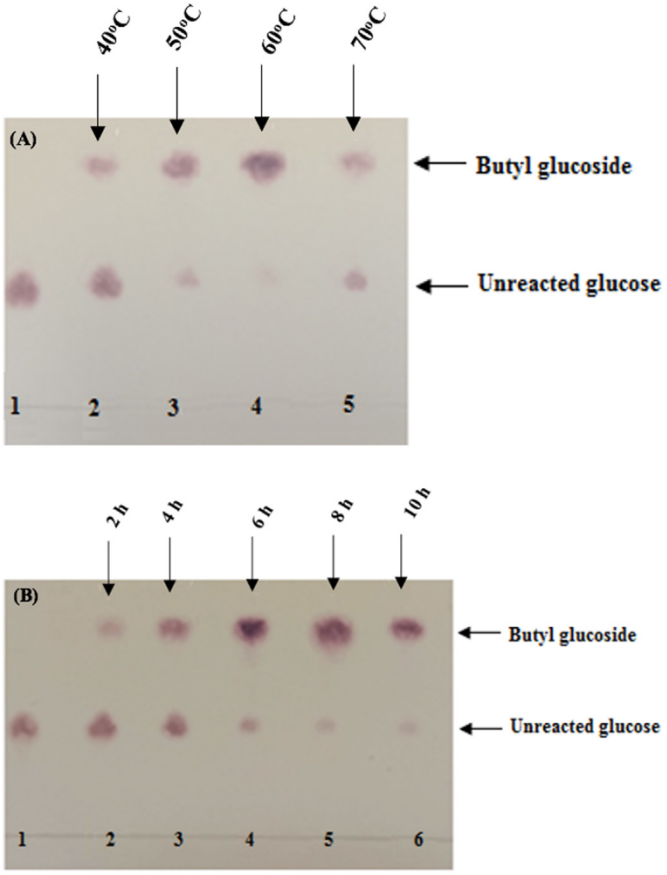


Fig. 6. **(A)** TLC analysis of the effect of temperature on butyl glucoside synthesis. The synthesis was catalysed by 215 U/mL *Sgβgl* using 100 mM glucose, 80% (v/v) butanol, 20% (v/v) DES (ChCl:Gly) at 50 °C, 220 rpm, at different temperatures (40 °C, 50 °C, 60 °C, 70 °C respectively) for 6 h. The control, lane 1, was the same reaction components, run at 50 °C and without enzyme. **(B)** Time course for butyl glucoside synthesis. The synthesis was catalysed by 215 U/mL *Sg*βgl, using 100 mM glucose, 80% (v/v) butanol, 20% (v/v) DES (ChCl:Gly) as co-solvent, at 60 °C over a period of 10 h and samples were taken at 2 h time intervals. Lane 1, control; lane 2, for 2 h; lane 3, for 4 h; lane 4, for 6 h; lane 5, for 8 h; lane 6, for 10 h. The control was run without enzyme.

