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RESEARCH ARTICLE

BIOTECHNOLOGY PROGRESS

Matrices (re)loaded: Durability, viability, and fermentative capacity of yeast encapsulated in beads of different composition during long-term fed-batch culture

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Abstract

Encapsulated microbes have been used for decades to produce commodities ranging from methyl ketone to beer. Encapsulated cells undergo limited replication, which enables them to more efficiently convert substrate to product than planktonic cells and which contributes to their stress resistance. To determine how encapsulated yeast supports long-term, repeated fed-batch ethanologenic fermentation, and whether different matrices influence that process, fermentation and indicators of matrix durability and cell viability were monitored in high-dextrose, fed-batch culture over 7 weeks. At most timepoints, ethanol yield (g/g) in encapsulated cultures exceeded that in planktonic cultures. And frequently, ethanol yield differed among the four matrices tested: sodium alginate crosslinked with Ca^{2+} and chitosan, sodium alginate crosslinked with Ca^{2+}, Protanal alginate crosslinked with Ca^{2+} and chitosan, Protanal alginate crosslinked with Ca^{2+}, with the last of these consistently demonstrating the highest values. Young's modulus and viscosity were higher for matrices crosslinked with chitosan over the first week; thereafter values for both parameters declined and were indistinguishable among treatments. Encapsulated cells exhibited greater heat shock tolerance at 50°C than planktonic cells in either stationary or exponential phase, with similar thermotolerance observed across all four matrix types. Altogether, these data demonstrate the feasibility of re-using encapsulated yeast to convert dextrose to ethanol over at least 7 weeks.

KEYWORDS
alginate, chitosan, fermentation, immobilization, yeast encapsulation

1 | INTRODUCTION

Cellular encapsulation was first achieved by Vincenzo Bisceglie in 1933, who showed that tumor cells embedded in a heterochthonous matrix maintained high viability.[1] Since then, microbial, plant, and metazoan cells have been encapsulated within different structures,[2] including a variety of natural gels like agarose, alginate, chitosan, collagen, fibrin, gelatin, and hyaluronic acid,[3,4] as well as within synthetic hydrated polymers and inorganic substrates, such as silica gels, sintered glass, and ceramic "beads."[5] In these matrices, encapsulated cells have been used to produce a variety of commercial products, including amino acids,[6-8] lactic acid,[9,10] beer and cider,[11-13] wine,[14,15] sparkling wine,[16,17] sake,[18,19] soy sauce,[20,21] probiotics for yogurt,[22] orange juice debittering,[23] vanillin,[24] and methyl ketone,[25] among others.

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In the realm of biomanufacturing, encapsulated cells offer two important advantages over free-floating or planktonic cells: higher service life and higher product yield per cell.[26-31] Extended service life has been attributed to encapsulated cells' resistance to acids,[32,33] organic solvents,[34,35] ethanol,[36] and osmotic and thermal stress.[37,38] These features are likely to be consequences of altered cell wall and plasma membrane composition following encapsulation,[39] and possibly also protection from shear forces afforded by the encapsulating matrix.[5] Encapsulated cells typically achieve higher product yield than planktonic cells as they divert less substrate to the formation of new biomass,[40,41] enabling them to more efficiently process feedstock.

Nagarajan et al encapsulated the yeast *Saccharomyces cerevisiae* in Ca^{2+}-alginate, which uncoupled reproduction from metabolism, allowing them to study chronological lifespan in active, nondividing cells. Their study provided evidence that, when continuously fed ad libitum, encapsulated cells are much longer lived than planktonic cells,[42] all the while exhibiting a stable pattern of gene expression that is distinct from either growing or starving planktonic cells. Relative to planktonic yeast grown in chemostats or planktonic yeast in exponential or stationary phase, continuously fed encapsulated yeast exhibits increased transcript levels of genes involved in cell wall remodeling, glycolysis, and stress resistance, and diminished transcript levels of tricarboxylic acid cycle genes and those regulating the cell cycle. Encapsulated cells are thus physiologically distinct from planktonic cells and age differently.[42]

Encapsulated cells have been studied in both continuous and repeated fed-batch culture systems. At present, because continuous culture systems do not easily scale up, repeated fed-batch culture is the favored means by which yeast is used to produce bioethanol[43-45] and other valuable commodities.[46,47] In repeated fed-batch systems using encapsulated yeast, high (85–90% g/g) yields of ethanol have been demonstrated after 5,[48] 24,[49] and 28 days.[50] This is higher efficiency than has been demonstrated using planktonic yeast in repeated fed-batch systems, which achieve up to 75% yield over 30 days.[51] Continuous culture systems offer more precise control over growth conditions, and this can translate into higher yields.[52,53] Indeed, prior studies have shown that continuously fed encapsulated yeast produces ethanol at 88–100% efficiency for 1 week,[54] 3 weeks,[55] and up to 3 months.[56] Thus, while multiple studies suggest that encapsulated cells could be re-used over several weeks, few[57,58] have explored whether and how different encapsulation matrices impact fermentation capacity and related parameters over longer time periods.[41]

To fill these knowledge gaps, glucose consumption and ethanol production by *S. cerevisiae* were monitored over the course of 7 weeks, comparing the performance of cells in planktonic culture with cells encapsulated as ~4 mm "beads" composed of four different matrices. To simulate industrial fermentation, a fed-batch system was used instead of the continuously fed system like that described by Nagarajan et al. We evaluated parameters related to bead resilience (Young's modulus, viscosity, size, and mass) and biological parameters related to fermentation capacity (ethanol yield, cell number/viability, and thermotolerance). Encapsulation matrices varied most in terms of viscosity and bead swelling over time, with the presence or absence of chitosan impacting results more than the type of alginate used. As a whole, encapsulated cells generated higher ethanol yield (g/g from dextrose) than planktonic cells and had greater heat shock resistance. These results demonstrate the potential for re-using encapsulated yeast in successive fed-batch cultures lasting at least 7 weeks and allow for comparisons to be made about long-term behavior of different encapsulation matrices.

2 | MATERIALS AND METHODS

2.1 | Strains and culture conditions

All experiments were performed using the same strain, *Saccharomyces cerevisiae* Ethanol Red®, an ethanol-tolerant (up to 18%[59]) industrial strain obtained from Leaf Lessafre (Marcq-en-Barœul, France; https://lesaffreadvancedfermentations.com/wp-content/uploads/2017/09/ER_EN_V3.pdf). Yeast were cultured in 250 ml of medium at 30°C in 250 ml screw-cap Erlenmeyer flasks placed on a gyrorotary platform shaking at 50 rpm, without pH control. To mimic the high sugar content typically used in biorefinery feedstock,[60,61] YEP medium (1% yeast extract, 2% peptone) was employed, amended with 15% dextrose (fermentation medium). Cells were maintained at −80°C in 30% (vol/vol) glycerol stocks and then plated on YPDA (2% dextrose, 2% peptone, 1% yeast extract, and 1.5% agar) prior to inoculation of single colonies into liquid culture.

Often, industrial-scale bioreactors are fed-batch, with new substrate supplied and product removed on 2- to 5-day cycles,[62] each cycle being re-pitched with fresh yeast. To mimic conditions encapsulated cells would encounter through multiple cycles of re-use, all cultures were provided fresh medium twice weekly, with spent medium discarded and fresh fermentation medium added. In both planktonic and encapsulated cell fermentations, the same cells were retained throughout the experiment. Spent medium was removed from encapsulated cultures by sieving it through a sterile brass sieve of 3-in. diameter and mesh size 10 (2 mm; McMaster Carr, Elmhurst, IL; Catalog no. 34735K216). Fresh sterile fermentation medium (250 ml final volume) was then placed into the same flask, containing the same beads, without cleaning/sterilization of the flask or beads. Spent medium was removed from planktonic cultures by decanting the entire 250 ml culture volume into 50 ml Falcon tubes, centrifuging these at 2000*g* for 2–3 min, then discarding spent medium. Pelleted cells were resuspended in fresh fermentation medium to a final culture volume of 250 ml, also without any cleaning or sterilization of the flask. Following addition of fresh medium, encapsulated and planktonic cultures were routinely checked for contamination by plating a sample of dilute cells on solid YPD (containing 1.5% agar).

