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Encapsulation enhances protoplast fusant stability

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Abstract

A barrier to cost-efficient biomanufacturing is the instability of engineered genetic elements, such as plasmids. Instability can also manifest at the whole-genome level, when fungal dikaryons revert to parental species due to nuclear segregation during cell division. Here, we show that by encapsulating *Saccharomyces cerevisiae-Pichia stipitis* dikaryons in an alginate matrix, we can limit cell division and preserve their expanded metabolic capabilities. As a proxy to cellulosic ethanol production, we tested the capacity of such cells to carry out ethanologenic fermentation of glucose and xylose, examining substrate use, ploidy, and cell viability in relation to planktonic fusants, as well as in relation to planktonic and encapsulated cell cultures consisting of mixtures of these species. Glucose and xylose consumption and ethanol production by encapsulated dikaryons were greater than planktonic controls. Simultaneous co-fermentation did not occur; rather the order and kinetics of glucose and xylose catabolism by encapsulated dikaryons were similar to cultures where the two species were encapsulated together. Over repeated cycles of fed-batch culture, encapsulated *S. cerevisiae-P. stipitis* fusants exhibited a dramatic increase in genomic stability, relative to planktonic fusants. Encapsulation also increased the stability of antibiotic-resistance plasmids used to mark each species and preserved a fixed ratio of *S. cerevisiae* to *P. stipitis* cells in mixed cultures. Our data demonstrate how encapsulating cells in an extracellular matrix restricts cell division and, thereby, preserves the stability and biological activity of entities ranging from genomes to plasmids to mixed populations, each of which can be essential to cost-efficient biomanufacturing.

KEYWORDS
cell encapsulation, cellulosic fermentation, *dikaryon*, genomic stability, *Pichia stipitis*, protoplast fusion, *Saccharomyces cerevisiae*

1 | INTRODUCTION

Genomic instability frequently reduces yield in biomanufacturing (Arkin & Fletcher, 2006). Many products of commercial interest are derived from accessory elements such as plasmids that are susceptible to segregational loss during cell division (Flores, de Anda-Herrera, Gosset, & Bolivar, 2004). Indeed, up to 99% of plasmid-containing cells can be lost in as few as 40 generations of continuous culture (Flores et al., 2004), with loss occurring every 10^2–10^7 cell divisions (S. Chen, Larsson, Robinson, & Chen, 2017). Not only does plasmid loss reduce product yield, it can also cause tank fouling as non-plasmid-bearing microbes outcompete plasmid bearers due to their lighter metabolic burden

(Glick, 1995). Steps taken to avert or delay plasmid loss include integrating circuits of interest into the host chromosome or placing those circuits under inducible promoters to reduce their metabolic burden (Cooper & Lenski, 2000; Cooper, Schneider, Blot, & Lenski, 2001; Sleight, Bartley, Lieviant, & Sauro, 2010).

Genomic instability is also a concern for bioprocesses that do not utilize plasmids, such as those driven by protoplast fusants or by cultures consisting of multiple species; both of these have been proposed as means to improve cellulosic ethanol production (Y. Chen, 2011; Gupthar, 1992). In addition to 6-carbon sugars like dextrose, which can readily be fermented by yeast like *Saccharomyces* spp. (Favaro, Jansen, & van Zyl, 2019), cellulosic feedstocks contain significant amounts (20–60%) of 5-carbon sugars like pentose (Kuhad, Gupta, Khasa, Singh, & Zhang, 2011). Although no naturally occurring microbe has been discovered that co-ferments 5- and 6-carbon sugars (5C/6C), some, like *Pichia stipitis* , will first ferment 6C sugars and then 5C sugars. In principle, a genetically stable dikaryon, containing the nuclear genomes of both *Saccharomyces cerevisiae* and *P. stipitis*, would be able to readily ferment both types of sugar, reducing cellulosic ethanol production costs. Previous efforts to solve the 5C/6C cofermentation conundrum have relied on genetically modified bacteria (Dien, Nichols, O'bryan, & Bothast, 2000; C. G. Liu et al., 2019; Mohagheghi, Evans, Chou, & Zhang, 2002; Ohta, Beall, Mejia, Shanmugam, & Ingram, 1991; Yomano, York, & Ingram, 1998) or yeast (Fujita et al., 2002; Lawford & Rousseau, 2003; E. Liu & Hu, 2010; Sreenath & Jeffries, 2000; Tantirungkij, Izuishi, Seki, & Yoshida, 1994), coculture of two organisms that, respectively, digest 5- and 6C sugars (De Bari et al., 2013; Fang, 2010; Zhu et al., 2016), or fusion of the same (Heluane, Spencer, Spencer, De Figueroa, & Callieri, 1993; Lin, Hsieh, Mau, & Teng, 2005). Combinations of these methods have also been investigated, including coculture of immobilized cells (De Bari, Cuna, Nanna, & Braccio, 2004; Fu, Peiris, Markham, & Bavor, 2009; Grootjen, Meijlink, van der Lans, & Luyben, 1990; Lebeau, Jouenne, & Junter, 2007), immobilization of recombinants (Zhao & Xia, 2010), fusion of recombinants (Pasha, Kuhad, & Rao, 2007), and coculture of recombinants (Y. Chen, Wu, Zhu, Zhang, & Wei, 2018; C. R. Lee et al., 2017; F. Liu et al., 2017; Zhang et al., 2017).

Here, we investigate ethanologenic fermentation of glucose and xylose by four types of yeast: (#1) hygromycin-resistance plasmid-bearing *S. cerevisiae* (*hyg*^{r}), (#2) geneticin-resistance plasmid-bearing *P. stipitis* (*G418*^{r}), (#3) 1:1 mixed culture of *hyg*^{r} *S. cerevisiae* and *G418*^{r} *P. stipitis*, and (#4) protoplast fusants of *hyg*^{r} *S. cerevisiae* and *G418*^{r} *P. stipitis*. Each type was cultured in triplicate for 19 days under two different culture conditions, planktonic or encapsulated within a Ca^{2+}-alginate matrix (Figure S1). Under both conditions, yeasts were supplied with fresh YPDX (2% dextrose, 2% xylose, 2% peptone, and 1% yeast extract) medium every 5 days. Although seed populations were grown for 24 hr under drug selection, antibiotic drugs were omitted for the duration of each fermentation experiment, enabling us to compare, in encapsulated and in planktonic cultures, the relative stability of drug-resistant plasmids (#1 and #2), the ratio of different species in mixed culture (#3), and the long-term stability of dikaryons formed by protoplast fusion (#4).

We did not observe cofermentation of glucose and xylose in any treatment containing both *P. stipitis* and *S. cerevisiae*, either as *bona fide* species or as dikaryons harboring both genomes. Instead, we observed preferential uptake and fermentation of glucose followed by that of xylose. Fusant dikaryons and mixed cultures of *P. stipitis* and *S. cerevisiae* produced similar amounts of ethanol, with encapsulated cultures producing much more ethanol on a per cell basis than their planktonic counterparts. Even in the absence of drug selection, encapsulation-stabilized plasmids bearing antibiotic-drug-resistant genes maintained a fixed ratio of the two species in mixed populations, and preserved the genomic integrity of dikaryons. To the best of our knowledge, we are the first to demonstrate long-term (19 days) preservation of bioactive yeast dikaryons in an encapsulated system, potentially paving the way for their use in biomanufacturing.