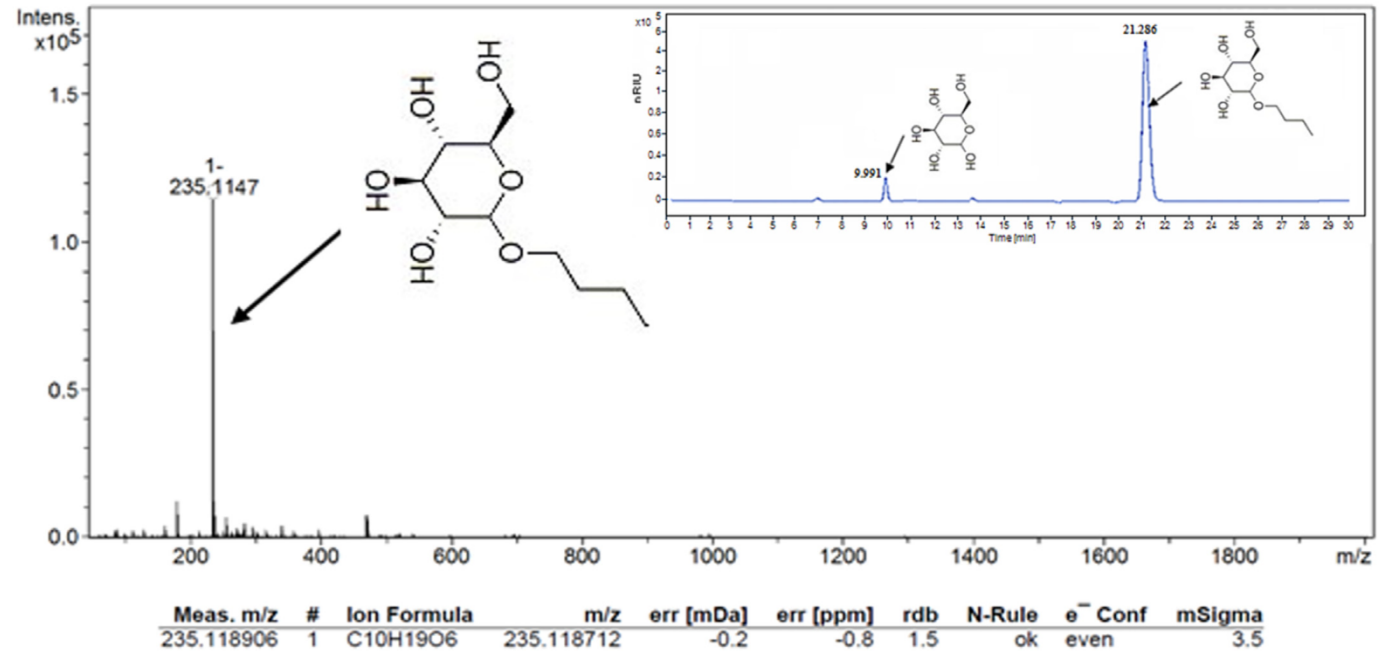
[46] reported that the hydrophilic characteristics of DES can influence thermodynamic water activity, which is required for enzyme activity. The advantages of various DES for enzymes' activity and stability have been documented [14,47–49]. The enhanced stability observed for *Sgβgl* enzymes in DES suggests the possibility of performing reactions at higher temperatures over a long time period to achieve enhanced product yield. It was of interest to examine the possible impact of DES on the synthetic activity of *Sgβgl*, and this was explored through alkyl glucoside synthesis as a model reaction.

3.3. Alkyl glucoside synthesis by Sgβgl using a DES co-solvent

Alkyl glucoside synthesis by *Sgβgl* by reverse hydrolysis using DES (ChCl:Gly) as a co-solvent was explored. The synthesis was initially carried out by screening various alcohols (butanol, pentanol, hexanol and heptanol) as glycosyl acceptors and glucose as glycosyl donor. This synthesis is normally approached in the presence of a vast excess of the alcohol [50] to shift the reaction equilibrium towards condensation resulting in higher yield [51]. The large excess of the solvent further dilutes the DES giving a final of 8% (v/v) in the reaction medium. Thus, it is acting as a co-solvent, or adjuvant, in this arrangement. The enzyme was dissolved in 50 mM phosphate buffer pH 7.0 containing 40% ChCl:Gly (1:1) and this was added to the substrates to initiate the reaction. The effect of DES (ChCl:Gly) on product formation was monitored for alcohols of different chain lengths by TLC (see Fig. 5A). In this arrangement, *Sgβgl* produced a single product with no apparent interference from the DES components. Butanol was found to give the greatest yield, echoing previous studies [52]. Previous researchers reported a lower yield of alkyl glycosides for longer-chain alcohols [53–56]. Fig. 5B shows the dramatic effect of DES as a co-solvent in enhancing product yield. In this experiment, acetonitrile was used as the comparison co-solvent since it was known to facilitate superior alkyl glucoside synthesis with *Sgβgl* [34]. It was clear that DES was better than acetonitrile and aqueous solution alone for butyl glucoside synthesis using *Sgβgl*.

