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1 **STABILIZING EFFECTS OF CATIONS ON LIPASES DEPEND ON THE**
2 **IMMOBILIZATION PROTOCOL**

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27 **Abstract**

28 The effect of an additive on enzyme stability use to be considered an intrinsic feature of
29 the lipase. However, in this paper we have found that the effect of additive on enzyme stability
30 is depended on the immobilization protocol. After assaying the effects of diverse chloride salts
31 with different cations on different lipases activity, no relevant effect was detected. Free enzymes
32 or the covalently immobilized enzymes are not stabilized by these cations for any of the studied
33 lipases. However, Mn^{2+} and Ca^{2+} (at a concentration of 5 mM) are able to greatly stabilize the
34 lipases from *Rhizomucor miehei* (RML) and *Candida rugosa* (CRL) when they are present
35 during the inactivation, but only if the enzymes are immobilized on octyl-agarose (stabilization
36 factor ranging from 20 to 50). The effect was only detected using more than 2.5 mM of the
37 cations, and reached the maximum value at 5 mM, suggesting a saturation mechanism of action.
38 The stabilization seemed to be based on a specific mechanism, and required to have the
39 recognition sites saturated by the cations. Mg^{2+} has no effect on enzyme stability for both
40 enzymes, but it is able to suppress the stabilization promoted by the other two cations using
41 CRL; while it has no effect on the cation stabilization when using RML.

42 This is the first report of a cation induced enzyme stabilization effect that depends on the
43 lipase immobilization protocol.

44

45 **Key words:** Lipase stabilization, octyl-agarose, interfacial activation, additives, cations.

46

47 1.- Introduction

48 Lipases are very promising catalysts, with the capability to catalyze a broad range of
49 reactions ranging from ester hydrolysis to amide synthesis¹⁻⁷, lipases are also able to catalyze
50 some promiscuous reactions unrelated to their biological activity and even to their active center
51⁸⁻¹². Lipases are robust in different reaction media, do not require any cofactor and admit many
52 different substrates while exhibiting a high enantio or regio specificity and selectivity¹⁻⁷.

53 The improvement of enzyme stability is always a way to enhance the handling of the any
54 enzyme, lipases included. An enhanced lipase stability also increases the range of conditions
55 where the lipase may be used and the operational lifetime of the biocatalyst^{13,14}. Lipase stability
56 improvement may be achieved via genetic tools (site-directed mutagenesis or directed evolution)
57¹⁵⁻¹⁸, chemical modification or immobilization¹⁹⁻²⁵, or the use of some stabilizing additives²⁶⁻
58²⁹. The use of additives presents some economical and process development drawbacks if used
59 during the final industrial reaction catalyzed by the lipase; they need to be added during each
60 reaction cycle and may complicate the final reaction product purification. These problems are
61 lower during lipase handling, and in fact most industrial soluble enzyme preparations are
62 stabilized by mixtures of different compounds, e.g., saccharides of different nature³⁰⁻³².
63 Immobilization of enzymes is performed in many instances in the presence of inhibitors or other
64 enzyme stabilizers to avoid the loss of activity associated in some instances to the different steps
65 of an immobilization protocol³³⁻³⁵.

66 Cations may be very simple and cheap additives. The effect of cations on enzyme
67 activity is systematically analyzed in the characterization of new enzymes in many instances³⁶⁻
68³⁹. However, the effect of cations on enzyme stability, mainly if the actions lack of effect on
69 enzyme activity, is not usually considered, although in some cases the cation effects are quite
70 relevant⁴⁰⁻⁴³. Ions may be very relevant to stabilize the enzyme surface ionic net, forming some
71 ionic crosslinking on specific areas or stabilizing concrete areas by ionic bridges. There are

72 many reports on enzymes that are stabilized by some specific cations, mainly multimeric
73 proteins where the cation plays a critical role in stabilizing the multimeric structure^{44,45}, and
74 other cases where a stabilization is achieved by the use of a high ionic strength, independently of
75 the ions used. Nevertheless, it may be assumed that if a low concentration (e.g., 5 mM) of an ion
76 has a great positive effect on enzyme stability, this may be used under any circumstance without
77 great problem.

78 Moreover, the stabilization effect of adequate cations may be coupled to the
79 stabilization achieved via other strategies, like immobilization. In fact, in some instances the
80 role of an ion on enzyme stability could only be disclosed when some inactivation causes were
81 eliminated via immobilization (e.g., preventing multimeric enzyme subunit dissociation)⁴⁶.

82 On the other hand, lipases exist in two different forms. The closed form has the active
83 center isolated from the environment by a polypeptide chain, called lid or flat. The lid has a very
84 hydrophobic internal face that interacts with the hydrophobic areas surrounding the active
85 center. In the open and active form of lipases, the lid moves and exposes the active center to the
86 medium. This open form tends to become adsorbed on hydrophobic surfaces and become
87 stabilized (the so-called interfacial activation)⁴⁷⁻⁵⁰. Thus, it is likely that the effect of the cations
88 may differ depending on the use of a lipase interfacially activated versus a hydrophobic surface
89 or lipase formulations where the conformational equilibrium exists. Free enzyme always has the
90 risk of precipitation in the presence of multivalent cations, where lipases also have a tendency to
91 form bimolecular aggregates^{51,52} and conclusions may be difficult to reach with these enzyme
92 formulations.

93 In this paper, the effect of different cations on the stability of 3 different preparations
94 of two of the most popular lipases, those from *Rhizomucor miehei* (RML)^{53,54} and from
95 *Candida rugosa* (CRL)^{55,56} have been studied.

96 The effects of the cations on the free enzymes, the enzymes covalently immobilized,
97 where the conformational equilibrium is maintained, and on octyl-agarose, where the open form
98 of the lipases becomes stabilized⁵⁷ have been analyzed. The main objective of the study is too
99 analyze if the effect of an additive is an inherent property of an enzyme, or may be greatly
100 modulated if the immobilization protocol produce different enzyme conformations, as in the
101 current research effort.

102

103

104 2.- MATERIALS AND METHODS

105

106 2.1. Materials

107 Solutions of lipases from *Candida antarctica* (isoform A) (9.5mg of protein /mL)
108 (CALA), *Thermomyces lanuginosus* (TLL) (36 mg of protein /mL), Lecitase (16 mg of protein
109 /mL) and RML (13.7 mg of protein /mL) were a kind gift from Novozymes (Spain). Cyanogen
110 bromide crosslinked 4 % agarose (BrCN) beads and octyl-agarose beads were from GE
111 Healthcare. *p*-Nitrophenyl butyrate (*p*-NPB), diethyl *p*-nitrophenylphosphate (D-*p*NPP) and
112 CRL (in powder form, 40% of protein content) were from Sigma Chemical Co. (St. Louis, MO,
113 USA). All reagents and solvents were of analytical grade. All experiments were performed by
114 triplicate and the results are given as an average with the standard deviation.

115

116 2.2. Determination of enzyme activity

117 This assay was performed by measuring the increase in absorbance at 348 nm produced
118 by the released *p*-nitrophenol in the hydrolysis of 0.4 mM *p*-NPB in 25 mM sodium phosphate
119 at pH 7.0 and 25 °C (ϵ under these conditions is $5150 \text{ M}^{-1} \text{ cm}^{-1}$). To start the reaction, 50–100
120 μL of lipase solution or suspension were added to 2.5 mL of substrate solution. One
121 international unit of activity (U) was defined as the amount of enzyme that hydrolyzes 1 μmol of
122 *p*-NPB per minute under the conditions described previously. Only to assess the effect of the
123 cations on enzyme activity, different salts (5 mM) were added to check the effect on the enzyme
124 activity. Protein concentration was determined using Bradford's method⁵⁸ and bovine serum
125 albumin was used as the reference.

126

127 2.3. Immobilization of enzymes

128 The enzymes were immobilized under conventional conditions; the cations used in this
129 study were not added in this step.

