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Compressive properties of yeast cell-loaded Ca-alginate hydrogel layers: Comparison with alginate–$CaCO_3$ microparticle composite gel structures

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ABSTRACT

The mechanical strength of alginate gel layers containing varying amounts of yeast cells was assessed by static uniaxial compression tests and compared to that of gel structures filled with equivalent quantities of inert mineral microparticles. Suitable gelation conditions were first determined by compression experiments on neat gel structures: alginate concentration, 2% w/v; Ca^{2+} ion concentration of the cross-linking solution, 100 mM; gelation time, 2 h. The presence of yeast cells in alginate disks led to the weakening of the gel structures, this effect increasing with the immobilized-cell content. By contrast, calcium chloride microparticles showing granulometric characteristics similar to those of yeast cells induced gel strengthening. The storage for 3 weeks at 4 °C in phosphate-free buffer induced noticeable weakening of alginate structures, whether filled with yeasts or not. These results are discussed in light of literature data on composite materials, in particular matrix–filler interactions.

1. Introduction

Immobilized cell technologies have widely developed since the early 1980s [1]. The artificial entrapment of microbial [or eucaryotic] cells in porous matrices prevails among the variety of immobilization procedures detailed over the last 20 years. Polysaccharide gel matrices, more particularly Ca-alginate hydrogels [2,3], are by far the most frequently used materials for harmless cell entrapment.

Alginates are anionic, linear copolymers composed of 1,4-linked β-D-mannuronic acid (M-block) and α-L-guluronic acid (G-block) units [2,4,5]. These naturally derived macromolecules display various compositions and sequences of M and G residues according to their origins (mainly brown seaweeds). Aqueous solutions of alginates are known to form hydrogels in the presence of divalent cations such as Ca^{2+} ions, which act as intermolecular cross-linkers between the functional groups of alginate chains, i.e., the negatively charged carboxyl groups of G-blocks.

Microbial, vegetal and animal cells have been entrapped in alginate gel particles to perform a wealth of bioprocesses including biosyntheses/bioconversions, biodegradation and bioremediation processes, food and beverage processing, and biomedical engineering. Some examples are the production of secondary metabolites by plant cells [6] and antibiotic production by fungi [7], wastewater treatment by microalgae [8] and soil bioremediation by bacteria [9], alcohol beverage production by yeasts [10], tissue engineering using mammalian cells [11].

A major problem that arises during implementation of alginate gel structures in operational conditions is their relatively low mechanical stability compared in particular to synthetic gels such as polyvinyl alcohol and polyethylene glycol gels [12]. In addition to inherent weakness of the ionically cross-linked alginate network, the presence of viable microbial cells, whose concentration is likely to increase with operation time as a consequence of cell growth in favourable incubation conditions [13] (i.e., growth-sustaining environments in terms of temperature, pH, concentrations of carbon and energy sources), may modify the mechanical behaviour of alginate gel structures [14]. Quantitative investigations of the effects of microbial cells on the mechanical properties of cell-loaded alginate particles are very scarce, however [15,16].

In the present work, the mechanical properties of alginate gel disks loaded with varying amounts of yeast cells were assessed by static uniaxial compression tests. The compression parameters of yeast-loaded gel structures were compared to those obtained using inert mineral microparticles [calcium carbonate] as filler. Preliminary experiments showing the influence of gelling conditions (i.e., alginate concentration, Ca^{2+} ion concentration of the cross-linking solution and gelation time) on the compressive parameters are reported. The effects of storage in a biological buffer on gel particle strength are also investigated.

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2. Materials and methods

2.1. Alginate samples and their characterization

Commercial sodium alginates were purchased from Prolabo (VWR Prolabo, Fontenay-sous-Bois, France) and Acros Organics (Geel, Belgium). The absolute average molecular weight and polydispersity of these alginates were determined by size-exclusion chromatography (SEC; polyhydroxymethylmetacrylate gel-packed columns OHPAK SB 804 and 806 HQ from Shodex Showa Denko K.K., Tokyo, Japan) coupled with multiangle laser light scattering (MALLS) detection (DAWN-EOS photometer, Wyatt Technology Inc., Santa Barbara, CA, USA) as described by Rousseau et al. [17]. Their *M*/*G* ratio was estimated by circular dichroism (CD6 spectrometer, Jobin Yvon, Longjumeau, France) according to Morris et al. [18].