The effect of temperature on this synthesis of butyl glucoside is shown in Fig. 6A. The synthesis reaction is sustained up to 60 °C. However, at 70 °C synthesis had declined and mirrored the thermostability of *Sgβgl* in DES (see Fig. 6A). Having established that 60 °C provided a detectable product yield, the effect of reaction duration was explored at this temperature. Fig. 6B shows the formation of butyl glucoside as a function of time.

The final product was analysed by UHPLC (Fig. 7A) and Mass Spectroscopy (Fig. 7B) to confirm identity.



Meas. m/z	#	Ion Formula	m/z	err [mDa]	err [ppm]	rdb	N-Rule	e⁻ Conf	mSigma
235.118906	1	C10H19O6	235.118712	-0.2	-0.8	1.5	ok	even	3.5

Fig. 7. Commercial LC-MS analysis of the synthesised butyl glucoside with a molecular mass of 235.1147. The inset depicts the UHPLC analysis of synthesised butyl glucoside: the reaction mixture of butyl glucoside showing unreacted glucose with a RT:9.991, and butyl glucoside with a RT:21.286.

3.5. General discussion

In this study, initial observations demonstrated that the deep eutectic solvents containing choline chloride and glycerol were most benign in supporting enzyme activity. Conversely, DES containing both urea and glucose were noted as inhibitory. This is not surprising since product inhibition is common for glucosidases and has been previously reported for this enzyme [38]. Urea is a known protein denaturant, although the surprising stability of enzymes in urea has been noted [45]. Interestingly, the presence of each DES tested had an inhibitory effect on *Sgβgl* activity at low concentration, but showed activation as DES concentration increased. At higher levels, above 40% (v/v), activity was again inhibited. This indicates a "sweet spot" where the DES has an optimum positive effect. It is difficult to interpret these observations in terms of a bulk solvent effect, since the DES in this case has it's greatest effect at only 40% (v/v). What is clear is that there is a tension between activation and inhibition of activity that seems to favour activation over a narrow DES concentration range. Glycerol is known to generally stabilise enzymes but, in the case of glucosidases, it might possibly inhibit by binding to the glucose site. However, this effect would not be expected to cause the observed activation with increasing glycerol levels. Choline chloride may be acting as an enzyme activator, but a model to explain these effects must account for the fact that it increases enzyme activity at low concentrations and subsequently inhibits activity at higher concentrations. It is possible that we are observing a mixture of two processes where activation exceeds inhibition at low DES levels. At higher levels activation is at a maximum and inhibition becomes more prominent.

The stabilisation in the presence of DES was striking. Thus, particularly at higher temperatures, the protective effect of DES inclusion was evident even for DES where the components were previously noted as inhibitory (ChCl:Urea). This suggests that the loss of activity in some DES is due to interference with catalysis, rather than enzyme denaturation, since the inhibition did not prevent stabilisation. Thus, the DES containing urea inhibited activity but still preserved thermostability (see Fig. 4).

When the effect of DES on alkyl glucoside synthesis was explored, *Sgβgl* was shown to readily synthesise alkyl glucosides in the presence of DES (ChCl/Gly). This synthesis initially increased with increasing temperatures but decreased at higher temperatures. Due to the commercial interest in butyl glycoside synthesis, some recent studies have sought to synthesise such compounds using chemoenzymatic routes. Most recently, an immobilised *Aspergillus oryzae* β-galactosidase was shown to catalyse the synthesis of butyl galactoside, a closely related compound, in a 3-phase system using acetone as co-solvent [58]. The conversion fraction of 0.98 mol mol^{-1} was greater than the present study, although requiring enzyme immobilisation and use of solvents. Furthermore, the higher temperatures used (over 50 °C) rapidly inactivated the enzyme. Synthesis of butyl galactoside by β-galactosidases from *A. oryzae* gave a conversion of 0.76 mol mol^{-1}, which is similar to the present study [58].