2.2 | Preparation of encapsulation matrices and cell encapsulation

Four encapsulation matrices were tested: sodium alginate, sodium alginate and chitosan, Protanal LF 10/60, and Protanal LF 10/60 and chitosan (Table 1). Matrices were prepared as described by Takka and

TABLE 1 Types of matrices utilized in this manuscript

Alginate	Crosslinker	Abbreviation
Sodium alginate	0.2 M $CaCl_2$	NaAlg
Sodium alginate	1% Acetic acid + 0.2 M $CaCl_2$ + 0.25% chitosan	NaAlgCh
Protanal LF 10/60	0.2 M $CaCl_2$	Pr
Protanal LF 10/60	1% Acetic acid + 0.2 M $CaCl_2$ + 0.25% chitosan	PrCh
None (planktonic)	N/A	-

Note: Five replicates of four types of alginate matrix, as well as five planktonic controls, were tested. All beads were approximately 4 mm in diameter.

Gürel (Figure S1).[63] Sodium alginate was sterilized by combining 60 g of alginic acid sodium salt (sodium alginate; Sigma Aldrich; Catalog no. 180947) with approximately 300 ml of 95% ethanol; this mixture was left overnight. This approach was utilized as other sterilization methods can alter the viscosity of alginate.[64] This same procedure was repeated with the Protanal LF 10/60 (Protanal; gift from FMC Biopolymer). After mixtures sat overnight, ethanol was separated from the alginate by use of a 0.2-μm bottle top vacuum filter unit and the alginate was allowed to dry overnight at room temperature on the top of the filter.

To avoid pseudo-replication, 20 separate batches of encapsulation matrices were made in two rounds, the first round consisting of 10 batches using sodium alginate and the second consisting of 10 batches using Protanal; 110 ml of sterile distilled water and 6 g of dry, sterile sodium alginate were added to each of 10 sterile plastic 1 L beakers. Alginate was mixed into water using a Jiffy Mixing Blades Power Tool Attachment (purchased from Home Depot; Catalog no. DC408) and a standard power drill. Next, 80 ml of stationary-phase (24 hr) yeast were centrifuged at 2000*g* for 5 min, after which the supernatant was discarded and cells re-suspended in 40 ml of fresh fermentation medium. The suspension of yeast and medium was gently mixed into the alginate–water mixture, creating a 4% alginate-and-yeast suspension.

Ten sterile 60 ml plastic syringes were each filled with alginate and yeast solution. The syringes were 13 cm high with a 2.5 cm internal diameter and a standard size Luer lock with a 4.3 mm internal opening (Fisher Scientific; Catalog no. 22-031-375).[65] Needles were not attached to the syringes, thus the yeast–alginate suspension dripped directly through the Luer fittings aperture. Five of the syringes dripped into a 500 ml beaker containing 350 ml of the crosslinking solution 0.2 M $CaCl_2$, and the other five dripped into a 500 ml beaker containing 350 ml of the crosslinking solution 0.16 M acetic acid with 0.2 M $CaCl_2$ and 0.25% (wt/vol) chitosan (Sigma Aldrich; Catalog no. 448869). After the entire volume of alginate and yeast had dripped into the crosslinking solution, all equipment was sterilized and the procedure repeated, producing 10 batches of Protanal beads that each contained 4.5 g (3%) Protanal. Both the sodium alginate and Protanal beads were hardened in the crosslinking solutions overnight at 4°C before being transferred to 250 ml screw-cap Erlenmeyer flasks that contained fermentation medium up to 250 ml. This process produced approximately 1,750 beads per replicate, each of which initially contained ~1.1 x 10^6 cells. All 1,750 beads were placed in a 250 ml flask, resulting in approximately 2 x 10^9 total cells per flask at the start of the experiment. Planktonic control cultures were treated similarly, with each replicate receiving 80 ml of stationary-phase yeast re-suspended in 40 ml fermentation (the same starting amount of yeast as encapsulated cultures). So as to be comparable to encapsulated yeast, planktonic yeast suspensions also sat at 4°C overnight before being placed in fresh fermentation medium in 250 ml screw-cap Erlenmeyer flasks.

2.3 | Estimation of Young's modulus, viscosity, and bead size via Universal Testing Machine

A Zwick Roell Z010 Universal Testing Machine (UTM; Zwick Roell, Ulm, Germany) was used to measure bead Young's modulus, viscosity, and bead size. Specifically, three replicate beads from each of the 20 encapsulated populations were tested over the course of 7 weeks by programming a probe (5 N maximum) to push down against the beads until the probe recorded 0.3 N of resistance. The probe then maintained that position for 50 s as it measured how the bead relaxed against constant force. A linear trendline was then applied to a graph of standard force (N) versus strain (mm), with the slope of this line, multiplied by $\frac{2}{\Pi r}$, where r is the radius of each bead, reading out the Young's modulus (Figure S2A). An exponential fit of the last 10 s of a graph plotting standard force (N) versus time (s) was used to measure the decay constant. The Young's modulus was divided by the absolute value of the decay constant to calculate viscosity (Figure S2B). The UTM was also used to estimate bead diameter, by recording the tool separation distance when the 5 N probe first felt resistance.

2.4 | Estimation of wet weight bead mass

At each timepoint, and for each of the 20 replicates containing encapsulated cells, the mass of 10 beads was measured as a group using a Mettler Toledo MS104TS analytical balance.[66]

2.5 | Fermentative capacity

Over the course of 7 weeks, the fermentative capacity of planktonic and immobilized cells was monitored 12 hr after media exchange, in other words, 12 hr after each culture was resuspended in fresh fermentation medium containing 15% dextrose; 12 hr was chosen because we determined empirically that glucose was never exhausted until >24 hr. Thus, the 12 hr timepoint was well before cells underwent the diauxic shift and began to consume ethanol. Glucose consumed and ethanol produced were assayed using the EnzyChrom™ Glucose Assay Kit (BioAssay Systems, Hayward, CA; Catalog no. EBGL-100) and the Ethanol Test Kit (Fisher Scientific, Waltham, MA; Catalog #NC9508587), in both cases using methods provided by the manufacturer. Specifically, ethanol yield was calculated as the

amount of ethanol produced (g/L) divided by the amount of glucose consumed (g/L), with a theoretical max of 0.51.

2.6 | Cell enumeration and cell viability

To estimate cell number, yeast encapsulation matrices were dissolved by placing five beads into 10 ml of 10% (wt/vol) sodium metaphosphate solution (Fisher Scientific, Hampton, NH; Catalog no. 10124-56-8), a calcium chelating agent. Beads were agitated overnight at room temperature in a tissue drum rotator (New Brunswick Scientific, Edison, NJ; Item no. TC-6). The following day, any remaining bead particles were mechanically disrupted by manual pipetting. Following dissolution, optical density (OD) of cell suspensions was determined at λ = 600 nm using a Synergy HTX multi-mode UV/VIS spectrophotometer (BioTek Instruments, Winooski, VT; Catalog no. 16022315). OD values were converted to cells/ml using a standard curve plotting OD600 against Ethanol Red cell number estimated using a Multisizer 4e Coulter Counter (Beckman, Indianapolis, IN; Catalog no. B43905; Figure S3). Planktonic cells, including those that had escaped encapsulation within encapsulated cultures (escaped cells), were enumerated in the same manner, though without overnight incubation in 10% sodium metaphosphate.