2.2. Preparation of calcium alginate gel layers

Disk-shaped samples of ionically cross-linked alginate hydrogel were prepared adapting a diffusion-controlled procedure already described [19]. Sodium alginate powder was dissolved in deionized water to yield the desired concentration of alginate, i.e., 0.5, 1.0, 2.0, or 3.0% w/v, and the solution was degassed under vacuum for 2 h to eliminate most air bubbles. The polysaccharide solution was next poured into the cylindrical opening (diameter, 15 or 33 mm) of a Plexiglas plate (2.5 mm thickness) fastened to another plastic plate. The cavity was covered with a microporous membrane (GS filter from Millipore, Freehold, NJ, USA; pore size, 0.22 μm; porosity, 75%) that was held in position by a third perforated plastic plate. The whole structure was immersed for 2 h in calcium chloride solution (50, 100, or 300 mM) under gentle agitation to allow gel formation by diffusion of Ca^{2+} ions through the microporous filter. The disk-shaped gel could then be removed form its housing and stocked for 24 h in 100 mM $CaCl_2$ solution at 4 °C before the compression test. A set of at least 12 identical alginate disks was constructed for each test. No significant decrease in gel disk volume due to water loss (syneresis) during the gelation and storage periods in $CaCl_2$ solution was observed.

2.3. Preparation of particle-filled gels

A commercial freeze-dried baker's yeast (*Saccharomyces cerevisiae*; Vahiné, Monteux, France) was used. Varying amounts of yeast lyophilisate were weighted, suspended in sterile deionized water and thoroughly homogenized by magnetic stirring. The cell concentrations in these suspensions were estimated using a calibration curve relating biomass dry weight to CFU (Colony-Forming Units) number. CFU numbers were obtained by standard plate counts on nutrient agar (WL Difco Nutrient Medium, BD Diagnostics, Franklin Lakes, NJ) after incubation of the plates for 24 h at 37 °C. A suitable volume of calibrated cell suspension (i.e., known dry weight and cell concentration) was added to an aqueous alginate solution (typically 2.5% w/v) to yield the requested yeast concentration (0.047–5.0% dry w/v, 3.1×10^6–3.9×10^8 CFU cm^{-3} gel) and an alginate concentration of 2% w/v. The yeast-alginate mixture was processed as described above to give cell-loaded Ca-alginate gel disks.

Calcium carbonate powder (Prolabo) was suspended in deionized water and the $CaCO_3$ particles were dispersed ultrasonically for 10 min to form a homogeneous suspension. Calcium carbonate particles were recovered by microfiltration and dried at 100 °C till constant weight was reached. Varying quantities of dried particles were weighted and mixed thoroughly in deionized water. These calibrated suspensions were incorporated to alginate solutions and the resulting mixtures processed as described above to obtain $CaCO_3$-loaded gel disks (2% w/v alginate, 0.04–5.0% w/v $CaCO_3$).

2.4. Particle sizing

The volume and size distributions of yeast cells and $CaCO_3$ particles were determined using a Coulter Multisizer (Coultronics, Margency, France).

2.5. Compression tests

The mechanical properties of alginate discs were evaluated at room temperature by uniaxial compression between two parallel, stainless-steel plates using Universal Testing Machines from Instron (Canton, MA, USA). First tests were performed using Model 4301 equipped with a 500 N load cell. Then Model 5543 equipped with a 100 N load cell was used. A constant compression speed of 2.54 mm min^{-1} was applied in all experiments. After data treatment with specific software developed by the manufacturer (Instron Series IX, version 8.13.00, or Merlin V22082), compression (stress–strain) curves were constructed, from which the mechanical characteristics of alginate gel samples [strain, stress and energy at failure, Young's elastic modulus] were obtained (see Fig. 1).

2.6. Storage of alginate disks

Considering the destructive effect of the complexing phosphate anion against Ca-alginate gel, a phosphate-free biological buffer was used as liquid storage medium. A series of cell-free or cell-loaded gel disks were distributed among three sterile flasks supplied with Tris buffer [0.1 M tris(hydroxymethyl)-aminomethane adjusted to pH 7.0 with 0.1 M HCl; Acros Organics product] on the basis of about 20 disks per flask – all disks being completely immersed in the buffer. The flasks were stored at 4 °C. Every week over 3 weeks, one of the flasks was taken and its gel disk contents submitted to compression tests.

