Original Article

Biofpr Biofuels Bioproducts & Biorefining

Sustainable recovery of a chitin–glucan complex–protein biocomposite from yeast biomass using biocompatible ionic liquids

Inês C. Ferreira, LAQV-REQUIMTE, Chemistry Department, NOVA School of Science and Technology, Universidade NOVA de Lisboa, Caparica, Portugal; i4HB – Institute for Health and Bioeconomy, NOVA School of Science and Technology, Universidade NOVA de Lisboa, Caparica, Portugal; UCIBIO – Research Unit on Applied Molecular Biosciences, Chemistry Department, NOVA School of Science and Technology, Universidade NOVA de Lisboa, Caparica, Portugal
Cristiana A. V. Torres, i4HB – Institute for Health and Bioeconomy, NOVA School of Science and Technology, Universidade NOVA de Lisboa, Caparica, Portugal; UCIBIO – Research Unit on Applied Molecular Biosciences, Chemistry Department, NOVA School of Science and Technology, Universidade NOVA de Lisboa, Caparica, Portugal
Carlos A. M. Afonso, Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal
Luísa A. Neves, LAQV-REQUIMTE, Chemistry Department, NOVA School of Science and Technology, Universidade NOVA de Lisboa, Caparica, Portugal
Filomena Freitas, i4HB – Institute for Health and Bioeconomy, NOVA School of Science and Technology, Universidade NOVA de Lisboa, Caparica, Portugal; UCIBIO – Research Unit on Applied Molecular Biosciences, Chemistry Department, NOVA School of Science and Technology, Universidade NOVA de Lisboa, Caparica, Portugal

Received August 14 2025; Revised October 15 2025; Accepted November 3 2025;
View online at Wiley Online Library (wileyonlinelibrary.com);
DOI: 10.1002/bbb.70093; *Biofuels, Bioprod. Bioref.* (2025)

Abstract: Chitin–glucan complex (CGC) is typically extracted from fungal or yeast biomass using harsh alkaline treatments that not only generate high-salt, organic-rich effluents, but also negatively impact the structural and functional properties of the recovered copolymer, while wasting the yeast protein fraction, which is discarded. In this work, we present an innovative, environmentally friendly procedure based on the use of cholinium acetate ([Cho][OAc]), a biocompatible ionic liquid (IL), as a novel solvent to directly recover a natural CGC–protein biocomposite from *Komagataella pastoris* cells. The process was optimized *via* response surface methodology, achieving an overall recovery yield exceeding 40 wt% under mild thermal conditions (82–116 °C) and moderate biomass loadings (7.5–15 wt%), with reaction time having minimal effect. Reaction at 90 °C for 24 h, from a 11 wt% yeast biomass suspension, resulted in the recovery of

Correspondence to: Filomena Freitas, Department of Chemistry, NOVA School of Science and Technology, Universidade NOVA de Lisboa, 2829-516 Caparica, Portugal. E-mail: a4406@fct.unl.pt

a CGC–protein biocomposite comprising 64 wt% CGC and 36 wt% protein, with minimal inorganic salts contamination (0.8 wt%). This represents a novel, value-added biomaterial, leveraging the Generally Recognized as Safe status of *K. pastoris* and combining the bioactive properties of both CGC and yeast-derived proteins. The developed IL-based method offers a sustainable, safe, and efficient alternative to traditional approaches and opens new possibilities for the valorization of yeast biomass and the use of CGC–protein biocomposites in food, cosmetic, and biomedical applications. © 2025 The Author(s). *Biofuels, Bioproducts and Biorefining* published by Society of Industrial Chemistry and John Wiley & Sons Ltd.

Key words: chitin-glucan complex (CGC); cholinium acetate; biocompatible ionic liquids; biocomposite; protein; yeast

Introduction

Chitin–glucan complex (CGC) is a biodegradable and biocompatible biopolymer that combines the properties of chitin and β-glucans.[1,2] Its bioactive properties[3–5] render CGC of interest for use in pharmaceuticals,[6] wound healing,[7,8] food products and processes,[9,10] cosmetics,[11] water treatment[12,13] and wine clarification.[14] It is extracted from fungal and yeast biomass by procedures that commonly involve cell-wall fractioning, followed by CGC isolation and purification. Cell-wall fractioning is usually carried out by treatment with hot alkali (e.g. NaOH 0.1–5 M; 40–121 °C),[15] which disrupts the cell wall and solubilizes most cellular components (proteins, mannans and alkali-soluble β-glucans). Chitin–glucan complex is then isolated and purified from the resulting insoluble material by treating it with phosphate-buffered saline solution,[1] hydrochloric acid (1 or 6 M),[16] acetic acid (2%, v/v),[17] and water and/or ethanol.[1,16,18] However, such procedures generate high-salinity effluents with a high organic load, whose handling/disposal considerably increases the overall process cost. Moreover, the harsh conditions used during extraction (high temperature in an alkaline medium) often impact on the biopolymer's final properties.[19] Such drawbacks have driven investigation toward novel more environmentally friendly procedures for CGC extraction from yeast biomass. Furthermore, *Komagataella pastoris* protein is discarded during such procedures, wasting a valuable material that could find additional uses given the GRAS (Generally Recognized as Safe) status of the microorganism.[9,16]

Ionic liquids (ILs) are organic salts with a melting point below 100 °C, composed of ions (an organic cation and an organic or inorganic anion), whose chemical and physical properties can be tuned by suitable cation and anion selection, according to the desired application.[20,21] This characteristic makes them attractive for several uses, including the dissolution and processing of polysaccharides such as cellulose, chitin and CGC.[22–24] For example, 1-ethyl-3-methyl-imidazolium acetate ([C_2mim][OAc]) and 1-allyl-3-methyl-imidazolium bromide ([Amim]Br) were used for chitin extraction from crustacean.[19,25] Another example is the preparation of fibers using chitin and the IL [C_2mim][OAc].[25] Cholinium-based ILs (cholinium acetate, cholinium propionate and cholinium hexanoate), which are biodegradable and possess low toxicity,[26] were used to dissolve CGC and prepare films and gels.[22]

This study introduces, for the first time, the use of the biocompatible IL cholinium acetate ([Cho][OAc]) for the recovery of a CGC–protein biocomposite from *K. pastoris* cells, aiming to enhance the valorization of this yeast biomass into value-added products. To optimize the procedure and maximize the CGC content in the biocomposite, a statistically robust design of experiments approach was applied, systematically evaluating the influence of key variables (reaction time, temperature, and biomass load) on recovery efficiency. The resulting CGC-rich material was comprehensively characterized with respect to composition, structure and physicochemical properties, which were compared with that of neat CGC obtained *via* conventional alkaline extraction.

Experimental

Materials

Cholinium hydroxide (46 wt% in H_2O, Sigma-Aldrich) and glacial acetic acid (Fluka™, Honeywell, Germany) were used as received. [Cho][OAc] was synthesized as reported by Ferreira *et al.*[22] Briefly, glacial acetic acid (1 mol) was added to a cholinium hydroxide aqueous solution (1 mol) and stirred for 24 h, at 20 °C. [Cho][OAc] was obtained after water removal by rotary evaporation and subsequent drying under vacuum, and its purity was confirmed by ^{1}H NMR analysis (>95%). The water content (19 wt%) was determined using the Karl-Fischer titration method (model 831 KF Coulometer, Methrom AG, Switzerland).