2 | MATERIALS AND METHODS

2.1 | Strains and culture conditions

Experiments were conducted with *S. cerevisiae* Ethanol Red, an ethanol-tolerant (up to 18% [Fermentations, 2019]) industrial strain obtained from Leaf LeSaffre (Marcq-en-Barœul, France) and *P. stipitis* (strain background unknown) obtained from Designer Energy (Rehovot, Israel). To confer resistance to the antibiotic hygromycin, we transformed *S. cerevisiae* with the 6,319 base pair plasmid p-CORE-Hp53 (Storici & Resnick, 2003), using the LiAc/ssDNA/PEG method of transformation (Gietz & Schiestl, 2007). To confer resistance to the antibiotic geneticin, we transformed *P. stipitis* with the 6,073 base pair plasmid p-CORE-Kp53 (Storici & Resnick, 2003), also using the LiAc/ssDNA/PEG method of transformation (Table 1). Antibiotic-resistant mutants were created in preparation for protoplast fusion, as a method for selecting fusants consisting of both yeast species.

Yeast was grown in 250 ml of YPDX medium (2% dextrose, 2% xylose, 2% peptone, 1% yeast extract) in 250-ml screw-cap

TABLE 1 Strains used in this manuscript.

Strain	Source	Abx resistance	Genomic content
Saccharomyces cerevisiae	Leaf Lesaffre	Hygromycin	pCORE-hp53
Pichia stipitis	Designer Energy	Geneticin	pCORE-kp53
S. cerevisiae/*P. stipitis* dikaryon	This manuscript	Hygromycin and geneticin	pCORE-hp53 and pCORE-kp53

Erlenmeyer flasks, while shaking at 30°C on a gyro-rotary platform at 50 rpm. Many industrial-scale bioreactors were fed-batch, with the new substrate being supplied and the product being removed on 2- to 5-day cycles (Qazizada, 2016), each cycle being repitched with fresh yeast. As encapsulated yeast is inherently more expensive to produce than unencapsulated yeast, with the former being commercially viable, they would need to be reused for multiple cycles. To mimic conditions, cells would undergo multiple cycles of reuse, all cultures were provided fresh medium every 5 days, 24 hr after xylose exhaustion, for 15 days. Measurements were taken up to 19 days, at which time the xylose from the day-15 addition was depleted. Every 5 days for 15 days, the spent medium was discarded and fresh YPDX medium was added.

Briefly, 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-inch diameter and mesh size 10 (2 mm, McMaster Carr, Elmhurst, IL; Catalog #34735K216). Fresh sterile YPDX 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 and, then, discarding spent medium. Pelleted cells were resuspended in fresh YPDX medium to a final culture volume of 250 ml, also without any cleaning or sterilization of the flask.

2.2 | Protoplast formation and fusion

Protoplasts were created by modifying the protocol described by Shalsh, Ibrahim, Mohammed, and Meor Hussin (2016) (Figure 1). Both *hyg*^{r} *S. cerevisiae* and *G418*^{r} *P. stipitis* were grown to stationary phase in the presence of 200 µg/ml hygromycin and 200 µg/ml geneticin, respectively. Then, 5 ml of each culture was placed into 15 ml Falcon tubes and centrifuged at 4,000*g* for 2 min, after which the supernatant was removed and 0.1 M phosphate buffer (pH 7.5) was added. This step was repeated, after which cells were resuspended in 10 ml protoplast solution (1.2 M sorbitol, 0.1 M Tris, 0.02 M ethylenediaminetetraacetic acid and pH 9.8) with 50 µl β-mercapto-ethanol (Sigma Aldrich; catalog #60-24-2) and allowed to incubate at room temperature for 15 min. Next, cells were washed with 1.2 M sorbitol, transferred to 1.2 M sorbitol amended with 0.075 mg/ml 20 T zymolyase (VWR; catalog #IC320921) and incubated at 30°C for 30 (*P. stipitis*) or 45 (*S. cerevisiae*) minutes.

Protoplasts were centrifuged at 100*g* for 10 min 15-ml Falcon tubes, and then, cells were washed three times in 10 ml buffer solution (0.1 M phosphate buffer and 1.2 M sorbitol), at the centrifugation speed of 100*g* for 10 min each time. After washing, the protoplasts of the two species were mixed at a 1:1 ratio, and then, resuspended in fusion buffer solution (0.6 M sorbitol; 10 mM Tris-HCl, 35% PEG, and 10 mM $CaCl_2$) and incubated at 30°C at 100 rpm for 30 min. Following this incubation, yeast were plated on YPD (2% dextrose, 2% peptone, 1% yeast extract) agar plates containing 200 µg/ml hygromycin, 200 µg/ml geneticin, and 0.2, 0.4, 0.6, 0.8, 1.0, or 1.2 M sorbitol. After 4 days of incubation at 30°C, resulting colonies from each sorbitol level were grown in liquid YPD containing 200 µg/ml hygromycin and 200 µg/ml geneticin, and, then, stained with propidium iodide (PI) and 4,6-diamidino-2-phenylindole (DAPI), as detailed below, to assess via flow cytometry and via light microscopy whether they were fusants harboring both parental nuclei.

2.3 | Verification of fusant ploidy by PI staining

PI (Thermo Fisher Scientific, Waltham, MA; Catalog #P1304MP; stock 10 mg/ml) was used to quantify fusant DNA content. In total, 21 colonies were randomly chosen and inoculated in liquid YPD medium with 200 µg/ml hygromycin and 200 µg/ml geneticin and allowed to reach exponential phase before fixing cells in 70% ethanol (Sazer & Sherwood, 1990). Controls consisting of *hyg*^{r} *S. cerevisiae*, *G418*^{r} *P. stipitis*, haploid *S. cerevisiae* strain BY4741, a diploid

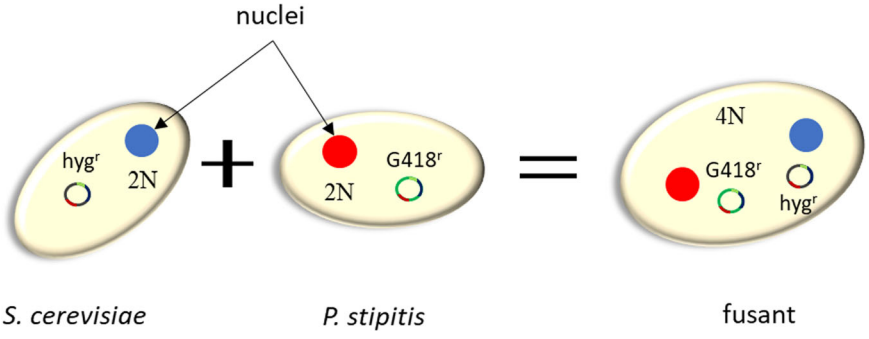

FIGURE 1 A scheme of protoplast fusion resulting in multidrug-resistant dikaryons. Fusants were created via protoplast fusion (Jassim Shalsh, Ibrahim, Mohammed, & Shobirin Meor Hussi, 2016) of the yeasts *Saccharomyces cerevisiae* and *Pichia stipitis* that were, respectively, resistant to hygromycin or to geneticin following transformation with drug-resistance plasmids. Fusant dikaryons were selected by plating cells on solid YEPD medium containing both drugs and, then, verified by DAPI staining as well as by flow cytometry, using haploid, diploid and tetraploid *S. cerevisiae* as controls. DAPI, 4,6-diamidino-2-phenylindole [Color figure can be viewed at wileyonlinelibrary.com]