2.7. Enumeration of gel-entrapped yeast cells

Yeast cells were released from gel disks by complete disruption of disks in 0.1 M, pH 7 phosphate buffer (a mixture of 17.42 g K_2HPO_4 and 13.61 g KH_2PO_4 per liter of deionized water) using a blender (Stomacher Lab blender 80, Seward, Worthing, UK). Suspended

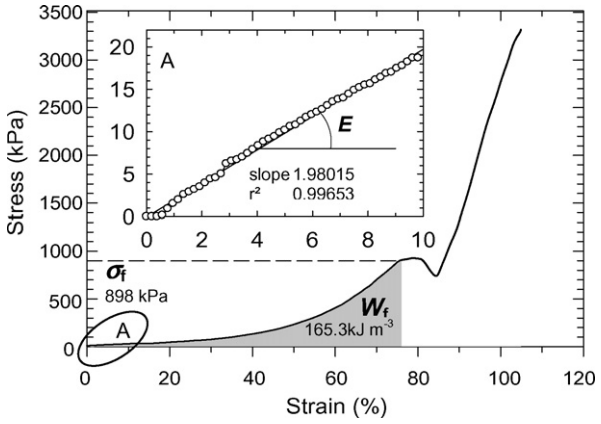

Fig. 1. Typical stress vs. strain compression curve of an alginate gel disk. Illustrative values of the parameters (σ_f, stress at failure; W_f, energy at failure; *E*, Young's modulus) were obtained for a 2% (w/v) algP (Prolabo alginate) gel disk hardened for 2 h at 4 °C in 0.1 M $CaCl_2$. The determination of Young's modulus is detailed in inset A.

Table 1
Physicochemical characteristics of the two commercial alginates (mean values from 2 experiments are given).

Alginate	$\bar{M}_n$ (g mol^{-1})	$\bar{M}_w$ (g mol^{-1})	I	M/G
Prolabo (AlgP)	127,000	185,000	1.46	1.71
Acros (AlgA)	203,000	341,000	1.68	1.61

$\bar{M}_n$ number average molecular weight; $\bar{M}_w$, weight average molecular weight; I, polydispersity index ($\bar{M}_n/\bar{M}_w$); M/G, M/G ratio (the ratio of mannuronic to guluronic units in alginate molecule).

cells were then enumerated by standard plate counts as described above.

2.8. Data processing and statistical analyses

Microsoft Office Excel (Microsoft Corporation, Santa Clara, CA) and Sigmaplot 10.0 (Jandel Scientific, Corte Madeira, CA) were used for compression data processing and descriptive statistics. Detection of outliers (Grubbs' test) was performed using the free GraphPad Software available at http://graphpad.com/qickcalcs/grubbs1.cfm.

3. Results and discussion

3.1. Alginate characteristics

The two commercial alginates used in this study were characterized by their molecular weight and M/G ratio (the ratio of mannuronic to guluronic units) (Table 1).

3.2. Influence of alginate concentration and gelation conditions

In preliminary experiments using AlgP alginate, we investigated the effects of some key parameters known to affect the mechanical properties of ionically cross-linked alginate gels, namely the alginate concentration, the Ca^{2+} ion concentration of the cross-linking solution ($CaCl_2$) and the hardening (gelation) time.

Compression magnitudes characterizing the mechanical strength of alginate gel disks were obtained from stress vs. strain curves (Fig. 1). Typical of viscoelastic gels, these deformation curves were composed of successive linear, non-linear and fracture regions, showing strain-hardening behaviour during the non-linear, large deformation region [20]. Energy at failure W_f was given by the area below the curve between origin and the breaking point (coordinates: ε_f and σ_f, strain and stress at failure, respectively). Elastic (Young's) moduli E were obtained from the slope of stress–strain curves during the linear region, limited to the first 10% of strain. Slopes of regression lines with r^2 value lower than 0.98 were eliminated. Significant outliers ($p < 0.01$) among experimental σ_f, W_f and E values were also removed before calculation of means and standard errors on the means. Very briefly, stress and energy at failure are representative of the gel strength and toughness, respectively, Young's modulus characterizes the stiffness (i.e., the resistance against the deflection of an applied force) and elastic behaviour of the gel and its compressive deformability is reflected by the failure strain.

3.2.1. Alginate concentration

At a concentration of 0.5% (w/v), gel structures were quite brittle and no consistent compression assays could be performed (not shown). For higher alginate concentrations, the strength and toughness (i.e., σ_f and W_f values) of gel disks logically increased with the polysaccharide concentration (Table 2). This effect is attributable to the increase in the number of junction zones between alginate

Table 2
Influence of alginate concentration on the mechanical characteristics of gel disks (calcium chloride concentration, 300 mM; gelation time, 2 h). Results are given as mean ± standard error.

