



**HAL**  
open science

## Biobased ionic liquids as solvents of paramylon

Frédérica Feuzing, Jean-Pierre Mbakidi, Florica Lazar, Luc Marchal, Eric Leroy, Sandrine Bouquillon

► **To cite this version:**

Frédérica Feuzing, Jean-Pierre Mbakidi, Florica Lazar, Luc Marchal, Eric Leroy, et al.. Biobased ionic liquids as solvents of paramylon. *Journal of Molecular Liquids*, 2022, 370, pp.120983. 10.1016/j.molliq.2022.120983 . hal-03953408

**HAL Id: hal-03953408**

**<https://hal.science/hal-03953408v1>**

Submitted on 24 Jan 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License

# Biobased Ionic liquids as Solvents of Paramylon

Frédérica Feuzing,<sup>a,c</sup> Jean-Pierre Mbakidi,<sup>a</sup> Florica Lazar,<sup>b</sup> Luc Marchal,<sup>c</sup> Eric Leroy,<sup>c</sup>  
Sandrine Bouquillon<sup>\*a</sup>

<sup>a</sup>Institut de Chimie Moléculaire de Reims, CNRS UMR 7312, Université de Reims  
Champagne-Ardenne, BP 1039, 51687 Reims Cedex, France

<sup>b</sup>Matériaux et Ingénierie Mécanique, MATIM, UFR Sciences, Université Reims Champagne  
Ardenne, 51687 Reims Cedex, France

<sup>c</sup>Université de Nantes, Oniris, CNRS, GEPEA, UMR 6144, F- 44470 Carquefou, France.

Correspondence: sandrine.bouquillon@univ-reims.fr; Phone: +33-(0)-3-26-91-89-73

## Keywords

Paramylon, solubility, ionic liquids, microalgae, choline, amino acids.

## Abstract

Paramylon is a linear glucan produced by *Euglena gracilis* microalgae as water insoluble intracellular granules. Its macromolecular structure contains only  $\beta(1-3)$  linkages, resulting in a stable triple helix conformation and a high crystallinity. It is soluble in very few solvents such as DMSO, harsh alkaline or acid solutions and imidazolium ionic liquids. In this paper we prepared biosourced more ecofriendly ionic liquids which can tolerate high amounts of water as a co-solvent and which are recyclable. The solubility of the paramylon was then evaluated depending of the nature of the ionic liquid and the structure and thermal properties of the biopolymer before and after solubilisation and regeneration were also discussed.

## Introduction

Paramylon is a storage polysaccharide, produced by *Euglena Gracilis* in autotrophic, heterotrophic or mixotrophic aerobic conditions [1]. It is accumulated as highly crystalline granules dispersed in the cytosol [2]. Paramylon is a linear polymer of glucose containing only  $\beta(1-3)$  glycosidic linkages. The macromolecules are assembled as triple helices, further packed in hexagonal crystalline cells arranged tangentially in the granules. The crystallinity approaches 90% and granules are insoluble in water [3]. The name paramylon actually comes from the fact that they are not colored blue-violet by iodinated water, contrary to starch.<sup>1</sup> Due to the strong intermolecular interactions between polymeric chains in the triple helices,

35 paramylon is only soluble in specific solvents like other linear polyglucans such as cellulose  
36 or starch [4]. Paramylon can be dissolved in basic [5] or acidic [6] aqueous solutions and also  
37 in organic solvents as DMSO [5a,7] or N,N-Dimethylacetamide these last being most of the  
38 time associated to salts like LiCl [5b,8]. Imidazolium ionic liquids (ILs) have also been used  
39 for the shaping of PM films for biomaterials applications by a process of solubilization  
40 followed by a regeneration [9]. Indeed, at the beginning of the 2000's, Rogers and Seddon  
41 reported the dissolution of natural polymers (cellulose) by using ionic liquids (ILs) [10]. 1-  
42 butyl-3-methyl-imidazolium chloride and 1-ethyl-3-methyl-imidazolium acetate were able to  
43 dissolve up to 15-20 % weight of cellulose. Indeed, ionic liquids show adapted physical and  
44 chemical properties such as tunable hydrophilic-lipophilic balance and basic properties that  
45 play an important role in the dissolution process of natural polymers [11,12]. But the major  
46 inconvenient of these ionic liquids is their eco- and cyto-toxicity [13].

47 In order to enhance a greener character, ionic liquids are for many years now prepared from  
48 renewable resources as starting materials such as acids, amino acids, amino alcohols, and  
49 sugars [14].

50 In this paper, we will use choline, fatty alcohols and amino acids as starting materials for the  
51 preparation of BIOBILs which will be used as solvents of PM dissolution.

52

## 53 **Experimental section**

54

### 55 **Materials**

56

57 Choline chloride, methanesulfonic acid, hexanoic, octanoic, decanoic acids, sodium  
58 perchlorate, potassium hydroxide, levulinic acid, potassium L-lactate solution 60%, Sigma,  
59 Glycine (Gly), L-alanine (Ala), L-serine (Ser), L-lysine (Lys), L-histidine (His), L-aspartic  
60 acid (Asp), L-valine (Val), L-threonine (Thr), L-arginine (Arg) (purity  $\geq 99$  %), Choline  
61 hydroxide (aqueous solution 46 wt%, Sigma) was purchased from Sigma Aldrich. Ethanol  
62 was of analytical grade and used without any further purification.

63  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded at 298 K on a Bruker AVANCE Digital operating at  
64 400 MHz. Solutions were prepared by dissolving 20-30 mg of each IL in 0.5 mL of  $\text{D}_2\text{O}$  or  
65 DMSO. IR spectra were recorded on a FTIR spectrometer (name) in the  $4000\text{--}400\text{ cm}^{-1}$  range.  
66 The decomposition temperatures (Td) were measured with a thermal gravimetric analyzer,  
67 with a heating rate of  $10\text{ }^\circ\text{C}/\text{min}$  under nitrogen. The Td is calculated from the intersection of  
68 the baseline weight (after the drying step) and the tangent line derived from the

69 decomposition curve. For reactions under ultrasounds, an ultrasonic bath Elmasonic S15 H is  
70 used and the power is 95W.

71

## 72 Synthesis

73

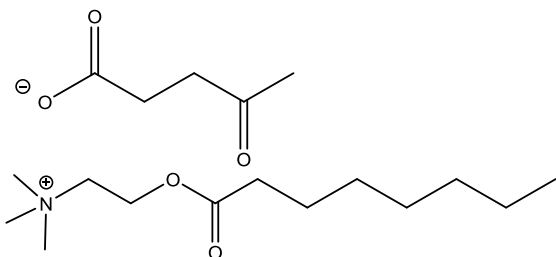
### 74 **Choline ester derivatives (CholC8X)**

75

76 In a 500 mL two-necked flask, Choline chloride (40 g; 0.286 mol; 1 eq.) and methane sulfonic  
77 acid (55.73 mL; 0.8589 mol; 3 eq.) were added with octanoic acid (90.65 mL; 0.572 mol).  
78 The reaction medium was heated at 110 ° C under reduced pressure (50-100 mbar) for 1 hour  
79 and a half. A brown solution was thus obtained and cooled at room temperature. Water (10  
80 ml) was added to the reaction; the resulting mixture was washed several times first with  
81 diethyl ether (200 ml) and then with ethyl acetate (50 ml) to remove the excess of carboxylic  
82 acid. In a second step, the aqueous phase recovered was introduced into a 250 ml Erlenmeyer  
83 flask. An excess of sodium perchlorate (3 eq.), previously dissolved in a minimum of water  
84 (20 mL), was then added. A white precipitate was obtained immediately and the reaction  
85 medium was left under stirring for 24 h in order to optimize the anionic metathesis between  
86 the chloride and perchlorate ions. Ethyl acetate was added to the reaction medium to dissolve  
87 the precipitate. After several washes with water to remove unreacted choline chloride and  
88 excess of methane sulfonic acid and sodium perchlorate, the organic phase was evaporated  
89 under reduced pressure. Finally, the addition of diethyl ether led to the precipitation of the  
90 perchlorate salt; after filtration, this white powder was dried under vacuum. This one was  
91 dissolved in 100 ml of ethanol. An aqueous solution of potassium levulinate or lactate (1 eq.;  
92 10 mL water) was added and the mixture was stirred at room temperature for 24 h. After  
93 evaporation of the ethanol, the aqueous phase is washed with ethyl acetate to remove the  
94 unreacted product. The resulting aqueous phase was reduced under vacuum and CholC8Lev  
95 was obtained as a viscous liquid of yellow-brown color.