S. cerevisiae YE658, and a tetraploid strain *S. cerevisiae* YE643 were also fixed. Fixed cells (300 µl) were washed in 1 ml of 50 mM sodium citrate, resuspended in 500 µl of 50 mM sodium citrate containing 0.1 mg/ml RNase A, and incubated at 37°C for 2 hr. Then, 500 µl of 50 mM sodium citrate with 8 µg/ml PI was added to the cells to stain DNA before analyzing the samples using a Sysmex CyFlow Space flow cytometer (Sysmex, Kobe, Japan; Catalog #1604063918). Controls containing diploid (2 N) or tetraploid (4 N) *S. cerevisiae* or a mix of such cells were used to establish proper gating to discriminate ploidy. Fluorescence intensity was measured via flow cytometer, utilizing a 488-nm laser and detected on the FL2 channel, log4 range with the gain set at 450. Control cell samples of known ploidy were used to gate fluorescence intensity to cellular DNA content, with an average of 10,000 events collected per sample.

2.4 | Verification of fusants using DAPI staining

Nuclear staining with the dye DAPI (Sigma Aldrich; Catalog #D9542) was performed, as described in Oberto et al. (2009). Briefly, exponential phase cells were fixed with 70% ethanol for 30 min and then washed twice with Phosphate-buffered saline (PBS). DAPI was added to a final concentration of 1 mg/ml, and cells were left in the dark for 5-10 min before washing cells again with PBS buffer. Cells were imaged using a Zeiss Axioskop epifluorescence microscope using ×100 magnification and a UV filter cube.

2.5 | Preparation of encapsulation matrices and cell encapsulation

Protanal LF 10/60 alginate (gift from FMC Biopolymer) was sterilized by combining 60 g of alginic acid sodium salt (sodium alginate; Sigma Aldrich Catalog #180947) with approximately 300 ml of 95% ethanol and left overnight. This approach was utilized as other sterilization methods can alter alginate viscosity (Leo, McLoughlin, & Malone, 1990). After the settlement of mixture overnight, ethanol was separated from the alginate using a 0.2-micron 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, 24 separate batches of the encapsulation matrix were divided into two groups of 12 (Figure S1). Sterile distilled water (70 ml) and dry, sterile Protanal alginate (4.5 g) were added to each of 12 sterile plastic 1-L beakers. Alginate was mixed into water using a Jiffy Mixing Blades Power Tool Attachment (purchased from Home Depot, Catalog #DC408) and a standard power drill. Next, 80 ml of stationary phase (24 hr) yeasts that had been grown to high density in YPDX medium amended with either 200 µg/ml geneticin (*G418^r P. stipitis)* or 200 µg/ml hygromycin (*hyg^r S. cerevisiae*), or both antibiotic drugs (fusants of *G418^r P. stipitis* and *hyg^r S. cerevisiae*) were gently mixed into the alginate–water mixture, creating a 3% alginate-and-yeast suspension.

Twelve 60-ml plastic syringes were each filled with the yeast alginate solution. The syringes were immersed into a 500-ml beaker containing 350 ml of the cross-linking solution 0.2 M $CaCl_2$. All types of equipment pieces were sterilized before repeating this procedure on the other 12 batches. Protanal beads were hardened in the cross-linking solution overnight at 4°C before being transferred to 250-ml screw-cap Erlenmeyer flasks that contained 250 ml YPDX medium lacking antibiotics drugs. This process produced approximately 1,750 beads per replicate, each bead initially containing approximately 3×10^6 cells. All 1,750 beads were placed in a 250-ml flask, resulting in ~ 5×10^9 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 (the same starting amount of yeast as encapsulated cultures) grown to high density under the selected conditions. So, to make it comparable to encapsulated yeast, planktonic yeast suspensions were also incubated overnight at 4°C before being placed in fresh YPDX medium without antibiotic drugs in 250-ml screw-cap Erlenmeyer flasks.

2.6 | Cell enumeration, viability, and population dynamics

To estimate the cell number in encapsulated yeast, the alginate matrix was dissolved by placing 5 beads in 5 ml of 10% (w/v) sodium metaphosphate solution (Fisher Scientific, Hampton, NH; Catalog #10124-56-8), a calcium chelating agent. Beads were agitated for 2-3 hr at room temperature in a roller drum (New Brunswick Scientific, Edison, NJ; Item #TC-6), after which any remaining bead particles were mechanically disrupted by manual pipetting. Following disruption, cells were diluted 1-50X in YPDX medium and enumerated using a modified Neubauer hemocytometer. Planktonic cultures were also diluted in YPDX medium and enumerated using a modified Neubauer hemocytometer (Sigma Aldrich, St. Louis, MO; Catalog#BR717810).

Cell viability was estimated by counting colony-forming units (CFUs) as well as by PI staining. Using cell counts obtained from the hemocytometer, cells were diluted in YPDX medium before scoring the number of colonies formed on YPDA (2% dextrose, 2% peptone, 1% yeast extract, and 1.5% agar). Moreover, this same dilution was placed on an additional plate type for each treatment. To assess the percentage of fusants remaining at various time-points, the fusants were also placed on YPDA containing both 200 µg/ml hygromycin and 200 µg/ml geneticin. It is expected that all colonies, even non-fusants, would grow on the YPD plates, but only fusants would be able to grow on plates with both antibiotics present. Though antibiotic drugs were present in the seeded cell populations, they were absent throughout the remainder of the experiment, enabling us to determine how encapsulation affected fusant stability. Similarly, to assess the percentage of cells with drug-resistance plasmids remaining, *S. cerevisiae* and *P. stipitis* pure cultures were also plated on both YPDA and YPDA containing either 200 µg/ml hygromycin or 200 µg/ml geneticin, respectively. Finally, to estimate the percentage of the mixed population that consisted of *P. stipitis*, mixed cultures were placed on both YPDA and YPXA (2% xylose, 2% peptone,

1.5% agar, and 1% yeast extract) plates, with all cells expected to grow on the YPD plates but only *P. stipitis* expected to grow on the xylose plates. All mixed cultures initially consisted of *P. stipitis* and *S. cerevisiae* at a 1:1 ratio.

Fusant stability was also assessed immediately after the addition of fresh medium by measuring the DNA content using the PI staining protocol described above. On Day 5, cell viability was also assessed using PI dye exclusion (Deere et al., 1998). Specifically, 2 ml of cell suspensions were diluted 1:200 in sterile water and, then, stained with 5 μg/ml PI (Thermo Fisher Scientific, Waltham, MA; Catalog #P1304MP; stock 10 mg/ml). At least 10,000 cells per sample were counted by flow cytometry. Controls containing heat-killed cells, live cells, and a mix of both were used to establish proper fluorescence 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 | Fermentation parameters

The glucose and xylose consumed and the ethanol produced were assayed using the EnzyChrom™ Glucose Assay Kit (BioAssay Systems, Hayward, CA; Catalog #EBGL-100), the D-Xylose Assay Kit (Megazyme, Bray, Ireland), and the Ethanol test kit, respectively, (Thermo Fisher Scientific, Waltham, MA; Catalog #NC9508587) in all cases using methods provided by the manufacturer.