130

131 2.3.1 Immobilization of enzymes on BrCN support

132 5 g of BrCN agarose beads was added to 50 mL of an enzyme solution (5 mg of
133 protein/g of wet support) in 25 mM sodium phosphate at pH 7 containing 0.1% (v/v) Triton X-
134 100 to have the monomeric form of the lipases^{52,59}. After 90 min at 4°C under gentle stirring,
135 the biocatalysts were washed with distilled water and incubated in a solution of 1 M
136 ethanolamine at pH 8 for 2 h to block the remaining reactive groups in the support. Finally, the
137 immobilized preparations were washed with abundant distilled water and stored at 4°C. The
138 biocatalysts thus prepared have an immobilization yield of 50% maintaining almost intact the
139 activity (as has been previously described in other examples using this support).⁵¹

140

141 2.3.2 Immobilization of enzymes on octyl (OC) supports

142 The immobilization was performed using 1 mg of protein per g of wet support. The
143 commercial samples of the enzymes were diluted or dissolved in the corresponding volume of 5
144 mM sodium phosphate at pH 7. Then, the OC support was added (10 g of support in 100 ml of
145 enzyme solution). The activity of both supernatant and suspension was followed using *p*-NPB.
146 After immobilization, the suspension was filtered and the supported enzyme was washed several
147 times with distilled water and stored at 4°C. The enzyme activity increased after immobilization
148 except in the case of CAL, as has been previously described^{53,57, 60-64}

149

150 2.4. Thermal inactivation of different enzyme preparations

151 This experiment was performed to determine the effect of the cations on the enzyme
152 stability. Free enzyme or 1 g of immobilized lipases were suspended in 10 mL of 5 mM of
153 sodium acetate at pH 5 or Tris buffer at pH 7 at different temperatures, containing different
154 concentrations of different salts. Periodically, samples were withdrawn and the activity was
155 measured using *p*-NPB. Half-lives were calculated from the observed inactivation courses.

156

157 2.5. Hydrolysis of R and S methyl mandelate

158 This experiment was performed to determine the effect of the cations on the enzyme
159 activity and specificity versus complex substrates of chiral nature. 50 mM solutions of R or S
160 methyl mandelate in 100 mM glycine at pH 7 containing or not 5 mM of CaCl₂ or MnCl₂ were
161 prepared. Reaction was initialized by adding 1 g of the lipase preparations in 5 mL of the
162 substrate solution. The suspension was maintained at 25°C under continuous stirring. The
163 reaction was followed by RP-HPLC (Spectra Physic SP 100 coupled with an UV detector
164 Spectra Physic SP 8450) using a Kromasil C18 (15 cm × 0.46 cm) column. Samples (20 µL)
165 were injected and eluted at a flow rate of 1.5 mL/min using acetonitrile/10 mM ammonium
166 acetate (35:65, v/v) at pH 2.8 as mobile phase and UV detection was performed at 230 nm. The
167 retention times of the acid was 3 min and of the ester was 7 min. The enzyme activity is given in
168 µmol of mandelic acid per minute under the conditions described above. Activity was
169 determined by triplicate, using a maximum conversion of 20–30%. The data are given as average
170 values

171

172 2.6. Irreversible inactivation of immobilized lipases by D-pNPP

173 Different lipase-immobilized preparations (1g) were suspended in 10 mL of different
174 concentrations of sodium phosphate buffer (from 10 mM to 1 M) solution at pH 7 and 25°C.
175 Then, D-*p*NPP was added up to a concentration of 1 mM. Samples of this suspension were
176 withdrawn periodically, and their activities were checked using the *p*-NPB assay.

177 3.-Results

178

179 3.1.- Effect of some cations on the stability of different lipase preparations.

180 Free and different immobilized preparations of some enzymes were inactivated at
181 several T and pH 7 in the presence of diverse chloride salts. OC preparations of TLL, CALB
182 and Lecitase kept their stabilities almost unaltered in the presence of the whole set of assayed
183 cations (Na^+ , K^+ , Mg^{2+} , Mn^{2+} , Ca^{2+}) (Fig 1 S).

184 Using RML and CRL results are quite surprising. While both free enzymes were not
185 stabilized by any of the cations (in fact the inactivation was slightly more rapid in presence of
186 the cations using RML and slight slower using CRL), the stabilization effect is very relevant
187 using the octyl preparations when 5 mM of Mn^{2+} or Ca^{2+} were present in the inactivation
188 suspensions (see Figures 1 and 2). OC-RML stabilization by both cations was really impressive,
189 when the enzyme inactivated in the absence of these cations were almost inactive, the cations
190 permitted the octyl preparations to retain over 90% of the activity (see Figure 1). The
191 stabilization observed with both cations were similar (OC-RML half-live improved around 50
192 folds using Mn^{2+} and 40 folds using Ca^{2+} . OC-CRL stabilization was slightly lower (25 folds
193 using Ca^{2+} and 30 folds using Mn^{2+}) (Figure 2). In any case, this stabilization permits a
194 qualitative jump: The enzyme in the presence of cations retained over 80% of the initial activity
195 while the reference had less than 20%.

196 To check if the lack of effect of the cations on the free enzyme was due to the
197 aggregation of the RML and CRL molecules in the presence of these bivalent cations, both
198 enzymes were covalently immobilized on BrCN. After immobilization, aggregation was
199 impossible, the mild covalent attachment still permitted the open/closed equilibrium, and the
200 effects of the cations should be directly reflected on enzyme stability. Using BrCN-RML
201 (Figure 1) or BrCN-CRL (Figure 2), both covalently immobilized enzyme preparations did not
202 become stabilized by any of these cations, results were even closer to the reference than using

203 the free enzyme. The inhibition of the free enzymes by D-*p*NPP was also very similar in
204 presence or absence of these salts, suggesting that the percentage of lipase open-closed
205 molecules remained unaltered.

206 The lack of effect of the cations on the stability of the free enzyme could be associated
207 to a promotion of enzyme aggregation, which could mask the stabilization effect. However, this
208 may not be the explanation using the covalently immobilized enzyme, where aggregation is no
209 longer possible. Thus, results suggested that the open forms of RML and CRL stabilized versus
210 the hydrophobic surface of octyl supports are the forms that are stabilized by Mn^{2+} or Ca^{2+} ,
211 while the non-absorbed lipases are not stabilized by them, even if the opening/closing equilibria
212 is possible. Stabilization using Mn^{2+} is slightly higher than using Ca^{2+} for both enzymes. The
213 lack of effect of Mg^{2+} suggested that the lipase stabilization produced by Mn^{2+} and Ca^{2+} may
214 follow a specific mechanism, perhaps involving adsorption of these cations on some specific
215 places of the enzyme surface.

216 To analyze if these stabilization effects could be extrapolated to other conditions, the
217 effect of these cations on the whole set of preparations of RML and CRL were assayed at pH 5.
218 Figures 3 and 4 show that the results were similar to those found at pH 7. Free RML inactivation
219 rate was accelerated in a clearer way at pH 5 than at pH 7 by the presence of all the cations
220 (Figure 3), while free CRL stability remained almost unaltered (Figure 4). BrCN-RML seemed
221 to be marginally stabilized by Mn^{2+} (a factor of lower than 2) (Figure 3), while BrCN-CRL
222 stability was not influenced by the studied cations (Figure 4). Again, a very clear stabilization
223 was observed using Mn^{2+} and Ca^{2+} for the OC preparations. Using OC-RML, stabilization was
224 lower than that detected at pH 7 (20 folds using Ca^{2+} and 25 folds using Mn^{2+}), while using
225 CRL stabilization seem to be very similar (28 times using Ca^{2+} and 32 folds using Mn^{2+} . At
226 alkaline pH values, both Ca^{2+} and Mn^{2+} presented a much reduced solubility; therefore we have
227 focused the study only in neutral and acid pH values.

228 The use of 5 mM of these cations during the activity assay did not promote any
229 relevant effect on the lipase activity on any of the different preparations and lipases assayed
230 (Table 1S). Thus, the main effect of the cations was just the improved stability of the octyl
231 preparation.

232 We further tried to characterize this stabilization effect.

233

234 **3.2.- Effect of the concentration of Mn²⁺ and Ca²⁺ on the stabilization effect**

235 The concentrations of both cations were varied from 0 to 25 mM at pH 7. For both
236 enzymes, the behavior was fairly similar with some differences. Figure 5 shows that at
237 concentrations of both cations of 1 mM or below, the stabilization effect were very small, at 2.5
238 mM the stabilization was 10-15 folds (except using Ca²⁺ and OC-RML), the increase of the
239 cation concentration to 5 mM permitted to greatly increase this stabilization effect while a
240 further increase of the cations concentrations to 25 mM produced a very slight increase in the
241 stabilizing effect. The highest increase in stability when going from 5 to 25 mM was using Mn²⁺
242 and OC-RML. In this case, the increase in stabilization factor was from 50 to 60. This result
243 suggests that the cation must saturate the stabilization place/places of the enzyme; otherwise the
244 stabilizing effect is not relevant. Moreover, after saturation of these sites, a further increase on
245 the cation concentration has no effect on the enzyme stability.