Komagataella pastoris biomass was obtained by the bioreactor cultivation (10 L, BioStat B-plus, Sartorius) of strain DSM 70877, as described by Farinha *et al.*[27] The biomass was recovered by centrifugation (11 712**g**, 15 min) and lyophilized (for 48 h, at – 110 °C, under vacuum). The dry biomass was kept in a closed vessel until further processing.

Recovery of CGC–protein biocomposite by processing the yeast biomass with [Cho][OAc]

Design of experiments

The reaction conditions were evaluated by response surface methodology (RSM).[28] A central composite rotatable design was used, comprising reaction time (X_1), temperature (X_2) and biomass content (X_3) as independent variables. Two observed responses were considered, namely, the overall extraction yield (Y_1) and the CGC extraction yield (Y_2). Seventeen experiments were performed: eight factorial design points (levels ±1), six axial level runs ($\alpha \pm 1.682$) and a central point with three replicas (Table 1). For each experiment, the lyophilized biomass was mixed with [Cho][OAc] (fixed mass of 5 g) and heated to the desired temperature, for different reaction times. After cooling to room temperature, the mixtures were centrifuged (13 257**g**, 20 min) to remove undissolved cell debris and the CGC–protein biocomposite was recovered from the clarified supernatant by dialysis (12–14 kDa Spectra/Por® molecular porous membrane tubing, Spectrum Laboratories Inc.) against deionized water. Upon removal of the IL during the dialysis process, the biocomposite precipitated and was recovered by centrifugation (13 257**g**, 20 min). The resulting pellet was lyophilized (48 h, at −110 °C, under vacuum) and kept in closed vessels until further characterization. The recovery yield (wt%) was determined as follows:

$$\text{Extraction yield (wt. \%)} = \frac{\text{mass of dried insoluble fraction}}{\text{mass of dry biomass}} \times 100\% \quad (1)$$

RSM model

The system's behavior was evaluated by fitting the experimental data to the following quadratic model:

$$Y_p = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \quad (2)$$

where Y_p is the predicted response; X_1, X_2, X_3 are the independent variables coded values; b_0 is the interception coefficient estimate; b_1, b_2 and b_3 are the linear terms; b_{11}, b_{22}

Table 1. Central composite rotatable design: independent variables time (*t*), temperature (*T*) and biomass concentration; overall extraction yield and chitin–glucan complex (CGC) extraction yield as the observed responses.

Run	*t* (h)	*T* (°C)	Biomass (wt%)	Biocomposite recovery yield (wt%)	CGC extraction yield (wt%)
	X_1	X_2	X_3	Y_1	Y_2
1	8	80	5	4.5	0.8
2	24	80	5	28.3	7.8
3	8	120	5	37.9	20.7
4	24	120	5	36.5	22.8
5	8	80	15	32.9	20.9
6	24	80	15	41.9	30.1
7	8	120	15	35.9	14.4
8	24	120	15	37.1	16.4
9	2.5	100	10	42.5	25.9
10	29.5	100	10	43.7	29.1
11	16	66.4	10	15.0	10.1
12	16	133.6	10	30.8	22.6
13	16	100	1.6	9.3	2.5
14	16	100	18.4	30.3	20.5
15	16	100	10	40.0	21.2
16	16	100	10	54.1	30.9
17	16	100	10	41.4	26.7

and b_{33} are the quadratic terms; and b_{12}, b_{13} and b_{23} are the interaction terms. MODDE 8.0 (Umetrics, Sweden) was used for the statistical analysis. Analysis of variance (ANOVA) and multiple linear regression (MLR) were used to assess the model's fitting to the experimental data. The obtained model was evaluated considering the correlation coefficient (R^2), statistical meaning (P-value <0.05) and no lack of fit ($P > 0.05$) at a 95% confidence level. Statistical and surface plot analysis were used to evaluate the effect of reaction time, temperature and biomass content in the extraction yield. The significance of all the factors and their interactions was assessed by the P-value at a 95% confidence level.

Model validation

The model was validated by conducting three validation experiments (three replicates each), as described in Table 2.

Characterization

The sample's chitin content (Q, %) was determined by Eqn (3):[29]

$$Q = 14.199 \times N \quad (3)$$

where N is the nitrogen content (%) of the sample determined by elemental analysis (Flash EA 1112 Series CHNS analyzer), after subtracting the nitrogen content corresponding to the protein content. The protein content was determined by mixing the sample (3.0–5.0 mg in 5.5 mL of deionized water) with 20% NaOH (1 mL), and incubating at 100 °C, for 5 min. After cooling on ice, 170 µL of $CuSO_4{\cdot}5H_2O$ (25%, w/v) was mixed in. The mixture was centrifuged (3500**g**, 5 min) and the supernatant's optical density was measured at 560 nm. Albumin (Sigma-Aldrich) solutions (0–2.0 g L^{-1}) were used as protein standards. The inorganic salts content was determined by subjecting the samples to pyrolysis (550 °C, 24 h).[16]

The Fourier transform infrared spectroscopy (FTIR) spectra of the samples (~2 mg) were acquired in the mid-infrared range of 400–4000 cm^{-1}, with a Perkin-Elmer Spectrum Two™, with a detector type L1600300 equipped with an attenuated total reflectance accessory. Twelve scans with a resolution of 4 cm^{-1} were acquired. Before performing the measurements, a background spectrum was recorded, in the same conditions as the sample spectrum. Thermogravimetric analysis was performed on a Setaram Labsys EVO, in which the samples (around 10 mg) were heated from 25 to 500 °C, at a heating rate of 10 °C min^{-1}, under an argon flow of 50 mL min^{-1}.

Results and discussion

Recovery of CGC–protein biocomposite from *K. pastoris* biomass

[Cho][OAc] was used for the recovery of the CGC–protein biocomposite from the yeast biomass, based on the knowledge that this IL efficiently dissolves the biopolymer.[22] Cholinium, a quaternary ammonium cation, is an essential nutrient for humans, necessary for the normal function and growth of the cells.[30] As reported by Petkovic *et al.*,[26] the cholinium cation when combined with alkanoate anions, results in biocompatible ILs with low toxicity, including [Cho][OAc].

Design of experiments

A design of experiments approach was applied to assess the reaction conditions that maximize the recovery yield of the biocomposite from *K. pastoris* biomass, while maximizing its content in CGC. For a fixed [Cho][OAc] mass of 5 g, biomass loads ranging from 1.6 to 18.4 wt% were subjected to different temperatures (66.4–133.6 °C) for different reaction times (2.5–29.5 h), according to the experimental design (Table 1). The independent variables' range was chosen taking into account that CGC solutions in [Cho][OAc] could be prepared at a concentration of 5 wt% by exposing the mixtures at 80 or 110 °C, during 24 h.[22] Also, previous works regarding crustacean chitin extraction using ILs reported reaction conditions that included temperatures from 70 to 110 °C, during 18–36 h, for raw crustacean biomass contents of 6.7–10 wt%.[19,25,31]

The procedure consisted in contacting the lyophilized *K. pastoris* biomass with [Cho][OAc] at high temperatures [Fig. 1(a)], followed by centrifugation of the mixture to

Table 2. Model validation experiments: comparison between the obtained and predicted overall and chitin–glucan complex (CGC) extraction yields (wt%) according to the model.