2.8 | Statistical analyses

Two-way ANOVA tests with Tukey's post hoc were used to compare treatments, using time and treatment as main effects. An α value of .05 was used as a cutoff for significance in all cases.

3 | RESULTS AND DISCUSSION

3.1 | Verification of dikaryons

Dikaryons of *G418^r P. stipitis* and *hyg^r S. cerevisiae* were created as described in *Protoplast formation and fusion* (Figure 1). As the nuclear genome of each parental species was diploid, protoplast fusion should have resulted in a tetraploid genome. We stained haploid, diploid, and tetraploid *S. cerevisiae*, as well as diploid *P. stipitis* with propidium iodide and then compared their fluorescence output with that of *S. cerevisiae-P. stipitis* fusants (Figure 2). This comparison confirmed the formation of tetraploid dikaryons that contained both genomes. Dikaryons were further verified by direct visual inspection of DAPI-stained cells using epifluorescence microscopy. This procedure revealed the presence of just one nucleus per cell in the parental strains *S. cerevisiae* and *P. stipitis* (Figure 3a,b) but two nuclei per cell in *S. cerevisiae-P. stipitis* dikaryons (Figure 3c).

3.2 | Encapsulation impacts cell viability over repeated fed-batch culture

At the onset of our experiments, viability, as assessed by CFUs, was more than 95% for all yeast strains (Figure 4), after which it declined steadily over the course of 19 days. When viability was assessed in terms of CFUs, 75% to 80% of cells in encapsulated populations were still viable after 19 days of repeated fed-batch culture as compared with 60% to 70% of cells in planktonic populations. However, these differences were not statistically significant ($p > .05$). In contrast, when PI staining was used to assess viability, we found that encapsulated strains were 90% to 95% viable after 19 days as compared with planktonic cells, which were 77% to 83%. These differences were significant ($F_{8,86} = 5.0$, $p < .001$, two-way ANOVA with Tukey's post hoc; Figure S2). The discrepancy between viability estimates can be attributed to the different methodologies used to make those estimates: PI staining records cells that have intact membranes but are not necessarily cultivable, whereas CFUs count cultivable cells only.

Encapsulation has been previously shown to confer tolerance to a variety of stressors including acids (Krisch & Szajani, 1997; Taipa, Cabral, & Santos, 1993), organic solvents (Desimone, Degrossi, D'Aquino, & Diaz, 2003; Qun, Shanjing, & Lehe, 2002), ethanol (Zaldivar, Nielsen, & Olsson, 2001), and osmotic and thermal shock (Z.-J. Sun et al., 2007; Ylitervo, Franzen, & Taherzadeh, 2011). This increased tolerance relative to planktonic cells may result from the altered cell wall and plasma membrane composition following encapsulation (Galazzo & Bailey, 1990), and possibly also protection from shear forces afforded by the encapsulating matrix (Nussinovitch, 2010). Using PI staining, we previously noted that encapsulated yeast grown in high-sugar (15% dextrose) medium sometimes exhibited lower viability than its planktonic counterpart, with both only being ~30% to 40% viable after 19 days (Gulli, Yunker, & Rosenzweig, 2019). In the lower sugar concentrations used here (2% dextrose and 2% xylose), population viability remained high for both treatments after 19 days. We attribute this finding to the fact that yeast in the experiments reported here underwent smaller fluctuations in pH (≥0.1 unit) and acetate levels (≥4 mM) over the course of each feeding cycle, relative to cells in high sugar medium, where pH varied by 1.2 units and acetate by ~15 mM over the course of each feeding cycle.

3.3 | Encapsulation reduces the accumulation of biomass

Population density in each treatment was estimated by direct cell count using a hemocytometer. In all cases, encapsulated cultures accumulated significantly less biomass than did planktonic cultures, evaluated in terms of cell number and assessed via two-way ANOVA with Tukey's post hoc ($F_{8,86} = 28.9$, $p < .001$). Specifically, although all treatments were initiated with approximately 5×10^9 cells in the entire culture volume, planktonic cultures had $1.6–2 \times 10^{11}$ cells