Alginate conc. (% w/v)	n[a]	Strain at failure (%)		Stress at failure (kPa)		Energy at failure (kJ m^{-3})		Young modulus			
								kPa		r^2	
1	17	50.09 ± 1.11 (4.59)[b]	*<0.0001*[c]	43.3 ± 1.3 (5.4)	*<0.0001*	5.95 ± 0.27 (1.12)	*<0.0001*		ND		
2	13	83.76 ± 3.42 (12.33)	*0.0238*	993 ± 132 (476)	*0.3215*	193.8 ± 29.3 (105.6)	*0.4499*	115.8 ± 14.0 (50.5)	*<0.0001*	0.9921 ± 0.0012 (0.0045)	
3	12	73.28 ± 2.56 (8.86)		1194 ± 149 (516)		227.4 ± 32.6 (112.8)		206.8 ± 9.1 (31.4)		0.9971 ± 0.0005 (0.0019)	

[a] Number of determinations.
[b] In parentheses: standard deviations.
[c] In italic: two-tailed P values of unpaired t-tests. Underlined values show differences that are not statistically significant.

chains in the polymer network. It has been widely reported in the literature for ionically cross-linked alginate gels obtained as in the present work by external diffusion of Ca^{2+} ions [21–24] or by internal release of chelating cations [25–28]. Here, however, the differences in σ_f and W_f values between 2% and 3% alginate gels were not statistically significant (Table 2). Gels displayed fair elasticity as shown by the high r^2 values of stress vs. strain correlation lines in the initial linear region, and stiffness (E value) significantly increased with alginate content. Since Young's elastic modulus is a measure of the resistance offered by the gel to the stretching of ionic bonds that bind the polymer chains, an increase in E value with the number of cross-linking sites is not surprising. However, alginate disks lost some deformability (ε_f value) when the polysaccharide concentration was increased from 2% to 3%. Such a decrease in ε_f value with increasing alginate concentration has already been described by Nussinovich et al. [25] while others [26,28] reported quasi-constant failure strain when varying the polymer concentration.

Considering these results, an alginate concentration of 2% w/v was sufficient to yield proper gel strength with minimal formulation costs.

3.2.2. Calcium ion concentration

The influence of the Ca^{2+} ion concentration of the cross-linking solution on the mechanical parameters of gel structures at constant alginate content is shown in Table 3. The gel strength and deformability were slightly affected when the Ca^{2+} concentration was reduced from 300 to 50 mM but the variations in σ_f and W_f values were not statistically significant. Martinsen et al. [21] already reported that the gel strength of alginate gel beads immersed in $CaCl_2$ solution remained constant for Ca^{2+} concentrations higher than c. 50 mM, indicating saturation of the sites for Ca^{2+} binding in the polysaccharide chains at the tested alginate concentration. Inversely, gel stiffness was improved by decreasing the Ca^{2+} concentration. A well-known drawback of alginate cross-linkage by Ca^{2+} ions diffusing from external solution inside the gel core is the appearance of inhomogeneities in the gel structure [29], namely a polymer concentration gradient develops between the gel/liquid interface and the inner areas as the gelation process is controlled by diffusion. As a consequence, the gelation kinetics is difficult to control, which makes it difficult to yield gel samples with standardized mechanical properties. Here, the dispersion of compression data, reflected by standard deviations (Table 3), was amplified at high Ca^{2+} concentration that also affected gel stiffness. However, it seems unrealistic to explain these results by the reaction-diffusion kinetics of Ca^{2+} ions since increasing the ratio of calcium to alginate concentration is likely to improve the gel homogeneity [29].

Consequently, the concentration of the calcium chloride cross-linking bath was set at 100 mM in the following experiments.

3.2.3. Hardening time

Another parameter that affects the gelation kinetics is the hardening time, i.e., the duration of gel immersion in the $CaCl_2$ bath – depending obviously on the $CaCl_2$ and alginate concentrations, but also on the size and geometry of gel structures. For spherical particles, which is the usual geometry for encapsulation of microbial cells, reported hardening times vary from 5 min to 2 h [30], most protocols recommending gel–$CaCl_2$ contact periods lower than 30 min. Our initial choice of a 2-h gelation period for the gel (3% w/v) in its mould during immersion in $CaCl_2$ (0.3 M) arose from visual observation of gel formation in the mould, revealed by the progressive whitening of the alginate solution: whitening was completed within 2 h. Then the alginate disk was released from the mold and stocked for 24 h in $CaCl_2$ solution. This procedure was applied to 2% w/v alginate solutions using 0.1 M $CaCl_2$ and varying gelation

Table 3
Influence of Ca^{2+} ion concentration on the mechanical characteristics of gel disks (alginate concentration, 2% w/v; gelation time, 2 h). Results are given as mean ± standard error.