Experiment	t (h)	T (°C)	Biomass (wt%)	Overall extraction yield (wt%)		Protein content (wt%)	CGC extraction yield (wt%)	
				Observed	Predicted		Observed	Predicted
1	8	119	10	41.8 ± 1.8	44.4	37.5 ± 1.1	26.1 ± 0.2	24.8
2	16	100	14	40.0 ± 5.5	44.2	36.9 ± 5.2	25.2 ± 1.9	26.3
3	24	90	11	46.8 ± 1.8	46.6	35.5 ± 1.1	30.2 ± 0.5	28.3

remove undissolved cell debris. Upon removal of the IL from the solution by dialysis against deionized water [Fig. 1(b)], the water-insoluble pellet [Fig. 1(c)] was recovered by centrifugation and, fanally, lyophilized [Fig. 1(d)].

Response analysis

The overall recovery yield varied between 4.5 and 54.1 wt%, as presented in Table 1. Both the temperature and the biomass content significantly influenced the biocomposite's recovery yield, with the highest overall values (≥40 wt%) being achieved for the runs performed at temperatures of 80 or 100 °C, with a biomass loads of 10 or 15 wt% (runs 6, 9, 10, 15, 16 and 17) (Table 1), while the lowest yields (<20 wt%) were observed for the runs conducted at either low temperatures (66.4 °C, run 11) or with lower biomass loads (1.6 or 5 wt%, runs 13 and 1, respectively). These results suggest that the biocomposite's recovery process is favored by higher temperatures and biomass contents. The reaction time, on the other hand, is apparently not relevant as such high extraction yields were obtained for runs conducted for periods of time ranging from 2.5 to 29.5 h. The samples' CGC contents ranged between 18.2 and 73.4 wt%, with protein contents within 26.6–81.8 wt%, corresponding to CGC extraction yields between 0.8 and 30.1 wt% (Table 1). The highest CGC extraction yields (>25 wt%) were achieved in runs 6, 9, 10, 16 and 17, for which the highest biocomposite recovery yields were also accounted for.

Response surface methodology modeling

Response surface methodology was used to evaluate the interactions between the three studied variables (reaction time, temperature, and biomass content) and their combined effect on the biocomposite recovery yield and the CGC extraction yield. The statistical model was also used to determine the variables' ranges that resulted in the highest extraction yields, for both studied responses. Correlation values of 0.899 and 0.939 were obtained for the overall extraction yield and the CGC extraction yield, respectively (Table 3). According to Lundstedt *et al.*,[28] a good fit corresponds to $R^2 > 0.90$, but $R^2 > 0.80$ is considered an acceptable fit. Both responses had significance as shown by the *P*-value below 0.05, with no evidence of lack of fit (*P*-value > 0.05), meaning that the pure errors of the responses and the model error are in the same range.

Influence of reaction time, temperature and biomass load on biocomposite recovery yield

As shown by the MLR analysis (Table 4), the biocomposite recovery yield was positively affected by the linear terms of temperature and biomass concentration (*P*-value < 0.05). The reaction time showed to have less effect on the studied response, being two times smaller than the biomass influence, with *P*-values > 0.05. On the other hand, despite the positive effect of linear terms of temperature and biomass, the interaction between them had a negative impact on the overall extraction yield (Table 4).

The 3D response surface plots (Fig. 2) showing the interactions between the three variables and their effect on the response agree with the MLR analysis results, in

Table 3. Analysis of variance (ANOVA) of the central composite design.

	P-value		R^2
	Model	Lack of fit	
Overall extraction yield (Y_1)	0.009	0.777	0.899
CGC extraction yield (Y_2)	0.002	0.885	0.939

CGC, Chitin–glucan complex.

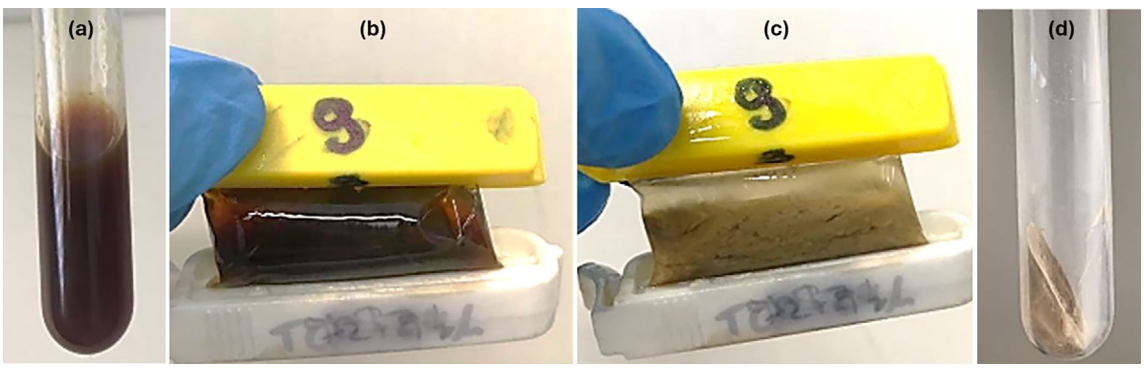

Figure 1. Extraction of chitin–glucan complex with [Cho][OAc]: (a) biomass suspended in [Cho][OAc]; (b) mixture after the removal of cell debris by centrifugation, placed in a dialysis membrane; (c) sample at the end of the dialysis against deionized water, evidencing the water-insoluble pellet; (d) freeze-dried sample obtained by centrifugation of the dialyzed sample.

Table 4. Multiple linear regression (MLR) analysis of the polynomial model.

Responses (Y)	Overall extraction yield (Y_1)		CGC extraction yield (Y_2)	
Effect	Coefficient	P-value	Coefficient	P-value
Constant	44.892	4.57×10^{-6}	26.346	3.06×10^{-6}
Linear				
Time (X_1)	2.535	0.173	1.880	0.081
Temperature (X_2)	4.860	0.023	2.616	0.025
Biomass (X_3)	5.559	0.013	4.391	0.002
Quadratic				
Time × time (X_{12})	0.218	0.909	0.161	0.879
Temperature × temperature (X_{22})	−6.922	0.007	−3.780	0.008
Biomass × biomass (X_{32})	−8.018	0.003	−5.495	0.001
Interaction				
Time × temperature (X_1X_2)	−4.125	0.101	−1.513	0.251
Time × biomass (X_1X_3)	−1.525	0.508	0.263	0.834
Temperature × biomass (X_2X_3)	−5.425	0.042	−6.888	0.001

CGC, Chitin–glucan complex.

which the biocomposite recovery yield was not significantly influenced by the reaction time [Fig. 2(a–c)], but the reaction temperature and the biomass load had a relevant impact [Fig. 2(d–f,g–i)]. Based on the RSM model, the parameters that would lead to higher biocomposite recovery yields, within the tested parameters range, were estimated: temperature within the range of 82–116 °C and biomass loads within 7.5 and 15 wt%, irrespective of the reaction time [Fig. 2(c)].