246 Next, we study the effect of the combined use of equimolecular mixtures of both
247 stabilizing cations. Using 5 mM of both cations, the results were very similar to the independent
248 use of each of the cations, for both enzymes (results not shown). These results suggest that the
249 effect of both cations were via the same mechanism and not additive. Using 2.5 mM of both
250 cations, where the theoretical site is not fully saturated with any of the cations, observed
251 stabilities were only marginally better than using the individual cations at 2.5 mM, and lower
252 than that obtained using 5 mM. Thus, 5 mM of Mn²⁺ or Ca²⁺ seemed to be necessary to take full
253 advantage of the stabilization produced by these cations.

254

255 3.3.- Effect of the concentration of Mg^{2+} on the stabilizing effects of Mn^{2+} and Ca^{2+}

256 Figure 6 shows the stabilizing effect of 5 mM of Mn^{2+} and Ca^{2+} in the presence of
257 growing concentrations of Mg^{2+} , the divalent cation that did not produce any stabilizing effect.
258 Using OC-CRL, the use of 5 mM de Mg^{2+} is able to fully suppress the stabilizing effect of both
259 cations at both pH values. However, the results are very different using OC-RML, where the
260 stability is almost unaffected in the presence of Mg^{2+} in the presence of 5 mM of the stabilizing
261 cations. This means that Mg^{2+} may compete for the site(s) where the stabilizing cations are
262 adsorbed using OC-CRL, suggesting that it may become adsorbed on the same place or in
263 another place that make the adsorption of Mn^{2+} and Ca^{2+} to the right place no longer possible
264 (by inducing some conformational change or blocking the entry of the other cations to the right
265 pocket). This hypothetic site of CRL will have an affinity higher than that of the enzyme for the
266 stabilizing cations. This cation did not modify the effect of the stabilizing cations using OC-
267 RML. Thus, or the Mg^{2+} cannot access to the RML sites where Mn^{2+} and Ca^{2+} have an
268 stabilizing role, or the affinity of these places of the enzyme by these cations is far higher than
269 that by Mg^{2+} (perhaps by this reason we have not found a stabilizing effect of this).

270

271 3.5.- Effect of the presence of cations in the specificity of RML and CRL.

272 It has been reported in many instances that the lipase specificity may be easily altered
273 by any strategy that may alter the final conformation of the enzyme, for example the
274 immobilization via different protocols⁶⁰⁻⁶². In the first section of this paper, we have showed
275 that the cations have not effect on the catalytic activity of the lipases in a very simple reaction,
276 the hydrolysis of pNPB. Now, the enzyme activity versus a more complex substrate is analyzed:
277 R or S methyl mandelate has been hydrolyzed using octyl-RML or CRL in presence or absence
278 of $CaCl_2$ or $MnCl_2$. This compound has an aromatic ring and a hydroxyl group in the alpha
279 position. Table 1 shows that neither RML nor CRL are greatly affected by these stabilizing

280 cations in their catalytic activities versus this substrates. This suggested that the stabilizing role
281 of these cations did not involve a conformational change of the octyl preparations, but just they
282 reinforce the overall enzyme structure.

283

284 **4.- Conclusion**

285 The immobilization protocol has been shown to have a critical role in the lipase
286 features. The same chemical modification or physical coating of differently immobilized
287 biocatalysts of lipases has been described to promote very different effects on the enzyme
288 properties, like selectivity or specificity^{51-54, 60-62}. However, this is the first report where a
289 simple additive, like a cation, is found to produce a high stabilization of only some immobilized
290 preparations of an enzyme and this effect is not found or is much less intense on other enzyme
291 preparations, including the free enzyme. This may be caused by the very different forms of the
292 two immobilized enzyme preparations: the OC-lipase has the open form adsorbed and stabilized
293 on the hydrophobic surface of OC supports⁵⁷, while BrCN immobilized lipase maintains the
294 equilibrium between closed and open conformation (non-stabilized by adsorption to a
295 hydrophobic surface). Thus, while studying the free enzyme, the researcher will deduce a lack of
296 stabilizing effect of Mn^{2+} or Ca^{2+} , correlated to a lack of effect on the enzyme activity and
297 specificity, using OC-immobilized enzymes the stabilization may be measured in dozens folds,
298 constituting a real qualitative stabilization. This becomes a new advantage of the immobilization
299 of these two enzymes on octyl supports⁵⁷.

300 Thus, the use of 5 mM of salts containing Mn^{2+} or Ca^{2+} may be used as a way to
301 greatly increase the enzyme stability in aqueous media during biocatalyst operation at pH values
302 ranging from 5 to 7; alkaline pH could not be analyzed due to problems in the salts solubility.
303 This low cation concentration may have a low economic impact used even during operation, and
304 in some cases may not be a great problem for the downstream of the final product. Moreover,
305 this low concentration of these cations may be used during storage and handling of the OC

306 immobilized RML or CRL, enlarging the range of conditions where the enzymes may be
307 utilized^{63,64}. This way, these results may open new possibilities for the use and handling of these
308 interesting immobilized enzymes. The mechanism of stabilization by cations of these OC-lipase
309 preparations require further studies to determine the real nature, perhaps using differential
310 scanning fluorimetry or by isothermal titration calorimetry.

311 From the results presented in this paper, it is more relevant that the researcher cannot
312 expect that a stabilizing (or perhaps a destabilizing) effect attributed to a particular compound is
313 an inherent feature of an enzyme in all their formulations. This makes necessary to perform the
314 study of stabilization/destabilization of an enzyme biocatalyst each time that the immobilization
315 protocol is changed. The results of this paper show that the extrapolation of one result from one
316 enzyme preparation to other preparation may be very risky.

317

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- 421

422 **Figure legends**

423 **Figure 1. Stability of different RML preparations in the presence of 5mM of different**
424 **chloride salts prepared in 5mM Tris HCl at pH 7.** Panel A: soluble RML, Panel B: OC-
425 RML, Panel C: BrCN-RML. The enzyme preparations were incubated at the indicated
426 temperatures: Soluble RML at 45°C, OC-RML at 45°C and BrCN-RML at 50°C. Other
427 specifications are described in section 2. Without addition of extra-cations (Pointed line),
428 Sodium (Stars), Potassium (Rhombus), Calcium (Triangles), Magnesium (Squares), Manganese
429 (Circles).

430 **Figure 2. Stability of different CRL preparations in the presence of 5mM of different**
431 **chloride salts prepared in 5mM Tris HCl at pH 7.** Panel A: soluble CRL, Panel B: OC-CRL,
432 Panel C: BrCN-CRL. The enzyme preparations were incubated at the indicated temperatures:
433 Soluble CRL at 50°C, OC-CRL at 60°C and BrCN-CRL at 55°C. Other specifications are
434 described in section 2. Without addition of extra-cation (Pointed line), Sodium (Stars),
435 Potassium (Rhombus), Calcium (Triangles), Magnesium (Squares), Manganese (Circles).

436 **Figure 3. Stability of different RML preparations in the presence of 5mM of different**
437 **chloride salts prepared in 5 mM sodium acetate at pH 5.** Panel A: soluble RML, Panel B:
438 OC-RML, Panel C: BrCN-RML. The enzyme preparations were incubated at the indicated:
439 Soluble RML at 45°C, OC-RML at 50°C and BrCN-RML at 65°C. Other specifications are
440 described in section 2. Without addition of extra-cation (Pointed line), Sodium (Stars),
441 Potassium (Rhombus), Calcium (Triangles), Magnesium (Squares), Manganese (Circles).

442 **Figure 4. Stability of different CRL preparations in the presence of 5mM of different**
443 **chloride salts prepared in 5 mM sodium acetate at pH 5.** Panel A: soluble CRL, Panel B:
444 OC-CRL, Panel C: BrCN-CRL. The enzyme preparations were incubated at indicated
445 temperatures: Soluble CRL at 50°C, OC-CRL at 58°C and BrCN-CRL at 60°C. Other
446 specifications are described in section 2. Without addition of extra-cation (Pointed line), Sodium

447 (Stars), Potassium (Rhombus), Calcium (Triangles), Magnesium (Squares), Manganese
448 (Circles).

449 **Figure 5. Effect of MnCl_2 and CaCl_2 concentrations on the stability of OC-RML (Pointed**
450 **line) and OC-CRL (Solid black line).** The unit of Relative Units is considered the half live of
451 the OC preparation in Tris without cations. The inactivation was performed in Tris HCL 5 mM
452 at pH 7 at the mentioned temperatures: RML assays were performed at 45°C and CRL assays at
453 60°C. Other specifications are described in section 2. CaCl_2 (Triangles), MnCl_2 (Circles).

454 **Figure 6. Effect of the concentration of MgCl_2 on the stabilizing effects of MnCl_2 and**
455 **CaCl_2 on OC-RML (Pointed line) and OC-CRL (Solid black line).** The unit of Relative
456 Units is considered the stability of the OC preparation in Tris without cations. Enzyme
457 preparations were incubated at specified temperatures: RML assays were performed at 45°C and
458 CRL at 60°C. Other specifications are described in section 2. CaCl_2 (Triangles), MnCl_2
459 (Circles).