Ca^{2+} conc. (mol dm^{-3})	n[a]	Strain at failure (%)		Stress at failure (kPa)		Energy at failure (kJ m^{-3})		Young modulus			
								kPa		r^2	
0.05	18	71.81 ± 1.97 (8.34)[b]	[c]	886 ± 103 (435)	*0.3296*	146.6 ± 17.9 (75.2)	*0.1676*	162.9 ± 5.7 (24.0)	*0.7715*	0.9934 ± 0.0006 (0.0027)	
			0.0577								
0.1	18	76.49 ± 1.34 (5.69)		1,015 ± 80 (339)		181.3 ± 17.1 (72.9)		160.0 ± 8.1 (34.5)		0.9955 ± 0.0007 (0.0032)	
			0.0353		*0.8814*		*0.6984*		*0.0069*		
0.3	13	83.76 ± 3.42 (12.33)		993 ± 132 (476)		193.8 ± 29.3 (105.6)		115.8 ± 14.0 (50.5)		0.9921 ± 0.0012 (0.0045)	

[a] Number of determinations.
[b] In parentheses: standard deviations.
[c] In italic: two-tailed P values of unpaired t-tests. Underlined values show differences that are not statistically significant.

times in the mould, extending from 2 to 10 h. Not surprisingly, no statistically significant variations could be observed between the mechanical parameter values of the different gel disks (not shown).

Gel disks obtained by applying the optimal gel preparation conditions to another commercially available alginate (i.e., AlgA) were submitted to compression tests. AlgA displayed macromolecular characteristics and composition that slightly differed from those of AlgP, namely a higher molecular weight and a little lower M/G ratio (Table 1). Higher molecular weight and lower *M*/*G* ratio are usually associated with better mechanical properties [21,27,31] owing to enhanced interchain physical interactions at high molecular weight and increased density of ionic cross-linking sites at low M/G ratio. In agreement with these well documented observations, Young's elastic modulus and strain at failure of AlgA disks were in the range of the values obtained for AlgP structures while stress and energy at failure were noticeably higher, i.e., over 3000 kPa and 600 kJ m^{-3}, respectively, compared to c. 900–1000 kPa and 150–200 kJ m^{-3} for AlgP (Table 3). As a result, considering the application of alginate gels to microbial cell immobilization, next experiments on organic or mineral filler incorporation and cell-loaded gel storage were performed using AlgA gel disks.

3.3. Influence of filling with microbial cells or inorganic particles

Fig. 2 illustrates variations in the values of gel compression parameters resulting from entrapment of increasing amounts of yeast cells. Stress at failure of cell-loaded alginate disks was slightly higher than the reference value (cell-free gel) at low cell content ($\leq$ 1% w/v) but endured significant decrease at higher cell load. The same evolution stood for strain and energy at failure at high cell load. The compression modulus was noticeably affected by the presence of yeast cells, all *E* values remaining below the cell-free reference, but no clear evolution with cell content could be highlighted. These results partly agree with data published by Nussinovitch et al. [16] who showed a progressive decrease in the strength and stiffness of alginate gel cylinders loaded with *S. cerevisiae* biomass at concentrations ranging from 10^5 to 10^9 CFU per ml gel, while ε_F values remained unchanged over the whole range of cell loads.

To try to clarify the reasons for this weakening action of entrapped yeast cells – more particularly, to investigate whether specific interactions occur between microbial cells and alginate chains, yeast cells were replaced with calcium carbonate particles as mineral filler in alginate gel structures. Commercial $CaCO_3$ particles were first submitted to ultrasonic treatment for varying times to adjust their number and volume size distributions to those of yeast cells (Fig. 3). The effects of increasing amounts of $CaCO_3$ particles on the compression parameters of alginate gel disks were markedly different from those observed with yeast-loaded gels (Fig. 4). As a general rule, the mechanical properties of gel structures were improved by the presence of inorganic particles. More precisely, stress and energy at failure significantly increased with $CaCO_3$ content in the 0.04–0.60% concentration range (w/v) and stabilized for higher $CaCO_3$ amounts. Young's modulus showed the same evolution but increased again at high calcium carbonate concentration. The σ_f, W_f and *E* values for $CaCO_3$-filled gels were always higher than the reference ones corresponding to particle-free alginate. Strain at fracture was unaffected by the presence of $CaCO_3$ particles, however.

