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# Fisetin yeast-based bio-capsules via osmoporation: effects of process variables on the encapsulation efficiency and internalized fisetin content

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**Abstract** Osmoporation is an innovative method that can be used with food-grade yeast cells of *Saccharomyces cerevisiae* as natural encapsulating matrices. This technique overcomes barriers that difficult encapsulation and enables the internalization of fragile bioactive molecules such as fisetin into yeasts. In the present study, we assessed the effects of concentration, osmotic pressure, and temperature on the encapsulation efficiency (EE) and internalized fisetin content (IF). Two different quantification strategies were investigated: direct extraction (DE) without cell washing or freeze-drying steps and indirect extraction (IE) performed after washings with ethanol and freeze-drying. Our results showed that osmoporation improved EE (33 %) and IF (1.199 mg). The best experimental conditions were found by using DE. High-resolution images showed that the yeast cell envelope was preserved during osmoporation at 30 MPa and 84 % of yeast cells remained viable after treatment. Washing cells with organic solvent led

to decreased EE (0.65 %) and IF (0.023 mg). This was probably due to either damages caused to yeast cell envelope or fisetin dragged out of cell. Overall, the results demonstrated the adequacy and relevant biotechnological potential of yeasts as encapsulating matrices for hydrophobic compounds. This fresh biotechnological approach has proven to be a promising tool for the production of bioactive-rich food products.

**Keywords** Fisetin · Encapsulation · *S. cerevisiae* · Osmoporation

## Introduction

The protection and vectorization of sensible molecules such as antioxidants, flavonoids, and vitamins using simple and efficient protocols have attracted great interest nowadays (Dias et al. 2015). In this context, the yeast *Saccharomyces cerevisiae* has been studied as a pre-formed protective biocapsule against deleterious interactions for substances internalized in its cytoplasm due to its favorable membrane conformation and cell wall (Pham-Hoang et al. 2013; Shi et al. 2008). The great potential of this strategy has been demonstrated in some successful studies using yeast cells to encapsulate curcumin (Paramera et al. 2011b), resveratrol (Shi et al. 2008), chlorogenic acid (Shi et al. 2007), and limonene (Normand et al. 2005). In addition, the food-grade yeast *S. cerevisiae* is an organism with wide application range in the food industry (Sundh and Melin 2011), making it an ideal carrier for encapsulation of food products (Blanquet et al. 2005). It seems that the key step for encapsulation of active molecules inside living microorganisms is to empty their intracellular compartments before having contact with the active ingredient (Pham-Hoang et al. 2015).

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The cell osmoporation is an innovative technique that has been applied to the encapsulation of water-soluble substances in *S. cerevisiae* yeasts. In this method, yeast cells are dehydrated by increasing the medium osmotic pressure, followed by rapid rehydration, which leads to instant internalization of the active ingredient diluted in the solution. Through osmoporation, it was possible to significantly increase the content of fluorescein isothiocyanate dextran (FITC-dextran, 20 kDa) in yeast cells (Pedrini et al. 2014). Other strategies have been proposed to improve encapsulation of bioactive molecules inside yeast cells. For example, the electroporation (pulse electric fields applied during milliseconds) is a well-known technique to enhance the diffusion of high-weight molecules across cell membranes; however, the efficiency of this technique is inversely proportional to cell viability (Tsong 1989). Chemicals (detergents, lithium acetate, toxic solvents, etc.) are widely used to improve encapsulation of active ingredients inside cells, although high-efficiency their applications are limited for food and many cosmetic or pharmaceutical products (Pham-Hoang et al. 2015; Stephens and Pepperkok 2001). Hydrophobic molecules have been successfully encapsulated inside yeast cells (Ciamponi et al. 2012; Normand et al. 2005; Paramera et al. 2011a; Shi et al. 2008). The best advantage of osmoporation technique is the instantaneous internalization of the bioactive molecule and could be a good strategy for industrial applications where time product processing is limited. Despite the successful previous applications of osmoporation, more information is still necessary in order to better understand the best parameters for encapsulation, the effect of the polarity of the active ingredient, and the impact of osmoporation on yeast cell structure.