Influence of reaction time, temperature and biomass load on the CGC extraction yield

Similar to the biocomposite recovery, the CGC extraction yield was positively affected by temperature and biomass load (P-value < 0.05), with the reaction time showing lower effect on the studied response, being also two times lower than the biomass influence, with P-values > 0.05 (Table 4). The parabolic form of the 3D plots [Fig. 3(a–c)] corroborates the influence of both linear and quadratic terms of temperature and biomass concentration on CGC extraction yield, shown by the MLR analysis. More specifically the effect of the quadratic terms of temperature and biomass load, shown by the P-value <0.05 (Table 4), represents that the optimal values for CGC extraction are not in the extremes of the experimental values but within it. Further, the interaction between temperature and biomass load had a more pronounced negative impact, meaning that the increase of temperature/biomass decreased the effect of biomass/temperature, respectively, on the CGC extraction yield. Therefore, from the analysis of the 3D response surface plots, it is possible to observe that the highest CGC extraction yields (>26.6 wt%) could be achieved for reaction times of 16 and 24 h. However, for the reaction time of 24 h, high CGC extraction yields can be achieved for a wider range of conditions [Fig. 3(c)], being the extraction favored by temperatures lower than 117 °C and biomass loads above 8 wt%.

Model validation

To validate the developed model, three experiments with different extraction conditions were performed and the obtained overall and CGC extraction yields were compared with the ones predicted by the model (Table 2). For each experiment, three replicates were performed. From the obtained results, a good correlation was found for both studied responses between the observed and the predicted values (relative error ≤ 10.5%), thus showing good accuracy of the model to the experimental data. As predicted, the conditions applied in experiment 3 (reaction time, 24 h; temperature, 90 °C; biomass, 11 wt%) led to the highest overall extraction yield (46.8 ± 1.8 wt%) and also to the highest CGC extraction yield (30.2 ± 0.5 wt%).

Characterization of the CGC–protein biocomposite

The biocomposite sample obtained under the optimal conditions of validation experiment 3 (24 h, 90 °C and 11 wt%) was characterized for its composition, structure, and thermal properties.

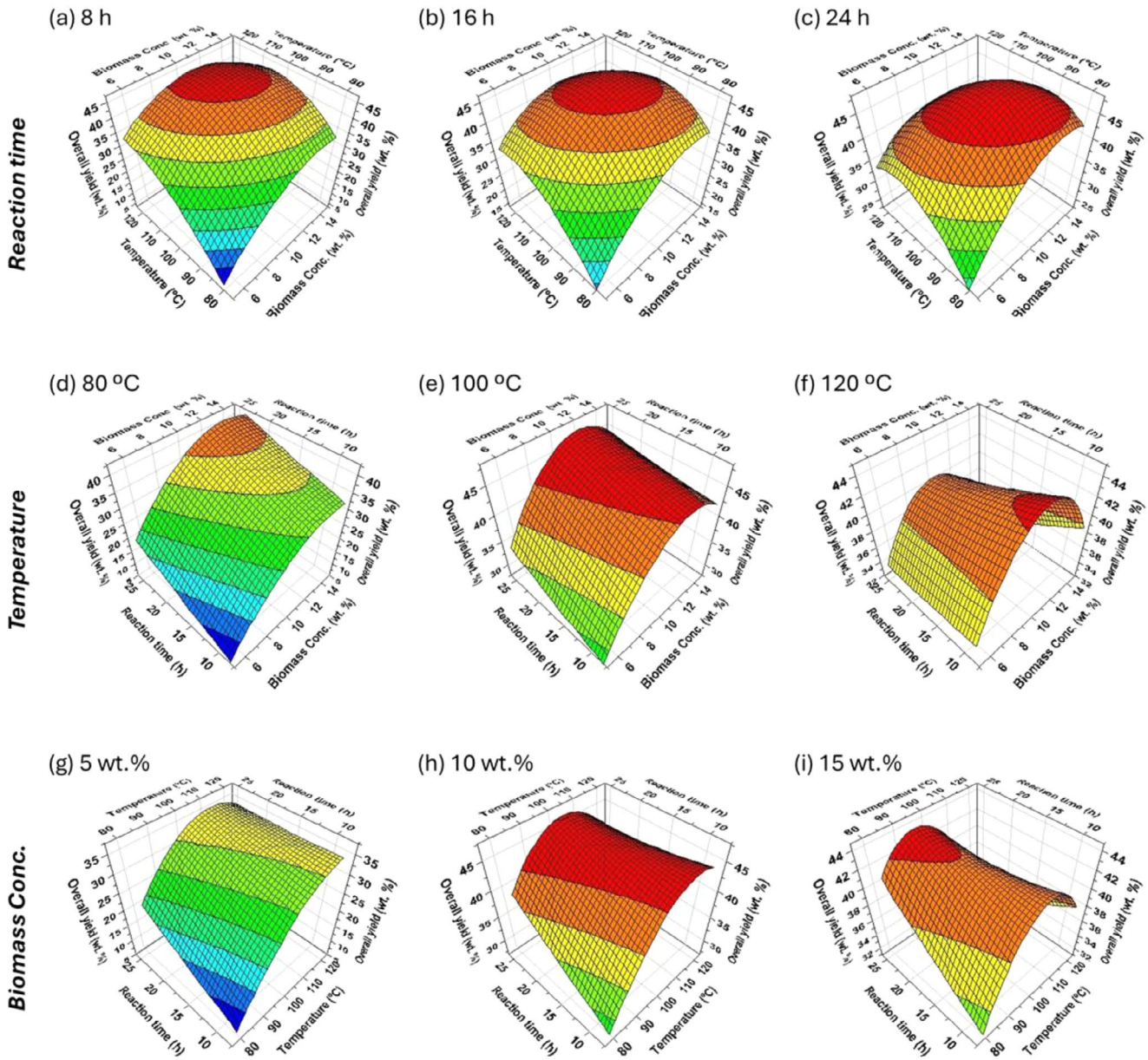

Figure 2. Response surface plots of the experimental design model for the overall extraction yield, (a–c) as a function of temperature and biomass concentration, (d–f) as a function of reaction time and biomass concentration, and (g–i) as a function of reaction time and temperature.

Composition

The sample had CGC and protein contents of 64 and 36 wt%, respectively, corresponding to a CGC extraction yield of 30.2 wt%. Inorganic salts were detected at trace amounts (0.8 ± 0.05 wt%), comparable with the values obtained by alkaline extraction with NaOH reported by Farinha *et al.* (<1 wt%),[16,27] and well below those reported by Roca *et al.* (15 wt%).[1] The biocomposite's chitin content, determined based on the elemental analysis data, was counted as 28%, which is within the range of the values reported for the copolymer extracted by the hot alkaline procedure (23.8–35.6%).[22,32,33] These results show that using [Cho][OAc] for treating *K. pastoris* biomass, although resulting in the recovery of a CGC–protein biocomposite, did not affect the copolymer's composition, maintaining its chitin content. Furthermore, given the GRAS status of *K. pastoris*, the high protein content of the obtained biocomposite renders it a biomaterial of additional value. Yeast biomasses are known