460

461 **Table 1. Effect of different cations on the octyl-lipase activity versus R or S methyl**
 462 **mandelate.** Enzyme reactions were carried out using as substrate R or S methyl mandelate (50
 463 mM) at pH 7 and 25 °C as described in Section 2.

| Biocatalyst(cation) | V_R Methyl mandelate* | V_S Methyl mandelate* | V_R / V_S |
|-----------------------------|--|--|--------------------------------------|
| OC RML (none) | 6.5 ± 0.32 | 7.0 ± 0.35 | 0.93 |
| OC RML (CaCl ₂) | 6.8 ± 0.34 | 6.1 ± 0.31 | 1.11 |
| OC RML (MnCl ₂) | 7.2 ± 0.36 | 7.1 ± 0.36 | 1.01 |
| OC CRL (none) | 0.5 ± 0.03 | 1.4 ± 0.07 | 0.36 |
| OC CRL (CaCl ₂) | 0.6 ± 0.03 | 1.3 ± 0.08 | 0.46 |
| OC CRL (MnCl ₂) | 0.6 ± 0.03 | 1.4 ± 0.06 | 0.43 |

464 *Activity (x10³)

465

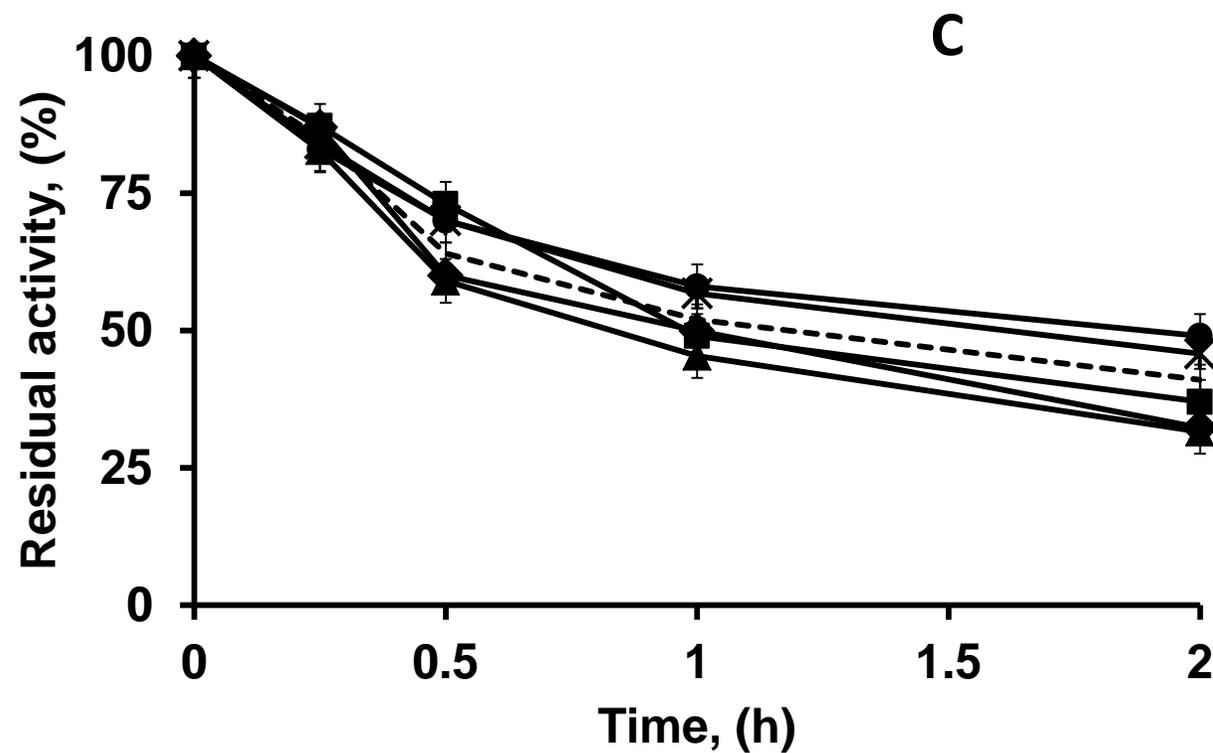
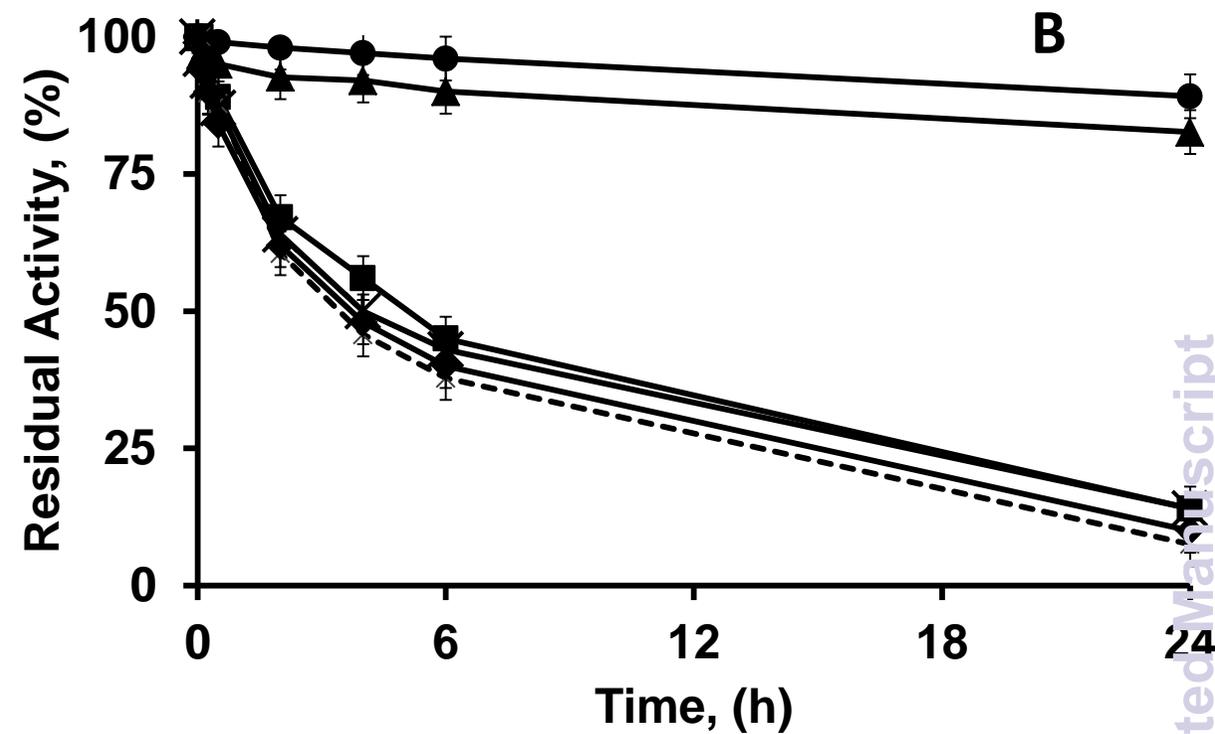
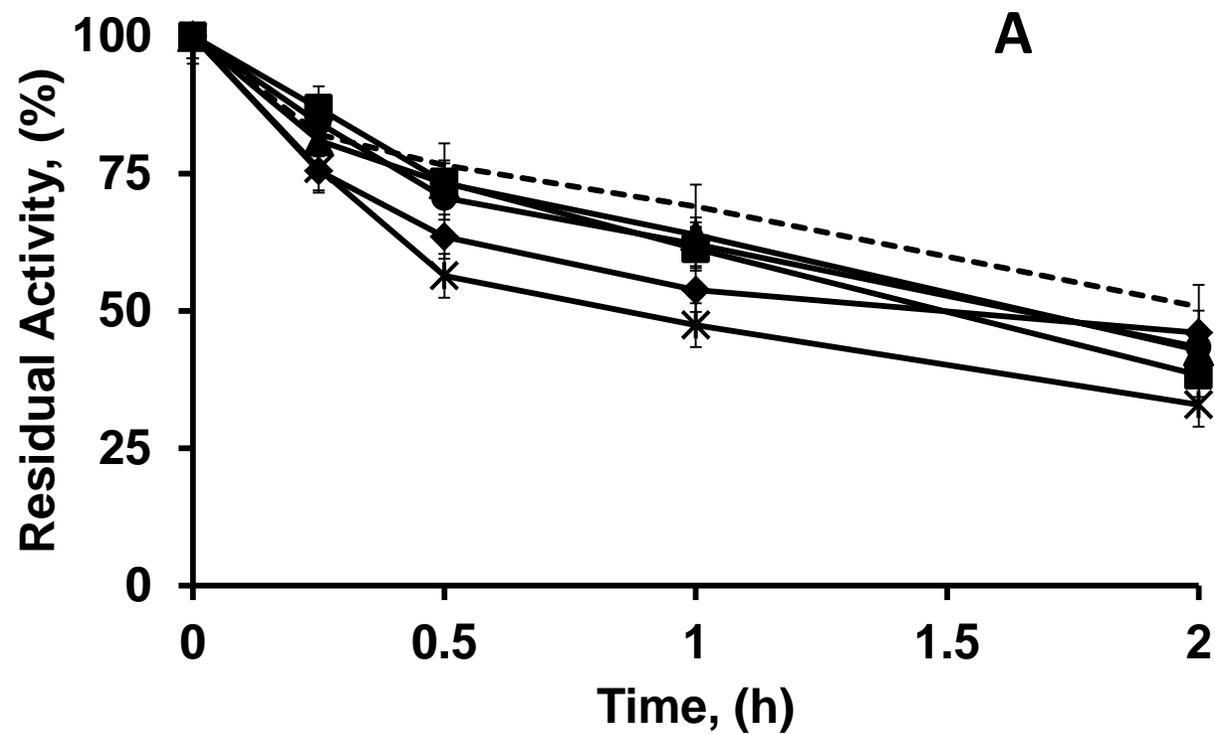