In the present work, we investigated the osmoporation technique using the hydrophobic flavonoid fisetin as a model compound. The fisetin molecule is a flavonoid with antiviral activity against herpes and dengue viruses (Lyu et al. 2005; Zandi et al. 2011) and recognized biological activity against prostate cancer (Khan et al. 2008). Moreover, this substance is able to decrease the inflammatory response in lung cells and connective tissue (Geraets et al. 2009; Park et al. 2008) and exhibits neuroprotective activity (Maher et al. 2006). Hydrophobic bioactive compounds, such as fisetin, have reduced bioavailability when directly applied in food, pharmaceutical, or cosmetic products, which is explained partially by their poor water solubility. In addition, these fragile active molecules may lose their functional properties when submitted to some processing conditions such as adverse temperatures, pH variations, and exposure to oxygen and light. Therefore, protective measures such as encapsulation strategies using biological matrices have been proposed. For example, Seguin et al. (2013) showed that the fisetin encapsulated into liposomes have a better bioavailability and efficiency against lung carcinoma. In addition, for several natural products, the encapsulation processes should avoid the use of any kind of toxic solvents or non-natural capsule materials during product

formulation (Pham-Hoang et al. 2015). In this case, yeast *S. cerevisiae* is a microorganism generally recognized as safe (GRAS) and the cell osmoporation process uses only water-glycerol solutions that are also GRAS (FDA 2015).

In order to evaluate the best experimental conditions of fisetin encapsulation, the encapsulation efficiency, and the fisetin internalized content in *S. cerevisiae* cells, two quantification strategies were assessed: (i) direct extraction (DE) without cell washing or freeze-drying steps and (ii) indirect extraction (IE) performed after washings with ethanol and freeze-drying. DE was evaluated by UV-visible spectrophotometry (UV) and IE by high-performance liquid chromatography (LC). The parameters considered in this study were fisetin concentration, level of osmotic dehydration, and process temperature. Scanning electron microscopy and cell viability assays were conducted to assess the impact of the process on the microbial structure and functionality, respectively. The fisetin intracellular location was determined by fluorescence confocal microscopy. Our results aim to elucidate the mechanisms involved in the osmoporation process and serve as a rational basis for the development of new technologies applied to encapsulation processes.

## Materials and methods

### Fisetin and glycerol solutions

Fisetin (purity  $\geq 98\%$ ; Sigma-Aldrich, USA) solutions (0.32, 1.00, 2.00, 3.00, or 3.68 mg mL<sup>-1</sup>) were prepared by dissolving fisetin in pure ethanol. The water-glycerol solutions with osmotic pressure ( $\pi$ ) of 1.4 (*iso*-osmotic), 4.8, 15.0, 30.0, 45.0, or 55.2 MPa were prepared by adding, respectively, 51, 179, 513, 1063, 1668, or 2045 g of pure glycerol to 1000 g of distilled water (25 °C). The osmotic pressure is linked to water activity ( $a_w$ ) by the following equation:

$$\pi = \frac{-RT \ln(a_w)}{V_w} \quad (1)$$

where  $R$  is the universal gas constant (J mol<sup>-1</sup> K<sup>-1</sup>),  $T$  is the temperature (K), and  $V_w$  is the partial molar volume of water (m<sup>3</sup> mol<sup>-1</sup>). The Norrish equation (Norrish 1966) was used to calculate the water activity:

$$a_w = (1 - X_s) \exp(-K X_s^2) \quad (2)$$

where,  $X_s$  is the solute molar fraction and  $K$  is the solute coefficient, for glycerol  $K = 1.16$  (Chirifé et al. 1980). The water activity of each solution was confirmed using a dew point hygrometer Aqualab<sup>®</sup> series 3 TE (Decagon Devices, Pullman, WA, USA).

## Cells and culture conditions

Three previous isolated colonies of commercial food-grade *S. cerevisiae* (Fleischmann<sup>®</sup>, Brazil) were transferred to 100 mL of modified Malt Wickerham (MW) medium (Dupont et al. 2010; Pedrini et al. 2014), in a rotary shaker TE-422 (Tecnal, Piracicaba, SP, Brazil) at 250 rpm, 25 °C for 48 h. Subcultures (1 mL) were transferred to 100 mL of the MW medium and cells grown overnight under the same conditions. The cell suspensions (40 mL) were centrifuged for 5 min at 2200×g and 25 °C (model SL 701, Solab, Piracicaba, SP, Brazil) and washed twice with *iso*-osmotic water-glycerol solution (1.4 MPa) and resuspended in 20 mL of the same solution (final cell density was approximately  $2 \times 10^8$  cells mL<sup>-1</sup>).

## Encapsulation of fisetin via osmoporation

The fisetin encapsulation was performed in two stages. (1) Osmotic dehydration: Cell suspension (1.5 mL) was centrifuged for 10 min at 5100×g and 25 °C, and 1.5 mL of water-glycerol solution at 4.8, 15.0, 30.0, 45.0, or 55.2 MPa was added. The cell suspension was kept in a rotary shaker at 250 rpm and 25 °C. (2) Rehydration and internalization: After 60 min and centrifugation for 10 min at 5100×g and 25 °C, cells were rapidly rehydrated (<1 min) by adding 1.5 mL of the water-glycerol 1.4 MPa *iso*-osmotic solution and 0.3 mL of fisetin solution (0.32, 1.00, 2.00, 3.00, or 3.68 mg mL<sup>-1</sup>). Time of internalization was <1 min.