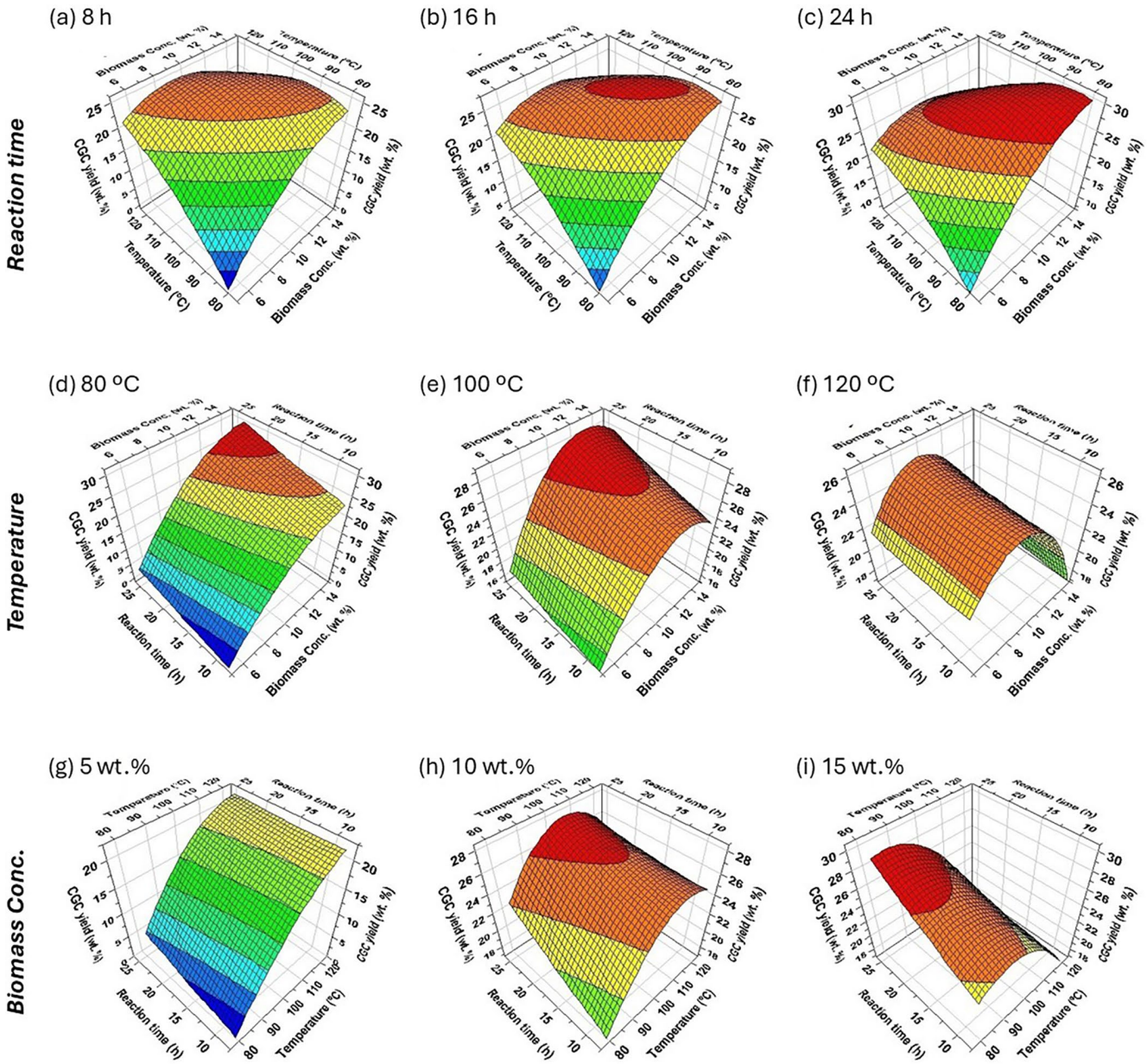

Figure 3. Response surface plots of the experimental design model for the chitin–glucan complex extraction yield, (a–c) as a function of temperature and biomass concentration, (d–f) as a function of reaction time and biomass concentration, and (g–i) as a function of reaction time and temperature.

to commonly have a wide spectrum of amino acids, with nutritional benefits making them suitable for use as food supplements.[34]

Fourier transform infrared spectroscopy

A structural analysis of the CGC–protein biocomposite was performed by Fourier transform infrared spectroscopy–attenuated total reflectance and compared with the CGC extracted by the hot alkaline procedure (CGC_NaOH) (Fig. 4). The CGC–protein biocomposite presented a spectrum similar to that of the CGC_NaOH, except for the two large bands appearing near 1630 cm^{-1} (instead of two peaks near 1653 and 1629 cm^{-1}) and 1532 cm^{-1} (instead of a peak near 1546 cm^{-1}), which can be related to the proteins present in the biocomposite (35.5 wt%). Characteristic peaks of proteins were reported to include the vibration frequencies of amide I, near 1650 cm^{-1}, and amide II, near 1550 cm^{-1}.[35] In this way, protein bands can be overlapped with the

characteristic bands of chitin, resulting in the two large bands observed in the CGC–protein biocomposite's spectrum. Nevertheless, the presence of chitin is confirmed by the peaks near 1455 and 1313 cm^{-1}, related to C-H deformation (asymmetric) and to the amide III, respectively,[36] while the peaks assigned to the β-(1,3)-glucans linkage near 1373, 1158 and 890 cm^{-1} are also observed.[37,38] The peak near 1744 cm^{-1}, which can be related to the presence of β-(1,6)-glucans linkage, is also observed.[37]

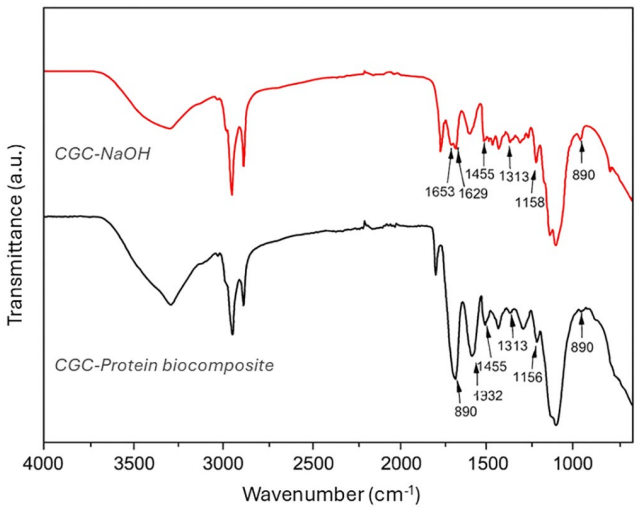

Figure 4. Comparison of Fourier transform infrared spectroscopy–attenuated total reflectance spectra of chitin–glucan complex (CGC)–protein composite obtained after extraction using ionic liquids, and the CGC obtained through the alkaline extraction with NaOH.

Thermogravimetric analysis

The thermogravimetric analysis of the CGC–protein biocomposite [Fig. 5(a)] comprised a first weight loss step corresponding to water evaporation[39] observed as the temperature increased from 35 to 135 °C ($T_{d,peak}$ = 83 °C), and a main degradation step occurring between 210 and 450 °C, with a weight loss of around 60%, corresponding to the degradation of the biocomposite components, which probably comprises an overlap of different degradation processes. The first degradation process can be related to the β-glucans and protein degradation, represented by a narrow peak in the derivative curve ($T_{d,peak}$ = 271 °C) [Fig. 5(a)]. These two components have been reported as the ones with the lowest degradation temperatures.[16,40] The second degradation process probably corresponds to the dehydration of the saccharide rings and decomposition of the chitin units,[41] represented by a small shoulder in the dTG curve ($T_{d,peak}$ = 303 °C). The final degradation process corresponds to the biocomposite's complete degradation, traduced through a peak ($T_{d,peak}$ = 362 °C) and small shoulder ($T_{d,peak}$ = 400 °C) in the dTG curve, with a char yield of 33%, at 550 °C.

