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Cutinase from Amycolatopsis mediterannei: marked activation and stabilisation in Deep Eutectic Solvents

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Abstract

Amycolatopsis mediterranei cutinase (AmCut) has potential biocatalytic applications in plastics degradation and ester synthesis. Deep Eutectic Solvents (DES) are next generation biodegradable solvents for biocatalysis. However, the behaviour of cutinase enzymes in DES is little studied. Herein, we examine the effect of selected DES, and their components, on AmCut activity and stability. Low amounts (10% v/v) of DES (choline chloride:glycerol; 1:1 mole ratio) caused striking activation of AmCut (over 2-fold). Further examination showed that the choline chloride component of DES caused the observed activation. This is the first report of activation of a cutinase by a small molecule. At higher concentrations (50% v/v), DES composed of a choline chloride with glycerol as hydrogen bond donor dramatically increased the thermostability of AmCut - the enzyme lost no activity after incubation at 50°C for 2 hours. The biotechnological utility and physiological relevance of choline chloride activation and stabilisation is discussed.

Keywords: Activation, *Amycolatopsis mediterannei,* Choline, Cutinase, Deep Eutectic Solvents, Stability.

1. Introduction

Cutinases and lipases are related α/β hydrolases that differ in their substrate specificity and structural features. A key difference between these enzymes is the presence of a lid structure covering the active site of lipases but not cutinases. In the case of lipases, the lid opens at a lipid/water interface to provide for enhanced substrate access and increased activity - a phenomenon known as interfacial activation. This type of conformational change is not a feature of classical cutinases and they do not exhibit interfacial activation. Indeed, the lack of a lid structure and the lack of interfacial activation are distinguishing characteristics of cutinases (see Nikolaivits et al., 2018: Chen et al., 2013; Nyyssölä, 2015). One exception is the report of a novel class of cutinase with a lid-covered active site which displays interfacial activation in the manner of a "true" lipase (Roussel et al., 2014).

Amycolatopsis mediterranei secretes an extracellular lipase-like enzyme (AmCut) that was shown to catalyse the synthesis of flavour esters (Dheeman et al., 2011). Structural studies revealed that AmCut had specificity for medium chain acyl moieties and lacked the lid structure found in "true" lipases. Therefore, it was classified as a cutinase (Tan et al., 2021). The open, surface-exposed, active site of cutinases has meant that they are often more appropriate than lipases for applications such as textile bioscouring, biomass processing, polyester hydrolysis, certain enantioselective synthesis reactions and plastics degradation (Martínez and Maicas, 2021; Su et al., 2018; Nyyssölä, 2015; Chen et al., 2013;). Recent studies in this laboratory showed that AmCut was capable of depolymerising certain plastics; polycaprolactone and polybutylene succinate but not polylactide, under mild conditions (Tan et al., 2021). The potential to use enzymes in plastics degradation is important since many of the existing and the emerging generation of biodegradable bioplastics are slow to degrade in landfill due to the lack of appropriate polyester degrading organisms in soils (Nikolaivits et

al., 2021; Kim et al., 2017). The activity and thermostability of AmCut is critical for these types of application.

Deep Eutectic Solvents (DES) are an emerging green chemistry medium for biocatalytic reactions. They are non-toxic, biodegradable, environmentally benign solvents. They are increasingly being explored as an alternative to commonly used toxic organic solvents. The effect of DES on enzyme activity is complex. They can activate or inhibit enzymes depending on their composition and concentration (Uhoraningoga et al., 2021; Mannu et al., 2021; Nascimento et al., 2019; Domínguez de María et al., 2018;). Many "true" lipases are activated in DES and the degree of activation reported varies widely, typically in the range 105% to 165% (but in one case 355%), depending on the enzyme and the nature of the DES employed (Elgharbawy et al., 2018; Kim et al., 2016).

