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

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

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8

9 ***Abstract***

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

22

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

25

26 **1. Introduction**

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

37

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

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

53 Deep Eutectic Solvents (DES) are an emerging green chemistry medium for biocatalytic
54 reactions. They are non-toxic, biodegradable, environmentally benign solvents. They are
55 increasingly being explored as an alternative to commonly used toxic organic solvents. The
56 effect of DES on enzyme activity is complex. They can activate or inhibit enzymes
57 depending on their composition and concentration (Uhoraningoga et al., 2021; Mannu et al.,
58 2021; Nascimento et al., 2019; Domínguez de María et al., 2018;). Many “true” lipases are
59 activated in DES and the degree of activation reported varies widely, typically in the range
60 105% to 165% (but in one case 355%), depending on the enzyme and the nature of the DES
61 employed (Elgharbawy et al., 2018; Kim et al., 2016).

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

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

82

83 **2. Material and Methods**

84 All chemicals were obtained from Sigma Aldrich.

85 *2.1 Enzyme purification:* The isolation of recombinant *Amycolatopsis mediterannei* cutinase
86 was carried out as previously described (Tan et al., 2021).

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

94 *2.2 DES synthesis:* The synthesis of ChCl based DES was carried out according to the
95 procedure as outlined in Tan et al., 2021. Briefly, the choline chloride salt and hydrogen bond
96 donors were mixed at the specific molar ratio in a beaker and heated to 100°C in a water bath
97 with stirring until a clear, homogenous liquid was formed. After cooling, the synthesised DES
98 were stored in a desiccator until required for use.

99 *2.3 Temperature stability:* Temperature stability was examined by adding DES to the buffer
100 component of the assay mixture. An initial activity measurement of AmCut in the presence of

101 the DES was taken (t= 0 h). At the end of the incubation at 50°C the activity was again
102 measured (t=2 h). The activity at t= 2 h was expressed as a percentage of activity at t= 0 h.

103 *2.4 Data Analysis:* All experiments were carried out as independent triplicates and the
104 differences between the mean of the reference and the samples were analysed using one-
105 tailed t-test (paired two samples for mean). Samples with a statistically significant difference
106 to the reference were marked with asterisks (**p<0.05).

107

108 **3. Results and Discussion**

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

114

115 **Table 1 here**

116

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

119

120 **Figure 1 here**

121

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

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

133

134 **Figure 2 here**

135

136 The inset of Figure 2 shows a direct comparison between choline chloride as a component of
137 a ChCl:Gly DES versus choline chloride alone in solution (6%, w/v). It was observed that, in
138 the ChCl:Gly (DES) formulation that AmCut activity was lower. This is probably due to the
139 inhibitory effect of the glycerol hydrogen bond donor (Figure 2, main diagram). Of course, a
140 hydrogen bond donor may be inhibitory at high concentrations (Urea) or, as in the case of
141 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 0.5 and 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 antarctica* 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

167 *3.1 Stabilisation of AmCut by DES*

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

175

176 **Figure 4 here**

177

178 This degree of stabilisation is significant. In the absence of DES, approximately 80% of
179 activity was lost following 2 hours incubation at 50°C. The presence of 10% v/v ChCl:Gly
180 reduces this loss in activity to approximately 60% of its initial activity. When the level of
181 DES was increased to 50% (v/v), no loss of activity was seen after 2 hours incubation at
182 50°C. Similar results were observed for ChCl:Glu; however, notably, ChCl:U did not have a
183 stabilising effect on AmCut under these conditions.

184 The individual components of those DES that provided the stabilisation were subsequently
185 examined for their stabilising effects on AmCut when incubated at 50°C for 2 hours (Figure
186 5).

187

188 **Figure 5 here**

189

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

192 DES due to the presence of the choline chloride moiety. To the best of our knowledge this is
193 the first example where stabilisation by DES has been ascribed to choline chloride.
194 This is a preliminary study, confined to a single cutinase, and is limited in that extent.
195 Nevertheless, it is of considerable interest that a cutinase can achieve an activated and
196 stabilised state in Deep Eutectic Solvents due to the presence of choline chloride. Given the
197 importance of such enzymes in a wide range of processing and chemoenzymatic processes
198 this study shows that the inclusion of DES is a useful consideration where cutinase thermal
199 stabilisation/activation is required.

200

201 *3.2 General Discussion*

202 The behaviour of enzymes in DES is complex with some enzymes being activated while
203 others are inhibited depending, not only on the enzyme, but also on the DES employed
204 (Uhoraningoga et al., 2021; Mannu et al., 2021; Nascimento et al., 2019). Several studies
205 have shown that the DES nanostructure is retained when water is present as a co-solvent at
206 levels up to 20% v/v. From 20% v/v up to 50% v/v, DES becomes a co-solvent and is present
207 as clusters dispersed in an aqueous phase. Above 50% v/v water, the solution changes into an
208 aqueous electrolyte-like mixture of DES components (see e.g., Domínguez de María et al.,
209 2019). In this work, activation was observed at 10% (v/v) DES. At this level, the individual
210 DES components are likely to be fully dissociated and, therefore, their components must be
211 causing the observed activation. This was confirmed by the observation that choline chloride
212 caused the observed activation when used alone.