Figure 1

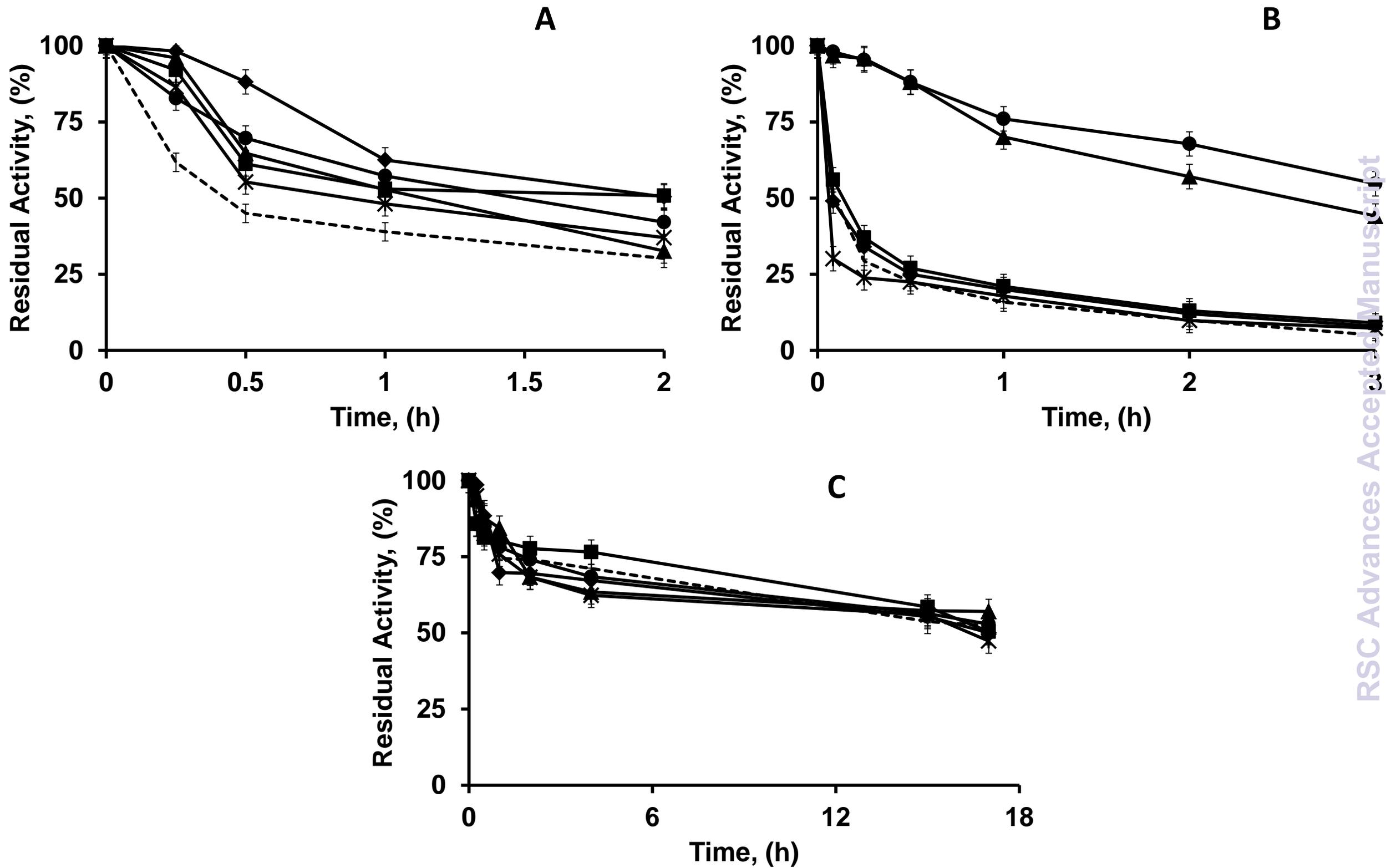


Figure 2

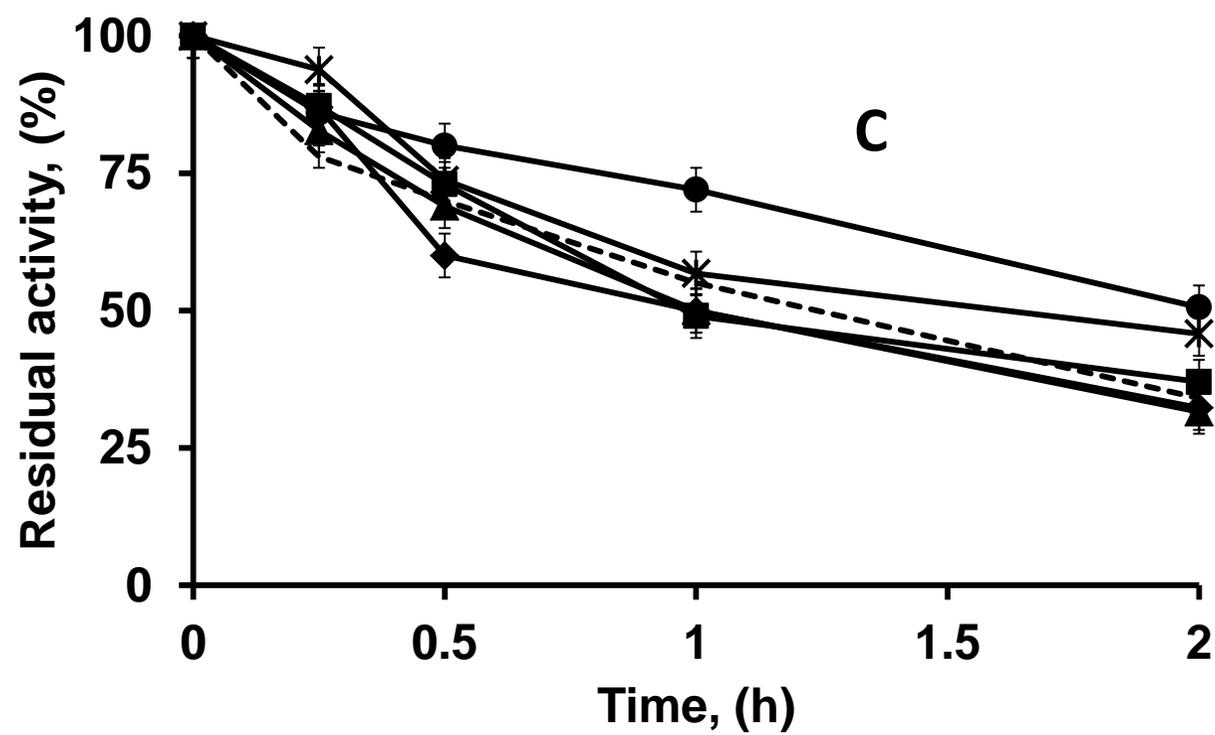