In general, increasing the stability and activity of enzymes used in biocatalytic processes is desirable. Due to their commercial importance, immobilisation, enzyme entrapment and site directed mutagenesis have all been used to improve cutinase stability and activity (Martínez and Maicas, 2021; Chen et al., 2013; Nyyssölä, 2015). The activation of lipases in DES is well known and has been explained by some researchers as being due to enhanced mobility of the lid region (Shehata et al., 2020) and in other cases to the direct participation of DES components in catalysis via H-bonding with active site moieties (Nian et al., 2019). There is, however, a lack of data on the behaviour of cutinases, and indeed many other biotechnologically significant enzymes, in DES. In this study, the activity of a wildtype, recombinant, AmCut was examined in the presence of three different cholinium DES preparations. Choline chloride was used as hydrogen bond acceptor (HBA) and glycerol, urea and glucose were the hydrogen bond donors (HBD). A 200% activation of AmCut in the presence of 10% v/v cholinium DES based on glycerol as HBD was observed and attributed to the presence of choline chloride. DES was also responsible for the stabilisation of AmCut.

Thus, AmCut lost no activity after incubation at 50^0C for 2 hours in 50% DES (choline chloride: glycerol; 1:1 mole ratio). The activity enhancement and stabilisation seen in the presence of DES will pave the way for AmCut, and perhaps other cutinases, to be used in the presence of this DES in a range of biocatalytic processes. Moreover, AmCut will be a useful model for exploring the mechanism of DES/choline chloride activation since the influence of a lid structure can be discounted.

2. Material and Methods

All chemicals were obtained from Sigma Aldrich.

2.1 Enzyme purification: The isolation of recombinant *Amycolatopsis mediterannei* cutinase was carried out as previously described (Tan et al., 2021).

Standard activity assay: Lipase activity was assayed as described previously (Dheeman, et al., 2011) using 1.0mM paranitrophenol palmitate (*p*-NPP) as the substrate at 37°C in 50 mM phosphate buffer pH 7.5. Briefly, assays were carried out in a Greiner CELLSTAR® 96 well plate UV spectrophotometer. Each well contained 230µl final substrate solution and 20µl lipase solution. Upon mixing, the plate was incubated for 10 mins at 37°C. The absorbance of the reaction at 400 nm was used to monitor the release of *p*-nitrophenol. All experiments were performed in triplicate.

2.2 DES synthesis: The synthesis of ChCl based DES was carried out according to the procedure as outlined in Tan et al., 2021. Briefly, the choline chloride salt and hydrogen bond donors were mixed at the specific molar ratio in a beaker and heated to 100°C in a water bath with stirring until a clear, homogenous liquid was formed. After cooling, the synthesised DES were stored in a desiccator until required for use.

2.3 Temperature stability: Temperature stability was examined by adding DES to the buffer component of the assay mixture. An initial activity measurement of AmCut in the presence of

the DES was taken (t= 0 h). At the end of the incubation at 50°C the activity was again measured (t=2 h). The activity at t= 2 h was expressed as a percentage of activity at t= 0 h. *2.4 Data Analysis*: All experiments were carried out as independent triplicates and the differences between the mean of the reference and the samples were analysed using one-tailed t-test (paired two samples for mean). Samples with a statistically significant difference to the reference were marked with asterisks (**$p<0.05$).

3. Results and Discussion

The hydrogen bond donors employed were an alcohol (glycerol; ChCl:Gly), an amide (urea; ChCl:U), and a sugar (glucose; ChCl:Glu). The DES synthesised with glucose was highly viscous and it was necessary to add some water to the solvent for ease of handling. This effectively diluted the DES and it was used in this dilute form. The composition of the DES used, and the mole ratio of their components, is shown in Table1.

Table 1 here

Figure 1 shows the activity of AmCut in the presence of a low amount (10%v/v) of the three DES mixtures examined: ChCl:Gly, ChCl:U and ChCl:Glu.