Calcium carbonate is very commonly used as filler in elastomer or thermoplastic polymer composites to reduce their cost and modify their properties, more particularly their mechanical properties. While conventional composites use mineral particles with dimensions in the micrometer range, more recent formulations privilege surface-treated nanoparticles to enhance further the stiffness, strength and/or toughness of filled polymers [32]. The intrinsic properties of the neat polymer, the filler type, its granulometry, surface state, concentration and dispersion in the polymer matrix are important parameters governing the mechanical properties of composite materials, together with potential matrix–filler interactions. In particular, it is well established that rigid inorganic filler particles are more efficient than ductile ones for reinforcing polymers as they increase both strength/stiffness and toughness of polymers while ductile fillers only enhance the ductility and

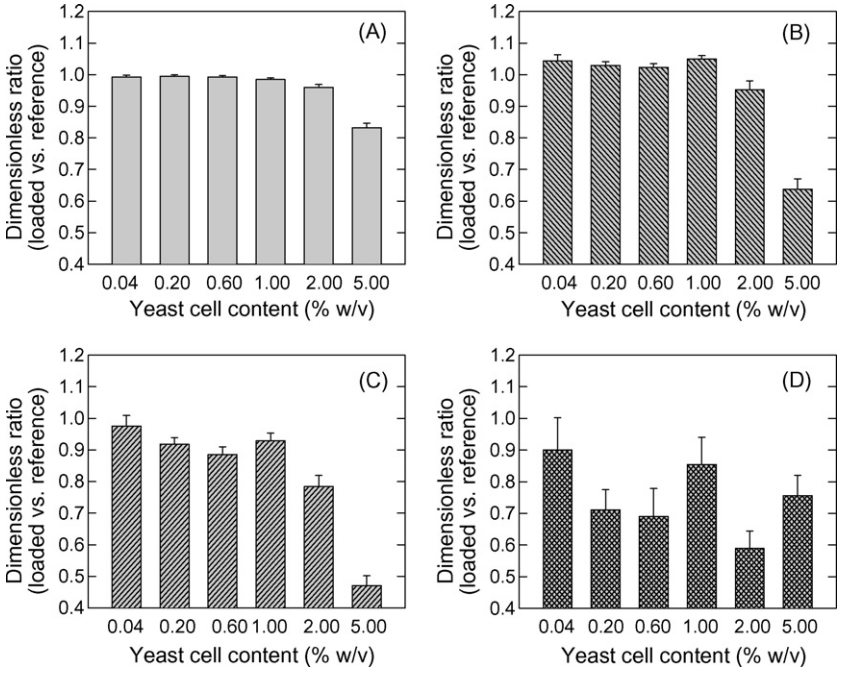

Fig. 2. Influence of yeast cell load on the mechanical properties of alginate gel disks (A, strain at failure ε_f; B, stress at failure σ_f; C, energy at failure W_f; D, Young's modulus *E*). Bars indicate errors on the means (*n* values ranging between 18 and 31).

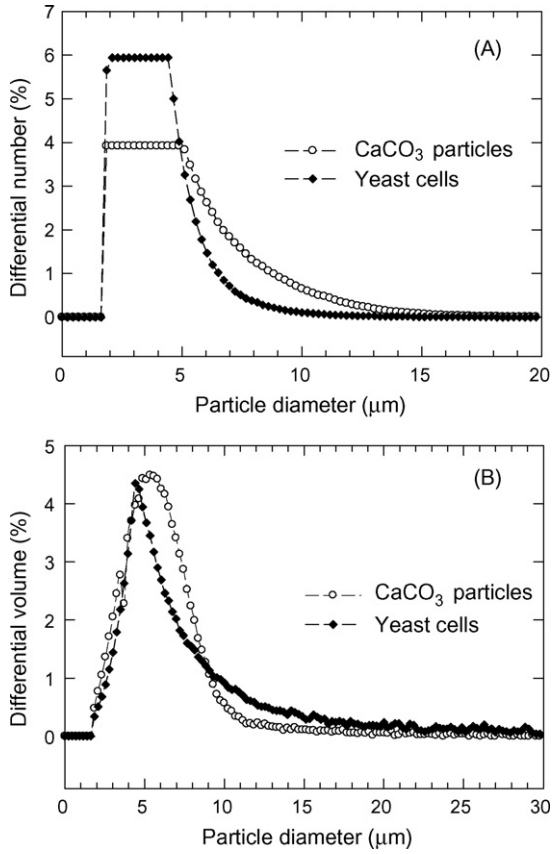

Fig. 3. Number (A) and volume (B) size distributions of yeast cells and $CaCO_3$ particles.

toughness of the composite materials [32]. Data showing the role of solid $CaCO_3$ particles as strengthening filler in alginate gel are very scarce, however.