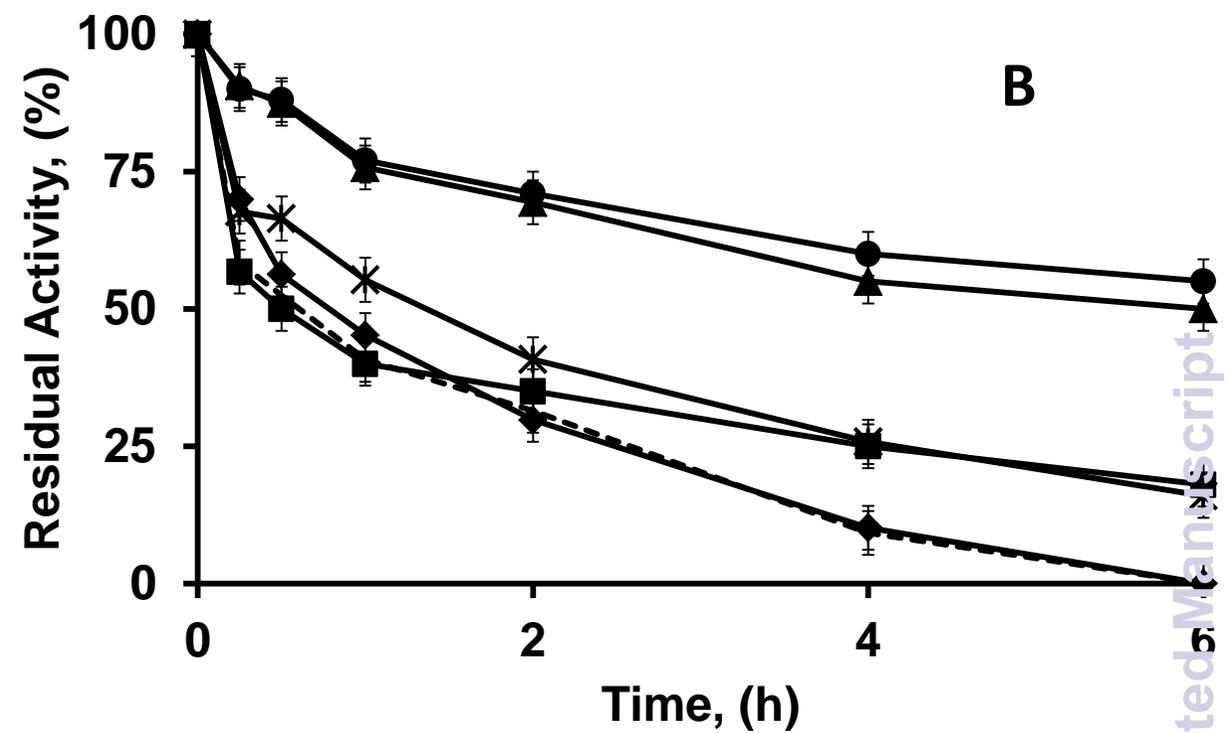
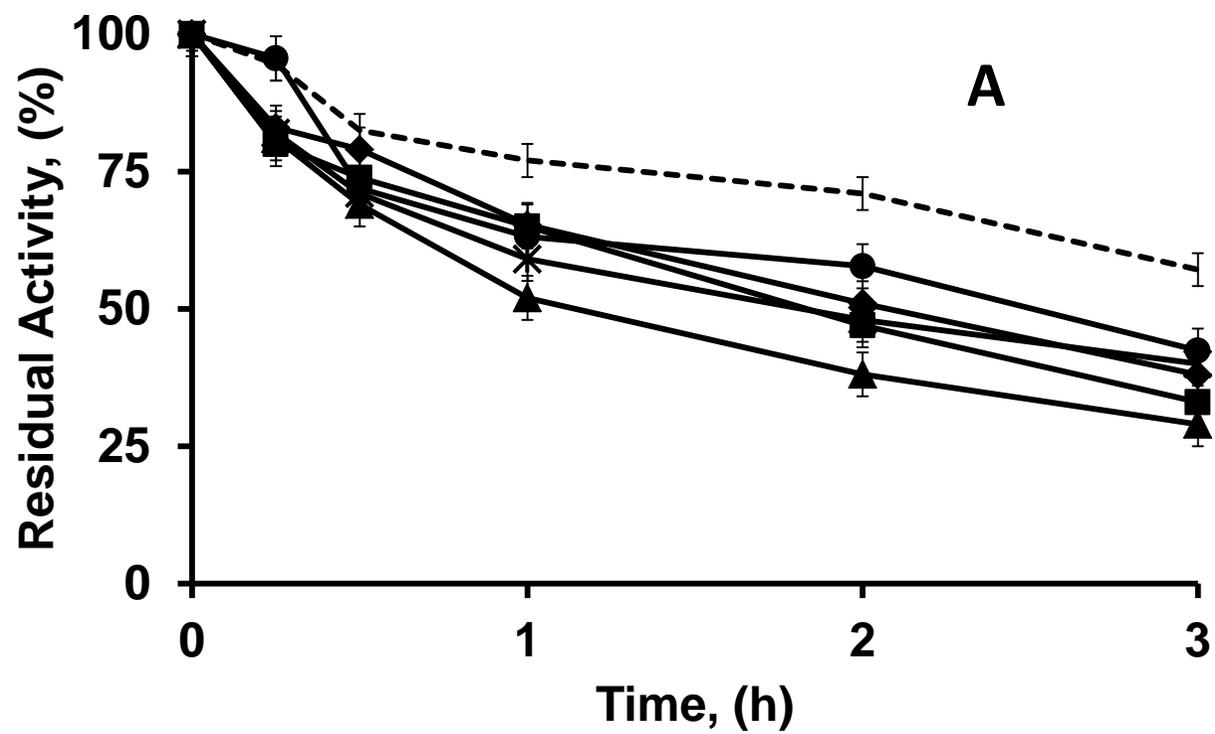