Cell viability was estimated by measuring plasma membrane permeability to propidium iodide (PI), a fluorogenic compound that binds stoichiometrically to nucleic acids, producing a fluorescence emission that is proportional to cellular DNA content.[67] Membrane permeability to PI is indicative of cells that are dead or dying.[68,69] Two milliliters of cell suspensions were diluted 1:200 in sterile water, then stained with 5 μg/ml PI (ThermoScientific, Waltham, MA; Catalog no. P1304MP; stock 10 mg/ml). At least 10,000 cells per sample were counted using a Sysmex CyFlow Space flow cytometer (Sysmex, Kobe, Japan; Catalog no. 1604063918). Controls containing heat-killed dead cells, live cells, and a mix of both were used to establish proper gating between live and dead cells, with a typical range for live cells consisting of 0.1–1 FL2(590–50) at 488 nm with a log4 gain of 450.

2.7 | Heat shock tolerance

Heat shock tolerance was assayed for yeast encapsulated in each of four matrices, as well as for two planktonic controls: one consisting of yeast in exponential phase and the other consisting of cells that had reached stationary phase (24 hr). Freshly encapsulated beads were prepared according to the procedure outlined above in Section 2.2, after which cells were provided with fresh YPD medium (2% dextrose, 2% peptone, 1% yeast extract) daily for 3 days to allow cells to recover from encapsulation. The planktonic control was exposed to exactly the same conditions (including overnight at 4°C to improve bead durability for encapsulated cultures) followed by three consecutive days of feeding.

Exponentially growing cells were obtained by diluting a stationary-phase yeast culture 1:100 into fresh YPD medium, then culturing the population for ~3.5 hr to an OD of 0.3–0.5 at λ = 600 nm. Three replicates for all four bead types were assayed, as well as three replicates for the two planktonic controls. Prior to heat shock, a baseline colony-forming unit (CFU)/ml was calculated for each culture by making eight 1:10 serial dilutions into sterile water and then plating six 5 μl spots of each dilution (undiluted to 10^{-7}) on a YPD agar plate.

Heat stress was applied by immersing each bead (or an equivalent number of planktonic cells) in 1 ml of prewarmed medium and then placing them in a 50°C incubator. Following high-temperature incubation at 5, 15, 30, 90, and 180 min, cells were cooled on ice for 1 min. Then, both planktonic and encapsulated cells were resuspended in 1 ml of 10% sodium metaphosphate for 15 min to dissolve beads; beads were further disrupted by pipetting up and down using a 1,000 μl pipet. The resulting cell suspensions were successively diluted 1:10 seven times, and from each of these dilutions six 5 μl spots were plated on a YPD agar plate.[70] Plates were incubated overnight at 30°C before scoring for cell growth.

2.8 | Statistical analyses

Bead viscosity, bead mass, bead diameter, ethanol yield, cell number, and cell viability were compared using analysis of variance (ANOVA) tests with Tukey's post hoc tests. An alpha value of 0.05 was used as a cutoff for significance in all cases. In some cases, a Pearson's coefficient was calculated to determine if a correlation existed between two parameters.

3 | RESULTS AND DISCUSSION

3.1 | Young's modulus and viscosity of alginate beads declined over time

The Young's modulus and viscosity of different types of alginate-containing beads were measured to determine how they structurally weakened over time. Cell encapsulation matrices differed in chemical composition in ways that could be expected to affect their durability in prolonged culture. Specifically, different types of alginates exhibit differences in viscosity and rigidity, both of which have been shown to impact the physical stress that beads can endure (Figure S4). Chitosan was added to two of four matrices as it has been previously shown to improve bead durability, perhaps by reducing calcium chelation.[63,71]

To estimate the mechanical and structural decline different matrices underwent as they aged, we calculated both the *Young's modulus*, a measure of the ability of a material to withstand changes in length when under lengthwise tension or compression, and *viscosity*, a parameter that integrates resistance to force and relaxation under a constant force.[72,73] Specifically, for each bead type, Young's modulus and viscosity were measured at eight timepoints by applying 0.3 N of force, then maintaining that position for 50 s. Young's modulus and viscosity were calculated from the resulting data (Figure 1a–d), and these data were compared across bead types.

Overall, similar trends were observed in both parameters. Both Young's modulus and viscosity revealed significant differences based on an ANOVA with Tukey's post hoc test on three out of eight timepoints (Tables S1 and S2). Over the initial phase of our

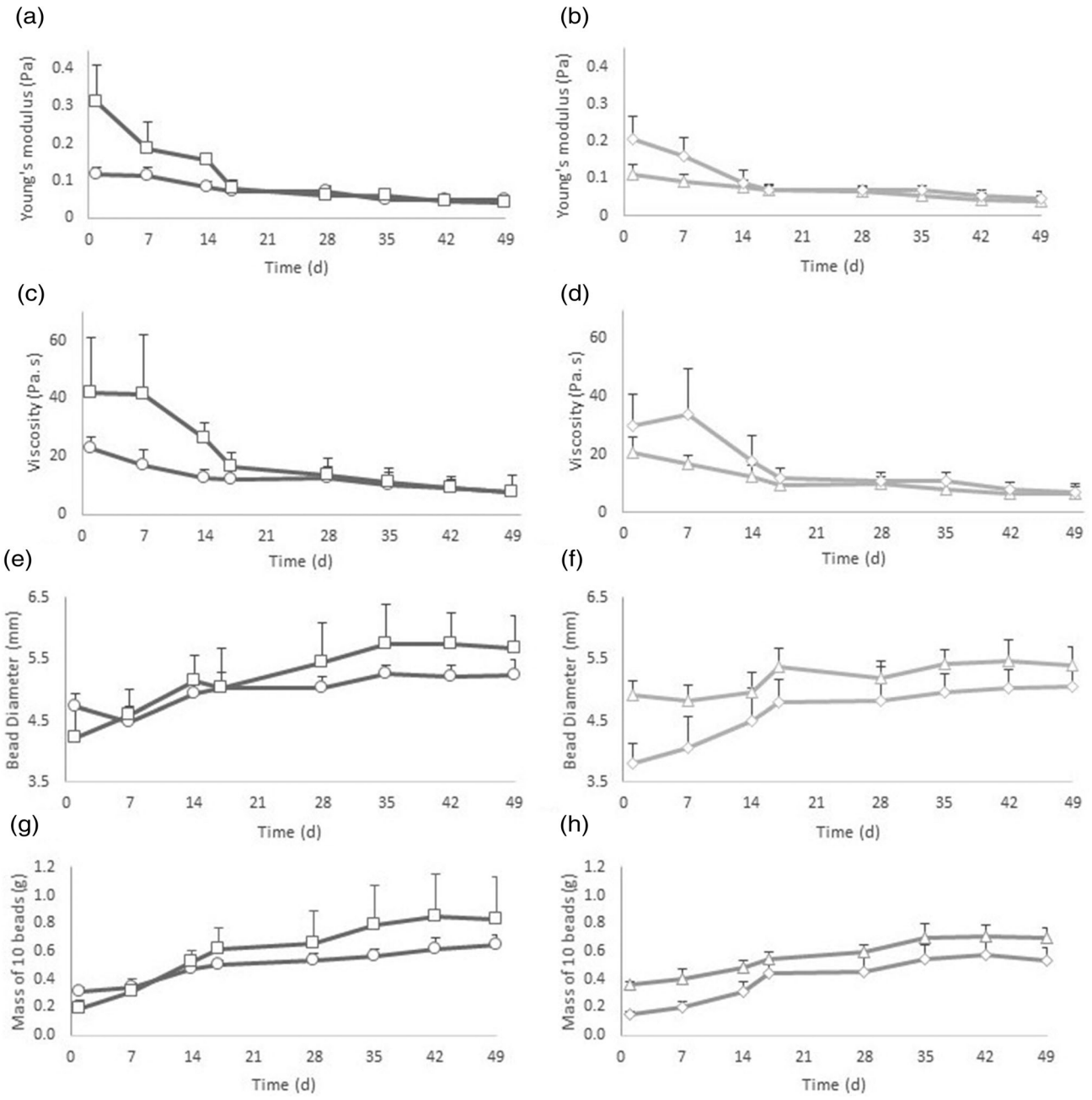

FIGURE 1 Bead durability and swelling. The Young's modulus and viscosity of three beads from each of 20 replicates were measured using a Universal Testing Machine. Over time, both Young's modulus (a,b) and viscosity (c,d) decreased. Beads increased in both diameter (e,f) and mass (g,h), becoming visibly larger as time progressed. Dark gray circles represent NaAlg beads, dark gray squares represent NaAlgCh beads, light gray triangles represent Pr beads, and light gray diamonds represent PrCh beads. Error bars represent 1 *SD*