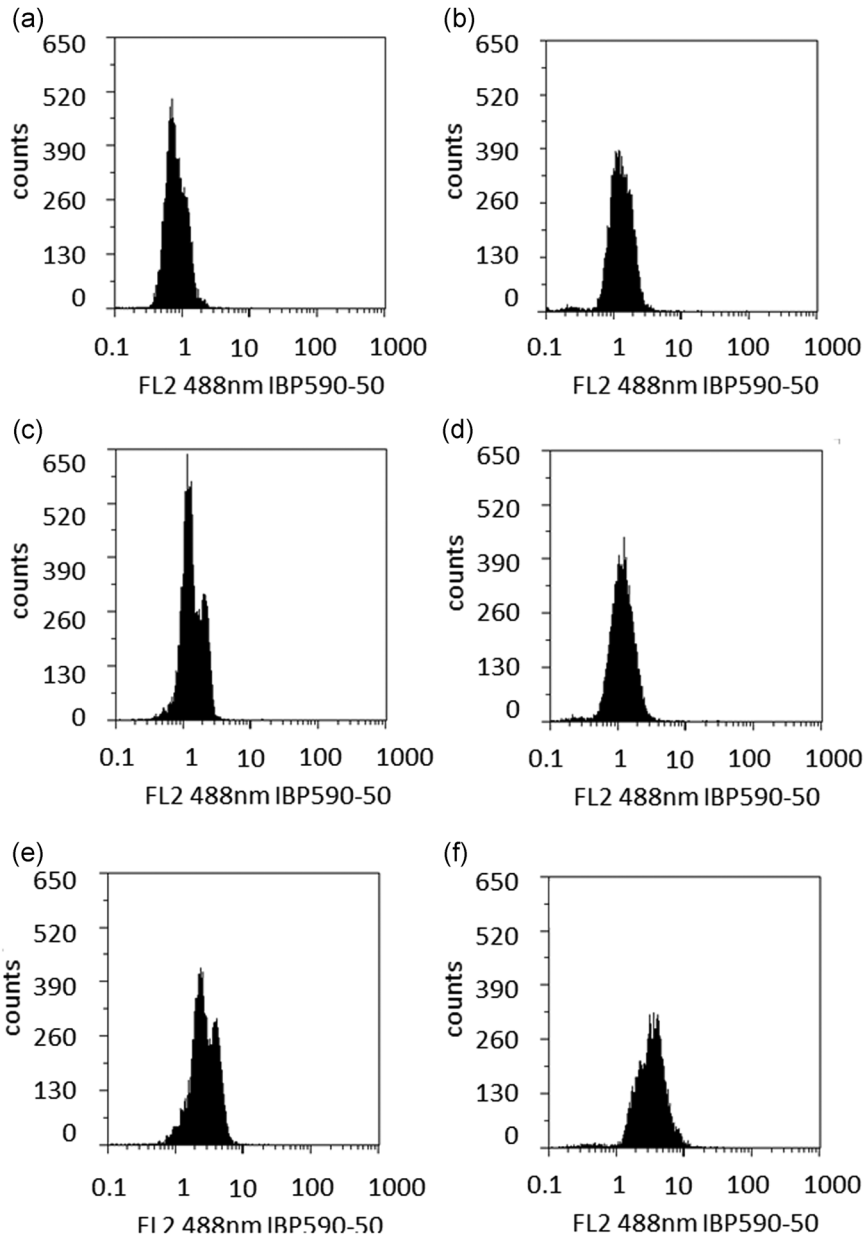

FIGURE 2 Verification of protoplast fusion via flow cytometry. The dye propidium iodide (PI), which binds to DNA, was used to verify protoplast fusion. A *Saccharomyces cerevisiae* haploid control (a) is left-shifted on a logarithmic axis as compared with a *S. cerevisiae* diploid (b), a diploid control, *S. cerevisiae* YE658 (c), and diploid *Pichia stipitis* (d). These diploids are in turn left-shifted with respect to both a tetraploid *S. cerevisiae* control (e) and the *S. cerevisiae P. stipitis* fusants (f)

after 19 days, roughly five cell doublings. In contrast, encapsulated cultures had 2.2 to 2.4×10^{10} cells after 19 days, about two cell doublings (Figure 5). Moreover, in encapsulated cultures, cell number increased only in the first 2 days, whereas in planktonic cultures cell number increased throughout the 19-day experiment.

Encapsulation has previously been shown to promote cell cycle arrest in *S. cerevisiae*, even when cells are continuously fed ad libitum (Nagarajan et al., 2014). Indeed, after an initial outgrowth period of ~ 72 hr, the great majority of cells in such populations remain virgin daughters for up to 3 weeks. Over this period, cells exhibit a stable pattern of gene expression that differs markedly from starving or growing planktonic yeast (Nagarajan et al., 2014). The carrying capacity of encapsulated treatments reported here is comparable with that previously reported for fed-batch cultures of encapsulated *S. cerevisiae* (Roukas, 1994).

3.4 | Encapsulation enhances fusant genome stability

Fungal protoplast fusion is notoriously unstable, with cell division frequently resulting in the segregation of a dikaryon's two nuclei (Gupthar, 1992; Yoon, Lee, Kim, Seo, & Ryu, 1996). Indeed, even though *S. cerevisiae-P. stipitis* fusants exhibit superior xylose utilization relative to their parental species (Yoon et al., 1996), their genomic instability led Jeffries and colleagues to conclude that such constructs are not suitable for industrial applications like cellulosic ethanol production (Jeffries & Jin, 2004). To test whether encapsulation could help overcome this problem, protoplast fusants were first grown to high density in the presence of both hygromycin and geneticin, which together served as fusants (i.e., for dikaryons containing nuclear genomes of both species). Half of this high-density

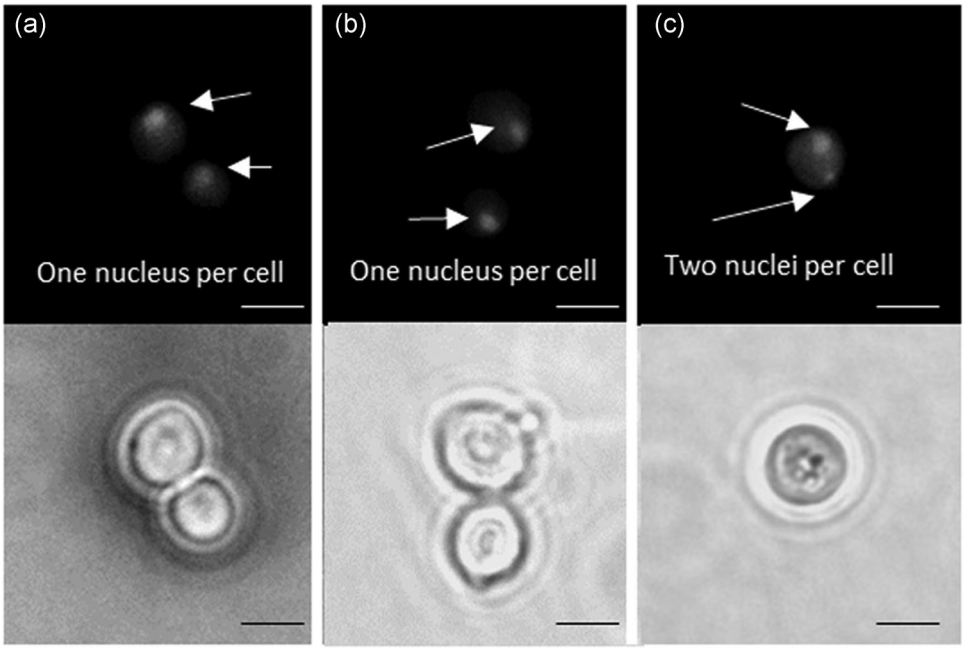

FIGURE 3 Verification of protoplast fusion via 4,6-diamidino2-phenylindole (DAPI). The dye DAPI, which binds to AT regions of dsDNA, was also used to verify protoplast fusion. Only one nucleus is detected in *Saccharomyces cerevisiae* (a) and *Pichia stipitis* (b), whereas two nuclei are visible in fusants (c). Top panel is DAPI staining, bottom panel is bright-field image. Scale bar is 5 μm. dsDNA, double-stranded DNA

fusant culture was encapsulated, and the other half was maintained as planktonic; both treatments were provided with fresh YPDX medium every 5 days for 19 days. Immediately after each addition of fresh medium, the percentage of cells that remained fusants was estimated by plating on hygromycin + geneticin YPD agar and by assaying cells' DNA content by PI staining.

Our results clearly show that encapsulation (Figure 5) can be exploited to stabilize dikaryons created by protoplast fusion (Figure 6a). In the first 5 days of outgrowth in the absence of drug selection, the percentage of encapsulated fusants declined, very likely due to segregational loss during cell division. Thereafter, the cell number remained remarkably constant, increasing only slightly