96

### 97 **CholC<sub>8</sub>Lev (76%)**



98

99 Liquid at room temperature

100 <sup>1</sup>H NMR (500 MHz ; DMSO – d<sub>6</sub>) δ (ppm) : 0.85 (3 H, t, J = 7.5 Hz) ; 1.24 (8 H, m) ; 1.53  
101 (2 H, t, J = 7.5 Hz) ; 2.06 (3 H, s) ; 2.21 (2 H, t, J = 7.5 Hz) ; 2.33 (2 H, t, J = 7.5 Hz) ; 2.51  
102 (2 H, t, J = 7.5 Hz) ; 3.16 (9 H, s) ; 3.72 (2 H, t, J = 7.5 Hz) ; 4.45 (2 H, t, J = 7.5 Hz)

103 <sup>13</sup>C NMR (62.5 MHz; DMSO – d<sub>6</sub>) δ (ppm): 14.1; 22.4; 24.2; 28.7; 29.2; 29.3; 29.3; 32.4;  
104 40.1; 53.1; 58.4; 63.1; 172.7; 176.6; 208.9

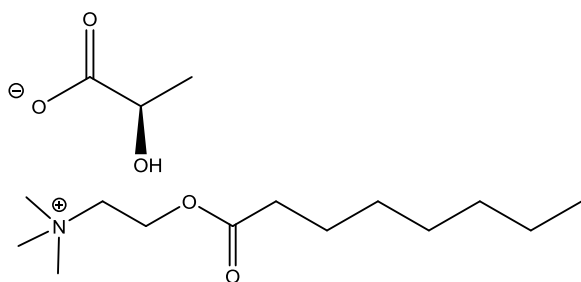
105 **Elemental Analysis** calcd.: C<sub>18</sub>H<sub>35</sub>NO<sub>5</sub>: C 62.58; H 10.21; N 4.05%; found: C 62.23; H 9.94;  
106 N 4.27%

107 IR: ν = 1746 cm<sup>-1</sup>

108 T<sub>d</sub>: 229°C

109 **CholC<sub>8</sub>Lact (80%)**

110



111

112

113 Liquid at room temperature

114 <sup>1</sup>H NMR (500 MHz ; DMSO – d<sub>6</sub>) δ (ppm) : 0.86 (3 H, t, J=7.5 Hz); 1.07 (3 H, d, J=7.5  
115 Hz); 1.25 (8 H, m); 1.54 (2 H, t, J=7.5 Hz); 2.34 (2 H, t, J=7.5 Hz); 3.16 (9 H, s); 3.47 (1 H, q,  
116 J=7.5 Hz); 3.71 (2 H, t, J=7.5 Hz); 4.45 (2 H, t, J=7.5 Hz) ppm

117 <sup>13</sup>C NMR (62.5 MHz; DMSO – d<sub>6</sub>) δ (ppm): 14.1; 18.2; 21.9; 22.5; 24.5; 29.2; 29.3; 41.2;  
118 53.2; 53.4; 53.7; 67.0; 172.5; 177.8

119 **Elemental Analysis** calcd.: C<sub>16</sub>H<sub>33</sub>NO<sub>5</sub>: C 60.16; H 10.41; N 4.38%; found: C 59.61; H 9.92;  
120 N 4.26%

121 IR: ν = 1746 cm<sup>-1</sup>

122 T<sub>d</sub>: 218°C

123

124 **ChAAILs**

125 The choline-based ILs were synthesized *via* an acid/base reaction between commercial  
126 Ch(OH) and amino acid (AA) in stoichiometric quantities. Water was added to ensure the  
127 complete dissolution of the AA and the reaction mixture was stirred for 24 h at room

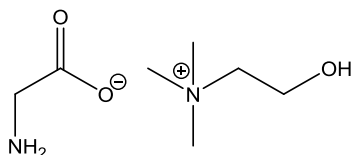
128 temperature under nitrogen atmosphere. Excess of water was removed under vacuum at 50°C.  
129 The ChAAILs were then precipitated by adding absolute ethanol. After filtration to remove  
130 any unreacted amino acid, the final product was dried under vacuum for 48h at 50°C.

131

### 132 **CholGly (95%)**

133 Liquid at room temperature

134



135

136 **<sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O)** δ/ppm: 3.04 (s, 9H, (CH<sub>3</sub>)<sub>3</sub>N), 3.07 (s, 2H, CH<sub>2</sub>NH<sub>2</sub>), 3.39 (m,  
137 2H, CH<sub>2</sub>OH), 3.92 (m, 2H, CH<sub>2</sub>CH<sub>2</sub>N)

138 **<sup>13</sup>C NMR (500 MHz, D<sub>2</sub>O)** δ (ppm): 44.7, 53.8, 55.5, 67.4, 180.7

139 **Elemental Analysis** calcd.: C<sub>7</sub>H<sub>18</sub>N<sub>2</sub>O<sub>3</sub>: C 47.17; H 10.18; N 15.72%; found: C 46.91; H  
140 9.91; N 15.38%

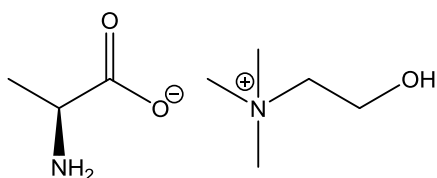
141 **IR:** ν= 3194, 2920, 1567, 1479, 1391, 1087, 953 cm<sup>-1</sup>

142 T<sub>d</sub>: 201°C

143

### 144 **CholAla (90%)**

145 Liquid at room temperature



146

147 **<sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O)** δ (ppm): 1.1 (d, 3H, CH<sub>3</sub>CH), 3.07 (s, 9H, (CH<sub>3</sub>)<sub>3</sub>N), 3.19 (q, 1H,  
148 CHNH<sub>2</sub>), 3.38 (m, 2H, CH<sub>2</sub>OH), 3.92 (m, 2H, CH<sub>2</sub>CH<sub>2</sub>N)

149 **<sup>13</sup>C NMR (500 MHz, D<sub>2</sub>O)** δ (ppm): 20.2, 51.4, 53.8, 55.5, 67.4, 183.6

150 **Elemental Analysis** calcd.: C<sub>8</sub>H<sub>20</sub>N<sub>2</sub>O<sub>3</sub>: C 49.98; H 10.49; N 14.57%; found: C 49.57; H  
151 9.98; N 14.38%

152 **IR:** ν=3187, 2962, 2863, 1565, 1479, 1395, 1085, 953, 838 cm<sup>-1</sup>

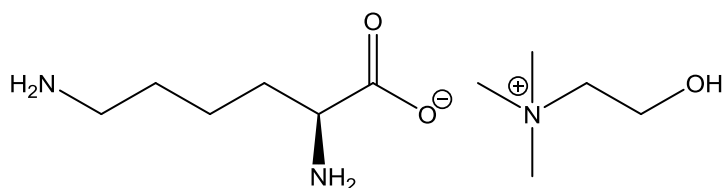
153 T<sub>d</sub>: 212°C

154

### 155 **CholLys (90%)**

156 Liquid at room temperature

157



158

159 **<sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O)** δ (ppm): 1.2 (m, 2H, CH<sub>2</sub>), 1.36 (m, 2H, CH<sub>2</sub>), 1.47 (m, 2H, CH<sub>2</sub>),  
160 2.29 (t, 2H, CH<sub>2</sub>), 3.1 (s, 10H, (CH<sub>3</sub>)<sub>3</sub>N, CH-N), 3.54 (t, 2H, CH<sub>2</sub>), 3.96 (m, 2H, CH<sub>2</sub>)

161 **<sup>13</sup>C NMR (500 MHz, D<sub>2</sub>O)** δ (ppm): 22.4, 31.3, 34.8, 40.3, 53.84, 55.9, 57.3, 67.5, 183.4

162 **Elemental Analysis** calcd.: C<sub>11</sub>H<sub>27</sub>N<sub>3</sub>O<sub>3</sub>: C 52.99; H 10.91; N 16.85%; found: C 53.15; H  
163 10.68; N 17.16%

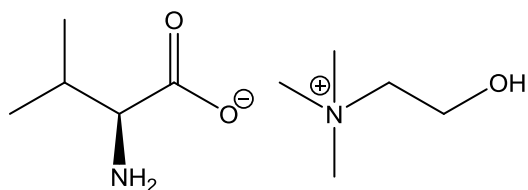
164 **IR:** ν= 3183, 2925, 2855, 1564, 1478, 1393, 1088, 953, 865 cm<sup>-1</sup>

165 T<sub>d</sub>: 199°C

166

### 167 CholVal (88%)

168 Liquid at room temperature



169

170 **<sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O)** δ (ppm): 0.75-0.76 (d, 3H, CH<sub>3</sub>), 0.82-0.83 (d, 3H, CH<sub>3</sub>), 1.81 (m,  
171 1H, CH), 2.92-2.93 (d, 1H, CH-N), 3.09 (s, 9H, (CH<sub>3</sub>)<sub>3</sub>N), 3.41 (t, 2H, CH<sub>2</sub>), 3.94 (m, 2H,  
172 CH<sub>2</sub>)