experiments, both matrices that incorporated chitosan exhibited significantly higher Young's modulus and viscosity than matrices that did not (Tables S1 and S2, ANOVA with Tukey's post hoc). However, by Day 14 those chitosan-related differences had largely disappeared, with differences in Young's modulus on Day 35 and viscosity on Day 17 unrelated to the presence or absence of chitosan. Both Young's modulus and viscosity declined by roughly half over 49 days (Figure 1a–d), with much of this change occurring between Days 1 and 17, and comparatively less between Days 17 and 49 (Figure 1a–d). One explanation for this observation relates to the behavior of alginate gels under different magnitudes of stress: under smaller stresses (<5 mN), they behave elastically, but under larger stresses, they behave viscoelastically, settling into a new steady state.[72] The gradual chelation of Ca^{2+} ions, which are essential to the structural integrity of alginate gels, likely also contributed to the observed decrease in mechanical stability over time.[74]

Alginates are extracted from brown algae and are composed of alternating 1-4 α-L-guluronic (G-block) and β-D-mannuronic (M-block) acid residues.[75] Alginates form hydrogels in the presence of divalent cations, including Ca^{2+} (used in this study), Ba^{2+}, Sr^{2+}, and Zn^{2+}.[76,77]

TABLE 2 Chemical composition of different alginates

Brown algae	Average no. of G-blocks
Laminaria hyperborea (stem)	17
Laminaria hyperborea (leaf)	9
Lessonia nigrescens	7
Macrocystis pyrifeira	6
Laminaria japonica	6
Laminaria digitala	6
Asophyllum nodosum	4

Note: A variety of brown algae species are used to produce alginates, each varying in the average length of guluronic acid (G)-blocks. Protanal is sourced from the stem of *Laminaria hyperborea*, whereas sodium alginate is sourced from *Macrocystis pyrifeira*. Table modified from FMC Biopolymer.

Specifically, Pr is derived from the stem of the algae *Laminaria hyperborea*, whereas NaAlg is derived from *Macrocystis pyrifeira*. These algae differ in the average length of G-block acids (Table 2), which affects bead rigidity (Figure S4). Based on these data, we expected that Pr beads would exhibit higher Young's modulus than NaAlg beads. Contrary to expectations, Pr beads displayed similar Young's modulus to NaAlg beads (Figure 1a–d). Earlier reports suggested that certain types of sodium alginate can have higher viscosity than Protanal LF 10/60 beads[76]; the higher alginate concentration used in sodium alginate preparation may also have contributed to higher viscosity of this bead type (Table 1).

Our data support the notion that crosslinking with chitosan improves bead viscosity in both NaAlg and Pr beads for up to a week. Chitosan is commonly derived from crustacean shells, and is a linear polysaccharide composed of D-glucosamine and *N*-acetyl-D-glucosamine units linked by β-(1-4) glycosidic linkages.[78] Chitosan, a polycation, strengthens beads by forming a complex with alginate, which may protect crosslinking Ca^{2+} from metal chelators.[63,71] Figure 1a–d indicates that the presence of chitosan significantly improved Young's modulus and viscosity for 7 days (Tables S1 and S2), consistent with previous studies noting increased durability with chitosan.[63,79]

3.2 | Bead size and mass increased over time

Bead swelling has been linked to structural instability, and is thus undesirable.[57] To investigate whether some bead types were more prone to swelling than others, bead diameter and mass were measured as a function of time. The UTM was employed as a caliper to measure bead diameter (Figure 1e,f), and a scale was utilized to measure the wet mass of 10 beads (Figure 1g,h). Significant differences among treatments were observed for bead diameter at each timepoint (Table S3; ANOVA with Tukey's post hoc). Alginate beads crosslinked with chitosan swelled more than twice as rapidly as beads lacking chitosan; NaAlgCh beads increased by approximately 1.4 mm in diameter over 49 days, PrCh beads by ~1.2 mm, and NaAlg and Pr beads both increased by ~0.5 mm. Similar patterns were observed for bead mass. NaAlgCh beads increased by approximately 0.65 g over 49 days, whereas PrCh beads increased by ~0.39 g, and NaAlg and Pr beads both increased by ~0.33 g. In general, fewer differences were observed among treatments based on bead mass than were observed based on bead diameter, with significant differences observed among matrices through Day 17 (Table S4; ANOVA with Tukey's post hoc).

Bead swelling and shrinking have been previously linked to changes in a variety of conditions, including pH,[77] temperature,[80,81] and crosslinking solution.[82] Regular changes in pH, from 5.8 to 4.6 over each 3.5-day feeding episode, were observed in this experiment and likely contributed to bead swelling. Swelling could also have been caused by gradual loss of calcium cations over time,[57] allowing beads to increase in size and develop seams (Figure S5) after 28 days. This swelling may have also contributed to time-dependent changes in Young's modulus and viscosity (Figure 1a–d). A Pearson's correlation coefficient of −0.45 revealed a strong association between larger beads and lower viscosity across all bead types. Interestingly, although chitosan improved Young's modulus and viscosity in the short term, it also increased the rate of bead swelling relative to beads not crosslinked with chitosan, potentially contributing to lower Young's modulus and viscosity in the long term. This is in agreement with studies indicating that chitosan contributes to bead swelling at low pH (~5) values,[63] conditions our beads experienced for 48 hr in each cycle after glucose was depleted and before fresh medium was added.

3.3 | Ethanol yield (g/g) from dextrose varied among treatments

To determine which treatment consistently resulted in the highest ethanol yield over successive fermentation cycles, glucose consumed and ethanol produced were measured 12 hr after adding fresh substrate. The 12 hr timepoint was chosen because it was prior to glucose exhaustion (and potential ethanol consumption), which never occurred until >24 hr. Depending on treatment and sampling day, 7.5–100 g/L of glucose remained at the 12 hr timepoint.

Ethanol yield was generally higher in encapsulated than in planktonic cultures (Figure 2), with concentrations ranging from ~10 to 75 g/L depending on treatment and time. While both NaAlg and Pr beads exhibited significantly higher ethanol yields than planktonic cultures on Day 1, yields dropped in both these matrices on Day 7. This decrease cannot be explained by a corresponding decline in viability or in cell number nor by an increase in escaped cells, but may instead be related to buildup of acetic acid in the medium (see Section 3.6 for further discussion), which reached ~15 mM. Cells in NaAlgCh and PrCh beads did not attain ethanol yields comparable to those achieved by cells in NaAlg and Pr beads until 14 days (Table S5; ANOVA with Tukey's post hoc). Since chitosan does not fully dissolve in water, 0.16 M acetic acid was used as a solvent. Thus, matrices crosslinked with chitosan were exposed overnight to acetic acid, whereas the other matrices and the planktonic control were not. Acetic acid is known to negatively impact cell viability[83,84] and could account for lower yields observed in those bead types early on. NaAlgCh and PrCh beads also exhibited a decline in ethanol yield