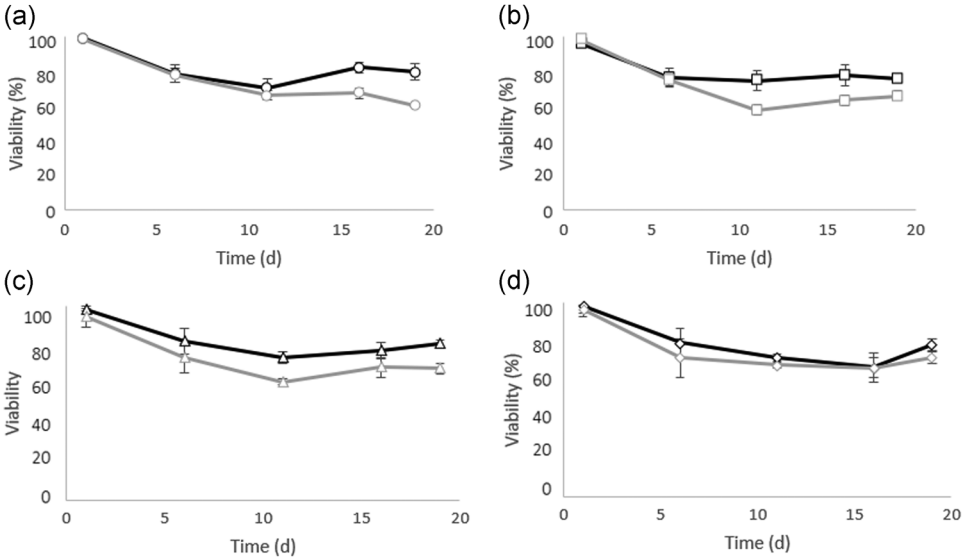

FIGURE 4 Cell viability, assessed in terms of colony-forming units (CFUs), was similar across treatment groups. Viability was assessed by counting CFUs immediately after cultures were supplied with fresh medium every 5th day. Black lines indicate encapsulated cultures, gray lines indicate planktonic cultures. Circles represent fusants (a), squares represent *Saccharomyces cerevisiae* (b), triangles represent *Pichia stipitis* (c), and diamonds represent mixed cultures of *S. cerevisiae* and *P. stipitis* (d). Error bars represent one standard deviation. No significant differences were found by a two-way ANOVA with Tukey's post-hoc ($F_{8,86} = 0.68$, $p > .05$)

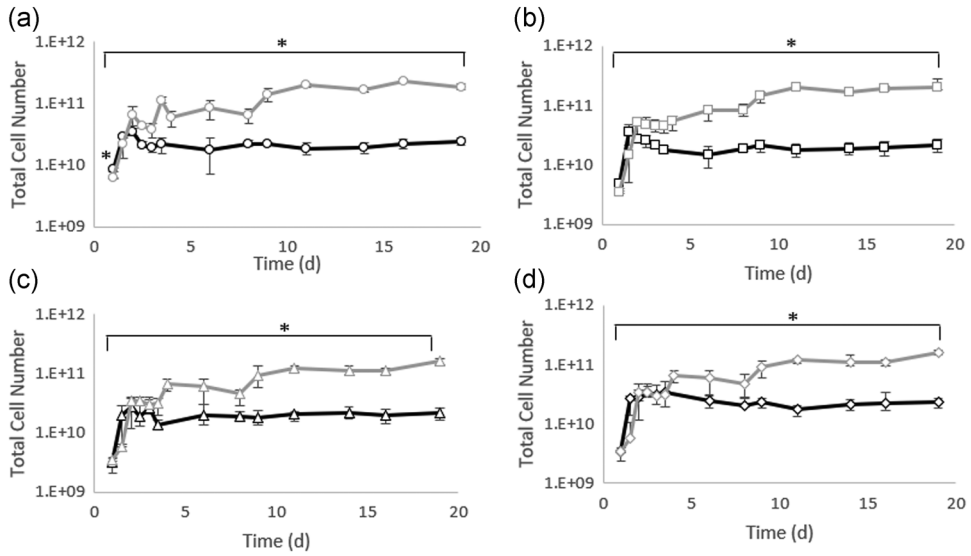

FIGURE 5 Encapsulation reduces biomass accumulation. Despite being seeded with equal cell numbers, planktonic cultures had more total cells (as assayed by a Neubauer hemocytometer) than encapsulated cultures at most points measured, increasing by roughly an order of magnitude more than encapsulated cells over 19 days. Black lines indicate encapsulated cultures, gray lines indicate planktonic cultures. Circles represent fusants (a), squares represent *Saccharomyces cerevisiae* (b), triangles represent *Pichia stipitis* (c), and diamonds represent mixed cultures of *S. cerevisiae* and *P. stipitis* (d). Error bars represent one standard deviation. * indicates a significant difference as assessed by a two-way ANOVA with Tukey's post-hoc ($F_{8,86} = 64.7$, $p < .0001$)

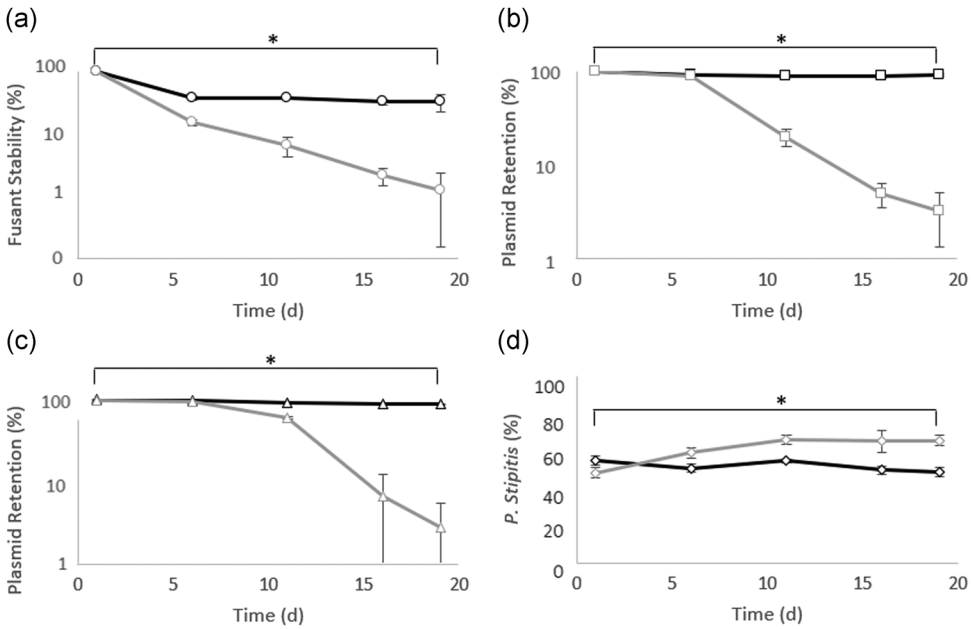

FIGURE 6 Encapsulation stabilizes fusant genomes, drug-resistant plasmids, and strain ratio in mixed cultures. Over the course of 20 days in medium not containing antibiotics, fusant stability (a) and antibiotic plasmid (p-CORE-Hp53, b; p-CORE-Kp53, c) retention was determined by comparing the ratio of colonies grown on selective YPD medium (plus antibiotic[s]) to that on YPD medium (permissive). Antibiotic drugs were absent from the regular growth medium, absence of selection resulted in both the percentage of fusants and plasmid-bearing cells to decline more quickly over time in planktonic than encapsulated cultures. Encapsulation also preserved a 1:1 ratio of *Saccharomyces cerevisiae* to *Pichia stipitis* cells (d), which increased to 65% *P. stipitis* in planktonic cultures. Black lines indicate encapsulated cultures, gray lines indicate planktonic cultures. Circles represent fusants, squares represent *S. cerevisiae*, triangles represent *P. stipitis*, and diamonds represent mixed cultures of *S. cerevisiae* and *P. stipitis*. Error bars represent one standard deviation. * indicates a significant difference as assessed by a two-way ANOVA with Tukey's post-hoc ($F_{8,86} = 26.6$, $p < .0001$)

between Days 5 and 19, during which time the proportion of fusants declined only slightly. In contrast, the cell number in the planktonic fusant treatment continued to increase throughout the experiment (Figure 5), resulting in just 1% of the population maintaining resistance to both antibiotic drugs at Day 19 (Figure 6a). These results accord with fusant stability assessed by PI staining (Figure S3), which shows that after 19 days, 32% of cells in encapsulated cultures were fusants compared to 7% of cells in planktonic culture. Thus, whether assessed by plating or by PI staining, encapsulation significantly ($F_{8,86} = 124.8$, $p < .001$, two-way ANOVA with Tukey's post hoc) enhanced the stability of fusant genome content (Figures 6a and S3). We speculate that this difference could be further magnified by maintaining antibiotic drug selection throughout the initial ~5-day outgrowth period.