Overall, the decomposition temperature range of the CGC–protein biocomposite [Fig. 5(a)] can be correlated with that of CGC reported in previous works [Fig. 5(b)], with a T_{deg} between 302 and 315 °C.[16,33] The main differences in the thermogravimetric analysis profiles of the CGC–protein biocomposite and the alkaline extracted CGC reside in the lower thermal stability of the first ($T_{d,peak}$ = 271 °C) that is probably related to the presence of proteins (35.5 wt%).

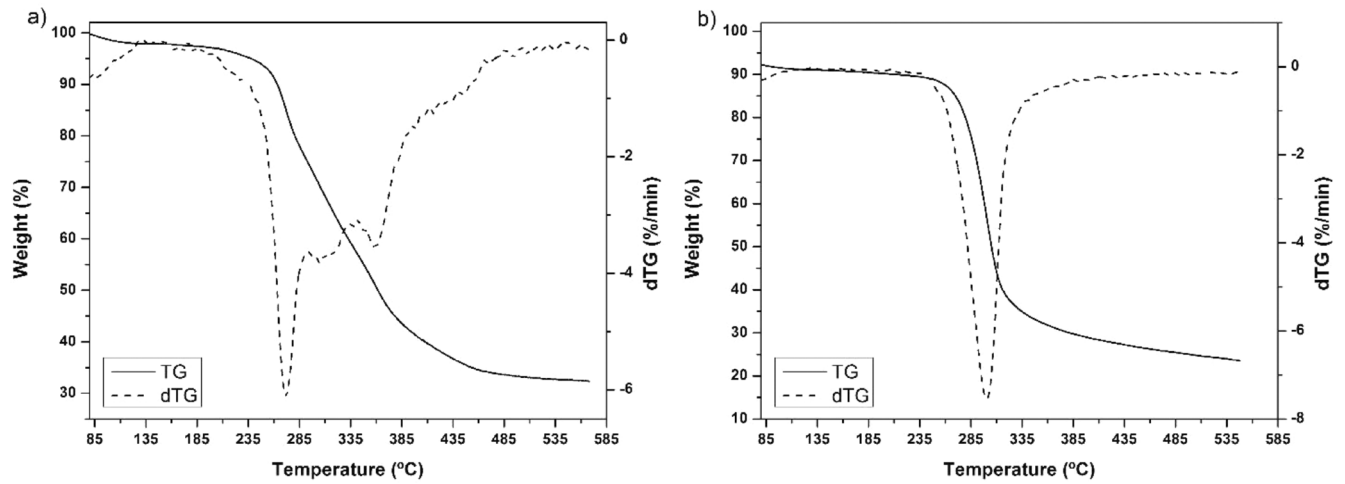

Figure 5. Thermogravimetric curve (TG; full line) and respective derivative (dTG; dashed line) for (a) chitin–glucan complex (CGC)–protein biocomposite (this work); (b) CGC (adapted from Araújo *et al.*[33]).

Overall assessment of the IL-based recovery procedure

The recovery of the IL used in the developed procedure is an important factor to account for since ILs are known to have a higher cost of production when compared with traditional organic solvents.[42] Hence, to guarantee the economic viability of the process, it is important that the IL is recovered and reused. Thus, an overall assessment of the proposed IL-based extraction procedure was made regarding the life cycle of the used [Cho][OAc], taking into account its synthesis, recyclability and disposal. When considering [Cho][OAc] synthesis (acid–base reaction of cholinium hydroxide with acetic acid), the only subproduct formed is water. After water removal, no further purification steps are required, and the IL can be used. Regarding the recovery, in general ILs can be recovered from aqueous mixtures by distillation owing to their low vapor pressure and high thermal stability.[19,42,43] In the developed IL-based procedure proposed here, [Cho][OAc] can be recovered from the dialysis water using a distillation process, such as vacuum evaporation, followed by a purification step using an organic solvent, if necessary, to remove dissolved impurities such as protein, salts or carbohydrates, that were present in the biomass and solubilized in the IL. Finally, another essential factor is the disposal of the IL and, consequently, its environment safety. It has been reported that cholinium-based ILs have a low cytotoxicity.[44] [Cho][OAc] was also reported to be readily biodegradable,[45] and also non-toxic to the activated sludge microbiota. Mena *et al.*[46] reported that [Cho][OAc] did not affect the microbial activity in activated sludge, being completely degraded, thus indicating that [Cho][OAc] is degraded in sewage treatment plants, which eliminates any environmental issues of the developed IL-based procedure.

Conclusions

In this study, a novel and sustainable procedure based on the use of the biocompatible IL cholinium acetate was successfully developed for the efficient recovery of a CGC–protein biocomposite enriched in CGC, from the biomass of the GRAS yeast *K. pastoris*. The procedure yielded a previously unreported natural biocomposite, primarily composed of CGC with a significant protein fraction (36 wt%). Importantly, the procedure preserved the chemical integrity and chitin content of the copolymer, while the co-extracted protein had no detrimental effect on the material's overall thermal stability, despite altering its degradation profile. The use of *K. pastoris* – a GRAS organism – alongside a green, solvent-free extraction strategy highlights the biocompatibility and safety of the final biomaterial. The synergistic combination of CGC and yeast-derived proteins enhances the functional value of the biocomposite, positioning it as a promising candidate for high-value applications in the food, cosmetic, and biomedical sectors.

Acknowledgement

Open access publication funding provided by FCT (b-on).

Funding information

This work was financed by national funds from FCT/MCTES – Fundação para a Ciência e a Tecnologia, I.P., Ministério da Ciência, Tecnologia e Ensino Superior, in the scope of the project UIDB/50006/2020 DOI 10.54499/UIDB/50006/2020 of the Associate Laboratory for Green Chemistry – LAQV, projects UIDP/04378/2020 and UIDB/04378/2020 of The Research Unit on Applied Molecular Biosciences; in Portuguese: Unidade de Ciências Biomoleculares Aplicadas, project LA/P/0140/202019 of the Associate Laboratory Institute for Health and Bioeconomy – i4HB, and projects UIDB/04138/2020 and UIDP/04138/2020 of iMed.ULisboa, and the national project PTDC/CTM-CTM/29869/2017. ICF acknowledges FCT, I.P. for financial support through PhD fellowship SFRH/BD/137636/2018. LAN acknowledges FCT/MCTES for the assistant researcher contract under Scientific Employment Stimulus (2022.00689.CEECIND). This work received support and help from FCT/MCTES (LA/P/0008/2020 DOI 10.54499/LA/P/0008/2020, UIDP/50006/2020 DOI 10.54499/UIDP/50006/2020 and UIDB/50006/2020 DOI 10.54499/UIDB/50006/2020), through national funds.