## Direct extraction and quantification

The yeast biocapsules were subdivided in two groups of equal volume (or mass): the first group was transferred to an ultrasonic bath 1440 plus (Odontobras, Ribeirão Preto, SP, Brazil) at 40 kHz and 25 °C for 10 min; the second group was not submitted to this treatment. Suspensions were completed up to 10 mL with pure ethanol and filtered through nylon membrane (0.45 μm). The filtrate (0.1 mL) was diluted in ethanol (1:100 v/v). The fisetin quantification was performed at 360 nm using a microplate UV-visible reader Asys UVM340 (Biochrom, Cambridge, UK) and external calibration. The EE was calculated by the following equation:

$$EE = \left[ \frac{(m_A - m_B)}{m_A} \right] \times 100 \quad (3)$$

where  $m_A$  is the mass of fisetin into cells treated by ultrasonic bath and  $m_B$  is the mass of fisetin into cells not submitted to this treatment.

## Indirect extraction and quantification

Before quantification, the yeast biocapsules were washed three times with pure ethanol, resuspended in 0.01 M phosphate-buffered saline (PBS, Sigma-Aldrich, USA) and freeze-dried in a Liotop L101 bench lyophilizer (Liobras, São Carlos, SP, Brazil). The fisetin extraction was performed by adding 5 mL 50 % (v/v) ethanol to 20 mg of dried biocapsules protected from light in rotary shaker at 250 rpm and 25 °C. After 60 min, the samples were filtered through a 0.45-μm nylon membrane. Quantification of extracted fisetin was performed using an LC-10ATvp HPLC (Shimadzu, Kyoto, Japan). A C18 column (Waters Symmetry, 150 × 3.9 mm, 5 μm) was used under isocratic conditions at 40 °C with a mobile phase consisting of water/methanol/acetic acid (440:550:10 v/v/v). The flow rate was 1.0 mL min<sup>-1</sup> and the volume injection 0.02 mL. Quantification was performed at 360 nm by external standard calibration. The EE was calculated by the following equation:

$$EE = \frac{m_e}{m_i} \times 100 \quad (4)$$

where  $m_e$  is the mass of encapsulated fisetin and  $m_i$  is the initial mass of fisetin.

## Determination of encapsulated fisetin mass

The mass of internalized fisetin (IF) into cells was estimated by regression of the values of EE using the following equation:

$$IF = \frac{m_i \times EE \times 1.8}{100} \quad (5)$$

where the final sample volume of each sample is 1.8 mL and  $m_i$  is the initial mass of fisetin.

## Fluorescence confocal microscopy

After encapsulation, cell suspension (0.2 mL) was centrifuged for 10 min at 5100×g and 25 °C and 0.2 mL of water-glycerol solution at 15.0 MPa and 4 °C was added to the cells to stop natural endocytosis (Dupont et al. 2010; Marechal et al. 1995; Pedrini et al. 2014). The samples were protected from light and kept in ice during analysis. A Nikon Eclipse TE 2000 U microscope (Nikon, Tokyo, Japan) with multispectral confocal head D Eclipse C1 was used to observe cells stained with fisetin. Excitation was performed at 488 nm with laser He/Ar, and the emission signal was recovered between 490 and 670 nm. Images were acquired with a ×100 (NA: 1.4) Plan

Apochromat oil-immersion objective (Nikon) and collected with NIS software 3.1 (Nikon).

### Scanning electron microscopy

Yeast *S. cerevisiae* cell suspensions in (i) water-glycerol at 1.4 MPa (*iso*-osmotic), (ii) osmotically dehydrated at 30.0 MPa, or (iii) rehydrated (with 1.4 MPa solution) cells were vacuum filtered through a 0.22- $\mu$ m cellulose acetate membrane. Samples were washed three times with 0.01 M PBS solution (pH 7.4) and fixed with 2 % (*v/v*) glutaraldehyde in 0.01 M PBS for 60 min at 4 °C. The dehydration was performed by applying ethanol gradients of 30, 50, 70, and 100 % (three cycles) *v/v* at 4 °C for 5 min each one. After 30 min, cells were freeze-dried. Cell fragments were fixed and coated with gold using a SC-701AT sputter-coater (Sanyu Electron, Tokyo, Japan). Images of cell surface were acquired at 12 kV using a SSX-550 scanning electron microscope (Shimadzu, Kyoto, Japan).