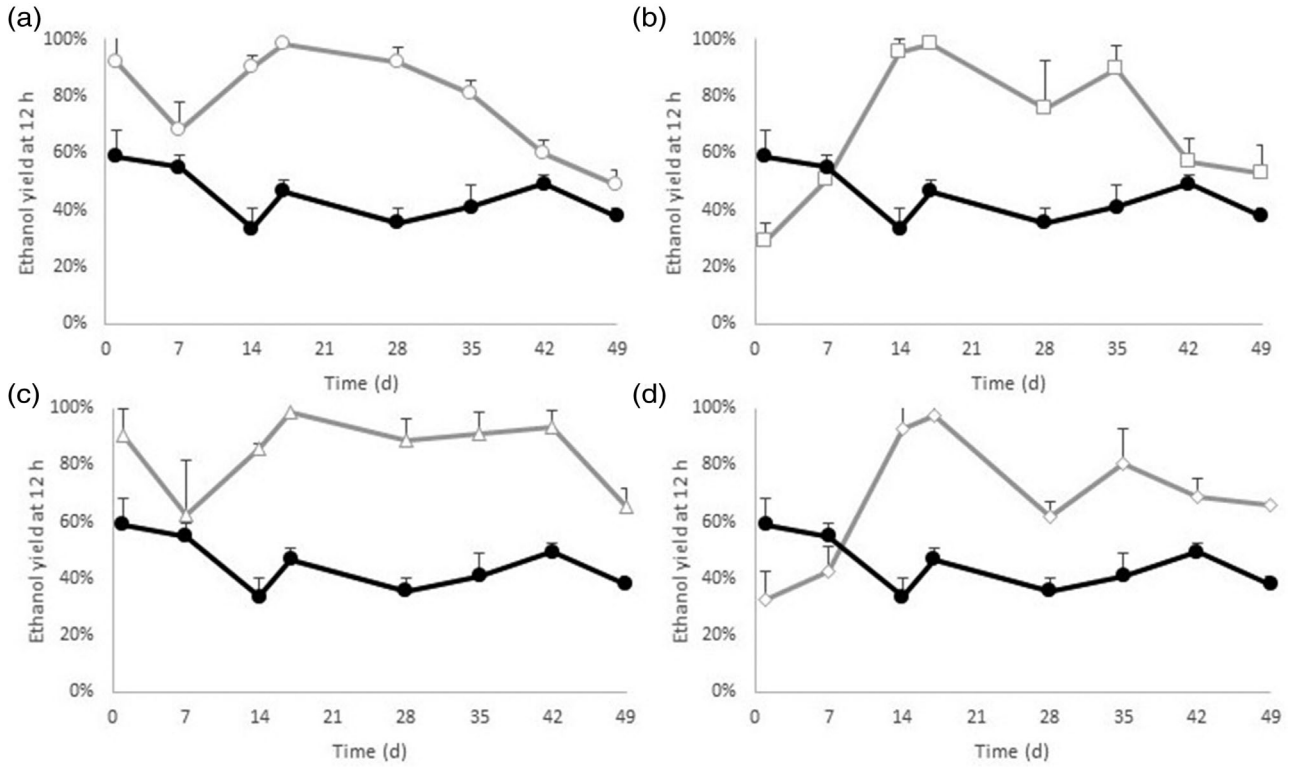

FIGURE 2 Ethanol yield over time in planktonic and encapsulated cultures. Encapsulated and planktonic cells were grown in 250 ml Erlenmeyer flasks with gentle (50 rpm) shaking at 30°C in a 250 ml culture volume of fermentation medium containing 150 g/L dextrose. The ethanol yield was calculated as a percentage of the theoretical maximum yield for the grams of ethanol produced from the grams of dextrose consumed after 12 hr. Yield was measured in five replicate cultures from each combination of alginate and cross linker: (a) NaAlg beads (gray circles), (b) NaAlgCh beads (gray squares), (c) Pr beads (gray triangles), and (d) PrCh beads (gray diamonds). The ethanol yield in five planktonic only controls is indicated by the black circles. Error bars represent 1 *SD*

on Day 28 (following a missed feeding on Day 24) that was absent in NaAlg and Pr beads. This observation could be attributed to a decline in cell viability also manifest on Day 28 (Figure 4). Yield in all four encapsulation matrices approached 100% (though with somewhat differing frequencies), and was generally high (>90%) between 14 and 35–42 days (depending on matrix type). By contrast, yield for the planktonic control never exceeded 60%.

The high yields observed for encapsulated cells here accord with previous findings using encapsulated *S. cerevisiae*. High (>80%) fermentation efficiencies have been demonstrated on starch[85] and 10% dextrose[54] for single batches, as well as high (>85%) yields from reused beads over 5 days,[86] 10 days,[87] 12.5 days,[88] and 15 days.[53] Others have noted no differences in ethanol production based upon the presence or absence of chitosan.[89]

Overall, our results indicate that encapsulated systems have higher ethanol yields than planktonically grown cells, in some cases for at least 49 days. Among encapsulated systems, significant differences were observed according to an ANOVA with Tukey's post hoc test on four out of eight timepoints, with Pr beads displaying the highest yields; indeed, Pr beads had significantly higher ethanol yields than planktonic cells even after 49 days of culture. Further, our results indicate that the culture system is robust to missed feedings; even after low yields were recorded following the missed feeding, the system was able to recover and again achieve yields and viabilities comparable to those observed before the missed feeding (Figure 2).

3.4 | Following outgrowth, total cell number was similar across encapsulated and planktonic cultures

A culture with more cells should, all else being equal, produce ethanol more rapidly than a culture with fewer cells. In order to assess ethanol production based on culture method (rather than on cell number), experiments were initiated at similar cell population sizes across encapsulated and planktonic treatments. Specifically, approximately 2×10^9 cells were added to 250 ml cultures of four bead types (Table 1; NaAlg, NaAlgCh, Pr, PrCh) as well as to the planktonic control. Total cell number in each flask was estimated immediately following (0 hr) and 12 hr after the addition of fresh medium at all eight timepoints across 49 days (Figure 3). Cell number was estimated indirectly via optical density of cell suspensions by regressing direct cell counts obtained by a Coulter Counter Multisizer 4e against OD600 ($y = 3 \times 10^7$–4×10^6, Figure S3).

Across all treatments, no appreciable changes in cell number were observed after the initial week-long outgrowth period (Figure 3). Analysis of absolute OD values by one-way ANOVA (Table S6) suggested