Figure 1 here

It was apparent that the presence of the DES containing choline chloride and glycerol had a remarkable activating effect on this enzyme while that with glucose also had a significant but lesser effect probably due to the presence of higher amounts of water in the ChCl:Glu mixture (due to its dilution for ease of handling, see Table 1). The DES composed of choline chloride and glycerol *doubled* the AmCut activity. While lipases have been shown to be activated by DES this, to the best of our knowledge, is the first time such activation has been reported for a cutinase.

To examine this activation further, the DES components were tested alone. From Figure 2 it was evident that the activation effect for AmCut was due to choline chloride and not the other DES components. Glycerol and urea were inhibitory with urea reducing activity to approximately 25% of the control. Glucose had no effect on activity.

Figure 2 here

The inset of Figure 2 shows a direct comparison between choline chloride as a component of a ChCl:Gly DES versus choline chloride alone in solution (6%, w/v). It was observed that, in the ChCl:Gly (DES) formulation that AmCut activity was lower. This is probably due to the inhibitory effect of the glycerol hydrogen bond donor (Figure 2, main diagram). Of course, a hydrogen bond donor may be inhibitory at high concentrations (Urea) or, as in the case of glucose, have little effect on activity.

142 The concentration of 1.0M choline chloride corresponds to a solution of 14% w/v. To
143 explore this phenomenon further the concentration dependence of choline chloride activation
144 was examined (Figure 3).
145
146 **Figure 3 here**
147
148 Clearly, choline chloride was responsible for considerable activation which reached a
149 maximum at levels between 0.5 and 1.5M (7 and 21%).
150 Activation by choline chloride appeared to be a saturable process and the activity increase
151 was seen to level out at concentrations between 0.5and 1.0 M and reach almost 2.5 times the
152 activity of the control. This is a high level of activation for enzymes of this kind. The
153 activation of lipases is a well-known phenomenon particularly at oil/water interfaces and is
154 due to the opening of a lid domain covering the active site. In the case of AmCut, this is not a
155 contributing factor in its activation since it does not possess an active site lid. A recent study
156 of activation of *Candida antartica* B lipase (CALB) in DES (it showed an activation of
157 115%) concluded that hydrogen bonding with active site bound substrate was the factor
158 contributing to activation (Nian et al., 2019). The latter model therefore, is one possible
159 explanation for the activation seen with AmCut.
160 AmCut shares a high level of sequence similarity with two known plastic degrading enzymes:
161 a PETase from *Ideonella sakaiensis* (67% similarity) and *Thermobifida fusca* (TfCut2)
162 cutinase (77% similarity; see Tan et al., 2021). Given the similarity between these enzymes
163 and AmCut it is possible that choline chloride activation is a common feature of these related
164 cutinases.
165
166

3.1 Stabilisation of AmCut by DES

The effect of DES on the stability of AmCut was also studied at 50°C over a period of two hours. In this experiment, AmCut was added to a solution containing the appropriate DES and its activity was measured. The enzyme was assayed again after 2 hours at 50°C. AmCut in buffer, without DES, was shown to lose activity under such conditions showing a reduction to roughly 20% of initial activity (see Figure 4). Remarkably, the presence of DES significantly stabilised the enzyme and the stabilisation was greater at higher levels (50%) DES.

Figure 4 here

This degree of stabilisation is significant. In the absence of DES, approximately 80% of activity was lost following 2 hours incubation at 50°C. The presence of 10% v/v ChCl:Gly reduces this loss in activity to approximately 60% of its initial activity. When the level of DES was increased to 50% (v/v), no loss of activity was seen after 2 hours incubation at 50°C. Similar results were observed for ChCl:Glu; however, notably, ChCl:U did not have a stabilising effect on AmCut under these conditions.

The individual components of those DES that provided the stabilisation were subsequently examined for their stabilising effects on AmCut when incubated at 50°C for 2 hours (Figure 5).