Here, inert microparticles of $CaCO_3$ were used with no surface treatment. While their size strongly minimized the risk of dispersion heterogeneities due to aggregation [33], no significant adhesion between the particles and the gel matrix could be expected to affect their strengthening effect, however. This is confirmed by comparing the compression data to several models according to which stress at failure increases with the volume ratio of non-adhesive particles in the composite [34]. Therefore, alginate gel reinforcement was mainly attributable to the intrinsic mechanical characteristics of the filler. For instance, an E value of 14 GPa has been reported for $CaCO_3$ particles [35], which yields a high phase modulus ratio to the composite [34]. In the same way, the improvement in mechanical properties of composite gel structures obtained by replacing yeast cells with $CaCO_3$ particles is likely due to the difference in rigidity between the two fillers: *S. cerevisiae* cells behave like an elastic material with a Young's modulus in the range of MPa for whole cell [36] and 100 MPa for isolated cell wall [37], i.e., several orders of magnitudes lower than that of $CaCO_3$ particles. On the contrary, the weakening effect of yeast cells on alginate gel (Fig. 2) cannot be explained by the mechanical characteristics of the microbial filler, whose E value is higher than that of the crude alginate gel matrix.

As already mentioned, the same effect has been reported by Nussinovitch et al. [15,16], not only for alginate/yeast composites, but also for alginate and agar gels containing bacteria or fungal spores, i.e., very different matrix/filler combinations and interactions, if any. The weakening effect occurred in alginate at lower microbial load than in agar [16], a difference these authors attributed to disturbances during alginate gel formation. The decrease in experimental σ_f value as a function of the volume ratio of microbial particles is in favour of a good interfacial adhesion between the filler and the matrix [34]. However, the cell surface of *S. cerevisiae* has been shown to be negatively charged over a wide range of pH and ionic strengths [38], this negative charge

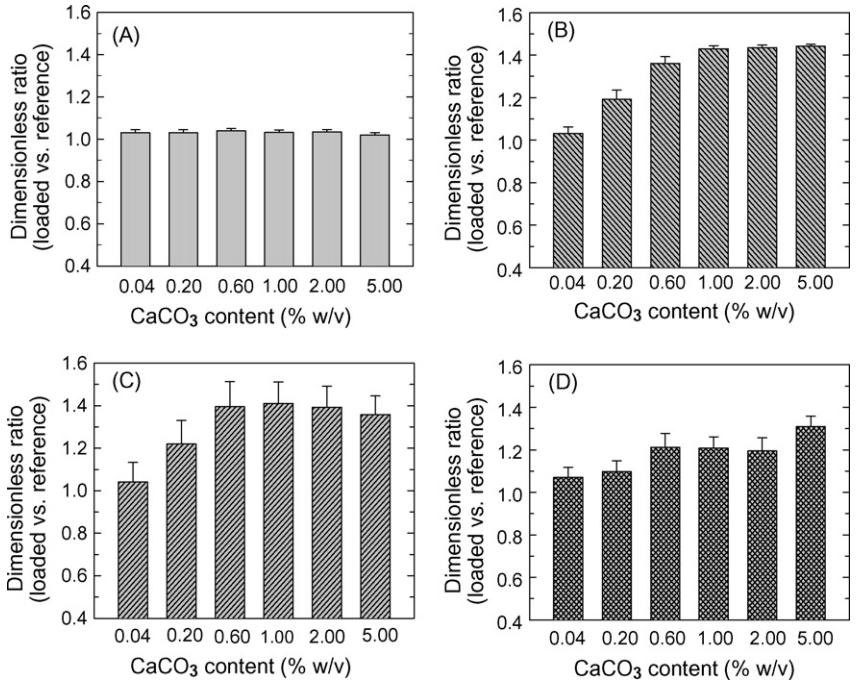

Fig. 4. Influence of $CaCO_3$ particle load on the mechanical properties of alginate gel disks (A, strain at failure ε_f; B, stress at failure σ_f; C, energy at failure W_f; D, Young's modulus E). Bars indicate errors on the means (n values ranging between 23 and 26).

Table 4
Time evolution of gel resistance and elasticity during storage in Tris buffer at 4 °C. AlgA gel samples were sterile or loaded with yeast biomass (0.5% dry w/v).