Figure 3

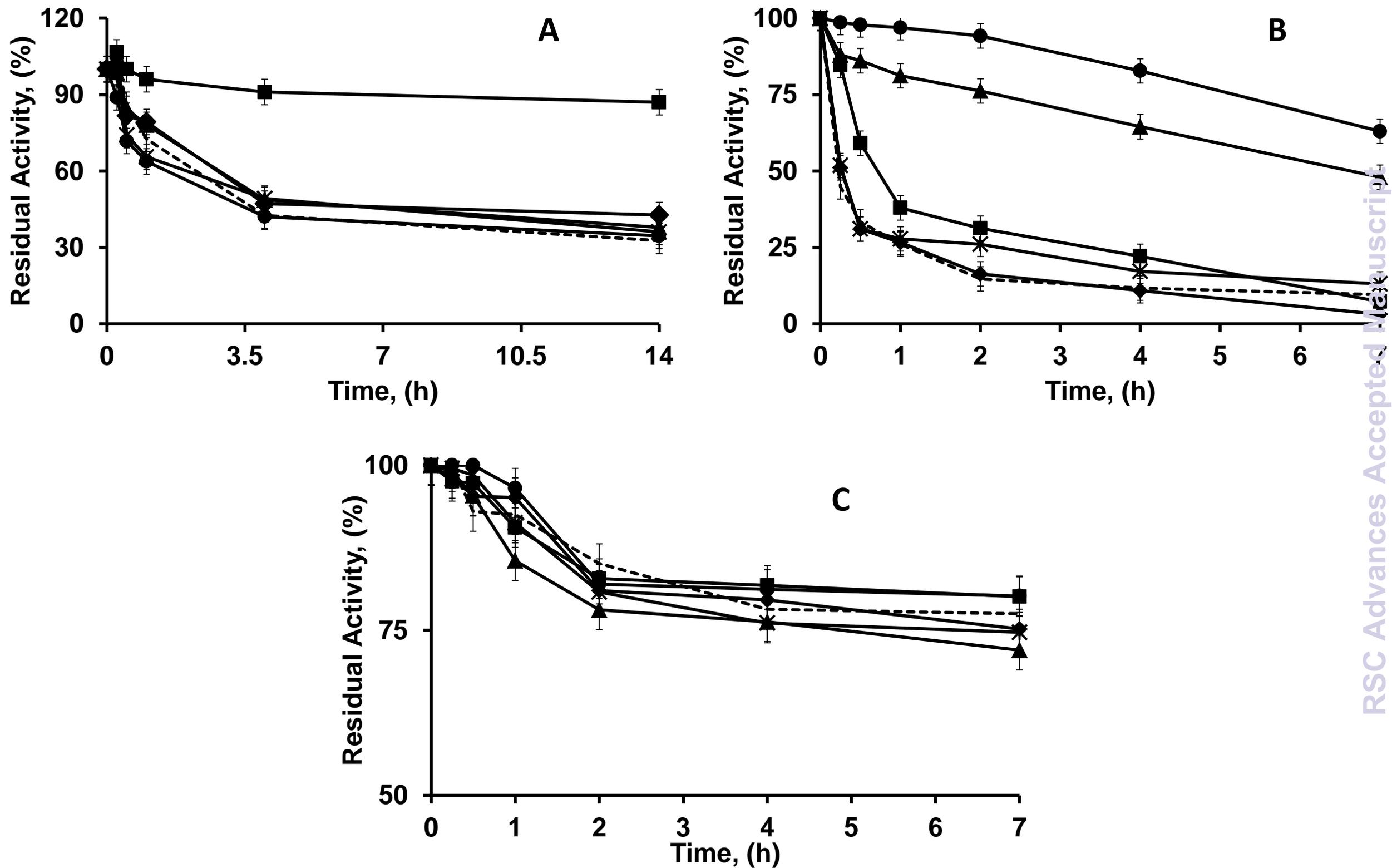


Figure 4

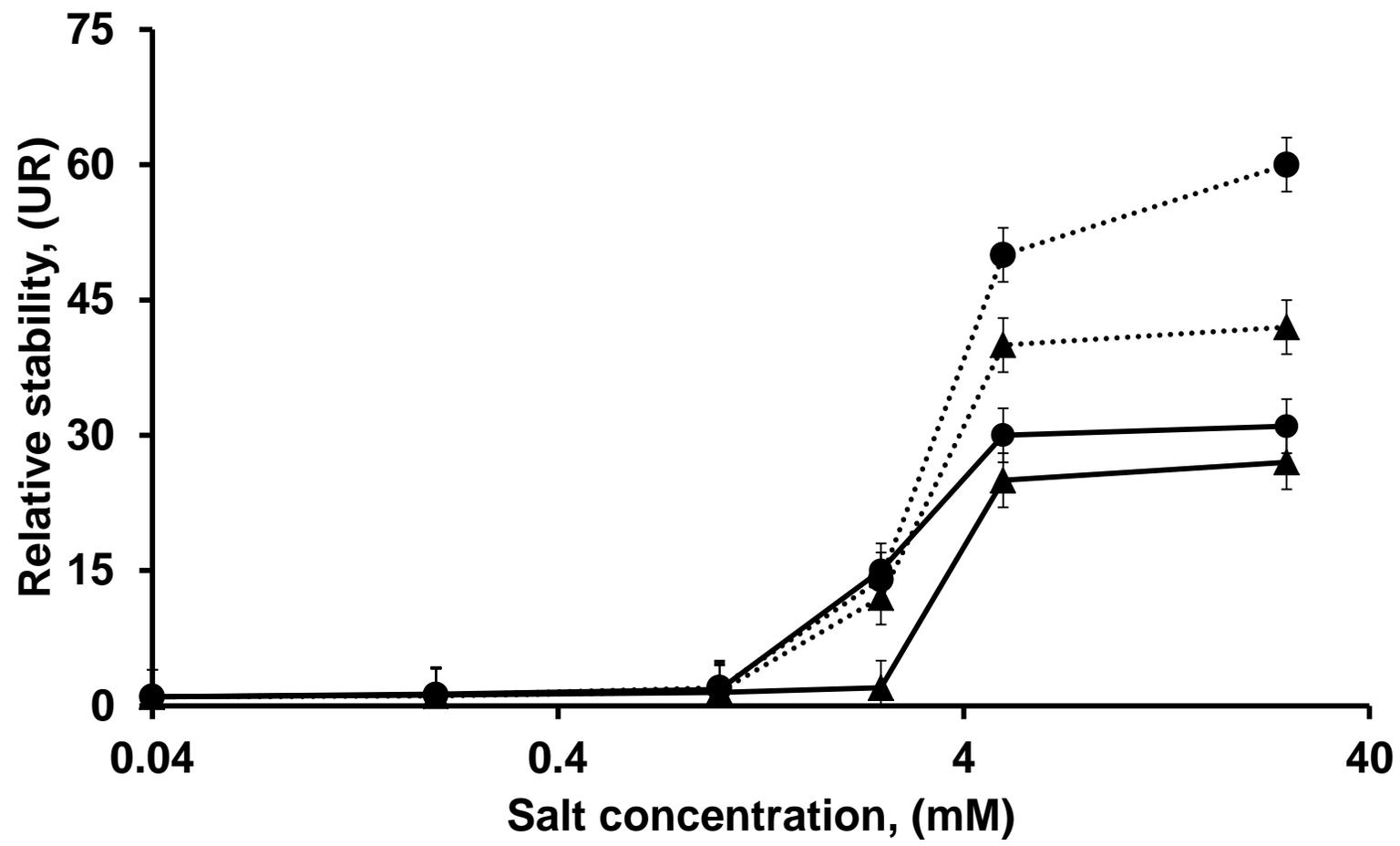


Figure 5

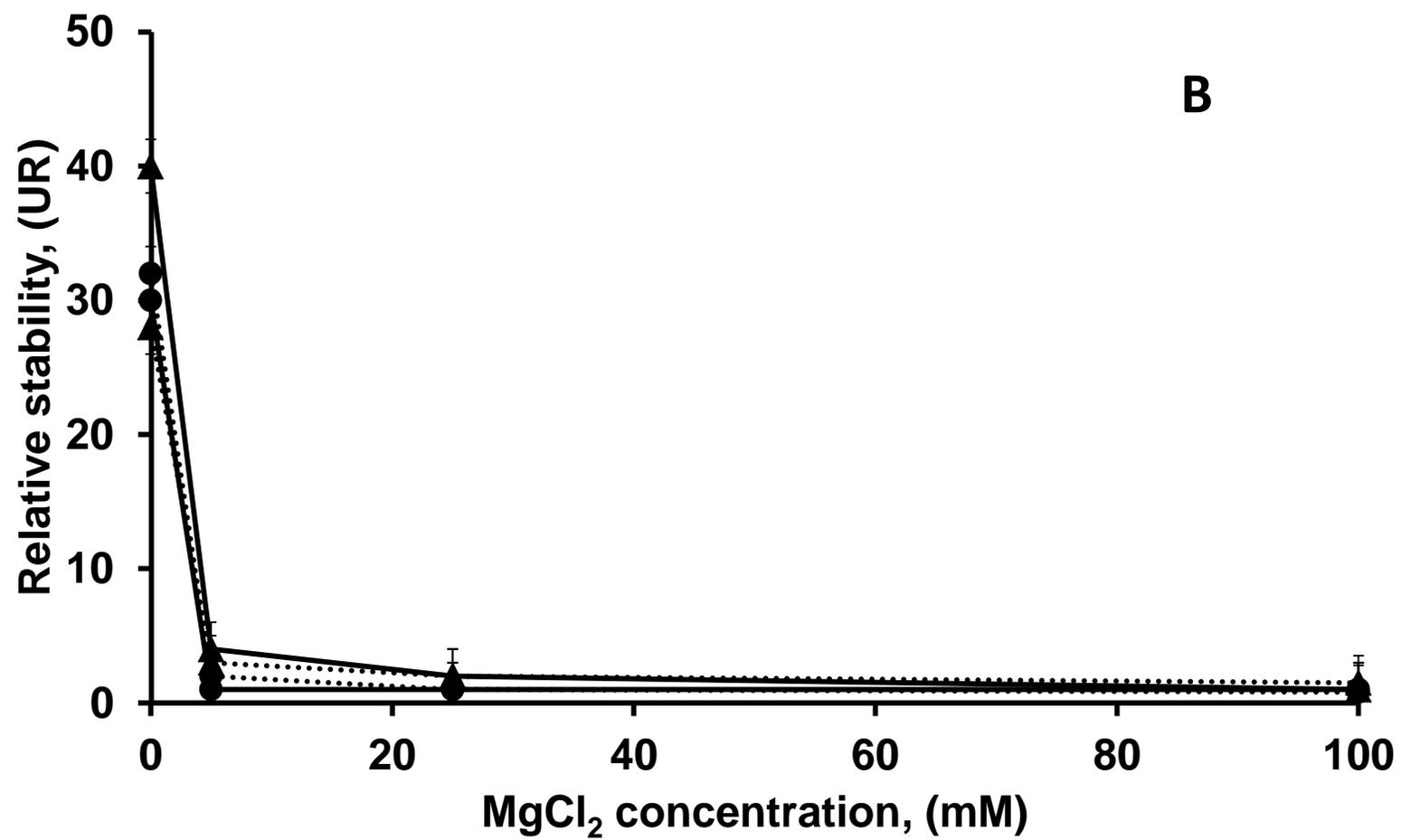
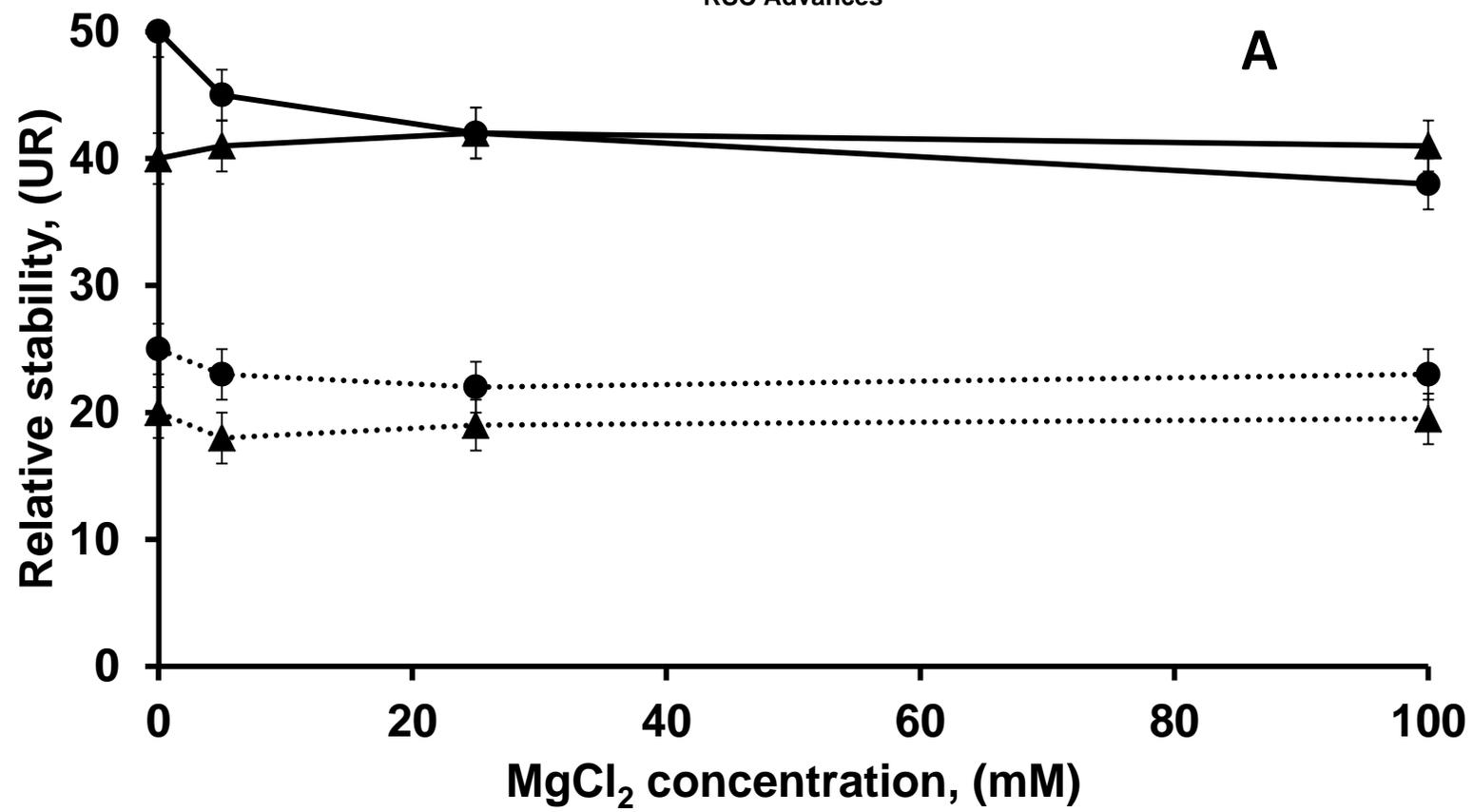
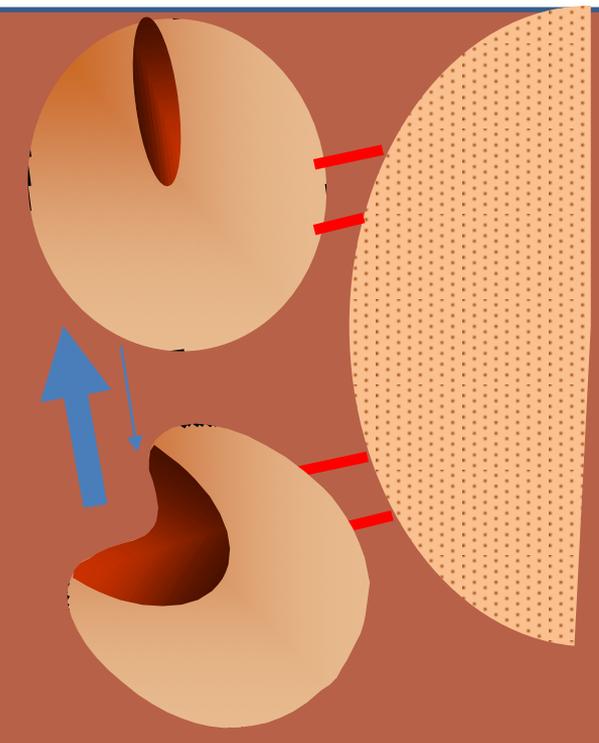
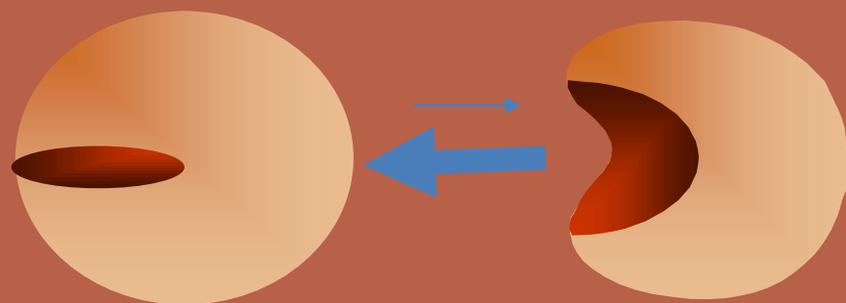