The mechanisms underlying the activating and stabilising effects of DES are not fully understood [1,3]. Activation and stabilisation are distinct and likely to be due to separate processes. Here, we suggest that the narrow range of activation seen for *Sgβgl* is as a result of competition between activation and inhibition, with inhibition becoming more prominent at higher DES concentrations. Recent experimental and molecular dynamics studies of *Candida antarctica* lipase B activation in DES showed that components of DES were directly taking part in catalysis by H-bonding with active site bound substrate [59]. A similar mechanism could apply for *Sgβgl*; it is perhaps not surprising given the high concentration of ionised species in DES that some effect on substrate ionisation might be observed. Thus, a direct interaction between DES components and substrate, active site residues or reaction intermediates might plausibly explain *Sgβgl* activation. It is also possible that, at higher levels, DES components might sterically hinder active site substrate access and cause the observed inhibition at DES (ChCl:Gly) levels over 40% (v/v).

Stabilisation of *Sgβgl* occurs at 40% (v/v) DES in aqueous buffer. It is well known that most enzymes in DES require a certain amount of water to support catalytic activity [1,3,6,60]. Surprisingly, studies have shown that the solvent nanostructure properties of DES are retained even when water is present as a co-solvent at levels up to 20% (v/v). At higher water levels, up to 50% (v/v), DES becomes the co-solvent and is present as clusters dispersed in an aqueous phase. Above 50% (v/v) water, the solution changes into an aqueous electrolyte-like mixture of DES components [60–62]. In the present case, stabilisation was observed at 60%

(v/v) water (40% v/v DES). At this level the individual DES components are likely to be largely dissociated. This suggests that the stabilisation of *Sgβgl* observed in this study is due to a direct interaction between DES components and the enzyme surface. Such an interaction was proposed for *Candida antarctica* lipase B which was stabilized by hydrogen (H)-bonding between surface amino residues of the enzyme and DES [59]. This interaction might prevent enzyme aggregation at elevated temperatures thereby enhancing thermal stability. Some researchers have referred to this stabilisation in DES as a type of coating or "supramolecular net" that protects the enzyme from unfavourable ionic interactions [41]. Such a coating may be adherent to the enzyme surface and retained even when the DES-treated enzyme is subsequently added to an organic solvent. Further experiments to fully understand DES effects on *Sgβgl* will be the focus of future work.

4. Conclusion

This work demonstrates that DES has a remarkable effect on *Sgβgl* in extending its stability and operating temperature range. As well as enhancing *Sgβgl* thermostability, DES (ChCh:Gly) was also shown to enhance the synthesis of butyl glucosides by reverse hydrolysis when used as a co-solvent. This enhanced thermostability along with the enhancement of synthetic activity in the presence of DES broadens the possible industrial applications of *Sgβgl*. DES offer a simple, effective and environmentally compatible means to enhance enzyme stability in processing environments. The use of DES as a substitute for, and/or an adjunct to, enzyme immobilisation or mutagenesis as a means of enhancing enzyme stability is worthy of further exploration.

Author contributions

All authors contributed substantially to this body of work, in the form of; Conceptualization, A.U., G.K.K., G.T.H. and B.J.R.; Data Curation, A.U.; Writing-Original Draft Preparation, A.U., G.K.K., G.T.H. and B.J.R.; Writing-Review & Editing, A.U., G.K.K., G.T.H. and B.J.R.; Supervision, G.K.K., G.T.H. and B.J.R.; Project Administration, B.J.R.; Funding Acquisition, B.J.R., G.T.H. and A.U.

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Declaration of competing interest

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

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