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

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

229

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

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

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

247

248 **4. Conclusions**

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

255

256

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260 **Author Contributions:**

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

264

265

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333 **Figure Captions:**

334

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

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

343 **Figure 3:** Effect of Choline Chloride (ChCl) concentration on the activity of AmCut. The enzyme
344 was assayed using the standard *p*NPP assay in the presence of 0 to 1.5M of choline chloride at 37°C in
345 50mM sodium phosphate buffer, pH 7.5. Relative activity was determined using the activity of
346 AmCut in buffer as the 100% reference.

347 **Figure 4:** Effect of DESs on the stability of AmCut at 50°C. The enzyme was incubated in phosphate
348 buffer pH 7.5 containing appropriate DESs at 50°C for 2 hrs and then assayed using the standard
349 assay at 37°C in pH7.5. The relative activity was calculated using the percentage of initial activity (t =
350 0h) of the sample and after the incubation (t = 2h). All percentages refer to v/v.

351 **Figure 5:** Effect of individual component of DES on AmCut activity at 50°C. The enzyme was
352 incubated in phosphate buffer (pH7.5) in the presence of DES components for 2 hrs and assayed using
353 the standard assay at 37°C. Relative activity was the percentage of initial activity (t = 0h) remaining
354 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

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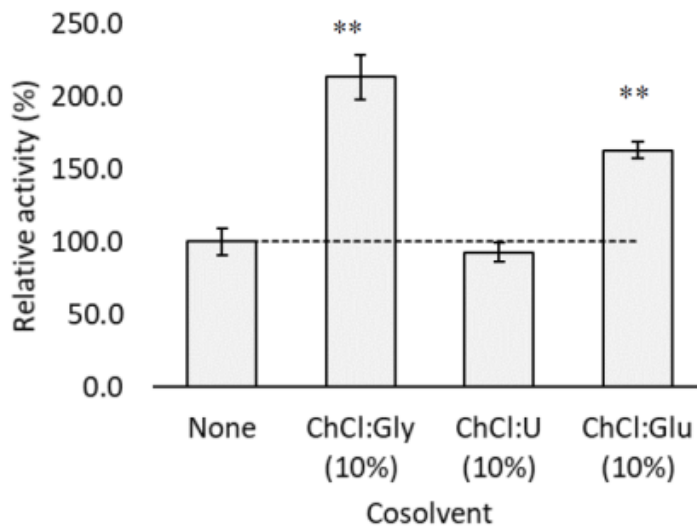
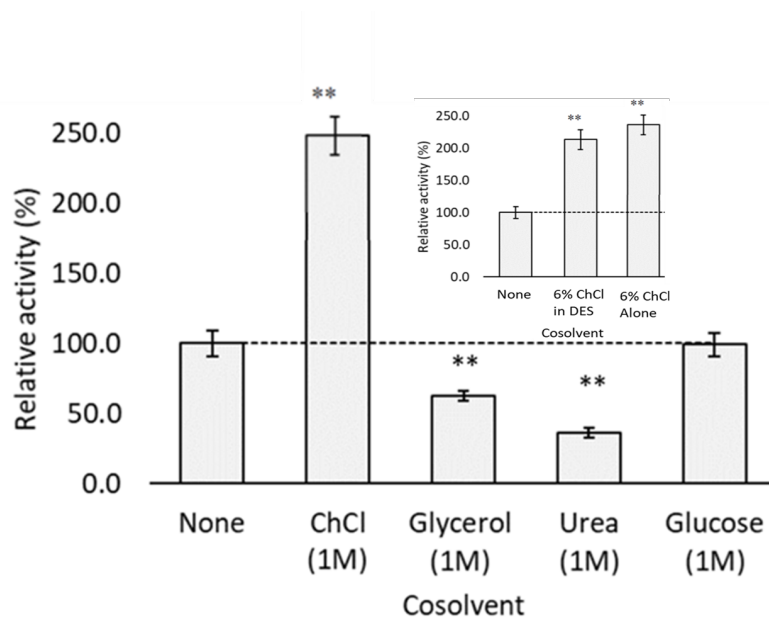


Figure 1: Effect of DES on the activity of AmCut. The enzyme was assayed at 37°C in 50 mM phosphate buffer pH7.5. Relative activity (%) was determined by setting the activity of AmCut in buffer as the 100% reference. All percentages refer to v/v. Note: ChClGlu was highly viscous and diluted with 27%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