Figure 5 here

It was clear that choline chloride (at 21% v/v or 1.5 M) had a pronounced effect on stabilisation of AmCut. These findings show the activation and stabilisation of AmCut in

DES due to the presence of the choline chloride moiety. To the best of our knowledge this is the first example where stabilisation by DES has been ascribed to choline chloride.

This is a preliminary study, confined to a single cutinase, and is limited in that extent. Nevertheless, it is of considerable interest that a cutinase can achieve an activated and stabilised state in Deep Eutectic Solvents due to the presence of choline chloride. Given the importance of such enzymes in a wide range of processing and chemoenzymatic processes this study shows that the inclusion of DES is a useful consideration where cutinase thermal stabilisation/activation is required.

3.2 General Discussion

The behaviour of enzymes in DES is complex with some enzymes being activated while others are inhibited depending, not only on the enzyme, but also on the DES employed (Uhoraningoga et al., 2021; Mannu et al., 2021; Nascimento et al., 2019). Several studies have shown that the DES nanostructure is retained when water is present as a co-solvent at levels up to 20% v/v. From 20% v/v up to 50% v/v, DES becomes a 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 (see e.g., Domínguez de María et al., 2019). In this work, activation was observed at 10% (v/v) DES. At this level, the individual DES components are likely to be fully dissociated and, therefore, their components must be causing the observed activation. This was confirmed by the observation that choline chloride caused the observed activation when used alone.

The inset of Figure 2 shows a direct comparison of choline chloride alone versus choline chloride as part of a DES at the same inclusion level (w/v). The hydrogen bond donor (glycerol) clearly serves to reduce overall AmCut activity. It is possible to make some observations from this data. The activation effect of the ChCl:Gly versus ChCl alone depends

on the hydrogen bond donor. From Figures 1 and 2, Urea is shown to be inhibitory. Glycerol is also somewhat inhibitory (Figure 2) and therefore, a DES with glucose as hydrogen bond donor would seem to be most appropriate for activation (albeit viscous and difficult to handle). There is also the question of inclusion level. At low % DES, the activation effect predominates but this is clearly a saturable process (see Figure 3). It is expected that inhibition by glycerol might increase at higher levels of DES (say >50%) while activation by choline chloride will reach a maximum. The net effect will be inhibitory at high DES inclusion levels. This data suggests that a DES of choline chloride and glucose might give the best overall performance (since glucose has no inhibitory effect) but clearly, there are a number of factors to consider. The choice of a DES will depend on factors such as enzyme stability, substrate solubility, inhibition by DES components, competing hydrolysis reactions and, of course, cost. Further studies are required to tease out these complex interactions.

It is difficult to explain the effect of choline chloride on both activity and stability and they may be separate processes. One possible explanation is that the binding of choline chloride triggers an AmCut conformational change to a form that is both higher in activity and stability. This could relate to its role *in vivo* where such a conformational change could enhance surface adhesion and binding to choline containing lipids, for example. Researchers have shown that the surface adhesion of cutinases greatly enhances their efficiency (Ribitsch and Guebitz, 2020). Further work is needed to understand this feature further.

The use of DES as an aid to tissue disruption when extracting bioactive compounds from natural sources is growing rapidly (Ivanović et al., 2020). This study shows that cutinase may be usefully employed as an adjunct to DES for this purpose to aid in the breakdown of biomass.

Given the considerable efforts undertaken to improve the activity and stability of cutinases via mutagenesis and immobilisation for specific applications, it will be of some interest that enhanced activity and stability may also be achieved by judicious choice of cosolvent. The use of choline chloride in conjunction with other means of stabilising cutinases such as glycosylation (Shirke et al., 2016) will extend the operating range of cutinases into new areas of application.