173 **<sup>13</sup>C NMR (500 MHz, D<sub>2</sub>O)** δ (ppm): 16.7, 19.1, 31.7, 53.8, 55.5, 61.9, 67.4, 182.6

174 **Elemental Analysis** calcd.: C<sub>10</sub>H<sub>24</sub>N<sub>2</sub>O<sub>3</sub>: C 54.52; H 10.98; N 12.72%; found: C 54.59; H  
175 10.91; N 12.35%

176 **IR:** ν= 3168, 2956, 2869, 1563, 1472, 1396, 1088, 954, 867 cm<sup>-1</sup>

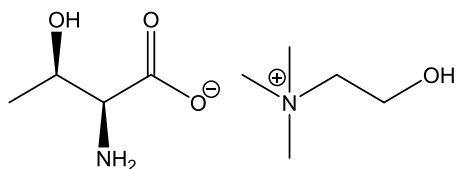
177 T<sub>d</sub>: 215°C

178

### 179 CholThr (92%)

180 Liquid at room temperature

181



182

183 **<sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O)** δ (ppm): 1.07-1.08 (d, 3H, CH<sub>3</sub>), 2.94-2.95 (d, 1H, CH-N), 3.07  
184 (s, 9H, (CH<sub>3</sub>)<sub>3</sub>N), 3.39 (m, 2H, CH<sub>2</sub>), 3.8 (m, 1H, CH<sub>2</sub>O), 3.94 (m, 2H, CH<sub>2</sub>)

185 **<sup>13</sup>C NMR (500 MHz, D<sub>2</sub>O)** δ (ppm): 19.3, 55.5, 61.9, 67.4, 69.5, 182.4

186 **Elemental Analysis** calcd.: C<sub>9</sub>H<sub>22</sub>N<sub>2</sub>O<sub>4</sub>: C 48.63; H 9.98; N 12.60%; found: C 49.12; H  
187 10.09; N 12.15%

188 **IR:** ν= 3216, 2967, 2926, 1557, 1478, 1394, 1085, 1060, 953 cm<sup>-1</sup>

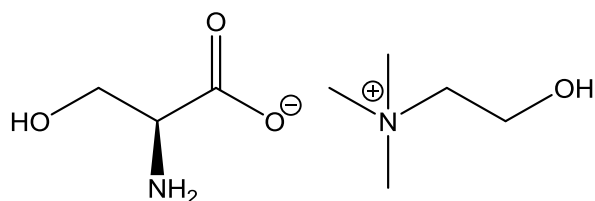
189 T<sub>d</sub>: 212°C

190

191 **CholSer (90%)**

192 Liquid at room temperature

193



194

195 **<sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O)** δ (ppm): 2.95 (s, 9H, (CH<sub>3</sub>)<sub>3</sub>N), 3.09 (t, 1H, CHN), 3.26 (t, 2H,  
196 CH<sub>2</sub>), 3.42-3.50 (dd, 2H, CH<sub>2</sub>), 3.78 (m, 2H, CH<sub>2</sub>)

197 **<sup>13</sup>C NMR (500 MHz, D<sub>2</sub>O)** δ (ppm): 53.5, 55.3, 57.4, 64.0, 67.2, 178.3

198 **Elemental Analysis** calcd.: C<sub>8</sub>H<sub>20</sub>N<sub>2</sub>O<sub>4</sub>: C 46.14; H 9.68; N 13.45%; found: C 46.38; H  
199 10.14; N 12.95%

200

201 **IR:** ν= 3195, 2856, 1557, 1478, 1398, 1335, 1084, 1044, 953 cm<sup>-1</sup>

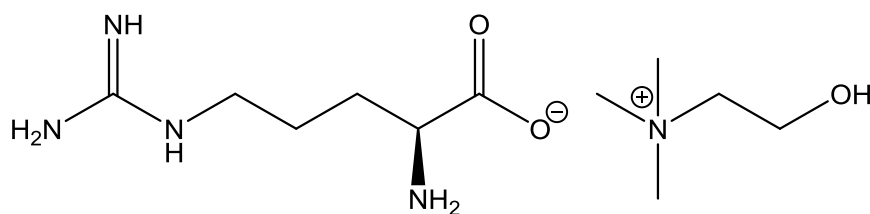
202 T<sub>d</sub>: 210°C

203

204 **CholArg (75%)**

205 Liquid at room temperature

206



207

208 **<sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O)** δ (ppm): 1.20 – 1.40 (t, 4H, CH<sub>2</sub>CH<sub>2</sub>), 2.85 (m, 11H, (CH<sub>3</sub>)<sub>3</sub>N,  
209 CH<sub>2</sub>), 2.96 (d, 1H, CH<sub>2</sub>N), 3.27 (q, 2H, CH<sub>2</sub>), 3.80 (s, 2H, CH<sub>2</sub>)



210  $^{13}\text{C}$  NMR (500 MHz,  $\text{D}_2\text{O}$ )  $\delta$  (ppm): 25.5, 27.7, 32.0, 40.4, 53.5, 55.3, 67.2, 161.1, 182.5

211 **Elemental Analysis** calcd.:  $\text{C}_{11}\text{H}_{27}\text{N}_5\text{O}_3$ ; C 47.63; H 9.81; N 25.25%; found: C 47.18; H  
212 10.12; N 24.95%

213 **IR:**  $\nu$ = 3193, 2940, 1566, 1476, 1388, 1087, 952, 549  $\text{cm}^{-1}$

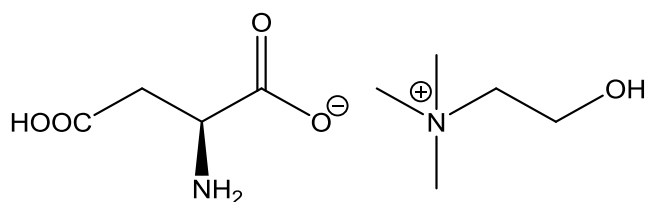
214  $T_d$ : 203°C

215

216 **CholAsp (75%)**

217 Liquid at room temperature

218



219

220  $^1\text{H}$  NMR (500 MHz,  $\text{D}_2\text{O}$ )  $\delta$  (ppm): 2.07 – 2.12 (m, 1H,  $\text{CH}_2$ ), 2.42 – 2.46 (m, 1H,  $\text{CH}_2$ ),  
221 3.01 (s, 9H,  $(\text{CH}_3)_3\text{N}$ ), 3.32 (m, 2H,  $\text{CH}_2$ ), 3.45(q, 1H,  $\text{CH-N}$ ), 3.85 (m, 2H,  $\text{CH}_2$ )

222  $^{13}\text{C}$  NMR (500 MHz,  $\text{D}_2\text{O}$ )  $\delta$  (ppm): 16.8, 42.5, 53.6, 55.4, 67.2, 179.2, 180.5

223 **Elemental Analysis** calcd.:  $\text{C}_9\text{H}_{20}\text{N}_2\text{O}_5$ ; C 47.75; H 8.53; N 11.86%; found: C 47.42; H 8.74;  
224 N 11.98%

225 **IR:**  $\nu$ = 3195, 2962, 1566, 1478, 1375, 1087, 1053, 953, 866  $\text{cm}^{-1}$

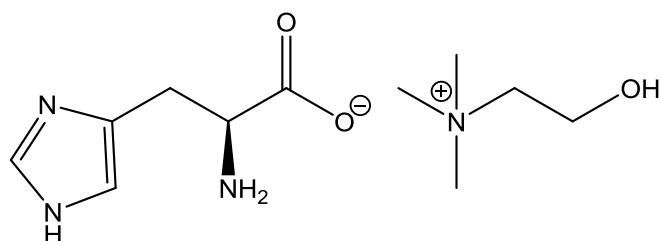
226  $T_d$ : 215°C

227

228 **CholHis (70%)**

229 Liquid at room temperature

230



231

232  $^1\text{H}$  NMR (500 MHz,  $\text{D}_2\text{O}$ )  $\delta$ (ppm): 2.20– 2.25 (m, 1H,  $\text{CH}_2$ ), 2.40– 2.45 (m, 1H,  $\text{CH}_2$ ), 2.51  
233 (s, 9H,  $(\text{CH}_3)_3\text{N}$ ), 2.82 (m, 3H,  $\text{CH}_2$ ,  $\text{CH-N}$ ), 3.36 (m, 2H,  $\text{CH}_2$ ), 6.35 (s, 1H, =CH), 7.10 (s,  
234 1H, =CH)

235  $^{13}\text{C}$  NMR (500 MHz,  $\text{D}_2\text{O}$ )  $\delta$ (ppm): 32.1, 54.8, 56.5, 67.1, 117.8, 133.3, 135.1, 180.8