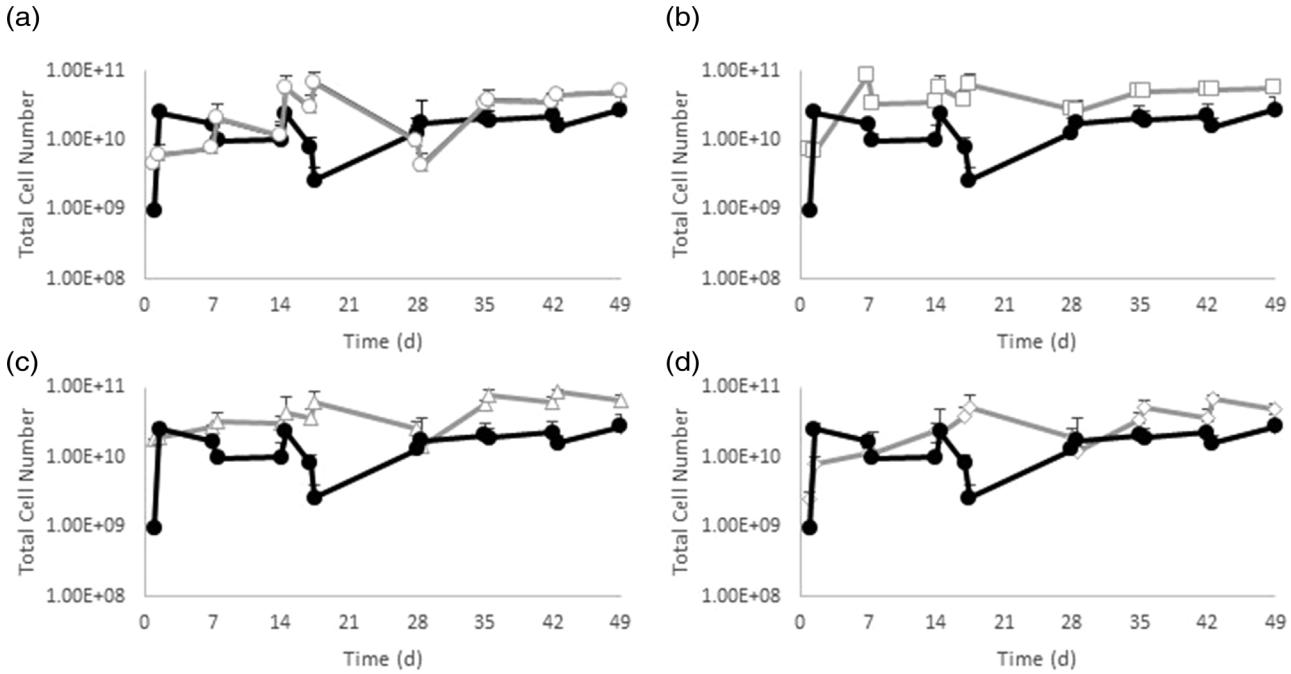

FIGURE 3 Encapsulated populations have similar cell numbers to planktonic populations. The number of encapsulated and planktonic cells before and 12 hr after the addition of fresh medium across 49 days was measured. The number of cells in encapsulated cultures is indicated by the gray lines. Black lines indicate the average number of cells in five planktonic replicates. Five replicates of each of two types of alginates were tested using two crosslinking solutions: (a) NaAlg (circles), (b) NaAlgCh (squares), (c) Pr (triangles), and (d) PrCh (diamonds). Error bars represent 1 *SD*

that while cell number remained constant from Days 7 to 28, cell number in the planktonic control did not increase as rapidly as it did in certain encapsulated cultures (Table S6; ANOVA with Tukey's post hoc), particularly the Pr matrix.

The carrying capacity of encapsulated treatments was comparable to values previously reported for fed-batch cultures *of S. cerevisiae*.[90] However, it should be noted that optical density measurements, which rely on light scattering, can be less accurate than direct enumeration by microscopy or Coulter count.[91] Variance in cell number was higher in these experiments than previously observed among alginate-encapsulated yeast continuously-fed ad libitum in immobilized cell reactors, with some treatments varying by as much as an order of magnitude even following outgrowth.[42] The latter observation could be attributed to the feast-and-famine nature of repeated fed-batch culture, which introduces variability in culture conditions not experienced by continuously fed cells.[92]

Among different matrix types, Pr beads contained more total cells than at least one other matrix type at 8 out of 16 timepoints (one-way ANOVA, $p < .05$), and fewer cells than any another matrix type at only one (Table S6). This higher cell number may have contributed to the higher ethanol yields observed for Pr beads, but does not explain this difference in and of itself. There is overlap at three timepoints where Pr beads exhibited greater yield and more cells than other bead types, but there was also one timepoint wherein Pr beads had higher ethanol yield but not more cells (Day 28), and another where they had more cells but not a higher yield (Day 35). Differences in rates of cell escape and in cell viability would also be expected to impact yield.

3.5 | Cell escape increased over time, except in Pr beads

Cell escape, where encapsulated cells exit the bead and become planktonic, is known to occur in alginate gels, where it can result in less efficient substrate utilization as well as competition with encapsulated cells for nutrients.[93,94] Total cell number reported for encapsulated treatment groups includes both encapsulated and escaped cells; "percent cell escape" represents the fraction of that total that was formerly encapsulated, but escaped the bead and became planktonic. Percent cell escape increased as beads aged (Figure 4; Table S7), with a strong correlation (Pearson's coefficient of 0.55) between declining viscosity and increased incidence of cell escape. The number of cells escaping was low in all matrices up until Day 28, after which Pr beads generally had fewer escapees (Figure 4); significant differences assessed by one-way ANOVA were detected only on Days 28 and 49 (Table S7) due to high variance among replicates. Although they did not have more cells than the other matrix types on Day 28, Pr beads had a significantly higher fraction of encapsulated cells, which may help account for higher ethanol yields observed in Pr beads on Day 28. This result is surprising as neither Pr Young's modulus, viscosity, bead diameter, nor mass could be distinguished statistically from that of other matrix types on those days, suggesting that some other

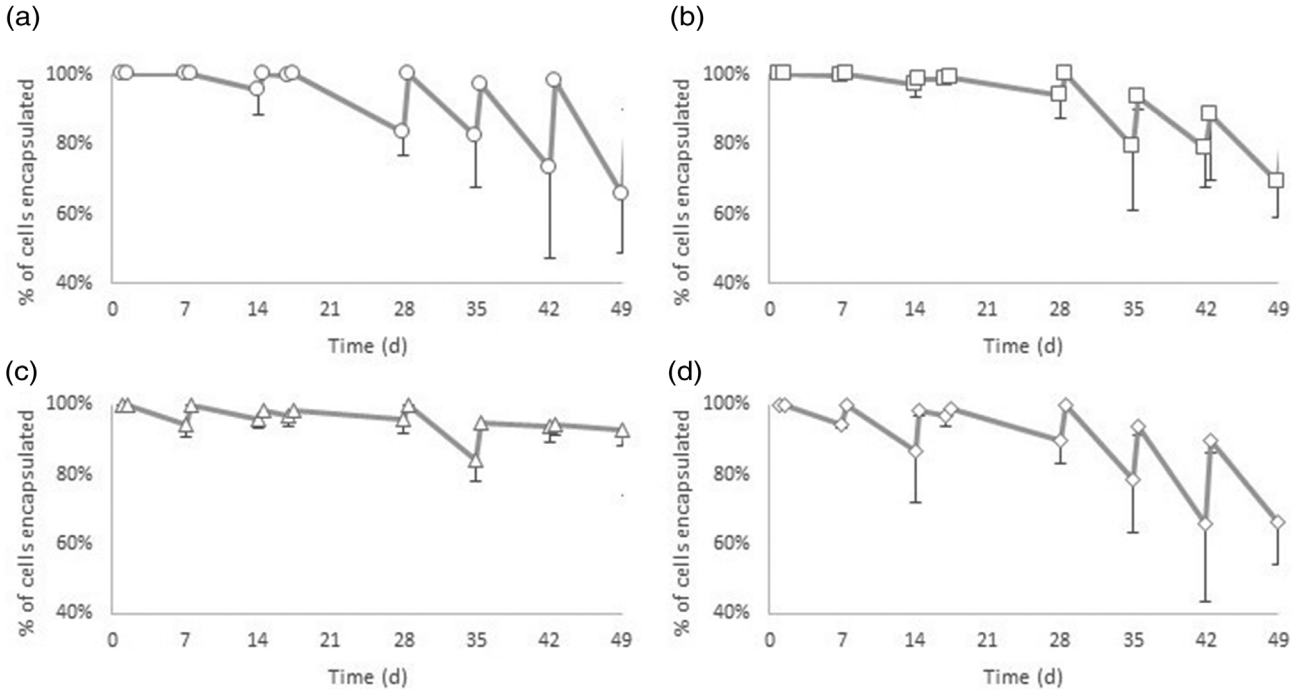

FIGURE 4 Cell escape over time. The number of encapsulated and planktonic cells before and 12 hr after the addition of fresh medium across 49 days was measured. The number of cells that escaped encapsulation to become planktonic was compared to the number of all cells (escaped and encapsulated) to find the percent of cells encapsulated at various timepoints. An average of five replicates from (a) NaAlg, (b) NaAlgCh, (c) Pr, and (d) PrCh matrices was utilized. Error bars represent 1 *SD*

factor not measured here, perhaps pore size, also contributes to cell escape, or lack thereof.[95]

3.6 | Cell viability declined over time in both encapsulated and planktonic cell cultures

As cells age, their viability declines.[96] To compare time-dependent changes in this parameter between encapsulated and planktonic cells, cell viability was assessed 12 hr after each addition of fresh medium by staining cells with PI and counting >10,000 individual cells via flow cytometry (Figure 5). Viability assessed by PI staining was compared to viability assessed by CFUs in both encapsulated and planktonic cultures at Day 1, after which PI alone was used. For both encapsulated and planktonic cells, viability assessed by PI staining was twice that estimated by CFUs, likely because the former method scores cells that are alive but unable to form colonies. Across all treatments, decline in viability was biphasic; viability declined rapidly during the first week of fed-batch culture, then slowly over the ensuing 6 weeks. A missed feeding on Day 24 helps to account for lower viability measured on Day 28; thereafter viability quickly recovered to prestarvation levels, indicating a robust culture system. When experiments were terminated at 7 weeks, cell viability in most treatments was ~20%, with planktonic yeast exhibiting only marginally greater viability than yeast that had been encapsulated (Figure 5; Table S8).