4. Conclusions

AmCut is activated and stabilised in certain Deep Eutectic Solvents, although not in all, due to the action of choline chloride. This activation will be useful in extending the operating range of this enzyme in biocatalytic applications. AmCut will serve as a useful model enzyme to examine the activation process. Finally, this study paves the way for the use of activated, stabilised AmCut in DES for biocatalysis and polyester degradation. Future studies will explore AmCut activation and its application in detail.

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Author Contributions:

YT; GTH; GKK; BJR: Conceptualization, Methodology. **YT**: Investigation. **YT:** Data curation, Writing. **GTH; GKK; BJR***:* Supervision. **GTH; GKK; BJR:** Writing- Reviewing and Editing.

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Figure Captions:

Figure 1: Effect of DES on the activity of AmCut. The enzyme was assayed at 37ºC in 50 mM phosphate buffer pH7.5. Relative activity (%) was determined by setting the activity of AmCut in buffer as the 100% reference. All percentages refer to v/v. Note: ChClGlu was highly viscous and diluted with 27% v/v water to allow for ease of handling (see Table 1). All percentages refer to v/v.

Figure 2: Effect of DES components on AmCut activity. AmCut was assayed at 37ºC in 50 mM phosphate buffer pH 7.5. Relative activity (%) was determined by setting the activity of AmCut in buffer as the 100% reference. The inset shows a direct comparison of ChCl as a component of DES (ChCl:Gly) at a level of 6% versus ChCl alone at 6% (w/v).

Figure 3: Effect of Choline Chloride (ChCl) concentration on the activity of AmCut. The enzyme was assayed using the standard *p*NPP assay in the presence of 0 to 1.5M of choline chloride at 37^0C in 50mM sodium phosphate buffer, pH 7.5. Relative activity was determined using the activity of AmCut in buffer as the 100% reference.

Figure 4: Effect of DESs on the stability of AmCut at 50ºC. The enzyme was incubated in phosphate buffer pH 7.5 containing appropriate DESs at 50ºC for 2 hrs and then assayed using the standard assay at 37ºC in pH7.5. The relative activity was calculated using the percentage of initial activity (t = 0h) of the sample and after the incubation (t = 2h). All percentages refer to v/v.

Figure 5: Effect of individual component of DES on AmCut activity at 50ºC. The enzyme was incubated in phosphate buffer (pH7.5) in the presence of DES components for 2 hrs and assayed using the standard assay at 37ºC. Relative activity was the percentage of initial activity (t = 0h) remaining after incubation (t = 2h).

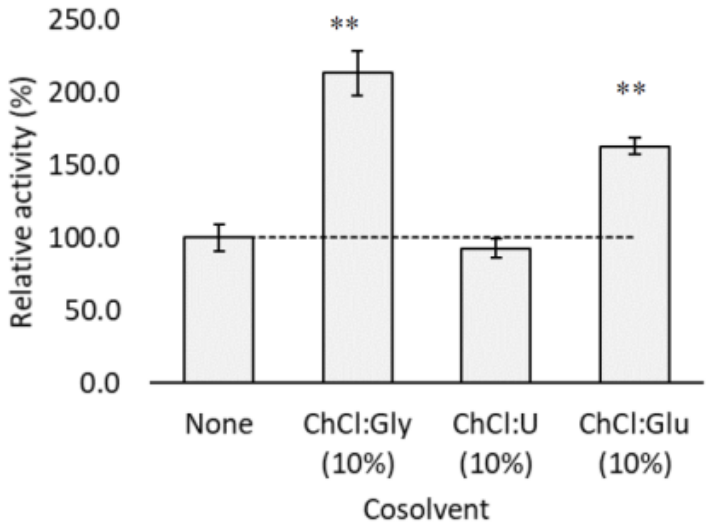
355 **Table 1**: Choline chloride-based DESs in this study with their components and mole ratios.