236 **Elemental Analysis** calcd.: C<sub>11</sub>H<sub>22</sub>N<sub>4</sub>O<sub>3</sub>: C 51.15; H 8.58; N 21.69%; found: C 51.46; H 8.94;  
237 N 21.95%

238 **IR:**  $\nu = 3194, 2958, 1342, 1048, 789 \text{ cm}^{-1}$

239 **T<sub>d</sub>:** 219°C

240

#### 241 **Dissolution procedure under conventional heating**

242

243 In a glass pillbox equipped with a cap is introduced the ionic liquid IL (4 mL). This one is  
244 heated 5 minutes at 80°C to reduce its viscosity. 80 mg of paramylon is then added (resulting  
245 to a concentration of 2%). The mixture is stirred for 1 to 2 hours depending of the nature of  
246 the IL. After cooling at room temperature, ethanol (15 mL) is added in order to precipitate the  
247 paramylon. After centrifugation (6000 rpm, 10 min), ethanol (15 mL) is added to the residue;  
248 the mixture is stirred during 5 minutes and centrifuged; the supernatant is removed. This  
249 operation is repeated 2 times. The alcohol solutions are collected and evaporated under  
250 reduced pressure to regenerate the ionic liquid. The ionic liquid is recovered with 97 to 99%  
251 and can be reused at least 5 times without loss of activity. The white residue is dried at room  
252 temperature and lyophilized, then characterized (NMR, IR and XRD). 70 mg of paramylon  
253 are finally obtained. Yield: 87.5%.

254

#### 255 **Dissolution procedure under ultrasounds**

256 In a glass pillbox equipped with a cap is introduced the ionic liquid IL (4 mL). This one is  
257 heated 5 minutes at 60°C under ultrasounds (95W) to reduce its viscosity. 80 mg of  
258 paramylon is then added (resulting to a concentration of 2%). The mixture is stirred for 2  
259 hours under ultrasounds (95W). Similar procedure as described above for recovering the  
260 paramylon is then employed.

261 72 mg of paramylon is finally obtained. Yield: 90%

262

#### 263 **X-ray diffraction (XRD)**

264 The structure of powders was characterized by X-ray diffraction, using a Bruker D8 Advance  
265 X-ray diffractometer with CuK $\alpha$  radiation ( $\lambda \text{ CuK}\alpha = 1.5418 \text{ \AA}$ ), operating voltage of 40 kV  
266 and a current of 20 mA. The data were collected using Bragg-Brentano configuration, for  $2\theta$   
267 ranging from 4° to 70°, with a step of 0.06° and a scan speed of 2s by step. The XRD analyses  
268 were carried out before and after each chemical treatment of the samples.

269

270 **Results and discussion**

271 ILs and their characterizations

272 The ILs, i.e. choline ester derivatives CholCnX (n= number of carbon on the ester chain, X=

273 lactate and levulinate) and cholinium aminobases (ChAAILs) were prepared at first.

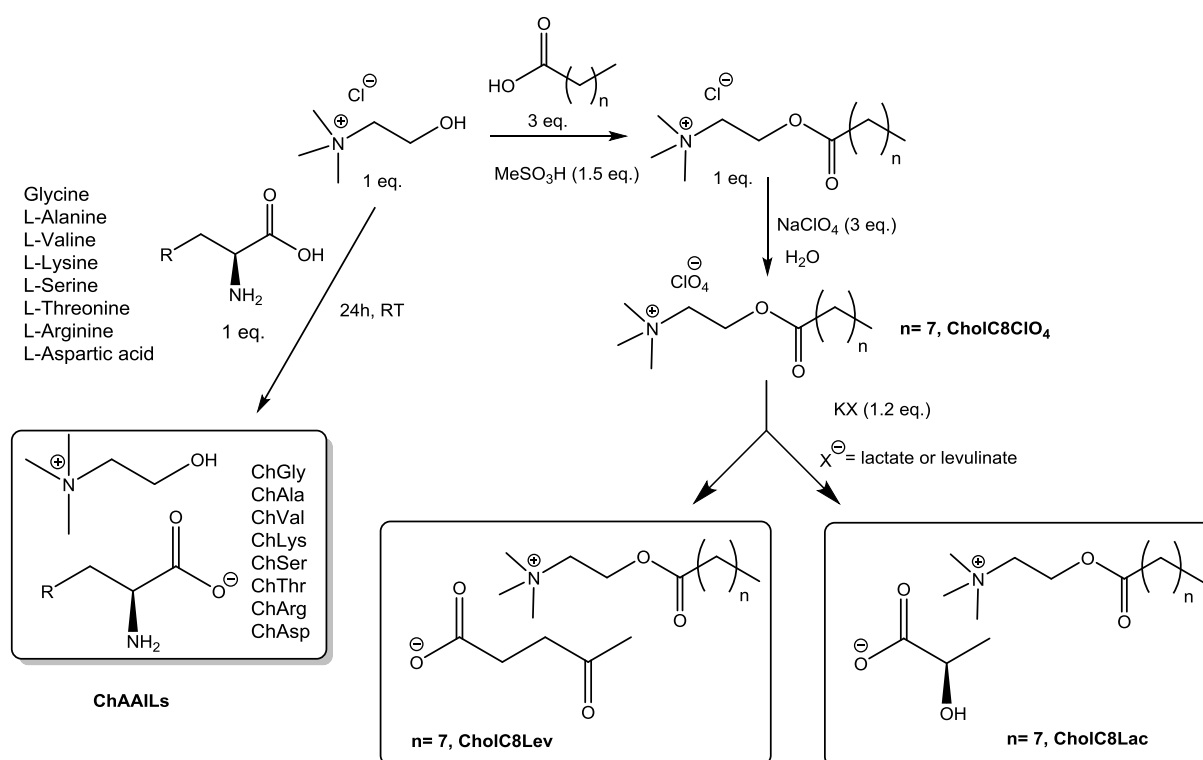
274 The choline esters derivatives were prepared in 3 steps according to an easy procedure we

275 recently performed (Scheme 1) [15]. For the present study, we prepared the Cho-C8-Lev and

276 Chol-C8-Lac with high yields; this IL was chosen for his good HLB value and his pH range

277 (9-10).

278



Scheme 1 – Synthesis of choline ester derivatives

ChAAILs were synthesized *via* an acid-base reaction between commercial Ch(OH) and amino acid in stoichiometric amounts with yields up to 95%. Purification of these ChAAILs was realized by precipitation in absolute alcohol and their characterizations (NMR, IR and TGA) were in total accordance with the ones reported by Zong and al. [16].

All these synthesized ionic liquids are relatively thermostable with degradation temperature above 200°C (Figure S1). Then these ILs and commercial ones were employed for the

290 solubilization of the PM at a massic concentration of 2% at 80°C for 1 hour. The results are  
 291 summarized in Table 1.

292 The results show that the commercial ionic liquids dissolved the paramylon except  
 293 [Bmim][Cl]. Concerning the choline esters derived ILs, only the ester containing a C8 carbon  
 294 chain and a levulation anion led to PM dissolution while all amino acid-based except  
 295 [Chol][Hist] (more hydrophobic nature of the IL with the unsaturated nitrogen ring) were  
 296 good solvent for PM. From these observations, we concluded that both viscosity and basicity  
 297 of ILs could influenced their dissolution power.

298 Thus as described in Figure S1, we can establish a solubility window of paramylon depending  
 299 of their viscosity and the pH values of the ILs solutions. Indeed, we began to observe partial  
 300 solubility between 10 and 13 pH values (corresponding to CholC8Lev and commercial ionic  
 301 liquids) and a complete PM solubility above a pH value of 13 corresponding to the major  
 302 ChAAILs. These first results show the importance of both pH of the reaction medium and the  
 303 nature of the associated anions and cations in ILs governing their viscosities.