Overall, few viability differences were observed among treatments. NaAlgCh and PrCh beads had lower viability than both other matrix types and planktonic cells on Day 1, likely due to their recent overnight exposure to 0.16 M acetic acid (necessary to dissolve chitosan).[83,84] Following the initially low viability of some cultures, which was likely due to encapsulation itself (and disregarding Day 28 as an artifact of a missed feeding), there were only 2 days in which significant differences were observed (one-way ANOVA with Tukey's post hoc, $p < .05$): Day 49, when some encapsulated populations had lower viability than the planktonic control, and Day 14, when all encapsulated treatments had higher viability than the planktonic control (Table S8; ANOVA with Tukey's post hoc). Significant differences among encapsulated treatments were noted only on Day 1, during the initial recovery period, indicating that the encapsulation matrices tested here do not significantly affect cell viability over time. As viability differences were largely absent, as has been reported under some conditions by others,[97] it is unlikely that this parameter contributed to differences in ethanol yields observed among different matrix types.

Other culture systems support higher yeast viability over prolonged incubations, even considering that viability here is estimated by PI staining, rather than by CFU. Cells in giant colonies grown on GM (1% yeast extract, 3% glycerol) agar retain 90% viability after 10 days, then slowly decline to 5% viability after 135 days.[98] Planktonic *S. cerevisiae* cultured in nutrient-limited retentostats retain 80% viability after 22 days,[99] and alginate-encapsulated yeast continuously fed ad libitum exhibited >90% viability after almost 3 weeks continuous culture.[42] The lower viability reported here may stem from cells being subjected to repeated cycles of feast and famine, each of which

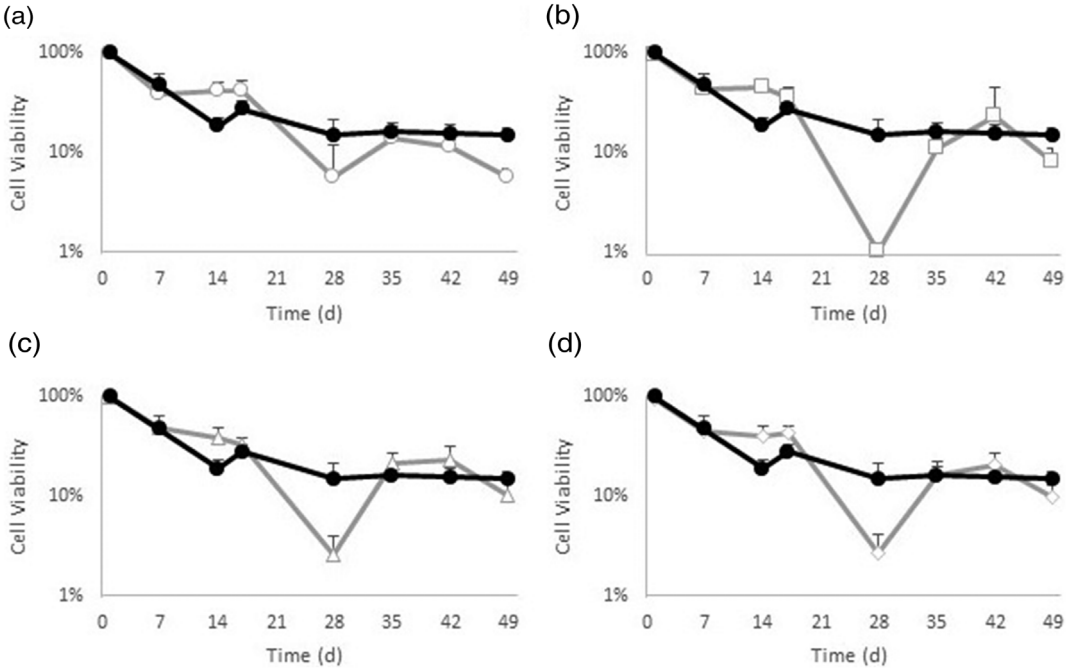

FIGURE 5 Encapsulated populations have lower viability than planktonic populations. 12 hr after the addition of fresh medium, cell viability was assessed by dissolving beads into sodium metaphosphate. Following matrix dissolution, cells were stained with propidium iodide, which can diffuse through damaged membranes; 10,000+ cells were counted for each replicate. Five replicates of each of two types of alginates were tested using two crosslinking solutions: (a) NaAlg beads (gray circles), (b) NaAlgCh beads (gray squares), (c) Pr beads (gray triangles), and (d) PrCh beads (gray diamonds). The viability of cells in five planktonic only controls is indicated by the black line. Error bars indicate 1 *SD*. A feeding was missed on Day 24

is attended by pronounced changes in pH, glucose, ethanol, and acetate concentrations. pH was found to change from 5.8 to 4.6 over 3.5 days, glucose from 15 to 0%, ethanol from 0 to 4–7.5%, and acetate from 0 to 15 mM. Prolonged exposure to 15 mM of acetate at pH of ~4.5 has been shown to negatively impact planktonic yeast cell viability and likely affected viability of encapsulated cells as well.[83,84] Since the *S. cerevisiae* strain used here is purported to be tolerant of ethanol up to 18%,[59] it is unlikely that a maximum theoretical concentration of 7.5% caused a significant decline in viability, making acetate and pH changes more plausible explanations. These fluctuating conditions stand in marked contrast with conditions experienced by colonies on agar, cells in retentostats, and cells in continuously fed immobilized cell reactors, all of which are relatively constant environments, albeit having different levels of substrate input.

Viability does not seem to impact cultures' fermentation capacity, at least over the first 12 hr measured for each timepoint. A Pearson's correlation coefficient relating viability to ethanol yield was only 0.168, indicating a weak association between viability and ethanol yield. Neither encapsulated nor planktonic cultures exhibited lower ethanol yield between Days 14 and 35, when PI-staining cells constituted ~80% of populations, than they did when viability was much higher. Our results are in line with other studies reporting a decrease in cell viability from 80 to 30% over five fed-batch cycles, even as yields remained constant at ~80%,[100] as well as an association between lower viability and pH changes.[101]

3.7 | Encapsulated cells are more heat-shock resistant than planktonic cells, but no one matrix confers greater heat-shock resistance than another

Simultaneous saccharification and fermentation has been widely implemented in the bioethanol industry due to its higher efficiency,[102,103] even though most enzymes operate optimally well above yeasts' preferred growing temperatures.[104,105] Thus, a more thermotolerant yeast could improve the efficiency of this step.[106,107] Moreover, since cooling costs for fermentation tanks can be costly in the summer, especially in warmer regions,[108] a more thermotolerant yeast could also help reduce cooling costs, and is thus doubly favored. Indeed, recent analyses suggest that for every 5°C increase in the fermentation temperature, in today's market approximately $30,000 per year could be saved for a 30,000 kl scale ethanol plant, not including reductions in initial investment costs.[109]