DES	Component	Mole ratio
ChCl:Gly	ChCl: glycerol	1: 1
ChCl:U	ChCl: urea	1: 2
ChCl:Glu	ChCl: glucose: water	1: 1: 0.75

356 ChCl: Choline chloride

357

358



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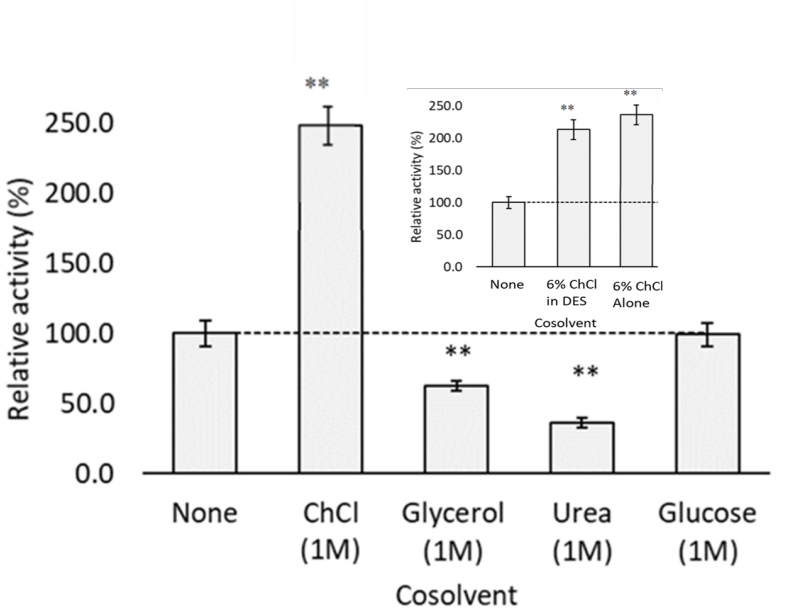

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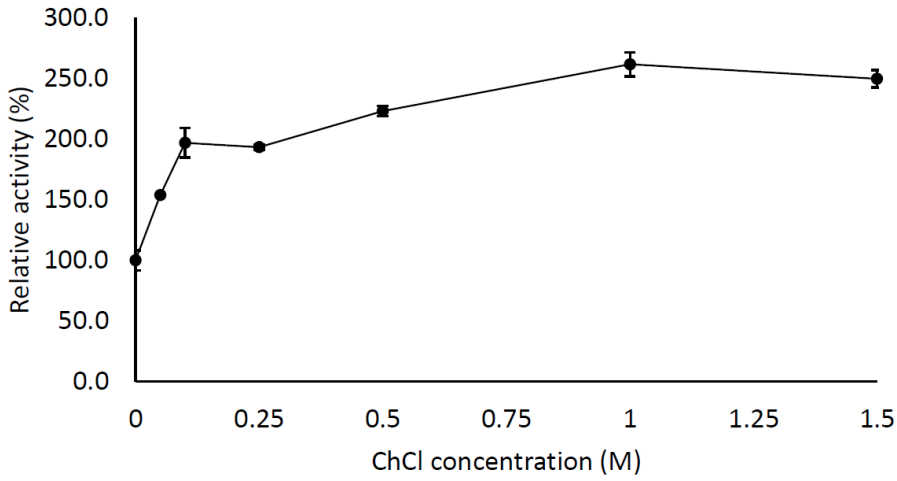