304

305 Table 1 - PM solubilization

306

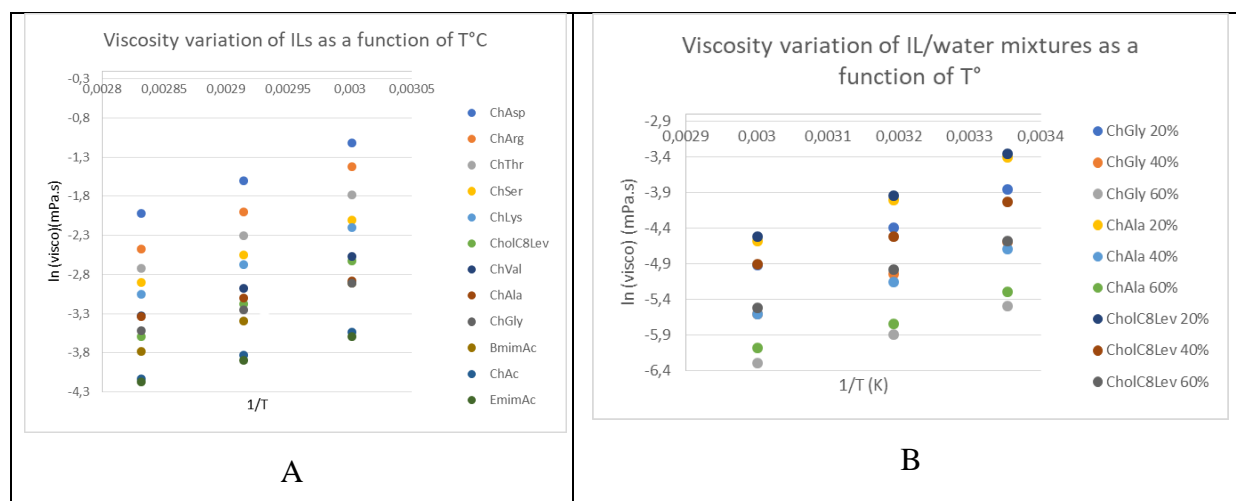
Solvents		Conditions	Viscosity ( $\eta$ /mPa s)		pH	PM Solubilisation <sup>a</sup>
			60°C	80°C		
Formic acid (pure)		15 min, RT	nd	nd	1	+ <sup>b</sup>
NaOH (0.5M)		5 min, RT	nd	nd	12	+ <sup>c</sup>
DMAc/LiCl		30 min under Argon, 110°C	nd	nd	nd	+ <sup>c</sup>
[Bmim] [Cl]		1h, 80°C	225	nd	nd	-
[Bmim] [Ac]	neat	1h, 80°C	54	23	13.8	+ <sup>c,d</sup>
	6 mL + H <sub>2</sub> O 4 mL	40 min, 80°C				+ <sup>c,d</sup>
[Emim] [Ac]	neat	1h, 80°C	27	15	12	+ <sup>c,d</sup>
	6 mL + H <sub>2</sub> O					+ <sup>c,d</sup>

	4 mL					
[Chol] [Ac]	neat	1h, 80°C	29	16	10	+ <sup>c,d</sup>
	7 mL + H <sub>2</sub> O 3 mL					+ <sup>c,d</sup>
[CholC6] [Lac]		1h, 80°C	42	21	8.3	-
[CholC8] [Lac]		1h, 80°C	68	28	9.5	-
[CholC10] [Lac]		1h, 80°C	nd	nd	nd	-
[CholC6] [Lev]		1h, 80°C	53	30	9.7	-
[CholC8] [Lev]	neat	1h, 80°C	73	34	10.3	+ <sup>c,d</sup>
	8 mL + H <sub>2</sub> O 2 mL					+ <sup>c,d</sup>
[CholC10] [Lev]		1h, 80°C	nd	nd	nd	-
[Chol] [His]		1h, 80°C	296	nd	13.8	-
[Chol] [Arg]	neat	1h, 80°C	239	84	> 15	+ <sup>c,d</sup>
	9 mL + H <sub>2</sub> O 1 mL	1h30, 80°C				+ <sup>c,d</sup>
[Chol] [Val]	neat	1h, 80°C	76	36	14.2	+ <sup>c,d</sup>
	5 mL + H <sub>2</sub> O 52 mL					+ <sup>c,d</sup>
[Chol] [Lys]	neat	1h, 80°C	111	47	13.6	+ <sup>c,d</sup>
	7 mL + H <sub>2</sub> O 3 mL					+ <sup>c,d</sup>
[Chol] [Ala]	neat	1h, 80°C	56	35	14	+ <sup>c,d</sup>
	4 mL + H <sub>2</sub> O 6 mL	45 min, 80°C				+ <sup>c,d</sup>
[Chol] [Gly]	neat	1h, 80°C	54	29	13.2	+ <sup>c,d</sup>
	4 mL	45 min, 80°C				+ <sup>c,d</sup>

	+ H <sub>2</sub> O 6 mL					
--	----------------------------	--	--	--	--	--

307 <sup>a</sup> -: no dissolution, +: dissolution, <sup>b</sup>: chemical modification (<sup>13</sup>C NMR), <sup>c</sup>: no chemical  
308 modification (<sup>13</sup>C NMR), <sup>d</sup>: XRD modification

309  
310 Next, to improve our choice of ILs for dissolution, we took into account the viscosity of these  
311 solvents because their use is often hindered by their excessive viscosity. So, we characterized  
312 the temperature dependence of the viscosities of the different ionic liquids and compared them  
313 to commercial ionic liquids commonly used. This relationship between the viscosity and the  
314 temperature follows the Arrhenius rule (Viscosity = K x exp (-Ea/RT)) because the  
315 ln(viscosity) is inversely proportional to the temperature (Figure 1) in a range of temperature  
316 between 60 and 80°C (Table S1).



317 Figure 1 - A -Variation of ln(viscosity) of for pure ILs towards 1/T accordingly to the  
318 Arrhenius's equation. B - Variation of ln(viscosity) of mixtures ILs/water towards 1/T  
319 accordingly to the Arrhenius's equation

320  
321  
322 Among commercial and "classical" (EmimAc, BmimAc or CholAc) ionic liquids, BmimAc  
323 being the most viscous considering the butyl chain on the imidazolium chain; their viscosities  
324 are reported in Table 1 to be compared to CholC8Lev and ChAAILs ones. Concerning the  
325 ChAAILs, the viscosity is depending on the carbon chain lengths and also on the presence of  
326 function like OH, COOH or NH-C(NH)-NH what H bonding in the medium. Indeed, the most  
327 viscous is the CholAsp followed by CholArg and CholSer. In general as attempted, the  
328 viscosity decreased with the temperature and more drastically for high viscosities even in a  
329 small temperature range (60-80°C). CholC8Lev is more viscous than EmimAc, BmimAc,

330 CholAc, CholAla and CholGly but his viscosity remains relatively low considering the  
331 number of carbon atoms on the ester chain. These results followed some previously described  
332 observations concerning the viscosities at various temperatures and their relationships towards  
333 the nature of cations and anions [17].

334 Furthermore, considering the values obtained at 60, 70 and 80°C, we used the Arrhenius's  
335 equation ( $\text{Viscosity} = K \times \exp(-E/RT)$ ) to determine the activation energy and so to predict  
336 the evolution of the viscosity at lower and higher temperatures. The predicted viscosities have  
337 been determined at 25°C and 100°C (Table S2) for all ILs and revealed that EmimAc, ChAc,  
338 BmimAc and CholC8Lev or CholGly, CholAla, CholVal with respectively 10 or 20 as  
339 predicted viscosities, could be used for an industrial development of the PM extraction from  
340 microalgae.

341 As ionic liquids are required to solubilize paramylon from microalgae in a reacting mixture  
342 containing often large proportions of water, we measured viscosities of ionic liquids with  
343 various percentages of water in order to understand the role of water. 3 ILs were used,  
344 CholGly, CholAla and CholC8Lev and 3 water percentages, 20, 40 and 60%. As attempted,  
345 the viscosities decreased with increasing water amounts and temperatures (Figure 1 and Table  
346 S3) and the predicted viscosities of these mixtures range from 1 to 4 ( $\eta/\text{mPa s}$ ) ( Table S4).