To discover whether certain encapsulation matrices offer more protection against thermal stress than others, yeast were subjected to heat shock at 50°C for 180 min. Planktonic cells, in either exponential or stationary phase, as well as cells encapsulated in each of four matrices, were exposed to 50°C for 5, 15, 30, 90, and 180 min (Figure 6), after which viability was assessed as CFUs following dilution and plating on rich agar. Consistent with prior studies, viability of planktonic exponential-phase cells declined more rapidly than that of stationary-phase cells.[110] Viability of encapsulated yeast consistently declined less rapidly than either planktonic treatment. However, few differences could be discerned among encapsulation matrices. Although significant

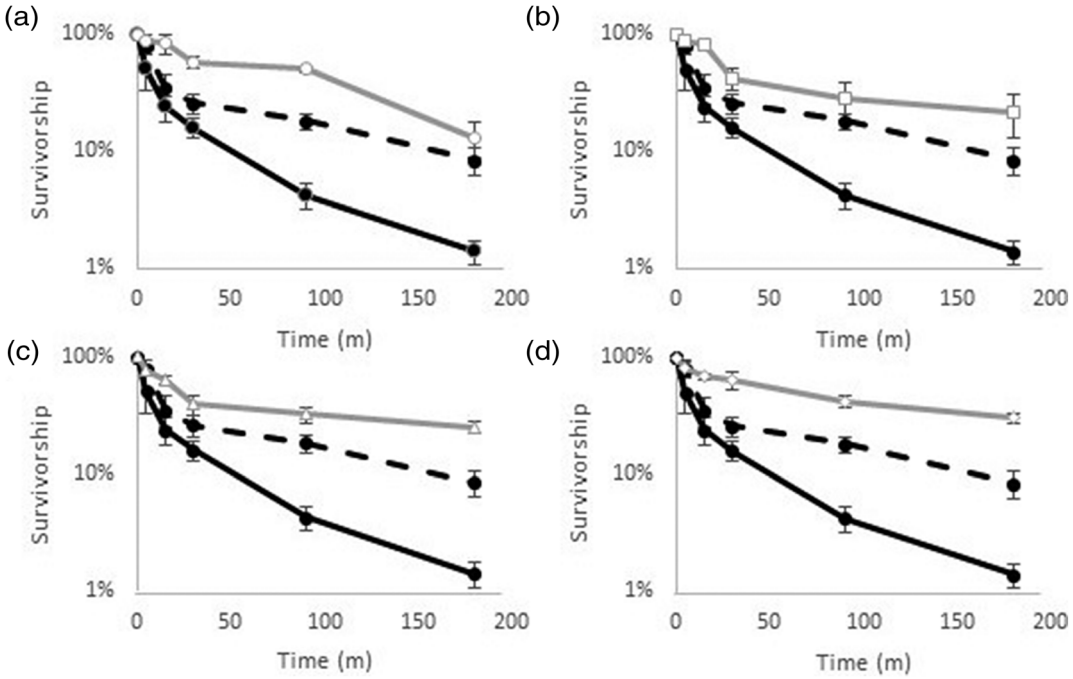

FIGURE 6 Survivorship of encapsulated and planktonic cells at 50°C. Three replicates of (a) NaAlg beads (gray circles), (b) NaAlgCh beads (gray squares), (c) Pr beads (gray triangles), and (d) PrCh beads (gray diamonds) as well as planktonic cells in the exponential (solid black line) and stationary (dashed black line) growth phases were exposed to 50°C for 3 hr. Survivorship was tracked by calculating colony-forming units/ml using the spot plate technique. Error bars represent 1 *SD*

differences were seen at the 30, 90, and 180 m timepoints (Table S9; ANOVA with Tukey's post hoc), no clear trend emerged to suggest one type of matrix conferred more thermal protection than another.

This result is perhaps to be expected given the known heat stability of alginates, which are stable at far higher temperatures than the yeast inside them could withstand.[111,112] Overall, heat-shock resistance in these matrices was similar to that previously reported for alginate-encapsulated *S. cerevisiae*.[42] Increased heat resistance displayed by encapsulated cells was once attributed to nutrient deficiency in cells close to the core of the bead, triggering a stress response similar to that observed for cells entering stationary phase.[28,113,114] Upon encapsulation and upon entry into stationary phase, yeast ceases to divide, which is accompanied by thickening of their cell wall and accumulation of reserve carbohydrates, both of which help to protect cells from stress.[115] Encapsulated cells are demonstrably heat-shock tolerant (Figure 6)[42,116]; others have shown that as much as 25% of this phenotype can be attributed to protection by the matrix itself.[42] However, we now know that it is physiological change brought on by encapsulation that provides much of the stress tolerance exhibited by such cells.[38] This physiological change is evident in the yeast transcriptome, where stress-related genes such as *YAP1*, *ATR1*, and *FLR1* are induced soon after encapsulation.[117]

3.8 | Encapsulation matrices vary in cost

Potential advantages to using encapsulated cells in biorefineries include higher service life and superior yields.[26,31,118] Cost of matrix components would contribute to the selection of one matrix over others for commercial applications. Matrices that do not contain chitosan are more economical than those that do. Currently, chitosan costs 83.6 cents/g, and an additional estimated 0.22 cents per bead (with beads formed according to Section 2.2), which translates to an additional $3.85 for the 250 ml volume used here (pricing from VWR; Item# 9012-76-4; 250 g quantity). Among alginates, sodium alginate currently costs 25.4 cents/g (pricing from Sigma Aldrich; Product #180947, 500 g quantity). Protanal is no longer available, but a comparable product also derived from *Laminaria hyperborea* costs 36.8 cents/g (pricing from Sigma Aldrich; Product #A1112; 500 g quantity), 45% more than sodium alginate. Purchases at larger scales would significantly reduce the cost per gram of both the alginates and chitosan, but are unlikely to reduce the cost of Pr below that of NaAlg.

Chitosan is a costly addition, and while it improved Young's modulus and viscosity for 7 days (Figure 1a–d), chitosan was also associated with increased bead swelling (Figure 1c–f) and decreased cell number (Figure 3) and viability (Figure 5), which may have negatively impacted ethanol yields (Figure 2). Therefore, our data do not support addition of chitosan to matrices for bioethanol production under repeated fed-batch conditions. Between Pr and NaAlg beads, Pr beads exhibited higher ethanol yields (Figure 2), possibly due to higher cell numbers therein (Figure 3) and lower percentages of cell escape (Figure 4). Critically, Pr beads demonstrated higher ethanol yields than NaAlg beads and planktonic cultures at Days 42 and 49, indicating that they could be re-used for longer than NaAlg beads and potentially for longer than 49 days. Cell viability and thermotolerance between the two alginate

types were also comparable. Therefore, even though Pr beads are more costly, our finding that they can be reliably used for at least 2 weeks longer than NaAlg beads recommends them as the best matrix for commercial applications calling for repeated, fed-batch culture.

4 | CONCLUSIONS

Our findings demonstrate that in extended fed-batch culture, encapsulated yeast have higher thermotolerance and ethanol yield than planktonic yeast, exhibit similar viability, and that they can be re-used for at least 7 weeks. PrCh beads were lightest and smallest throughout our experiment, making them suitable for situations where small bead size is desirable. NaAlgCh beads demonstrated the highest Young's modulus and viscosity early on, but also the poorest product yield of any bead type; experiments that are short in duration but require a highly durable bead should utilize this matrix. NaAlg beads were the most economical to produce and displayed average performance for many of the metrics examined here; they should be used for extremely price-sensitive applications. For the purposes of ethanol production from encapsulated cells, our data recommend Pr beads, which consistently demonstrate highest ethanol yields over repeated fed-batch culture.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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