References

1. Roca C, Chagas B, Farinha I, Freitas F, Mafra L, Aguiar F *et al.*, Production of yeast chitin-glucan complex from biodiesel industry byproduct. *Process Biochem* **47**:1670–1675 (2012). https://doi.org/10.1016/j.procbio.2012.04.004.
2. Hong Y and Ying T, Characterization of a chitin-glucan complex from the fruiting body of *Termitomyces albuminosus* (Berk.) Heim. *Int J Biol Macromol* **134**:131 (2019). https://doi.org/10.1016/j.ijbiomac.2019.04.198.
3. Chen A, Pan F, Zhang T, Yu C, Xiao Y, Li S *et al.*, Characterization of chitin-glucan complex from *Tremella fuciformis* fermentation residue and evaluation of its antibacterial performance. *Int J Biol Macromol* **186**:649–655 (2021). https://doi.org/10.1016/j.ijbiomac.2021.06.048.
4. Berecochea-Lopez A, Decordé K, Ventura E, Godard M, Bornet A, Teissèdre P-LL *et al.*, Fungal chitin-glucan from *Aspergillus niger* efficiently reduces aortic fatty streak accumulation in the high-fat fed hamster, an animal model of nutritionally induced atherosclerosis. *J Agric Food Chem* **57**:1093 (2009). https://doi.org/10.1021/jf803063v.

5. Dalonso N, Goldman GH and Gern RMM, β-(1→3),(1→6)-Glucans: medicinal activities, characterization, biosynthesis and new horizons. *Appl Microbiol Biotechnol* **99**:7893 (2015). https://doi.org/10.1007/s00253-015-6849-x.
6. Neyrinck AM, Possemiers S, Verstraete W, De Backer F, Cani PD and Delzenne NM, Dietary modulation of clostridial cluster XIVa gut bacteria (*Roseburia* spp.) by chitin-glucan fiber improves host metabolic alterations induced by high-fat diet in mice. *J Nutr Biochem* **23**:51–59 (2012). https://doi.org/10.1016/j.jnutbio.2010.10.008.
7. Abdel-Mohsen AM, Frankova J, Abdel-Rahman RM, Salem AA, Sahffie NM, Kubena I *et al*., Chitosan-glucan complex hollow fibers reinforced collagen wound dressing embedded with *Aloe vera*. II. Multifunctional properties to promote cutaneous wound healing. *Int J Pharm* **582**:119349 (2020). https://doi.org/10.1016/j.ijpharm.2020.119349.
8. Liu Y, Zhang C, Liu L, Zhang X, Hou Y and Zhao L, Characterization of chitin-glucan complex of *Ganoderma lucidum* extract and its application as hemostatic hydrogel. *Waste Biomass Valori* **13**:3297–3308 (2022). https://doi.org/10.1007/s12649-022-01711-2.
9. EFSA Panel on Dietetic Products Nutrition and Allergies, Scientific opinion on the safety of 'chitin-glucan' as a novel food ingredient. *EFSA J* **8**(1):1–17 (2010). https://doi.org/10.2903/j.efsa.2010.1687.
10. Zhang Z, Zhao L, Qu H, Zhou H, Yang H and Chen H, Physicochemical characterization, adsorption function and prebiotic effect of chitin-glucan complex from mushroom *Coprinus comatus*. *Int J Biol Macromol* **206**:255–263 (2022). https://doi.org/10.1016/j.ijbiomac.2022.02.152.
11. Gautier S, Xhauflaire-Uhoda E, Gonry P and Piérard GE, Chitin-glucan, a natural cell scaffold for skin moisturization and rejuvenation. *Int J Cosmet Sci* **30**:459–469 (2008). https://doi.org/10.1111/j.1468-2494.2008.00470.x.
12. Skorik YA, Pestov AV and Yatluk YG, Evaluation of various chitin-glucan derivatives from *Aspergillus niger* as transition metal adsorbents. *Bioresour Technol* **101**(6):1769 (2010). https://doi.org/10.1016/j.biortech.2009.10.033.
13. Yousefi N, Jones M, Bismarck A and Mautner A, Fungal chitin-glucan nanopapers with heavy metal adsorption properties for ultrafiltration of organic solvents and water. *Carbohydr Polym* **253**:117273 (2021). https://doi.org/10.1016/j.carbpol.2020.117273.
14. Bornet A and Teissedre PL, Chitosan, chitin-glucan and chitin effects on minerals (iron, lead, cadmium) and organic (ochratoxin A) contaminants in wines. *Eur Food Res Technol* **226**:681–689 (2008). https://doi.org/10.1007/s00217-007-0577-0.
15. Araújo D, Ferreira IC, Torres CAV, Neves L and Freitas F, Chitinous polymers: extraction from fungal sources, characterization and processing towards value-added applications. *J Chem Technol Biotechnol* **95**:1277 (2019). https://doi.org/10.1002/jctb.6325.
16. Farinha I, Duarte P, Pimentel A, Plotnikova E, Chagas B, Mafra L *et al*., Chitin-glucan complex production by *Komagataella pastoris*: downstream optimization and product characterization. *Carbohydr Polym* **130**:455 (2015). https://doi.org/10.1016/j.carbpol.2015.05.034.
17. Boureghda Y, Satha H and Bendebane F, Chitin–glucan complex from *Pleurotus ostreatus* mushroom: physicochemical characterization and comparison of extraction methods. *Waste Biomass Valor* **12**:6139 (2021). https://doi.org/10.1007/s12649-021-01449-3.
18. Smirnou D, Krcmar M and Prochazkova EVA, Chitin-glucan complex production by *Schizophyllum commune* submerged cultivation. *Pol J Microbiol* **60**:223 (2011). https://doi.org/10.33073/pjm-2011-031.
19. Setoguchi T, Kato T, Yamamoto K and Kadokawa J, Facile production of chitin from crab shells using ionic liquid and citric acid. *Int J Biol Macromol* **50**:861–864 (2012). https://doi.org/10.1016/j.ijbiomac.2011.11.007.
20. Petkovic M, Seddon KR, Rebelo LPN and Pereira CS, Ionic liquids: a pathway to environmental acceptability. *Chem Soc Rev* **40**:1383 (2011). https://doi.org/10.1039/c004968a.
21. Anthony JL, Brennecke JF, Holbrey JD, Maginn EJ, Mantz RA, Rogers RD *et al*., Physicochemical properties of ionic liquids, in *Ionic Liquids in Synthesis*, ed. by Wasserscheid P and Welton T. Wiley-VCH Verlag GmbH & Co. KGaA, Wheinheim, Germany, pp. 41–126 (2003).
22. Ferreira IC, Araújo D, Voisin P, Alves VD, Rosatella AA, Afonso CAM *et al*., Chitin-glucan complex – based biopolymeric structures using biocompatible ionic liquids. *Carbohydr Polym* **247**:116679 (2020). https://doi.org/10.1016/j.carbpol.2020.116679.
23. Swatloski RP, Spear SK, Holbrey JD and Rogers RD, Dissolution of cellose with ionic liquids. *J Am Chem Soc* **124**:4974–4975 (2002). https://doi.org/10.1021/ja025790m.
24. Shamshina JL, Chitin in ionic liquids: historical insights into the polymer's dissolution and isolation. A review. *Green Chem* **21**:3974 (2019). https://doi.org/10.1039/c9gc01830a.
25. Qin Y, Lu X, Sun N and Rogers RD, Dissolution or extraction of crustacean shells using ionic liquids to obtain high molecular weight purified chitin and direct production of chitin films and fibers. *Green Chem* **12**:968 (2010). https://doi.org/10.1039/c003583a.
26. Petkovic M, Ferguson JL, Gunaratne HQNN, Ferreira R, Leitão MC, Seddon KR *et al*., Novel biocompatible cholinium-based ionic liquids – toxicity and biodegradability. *Green Chem* **12**:643 (2010). https://doi.org/10.1039/b922247b.
27. Farinha I, Araújo D and Freitas F, Optimization of medium composition for production of chitin-glucan complex and mannose-containing polysaccharides by the yeast *Komagataella pastoris*. *J Biotechnol* **303**:30–36 (2019). https://doi.org/10.1016/j.jbiotec.2019.07.007.
28. Lundstedt T, Seifert E, Abramo L, Thelin B, Nystrom A, Pettersen J *et al*., Experimental design and optimization. *Chemom Intel Lab Syst* **42**:3 (1998). https://doi.org/10.1007/978-3-540-49148-4_3.
29. Barrera B, Beltran O and Diosa G, Chitin-glucan complexes and method for the preparation thereof from chitin-rich biomaterials. US Patent 20160122444A1 (2016).
30. Blusztajn JK, Cholinium, a vital amine. *Science* **281**:794 (1998). https://doi.org/10.1126/science.281.5378.794.
31. Tolesa LD, Gupta BS and Lee MJ, Chitin and chitosan production from shrimp shells using ammonium-based ionic liquids. *Int J Biol Macromol* **130**:818–826 (2019). https://doi.org/10.1016/j.ijbiomac.2019.03.018.
32. Araújo D, Rodrigues T, Roma-Rodrigues C, Alves VD, Fernandes AR and Freitas F, Chitin-glucan complex hydrogels: physical-chemical characterization, stability, *in vitro* drug permeation, and biological assessment in primary cells. *Polymers* **15**:791 (2023). https://doi.org/10.3390/polym15040791.
33. Araújo D, Alves VD, Marques AC, Fortunato E, Reis MAM and Freitas F, Low temperature dissolution of yeast chitin-glucan complex and characterization of the regenerated polymer. *Bioengineering* **7**:28 (2020). https://doi.org/10.3390/bioengineering7010028.
34. Jach ME, Serefko A, Ziaja M and Kieliszek M, Yeast protein as an easily accessible food source. *Metabolites* **12**:63 (2022). https://doi.org/10.3390/metabo12010063.
35. Montroni D, Sparla F, Fermani S and Falini G, Influence of proteins on mechanical properties of a natural chitin-protein composite. *Acta Biomater* **120**:81–90 (2021). https://doi.org/10.1016/j.actbio.2020.04.039.