Although several others (Hansen, Rocken, & Emeis, 1990; Spencer, Spencer, & Reynolds, 1988; Vidoli, Yamazaki, Nasim, & Veliky, 1982) have encapsulated fusants in alginate beads to assist in their recovery from protoplast fusion, we are the first, to the best of our knowledge, to investigate this system as a means for long-term stabilization of dikaryons created via protoplast fusion.

Encapsulation also significantly improved the stability of extrachromosomal genetic elements. 80% to 85% of encapsulated *S. cerevisiae* and *P. stipitis* transformants hosted plasmids after 19 days, as assessed by plating on YPDA and YPDA amended with 200 μg/ml of either hygromycin or geneticin. By comparison, planktonic transformant cultures were only 34% to 38% plasmid-bearing after the same period of time (Figure 6b,c). As with dikaryons, the most likely explanation for enhanced plasmid stability is less cell division—and reduced segregational loss—in encapsulated relative to planktonic cells (Figure 5). Even though nonplasmid bearers would be expected to out-compete plasmid bearers in the absence of antibiotic drug selection, the low incidence of cell division in encapsulated cultures (Nagarajan et al., 2014) offsets this advantage.

The use of encapsulation to stabilize plasmids was already well established by Barbotin (1994) (Berry, Sayadi, Nasri, Barbotin, & Thomas, 1988; Nasri, Sayadi, Barbotin, & Thomas, 1987; Nasri, Sayadi, Barbotin, Dhulster, & Thomas, 1987). Even earlier Sayadi, Nasri, Berry, Barbotin, and Thomas (1987) demonstrated that after 120 generations (counted in planktonic cultures) 88% of encapsulated *E. coli* cells retained plasmids compared to only 40% of planktonic cells (Sayadi, Nasri, Berry, Barbotin, & Thomas, 1987). Our data (Figure 6b,c) are consistent with these observations, as well as with more recent reports demonstrating that encapsulation enhances the stability of a wide variety of plasmids in both bacteria and yeast (Lú Chau, Guillán, Roca, Núñez, & Lema, 2000; Park & Chang, 2000).

Our results further indicate that cell cycle arrest stemming from encapsulation can be used to maintain the desired species ratios in mixed populations (Figure 6d). We seeded mixed cultures with *P. stipitis* and *S. cerevisiae* at the ration of 1:1. Over the course of 19 days, encapsulated cultures remained ~50% *P. stipitis*, whereas planktonic cultures saw an increase in the proportion of *P. stipitis* to ~65% (Figure 6d). Under certain conditions, the 1:1 ratio of these two species can be optimal for mixed sugar fermentation (Y. Chen, 2011). Indeed, modeling suggests that the ideal ratio of *S. cerevisiae: P. stipitis* is 1:1 when the glucose:xylose ratio is 3:1 (Unrean & Khajeeram, 2015). As our glucose: xylose ratio was 1:1, a higher relative amount of *P. stipitis* might have allowed for more rapid conversion of xylose to ethanol. We maintained a 1:1 species ratio in these experiments to keep results comparable to fusants, where the *P. stipitis* and *S. cerevisiae* nuclear genomes were present in a 1:1 ratio.

3.5 | Encapsulation increases ethanol production on a per cell basis

Ethanol production was examined following the addition of fresh YPDX medium, which was provided every 5 days for 19 days. Encapsulation was expected to increase ethanol yield, as nondividing encapsulated cells allocate scant resources to producing new biomass (Y. Chen et al., 2013; Jamai, Ettayebi, El Yamani, & Ettayebi, 2007; Kondo et al., 2002; K. H. Lee, Choi, Kim, Yang, & Bae, 2011; McGhee, Julian, Detroy, & Bothast, 1982; Watanabe et al., 2012). When considering overall ethanol production (in contrast to per-cell production), we observed statistically significant differences between treatments only when comparing fusant, mixed, or pure *P. stipitis* to pure *S. cerevisiae*, which produced less ethanol than the other treatments because it cannot utilize xylose (Figure S4). We did not observe any difference in ethanol production based on encapsulation, possibly due to differences in cell number between the two treatments; that is, even if planktonic cells are less efficient, their greater numbers may compensate for lower per cell conversion of fermentable substrate to product. This is confirmed by analysis of ethanol production on a per-cell basis (Figure 7), which shows that encapsulated cells produced much more ethanol than planktonic cells ($F_{8,86} = 6.4$, $p < .0001$, two-way ANOVA with Tukey's post hoc).

An examination of fermentation kinetics among treatments revealed no major differences in glucose consumption ($F_{8,86} = 0.11$, $p > .05$, two-way ANOVA with Tukey's post hoc); xylose consumption differed only between pure *S. cerevisiae*, which cannot utilize xylose, and all other treatments ($F_{8,86} = 15.3$, $p < .0001$, two-way ANOVA with Tukey's post hoc). Encapsulated cells, considered as an entire group, did not differ significantly in per-cell ethanol production, nor did planktonic cells if considered as a group; however, significant differences were observed when comparing per-cell ethanol production of encapsulated and planktonic cells ($F_{8,86} = 6.4$, $p < .0001$, two-way ANOVA with Tukey's post-hoc). Predictably, because *S. cerevisiae* cultures could not utilize xylose both planktonic and encapsulated *S. cerevisiae* produced less ethanol per cell than the other treatments as well as less ethanol overall. However, encapsulated Baker's yeast converted glucose to ethanol at near-theoretical levels (Figure 7c), consistent with previous findings (Gulli et al., 2019).

Mixed populations, consisting of one microbial species capable of fermenting 5C sugars and another capable of fermenting 6C sugars, have been previously evaluated as a means to cellulosic ethanol