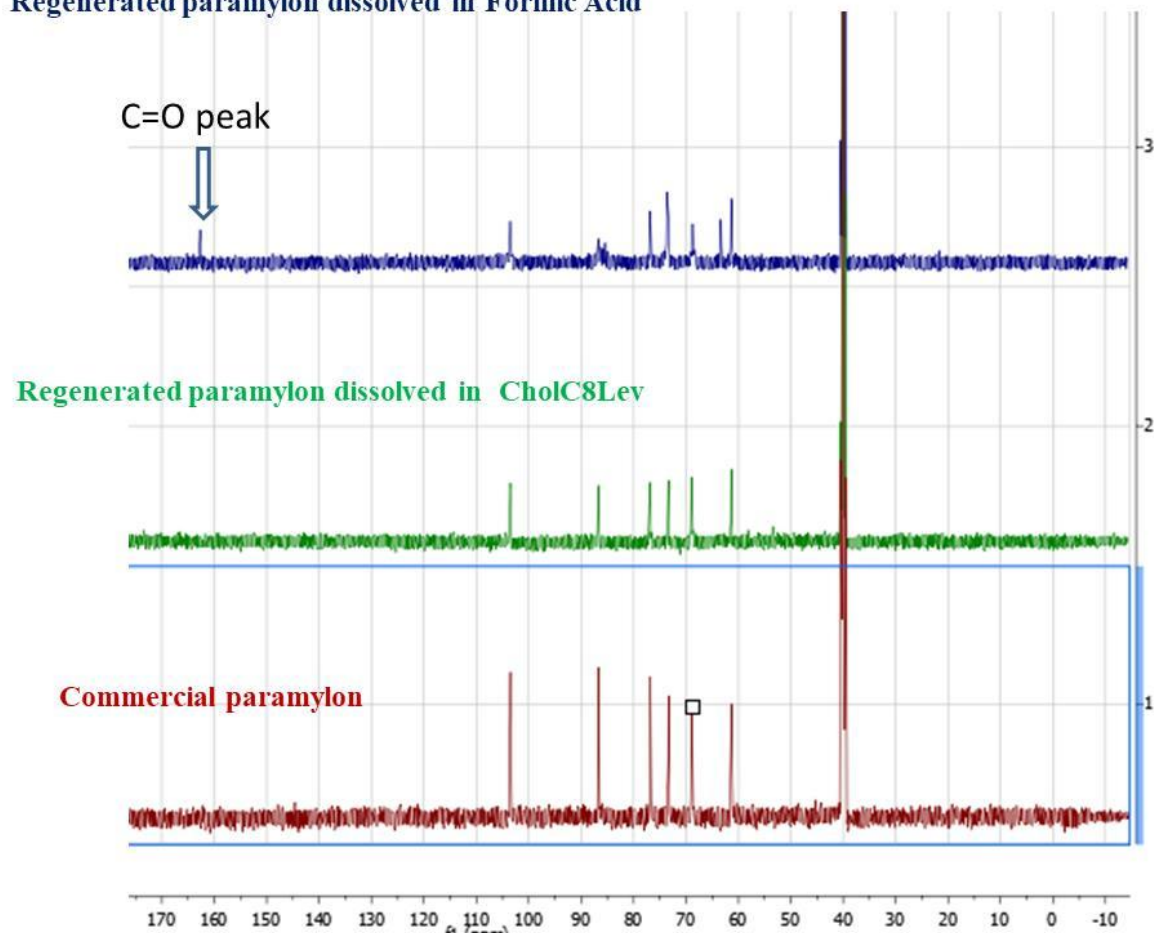
347  
348 To determine the highest quantity of water necessary to dissolve the paramylon but which  
349 could be also tolerated by the different ionic liquids, water content was increased until we  
350 obtained a turbid solution. Moderate to high water content can be tolerated by ionic liquids  
351 especially for ChAAILs which could tolerate a large amount of water ( $\geq 50\%$ ) without  
352 reducing the dissolution of the PM.

353 After identifying the most favorable ILs or water/IL mixtures, it was important to show if the  
354 nature of the PM could be affected by the dissolution step. Thus, the dissolution of PM in  
355 various solvents (acid, basic and commercial ones and commercial and synthetic ILs) was  
356 realized during 5 to 120 min at room temperature or at 80°C. After the dissolution, the PM  
357 was regenerated by adding EtOH and characterized first of all by  $^{13}\text{C}$  NMR. Indeed, we  
358 compared the  $^{13}\text{C}$  NMR spectra of the native and regenerated PMs. On Figure 2, the  
359 superposition of 3 NMR spectra clearly shows that the use of CholC8Lev did not modify  
360 chemically the structure of the PM which remains identical to that of the commercial one; in  
361 contrast, the dissolution of PM in the formic acid led to the functionalization of the PM which  
362 was proved by the detection of a new peak in  $^{13}\text{C}$  NMR at 160 ppm relative to a C=O bond.  
363 This information have been also confirmed by the infrared spectroscopy, on which we can

364 identify the characteristic absorption band of the aldehyde for the regenerated paramylon  
365 dissolved in formic acid while there was no change with the paramylon dissolved in  
366 CholC8Lev (Figure S2).

367

#### Regenerated paramylon dissolved in Formic Acid



368

369 Figure 2 -  $^{13}\text{C}$  NMR of PM and regenerated PMs

370

371 No chemical modification was also observed with all ILs mentioned in this study (Table 1).

372

373 While NMR analysis has shown that the polymer remains chemically unchanged after the  
374 dissolution in all ILs and BIOBILs, the XRD highlighted however a modification of the  
375 polymer structure (Figure 3).

376 The diffractograms of pristine paramylon present 3 main peaks [18,19]: i) a first and strongest  
377 peak at  $2\theta \approx 7^\circ$  which corresponds to the [100] planes of the hexagonal crystalline cell, in  
378 which paramylon triple helices are packed along the vertical axis; ii) a second strongest peak  
379 at  $2\theta \approx 19^\circ$  which can be ascribed to the [111] planes; and iii) a third peak at  $2\theta \approx 20.7^\circ$  which  
380 can be ascribed to [201] planes.



381 As can be seen on **figures 3a and 3b** (zoom of the right side on **Figures 3a BIS and 3b BIS**  
382 **in SI**), the [100] diffraction peak at  $7^\circ$  vanishes for all the regenerated paramylon samples,  
383 indicating that no hexagonal lateral packing of triple helices is present after solubilization in  
384 ILs. For the paramylon samples solubilized in amino acid based ILs, a new broad diffusive  
385 peak appears around  $2\theta \approx 6.2^\circ$ , corresponding to a characteristic distance of 1.42 nm. It may  
386 be ascribed to the presence of individual triple helices in regenerated paramylon. It is  
387 noteworthy that for these ILs, the two other characteristic peaks ([111] and [201]) of pristine  
388 paramylon also disappear, while a new peak appears at  $2\theta \approx 18.5^\circ$  (**Figure 3b**). The  
389 occurrence of such a peak was recently reported for paramylon samples hot-pressed above  
390  $230^\circ\text{C}$ , and ascribed to the melting and recrystallization of paramylon in an unknown  
391 crystalline structure, different from the native hexagonal packing [6]. Regarding the other  
392 regenerated paramylon samples (**Figure 3a**), this new peak at  $18.5^\circ$  is also present after  
393 solubilization in [Emim][Ace] and [Chol][Ace], while the native [111] peak remains at  $19^\circ$   
394 for [CholC8][Lev] and [Bmim][Ace]. Concurrently, the [201] diffraction peak around  $20.7^\circ$   
395 vanishes for these later, while a broad diffusion peak appears for [Emim][Ace] and  
396 [Chol][Ace].

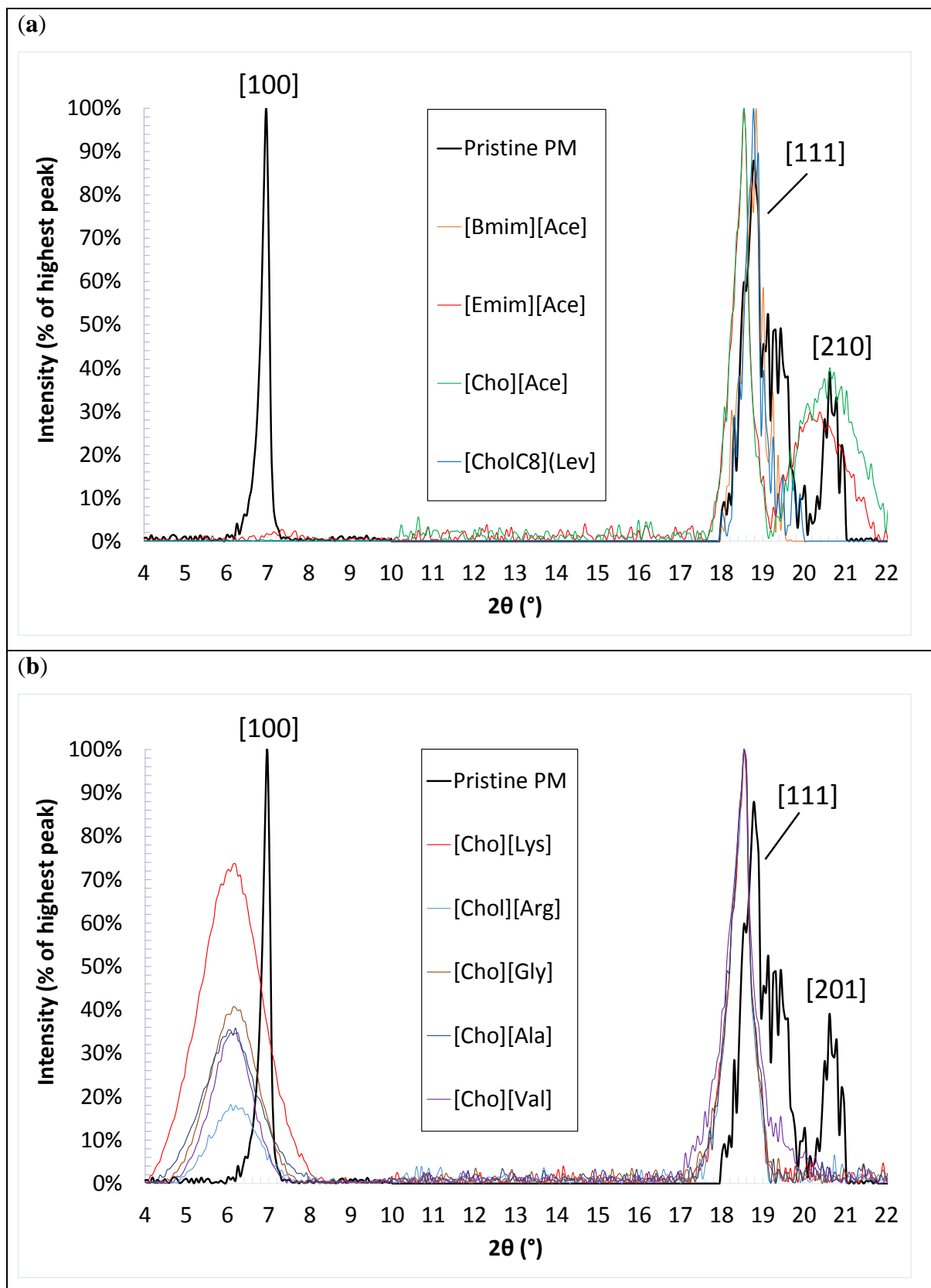
397 In all cases, the differences between the diffractograms before and after solubilization in ILs  
398 suggest a complete disruption of the crystalline structure by the solvents, with a  
399 recrystallization with a different crystalline packing after regeneration, and/or the presence of  
400 individual triple helices.