36. Lu Y, Sun Q, She X, Xia Y, Liu Y, Li J *et al*., Fabrication and characterisation of α-chitin nanofibers and highly transparent chitin films by pulsed ultrasonication. *Carbohydr Polym* **98**:1497 (2013). https://doi.org/10.1016/j.carbpol.2013.07.038.
37. Thanardkit P, Khunrae P, Suphantharika M and Verduyn C, Glucan from spent brewer's yeast: preparation, analysis and use as a potential immunostimulants in shrimp feed. *World J Microbiol Biotechnol* **18**:527–539 (2002). https://doi.org/10.1023/A:1016322227535.
38. Corradi da Silva ML, Fukuda EK, Vasconcelos AFD, Dekker RFH, Matias AC, Monteiro NK *et al*., Structural characterization of the cell wall D-glucans isolated from the mycelium of *Botryosphaeria rhodina* MAMB-05. *Carbohydr Res* **343**:793–798 (2008). https://doi.org/10.1016/j.carres.2007.12.021.
39. Kaya M, Sargin I, Tozak KÖ, Baran T, Erdogan S and Sezen G, Chitin extraction and characterization from *Daphnia magna* resting eggs. *Int J Biol Macromol* **61**:459–464 (2013). https://doi.org/10.1016/j.ijbiomac.2013.08.016.
40. Shuping Z, Yulong W, Mingde Y, Chun L and Junmao T, Pyrolysis characteristics and kinetics of the marine microalgae *Dunaliella tertiolecta* using thermogravimetric analyzer. *Bioresour Technol* **101**:359 (2010). https://doi.org/10.1016/j.biortech.2009.08.020.
41. Álvarez SPO, Cadavid DAR, Sierra DME, Orozco CPO, Vahos DFR, Ocampo PZ *et al*., Comparison of extraction methods of chitin from *Ganoderma lucidum* mushroom obtained in submerged culture. *Biomed Res Int* **2014**:169071 (2014). https://doi.org/10.1155/2014/169071.
42. de Jesus SS and Maciel Filho R, Are ionic liquids eco-friendly? *Renew Sustain Energy Rev* **157**:112039 (2022). https://doi.org/10.1016/j.rser.2021.112039.
43. Wang X, Li H, Cao Y and Tang Q, Cellulose extraction from wood chip in an ionic liquid 1-allyl-3-methylimidazolium chloride (AmimCl). *Bioresour Technol* **102**:7959 (2011). https://doi.org/10.1016/j.biortech.2011.05.064.
44. Muhammad N, Hossain MI, Man Z, El-Harbawi M, Bustam MA, Noaman YA *et al*., Synthesis and physical properties of cholinium carboxylate ionic liquids. *J Chem Eng Data* **57**:2191 (2012). https://doi.org/10.1021/je300086w.
45. Lotfi M, Moniruzzaman M, Sivapragasam M, Kandasamy S, Abdul Mutalib MI, Alitheen NB *et al*., Solubility of acyclovir in nontoxic and biodegradable ionic liquids: COSMO-RS prediction and experimental verification. *J Mol Liq* **243**:124–131 (2017). https://doi.org/10.1016/j.molliq.2017.08.020.
46. Mena IF, Diaz E, Palomar J, Rodriguez JJ and Mohedano AF, Cation and anion effect on the biodegradability and toxicity of imidazolium– and choline–based ionic liquids. *Chemosphere* **240**:124947 (2020). https://doi.org/10.1016/j.chemosphere.2019.124947.

Inês C. Ferreira

Inês C. Ferreira holds a PhD in Chemical and Biological Engineering from NOVA School of Science and Technology, NOVA University Lisbon (NOVA FCT), Portugal and is experienced in development of biopolymeric structures using biocompatible ionic liquids as solvents for biomedical applications.

Cristiana A. V. Torres

Cristiana A. V. Torres is a Researcher in Industrial Biotechnology at NOVA FCT, specializing in sustainable bioprocesses for producing biopolymers and fine chemicals and is experienced in microbial biotechnology, process optimization, and biopolymer characterization for value-added product development.

Carlos A. M. Afonso

Carlos A. M. Afonso is a Professor at the Faculty of Pharmacy, University of Lisbon, Portugal. His current research is mainly focused on synthetic methodologies, more sustainable chemistry, synthetic valorization of national natural resources and synthesis of bioactive molecules.

Luísa A. Neves

Luísa A. Neves is an Assistant Professor at NOVA FCT whose research focuses on membrane science and sustainable separation technologies, particularly involving ionic liquids, deep eutectic solvents, and CO_2 capture. Her work increasingly extends to biomaterials and environmentally friendly processing approaches.

Filomena Freitas

Filomena Freitas is an Assistant Professor at NOVA FCT and Senior Researcher at UCIBIO, focused on developing biotechnological processes and microbial bioproducts. She has experience in polymeric structures (hydrogels, emulsions, micro- and nanoparticles, scaffolds) from various biopolymers, including polysaccharides and polyhydroxyalkanoates, as well as their functional characterization.