Figure 6

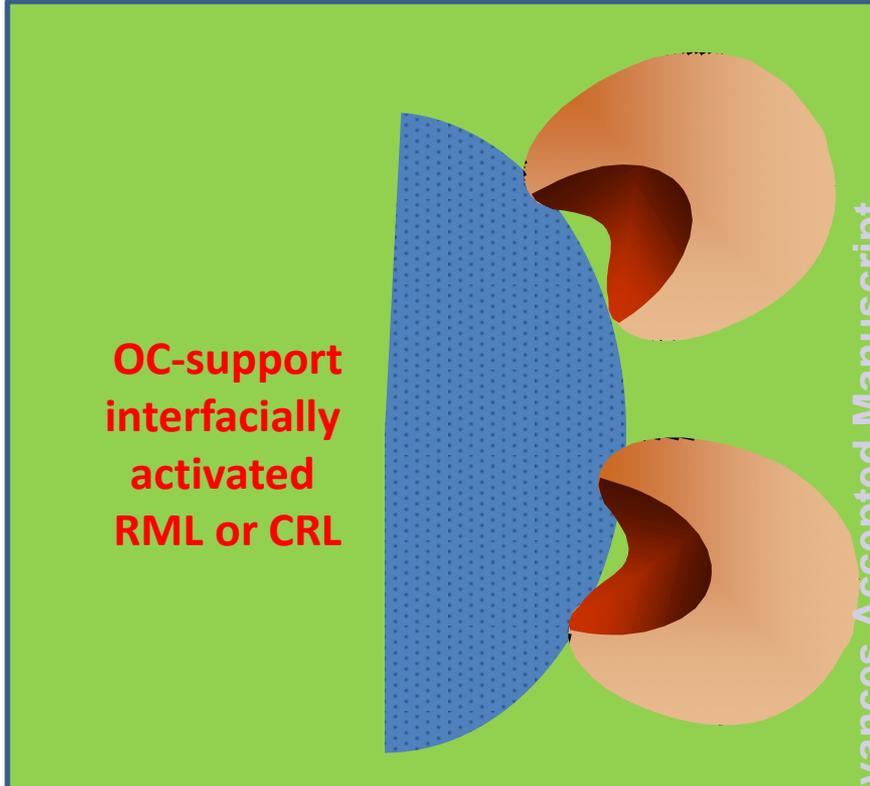


**Covalently
immobilized
RML or CRL**

**Ca²⁺ or Mn²⁺ have not effect
on lipase stability**



Free lipase



**OC-support
interfacially
activated
RML or CRL**

**Ca²⁺ or Mn²⁺ produced a
significant
lipase stabilization**