401 Summing up, XRD results showed that after their dissolution in ionic liquids at  $80^\circ\text{C}$ , the  
402 paramylon samples recrystallize upon regeneration but differently depending on the nature of  
403 the ionic liquid. All samples showed partial recrystallization, different from native paramylon  
404 but we have not clear correlation with pH values discriminating partial or total solubilization.

405 Thermal stability of regenerated paramylon samples was also performed by TGA  
406 measurements. The pristine paramylon presents a degradation temperature between  $290^\circ\text{C}$   
407 and  $320^\circ\text{C}$  (Figure 4a). For regenerated paramylon dissolved in ionic liquids, no change was  
408 observed with [CholAc] and [CholArg], while lower thermal stability was noticed when  
409 imidazolium ILs were used (Figure 4b). Two thermal degradation steps could be noted, one  
410 between  $250^\circ\text{C}$  and  $290^\circ\text{C}$  corresponding to the degradation of the mono helices and another  
411 one between  $290^\circ\text{C}$  and  $320^\circ\text{C}$  corresponding to the degradation of the triple helices as  
412 described for native curdlan by Okuyama and al. [20].

413

414



415 Figure 3 - XRD characterizations of pristine and regenerated paramylon samples.

416

417

418

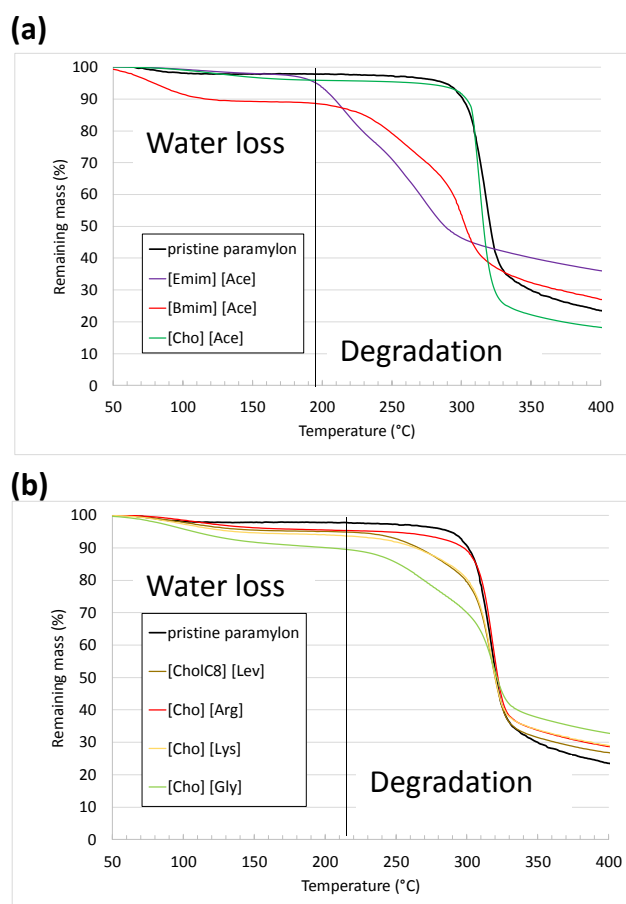


Figure 4 – Thermal stability of pristine paramylon and regenerated paramylon samples.

420

421

422

423 As the final aim of this study is to extract paramylon from microalgae, we wanted to  
 424 determine the maximum percentage of water that might not be harmful for the solubilization  
 425 of this biopolymer. We used imidazolium and cholinium based ILs and optimized the  
 426 conditions and the highest IL/ratio which could lead to the best dissolution of PM at 80°C.  
 427 The optimal conditions are summarized in Table 1.

428

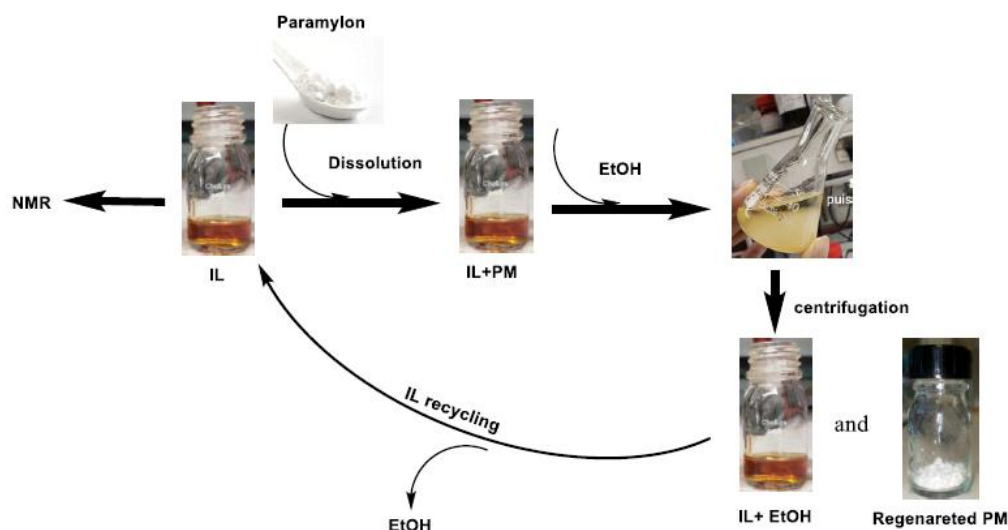
429 With commercial ILs (BmimAc and EmimAc), at 80°C during respectively 60 or 40 minutes,  
 430 the solubilization of PM could occur until a ratio IL/ water equal to 6/4. When the amount of  
 431 water is more important, a precipitate of PM was observed. Concerning the use of  
 432 CholC8Lev, a lower quantity of water was tolerated (ratio IL/ water 8/2), probably due to the  
 433 higher hydrophobic character of the IL containing a C8 carbon chain.

434 The behavior of PM in ChAAILs in the presence of water is really dependent on the nature of  
 435 the amino carboxylates. Indeed, a higher proportion of water was tolerated with CholGly and  
 436 CholAla (4/6) and an equal proportion (5/5) of water and CholVal which contains an

437 additional methyl group. When we considered the solubility of PM in CholAc/water and  
438 CholGly or CholAla/water mixtures, we proved the benefit of the NH<sub>2</sub> group on the solubility  
439 in IL/water mixtures. Finally, concerning the CholArg, the presence of the guanidine group  
440 required a lower water amount. . So moderate to very high water content can be tolerated for  
441 the dissolution of paramylon in BIOBILS, especially ChAAILs which could be used with a  
442 large amount of water ( $\geq 50\%$ ) without loss of PM dissolution power.

443  
444 Finally, in order to minimize the heating temperature as much as possible, activation by  
445 ultrasounds (95W during 2 hours) was used, thus allowing the temperature to be reduced from  
446 80 to 60°C. Similar dissolutions were observed with imidazolium ILs, CholAc and  
447 CholC8Lev as well as ChAAILs. No chemical modification was observed through NMR and  
448 same observations in DRX have been realized. Indeed, the technique using ultrasounds seems  
449 to be a very good method for solubilizing the paramylon because firstly, the temperature can  
450 be lowered and secondly, no any chemical modification of the paramylon structure occurred-

451  
452 The last step of our study was to enhance the major property of the ILs, namely their  
453 recycling. The recycling procedure is described on Figure 5 and was applied using  
454 conventional heating or US activation with all ILs previously mentioned.