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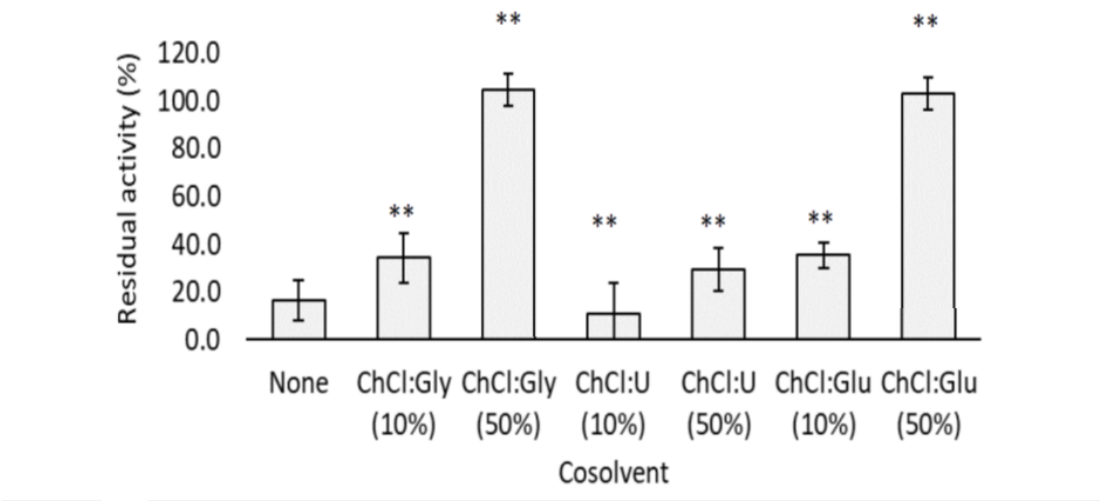

402

403 **Figure 4:** Effect of DESs on the stability of AmCut at 50ºC. The enzyme was incubated in phosphate buffer pH
404 7.5 containing appropriate DESs at 50ºC for 2 hrs and then assayed using the standard assay at 37ºC in pH7.5.
405 The relative activity was calculated using the percentage of initial activity (t = 0h) of the sample and after the
406 incubation (t = 2h). Note: ChClGlu was highly viscous and diluted with 27% v/v water to allow for ease of
407 handling (see Table 1). All percentages refer to v/v.

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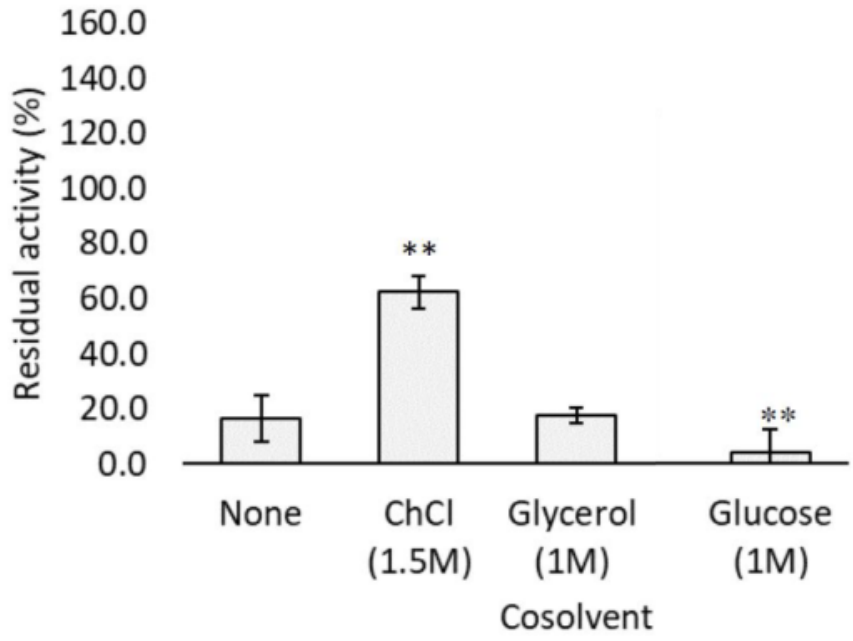

409

410 **Figure 5:** Effect of individual component of DES on AmCut activity at 50°C. The enzyme was incubated in
411 phosphate buffer (pH7.5) in the presence of DES components for 2 hrs and assayed using the standard assay at
412 37°C. Relative activity was the percentage of initial activity (t = 0h) remaining after incubation (t = 2h).

413