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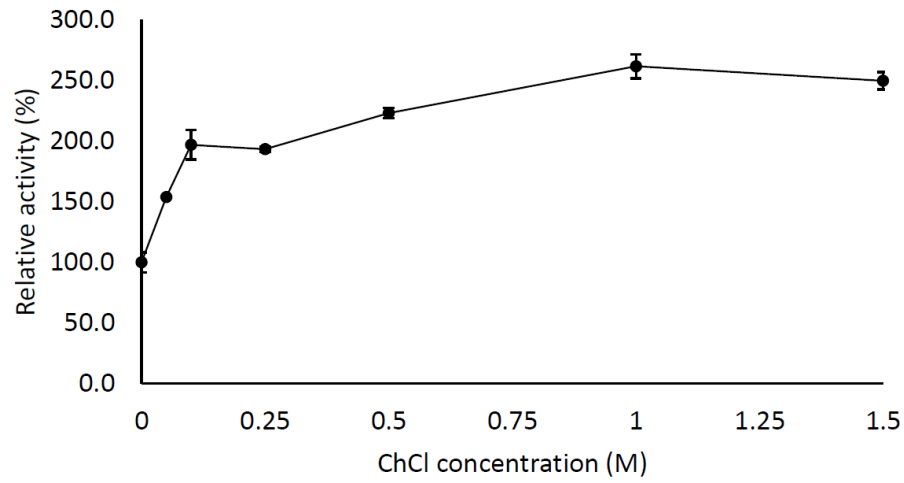


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379 **Figure 2:** Effect of DES components on AmCut activity. AmCut was assayed at 37°C in 50mM phosphate
380 buffer pH 7.5. Relative activity (%) was determined by setting the activity of AmCut in buffer as the 100%
381 reference. The inset shows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

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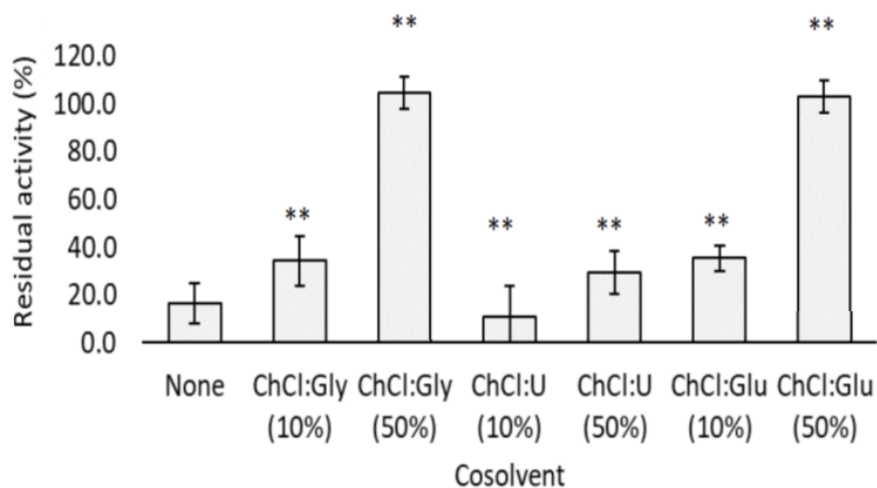
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Figure 3: Effect of Choline Chloride (ChCl) concentration on the activity of AmCut. The enzyme was assayed using the standard *p*NPP assay in the presence of 0 to 1.5M of choline chloride at 37°C in 50mM sodium phosphate buffer, pH 7.5. Relative activity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

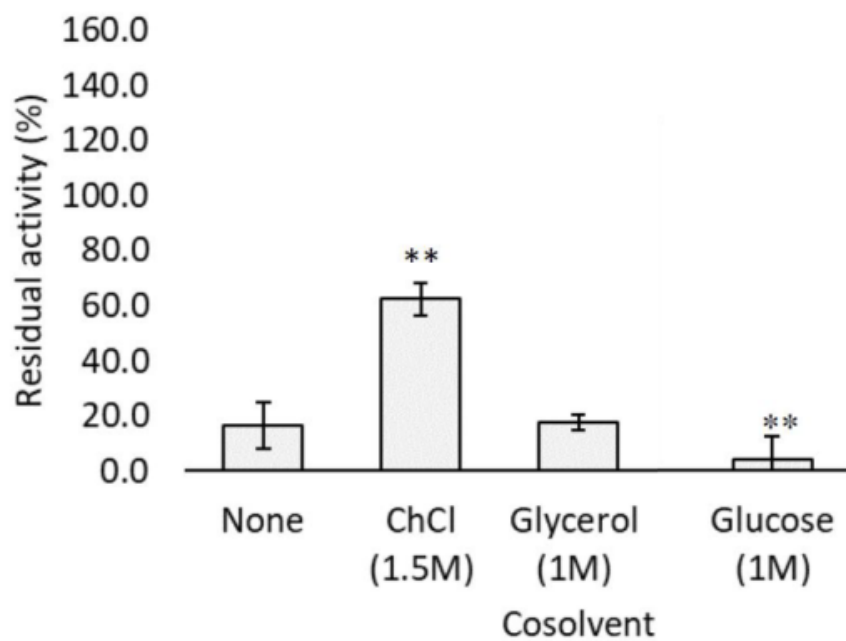
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403 **Figure 4:** Effect of DESs on the stability of AmCut at 50°C. The enzyme was incubated in phosphate buffer pH
 404 7.5 containing appropriate DESs at 50°C for 2 hrs and then assayed using the standard assay at 37°C in pH7.5.
 405 The relative 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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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).

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