455  
456  
457  
458 After each centrifugation, the supernatant alcohol solutions were collected and evaporated  
459 under reduced pressure to regenerate the ionic liquid whose unchanged structure was

460 confirmed by <sup>1</sup>H NMR (Figure S3). The ionic liquid was recovered with 97 to 99% yield and  
461 can be reused at least 4 times without loss of activity.

462

## 463 **Conclusions**

464

465 In this paper we described the solubilization of PM in choline based ionic liquids under  
466 conventional heating or by using ultrasounds activation. A concentration of 2% in PM was  
467 used and the solubilization could occur in various water amounts depending of the nature of  
468 the ILs. The recycling of the ionic solvents has been also proved including the non-  
469 degradation of the solvents and of the PM after each step by NMR and XRD analyses. The  
470 next step is the PM solubilization from microalgae biomass. These tests are currently in  
471 progress.

472

473

## 474 **References**

475 [1] J. Krajčovič, M. Vestega, S.D. Schwartzbach, Euglenoid flagellates: A multifaceted  
476 biotechnology platform, *J. Biotechnol.* 202 (2015) 135-145.

477 [2] J.Z. Kiss, A.C. Vasconcelos, R.E. Triemer, Structure of the Euglenoid storage  
478 carbohydrate, *Paramylon. Am. J. Bot.* 74 (1987) 877-882.

479 [3] R. Marchessault, Y. Deslandes, Fine structure of (1->3)-B-D-Glucans: Curdlan and  
480 Paramylon, *Carbohydr. Res.* 75 (1979) 231-242.

481 [4] a) A.P. Dadi, S. Varanasi, C.A. Schall, Enhancement of cellulose saccharification kinetics  
482 using an ionic liquid pretreatment step, *Biotechnol. Bioeng.* 95 (2006) 904-910. b) C. Mayer,  
483 R. Moritz, C. Kirschner, W. Borchard, R. Maibaum, J. Wingender, H.C., Flemming, The role  
484 of intermolecular interactions : studies on model systems for bacterial biofilms, *Int. J. Biol.*  
485 *Macromol.* 26 (1999) 3-16.

486 [5] a) Y. Daglio, M.C. Rodriguez, H.J. Prado, M.C. Matulewicz, Paramylon and synthesis of  
487 its ionic derivatives: Applications as pharmaceutical tablet disintegrants and as colloid  
488 flocculants, *Carbohydr. Res.* 484 (2019) 107779-107789. b) M. Shibakami, G. Tsubouchi, M.  
489 Nakamura, M. Hayashi, Polysaccharide nanofiber made from euglenoid alga. *Carbohydr.*  
490 *Polym.* 93 (2013) 499–505. c) R. Arashida, R. Nakano, K. Suzuki, E. Yoshida, E. Patent  
491 JP2011184592A, 2011-09-22.

492 [6] a) Y. Kawahara, A. Koganemaru, Development of Novel Film Using Paramylon Prepared  
493 from *Euglena gracilis*, *J. Appl. Polym. Sci.* 102 (2006) 3495-3497. b) M. Shibakami, G.

494 Tsubouchi, M. Nakamura, M. Hayashi, Polysaccharide nanofiber made from euglenoid alga,  
495 Carbohydr. Polym. 93 (2012) 499-505.

496 [7] J. Aketagawa, S. Tanaka, H. Tamura, Y. Shibata, H. Saitô, Activation of limulus  
497 coagulation factor G by several (1 $\rightarrow$ 3)-beta-D-glucans: comparison of the potency of glucans  
498 with identical degree of polymerization but different conformations, J. Biochem. 113(6)  
499 (1993) 683-686.

500 [8] a) M. Shibakami, G. Tsubouchi, M. Hayashi, Thermoplasticization of euglenoid  $\beta$ -1,3-  
501 glucans by mixed esterification, Carbohydr. Polym. 105 (2014) 90-96. b) M. Shibakami, G.  
502 Tsubouchi, M. Nakamura, M. Hayashi, Preparation of carboxylic acid-bearing polysaccharide  
503 nanofiber made from euglenoid  $\beta$ -1,3-glucans, Carbohydr. Polym. 98 (2013) 95–101.

504 [9] K. Yasuda, M. Ogushi, A. Nakashima, Y. Nakano, K. Suzuki, Accelerated Wound  
505 Healing on the Skin Using a Film Dressing with  $\beta$ -Glucan Paramylon, *In vivo* 32 (2018), 799-  
506 805.

507 [10] P.R.P. Swatloski, S.K. Spear, J.D. Holbrey, R.D. Rogers, Dissolution of Cellulose with  
508 Ionic Liquids, J. Am. Chem. Soc. 124 (2002) 4974–4975.

509 [11] a) H. Tadesse, R. Luque, Advances on biomass pretreatment using ionic liquids: An  
510 overview, Energy Environ. Sci. 4 (2011) 3913-3929. b) A. Salama, P. Hesemann, Recent  
511 Trends in Elaboration, Processing and Derivatization of Cellulosic Materials Using Ionic  
512 Liquids, ACS Sustain. Chem. Eng. 8 (2020) 17893–17907.

513 [12] (a) S.D. Zhu, Y.X. Wu, Q.M. Chen, Z. Yu, C. Wang, S. Jin, Y. Ding, G. Wu,  
514 Dissolution of cellulose with ionic liquids and its application: a mini-review, Green Chem.  
515 8(4) (2006) 325-327; (b) A. Pinkert, K.N. Marsh, S. Pang, M.P. Staiger, Ionic Liquids and  
516 Their Interaction with Cellulose. Chem. Rev. 109(12) (2009) 6712–6728.

517 [13] S. Magina, A. Barros-Timmons, S.P.M. Ventura, D.M. Evtuguin, Evaluating the  
518 hazardous impact of ionic liquids - Challenges and opportunities, J. Hazard. Mater. 412  
519 (2021) 125215.

520 [14] a) S. Hayouni, N. Ferlin, S. Bouquillon, Hydrogenation Catalysis in Biobased Ionic  
521 Liquids, “New Advances in Hydrogenation Processes—Fundamentals and Applications”;  
522 Ravanchi, M.T., Ed.; Intech Open Science: London, UK (2017) 15 and references therein. b)  
523 C. Chiappe, C. Cinzia, A. Marra, A. Mele, Synthesis and Applications of Ionic Liquids  
524 Derived from Natural Sugars, Top. Curr. Chem. 295 (2010) 177-195. c) S.T. Handy, M.  
525 Okello, G. Dickenson, Solvents from Biorenewable Sources: Ionic Liquids Based on  
526 Fructose, Org. Lett. 5 (2003) 2513-2515. d) S. Hayouni, N. Ferlin, S. Bouquillon, High

527 catalytic and recyclable systems for heck reactions in biosourced ionic liquids, Mol. Catal.  
528 437 (2017) 121–129.

529 [15] J.P. Mbakidi, S. Bouquillon, Composés de type liquide ionique, leurs procédés de  
530 préparation et leurs utilisations, PCT/EP2020/070365.

531 [16] Q.P. Liu, X.D. Hou, N. Li, M.H. Zong, Ionic liquids from renewable biomaterials:  
532 synthesis, characterization and application in the pretreatment of biomass, Green Chem. 14  
533 (2012) 304-307.

534 [17] A. Ahosseini, A. Scurto, Viscosity of Imidazolium-Based Ionic Liquids at Elevated  
535 Pressures: Cation and Anion Effects, Int. J. Thermophys. 29(4) (2008) 1222-1243.

536 [18] K. Kobayashi, S. Kimura, E. Togawa, M. Wada, S. Kuga, Crystal transition of  
537 paramylon with dehydration and hydration, Carbohydr. Polym. 80 (2010) 491-497.

538 [19] J.S. Kiss, J.M. Roberts, R.M. Brown Jr., R.E. Tremier, X-ray and Dissolution Studies of  
539 Paramylon Storage Granules from Euglena, Protoplasma 146 (1988)150-156.

540 [20] K. Okuyama, A. Otsubo, Y. Fukuzawa, M. Ozawa, T. Harada, N. Kasai, Single-Helical  
541 Structure of Native Curdlan and its Aggregation State, J. Carbohydr. Chem. 10(4) (1991) 645-  
542 656.

543

#### 544 *Acknowledgments*

545 This work was supported by the CNRS (CNRS Prime 80, PhD Fédérica Feuzing). We are  
546 grateful to the Public Authorities of Champagne-Ardenne and FEDER for material funds and  
547 the post-doc positions to Jean-Pierre Mbakidi. We are also grateful to the SATT Nord for an  
548 engineer position to Jean-Pierre Mbakidi.

549