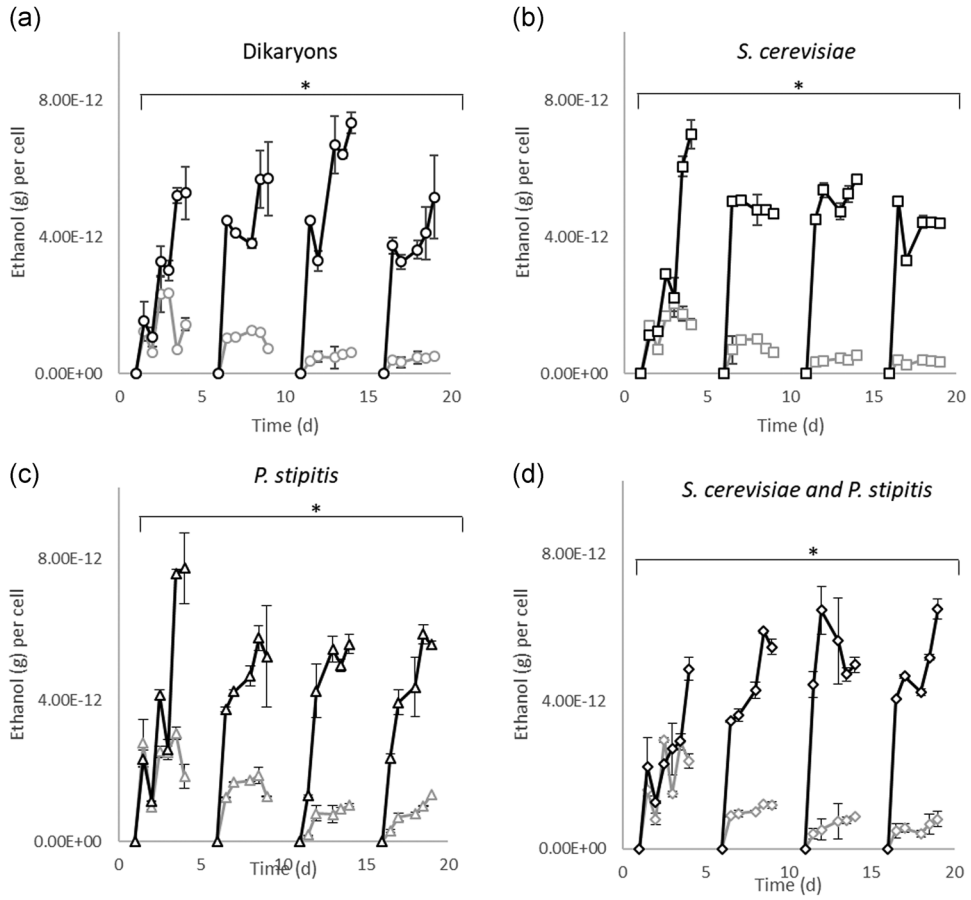

FIGURE 7 Encapsulation increases ethanol production on a per cell basis. Encapsulated cultures produced significantly more ethanol than their planktonic counterparts on a per-cell basis over most of the experiment. All cultures were provided with fresh medium every five days. Black lines indicate encapsulated cultures, gray lines indicate planktonic cultures. Circles represent dikaryons (a), squares represent *Saccharomyces cerevisiae* (b), triangles represent *Pichia stipitis* (c), and diamonds represent mixed cultures of *S. cerevisiae* and *P. stipitis* (d). Error bars represent one standard deviation. * indicates a significant difference as assessed by a two-way ANOVA with Tukey's post-hoc ($F_{8,86} = 5.3$, $p = .0065$)

production (Y. Chen, 2011; Taniguchi, Tohma, Itaya, & Fujii, 1997). Some have found deleterious effects arising from coculture (Grootjen, Jansen, van der Lans, & Luyben, 1991), while others have observed simultaneous cofermentation of 5C and 6C sugars (Gutiérrez-Rivera, Waliszewski-Kubiak, Carvajal-Zarrabal, & Aguilar-Uscanga, 2012; Unrean & Khajeeram, 2015). We did not observe simultaneous cofermentation by either dikaryons or mixed cultures; instead we noted in all experiments a lag phase following glucose consumption before onset of xylose metabolism (Figure S5). Previous work has shown (Slininger, Thompson, Weber, Liu, & Moon, 2011) that this lag phase varies in relation to the strain of *P. stipitis* employed, as well as the initial glucose concentration.

Xylose fermentation in *P. stipitis* is highly dependent on culture conditions, and is repressed or inefficient if ambient ethanol exceeds 3% (Agbogbo & Coward-Kelly, 2008; Y. Chen, 2011), or if excessive oxygen (Delgenes, Moletta, & Navarro, 1988; du Preez, van Driessel, & Prior, 1989; Klinner, Fluthgraf, Freese, & Passoth, 2005; Taniguchi et al., 1997), glucose (Grootjen et al., 1991; Panchal, Bast, Russell, & Stewart, 1988), or xylitol (Agbogbo & Coward-Kelly, 2008) is present. Since only moderate (2% glucose, 2% xylose) sugar concentrations were used here and cultures were regularly provided fresh medium, cells were not exposed to inhibitory levels of ethanol and acetate for prolonged periods, which is reflected in their high viability after 19 days of fed-batch culture (Figure 4). Oxygen levels were not measured, but were assumed to be low based on the minimal head space in our cultures (250 ml volume in a 250-ml flask), the screw-cap tops employed, and the slow rate of agitation (50 rpm); xylitol production is usually minimal under low oxygen conditions as well (Agbogbo & Coward-Kelly, 2008). It is unlikely, then, that this long lag time between glucose and xylose fermentation is due to repressive culture conditions, but may instead be a strain-specific effect.

4 | CONCLUSIONS

Our results demonstrate that encapsulation can be used to stabilize *P. stipitis-S. cerevisiae* dikaryons created by protoplast fusion (Figures 6 and S3). Dikaryons retained the metabolic capacity

encoded by both parental genomes in extended glucose + xylose fed-batch cultures. We did not observe simultaneous cofermentation of 5C and 6C sugars. Instead, like immobilized mixed cultures of *P. stipitis* and *S. cerevisiae* as well as like planktonic mixed cultures of these same species, we observed ethanologenic fermentation of glucose succeeded by that of xylose, with no difference among treatments in the kinetics of substrate-to-product conversion.

Although these experiments do not demonstrate utility for scaled-up biomass conversion, they do clearly demonstrate genomic stability among encapsulated dikaryons. Genomic stability in encapsulated cells results from placing limits on cell division. With little chance for their nuclei to segregate, encapsulated fusants are much more stable than has been reported previously (Kahar & Tanaka, 2014). This feature may facilitate their use in a variety of applications, ranging from the production of amylase enzymes (Pandey et al., 2000) to oil recovery (S. Sun et al., 2013) and even to microbial weed control (TeBeest, 2012).

Genome stabilization via encapsulation may have much wider implications for industrial microbiology. Synthetic microorganisms carrying heterologous genes are routinely designed to manufacture high-value products such as polypeptides, small bioactive molecules (antibiotics, food additives, and antimalarials) and other specialty chemicals (Daly & Hearn, 2005; Juturu & Wu, 2018; Spadiut, Capone, Krainer, Glieder, & Herwig, 2014; Wells & Robinson, 2017). However, synthetic microorganisms can be (and often are) genetically unstable, particularly if they harbor complex heterologous pathways that produce toxic compounds. Mutational inactivation of heterologous genes is also a critical challenge in synthetic biotechnology. Because nondividing, encapsulated cells are not subject to replication error and because they are inherently stress-resistant, heterologous pathways in such cells should be shielded from mutational inactivation. In a large scale-up, any appreciable genetic instability limits production yields, shortens production run time, and increases overall costs of production. Instabilty is, therefore, a major impediment to molecular design, one that necessitates genomic integration, or the use of expensive selective media. We show that encapsulation can stabilize even notoriously unstable molecular arrangements such as heterokaryonic fusants. The stabilized genomes, high stress resistance, and low biomass production of encapsulated cells may make them attractive platforms for continuous biomanufacturing in high-value, small-batch production systems.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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