Storage	n[a]	Strain at failure (%)		Stress at failure (kPa)		Energy at failure (kJ m^{-3})		Young modulus	
								kPa	r^2
Sterile									
No	20	98.91 ± 0.22		3190 ± 31		693.1 ± 7.0		152.7 ± 6.4	0.9951 ± 0.0013
Buffer for 1 week	15	81.21 ± 0.78	(0.5514)[b]	1664 ± 68	(0.0659)	296.1 ± 14.9	(0.0661)	112.3 ± 7.2	0.9907 ± 0.0021
Buffer for 2 weeks	13	80.42 ± 1.24		1470 ± 75		251.4 ± 18.2		83.5 ± 5.2	0.9887 ± 0.0026
Buffer for 3 weeks	14	75.37 ± 0.71		1184 ± 47		185.6 ± 9.4		83.6 ± 6.3	(0.9904) 0.9872 ± 0.0019
Cell-loaded									
No	16	83.36 ± 0.83		2130 ± 83		372.3 ± 21.1		102.3 ± 8.2	0.9869 ± 0.0015
Buffer for 1 week	13	75.49 ± 0.67	(0.1267)	1361 ± 61		211.1 ± 11.0		117.3 ± 5.4	(0.1582) 0.9939 ± 0.0013
Buffer for 2 weeks	8	74.00 ± 0.46	(0.9921)	1128 ± 58	(0.9881)	171.0 ± 9.5	(0.8663)	81.6 ± 6.7	(0.9737) 0.9846 ± 0.0038
Buffer for 3 weeks	10	74.01 ± 0.80		1129 ± 37		169.0 ± 7.2		82.0 ± 9.2	0.9893 ± 0.0023

[a] Number of determinations.
[b] In parentheses, P values of the t-test showing differences that are not statistically significant (r^2 values not included).

being attributable to surface phosphate rather than to amino or carboxyl groups which are also present in cell wall [39]. Therefore, electrostatic repulsive forces occurred between yeast cells and negatively charged alginate chains, minimizing the possibility of strong matrix–filler interactions. The presence of yeast particles probably affected the gel formation process by immobilizing a fraction of Ca^{2+} ions that adsorbed to yeast cell walls and screening part of the cross-linking sites (i.e., carboxyl groups of guluronate residues) on alginate chains.

3.4. Storage stability

Alginate disks containing or not yeast cells were stocked at 4 °C in phosphate-free buffer (pH 7) and were subjected to compression assays after storage for 1, 2 or 3 weeks. The values of the different mechanical parameters characterizing cell-free and cell-loaded gel structures showed fairly similar variations with storage time, i.e., a significant decrease during the first week followed by a progressive stabilization over the next two weeks (Table 4). After 3 weeks, the neat and yeast-filled gels showed statistically equivalent compression behaviours. The yeast cell content of gel disks also varied during the storage period, gradually decreasing during the first 2 weeks and stabilizing later (Table 5). Some cell leakage from gel structures occurred during storage, as revealed by a limited increase in optical density of Tris buffer (not quantified).

The storage stability of alginate gel structures has been widely investigated, most studies focusing on the survival of gel-entrapped microorganisms and the preservation of their biocatalytic efficiency. Air- or freeze-drying processes have been more particularly developed in environmental application fields for extended storage while maintaining high cell viability [9,40–42]. Much fewer studies are concerned with the storage of wet gel particles in liquid medium, e.g., demineralized water [12]. The present results show that storage for 3 weeks at 4 °C in Tris buffer induced noticeable weakening of gel disks, whether filled with yeast cells or not. The gel compressive properties were very probably altered during storage by progressive leakage of cross-linking Ca^{2+} ions diffusing in the biological buffer which did not contain phosphate anions (which have high affinity for calcium ions) and non-gel-inducing cations (which can compete with Ca^{2+} ions for the junction sites), but was also devoid of free calcium ions. About 40% of initially entrapped microorganisms were not recovered from the gel after storage for 2 weeks (Table 5), showing loss in cell viability (culturability on solid nutrient medium) or/and cell lysis in starvation condition, in addition to cell leakage from the gel structure. These modifications in overall cell content of yeast-filled gel had minor influence on the time evolution of gel compressive properties.

Table 5
Time evolution of gel-entrapped yeast cell population during storage in Tris buffer at 4 °C. Results are given as mean ± SEM (n = 3).

Incubation time (weeks)	$10^8 \times$ CFU g^{-1} gel
0	1.84 ± 0.09
1	1.43 ± 0.02
2	1.13 ± 0.04
3	1.13 ± 0.02

4. Conclusions and future prospects

This paper confirms that the mechanical properties of alginate gel structures filled with microbial cells are impaired by the presence of cells. This overall weakening effect, which may be attributed to the negative charge of those microbial particles affecting gel cross-linking by Ca^{2+} ions, increases with the amount of gel-entrapped cells. Considering these results, an additional task will be to monitor the compressive behaviour of cell-filled gel disks during incubation in a nutrient medium allowing yeast growth inside the gel matrix, i.e., in other words, to evaluate the operational stability of the immobilized-cell structures. In addition to cell proliferation, the mechanical characteristics of cell-loaded alginate structures during operation in bioreactors may be affected by exposure to media that are aggressive towards gel structure, e.g., media containing other divalent or monovalent cations than Ca^{2+} ions or cation chelating agents such as wastewater [12], seawater [43] or simple culture media [44].

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