### Cell viability

Cell viability was estimated by the colony-forming unit (CFU) method. After osmotic treatment and appropriated serial dilutions in 0.01 M PBS, cell suspension (0.1 mL) was spread onto 20 mL MW medium solidified with agar (15 g L<sup>-1</sup>) and incubated at 30 °C. After 48 h, isolated colonies were counted. Cell suspension without osmotic treatment was used as a control.

### Experimental design and statistical analysis

The optimal conditions for fisetin encapsulation were studied by a central composite design (CCD) and response surface (RS) methodology with three factors: fisetin concentration (*C*), dehydration osmotic pressure (*P*), and temperature (*T*). All experiments were performed in triplicate (*n* = 3). All statistical procedures were performed using the STATISTICA<sup>®</sup> software, version 7 (Statsoft, Tulsa, OK, USA). Data were tested using one-way analysis of variance (ANOVA). Tukey HSD post hoc test (95 % of probability level) was used for determining statistical significance (*p* < 0.05).

## Results

### Regression equations for EE and IF quantified after DE or IE

Using the RS obtained from an empirical mathematical model is possible to obtain the optimized conditions of a process (Box et al. 2005). In this work, the effects of three factors (fisetin concentration, osmotic pressure, and temperature levels) were assessed. The three-factor design and responses

are summarized in Table 1. The empirical observations were converted into four representative equations with three independent variables:

$$EE_1 = -132.7 - 24.2C - 2.6C^2 + 1.9P - 0.02P^2 + 13.3T - 0.3T^2 - 0.1CP + 1.2CT - 0.01PT \quad (6)$$

$$IF_1 = -5.9 + 0.2C - 0.2C^2 + 0.05P - 0.001P^2 + 0.5T - 0.01T^2 - 0.002CP + 0.03CT + 0.0002PT \quad (7)$$

$$EE_2 = 82.5 + 1.1C - 0.3C^2 + 0.2P - 0.003P^2 - 6.8T + 0.1T^2 + 0.01CP - 0.01CT + 0.0005PT \quad (8)$$

$$IF_2 = 3.0 + 0.1C - 0.02C^2 + 0.005P - 0.0001P^2 - 0.3T + 0.01T^2 + 0.001CP - 0.001CT - 0.00001PT \quad (9)$$

where EE is the encapsulation efficiency (%), IF is the mass of internalized fisetin (mg), *C* is the fisetin concentration (mg mL<sup>-1</sup>), *P* is the dehydration osmotic pressure level (MPa), and *T* is the temperature (°C). The subscripted numbers 1 and 2 (EE<sub>1</sub>, IF<sub>1</sub>, EE<sub>2</sub>, IF<sub>2</sub>) indicate the quantification strategies: (1) DE and (2) IE.

The statistical significance of Eqs. 6–9 was verified through ANOVA for the response surface quadratic models. The results (Table 2) indicate that almost all factors are statistically significant (*p* < 0.05). The second-order models accounted for response variability between 89.8 and 80.8 % (*R*<sup>2</sup>), which shows that the empirical model is in agreement with experimental data. For the DE values, the *F* model values (7.80 and 7.82) were twice higher than the standard value for the same degrees of freedom (3.39). In addition, no lack of fit was observed for these models (2.78 and 1.90 < 9.01), which demonstrates that the mathematical models are highly significant. However, the lack of fit for IE values (635.47 and 450.47) was statistically significant (*p* < 0.05), which suggests that these second-order models are not in agreement with observed data.

### Response surface analysis

The 3D surface plots of the empirical models (Eqs. 6–9) for all responses are presented in Fig. 1. The graphs were built by keeping one factor constant (center point; *C* = 2.00 mg mL<sup>-1</sup>, *P* = 30.0 MPa or *T* = 25.0 °C). The optimal area for the process, i.e., the best response, corresponds to the area with the highest slope in the response surface. The results obtained by DE showed that the fisetin concentration significantly affects the EE. An increase in EE was observed when fisetin concentration was reduced in the solution (Fig. 1a, b). On the other hand, the reduction of EE to *C* > 2.00 mg mL<sup>-1</sup> shows

**Table 1** CCD and responses for the encapsulation of fisetin via osmoporation using two quantification strategies (direct and indirect extraction)

Run	<i>C</i>	<i>P</i>	<i>T</i>	EE <sub>1</sub>	IF <sub>1</sub>	EE <sub>2</sub>	IF <sub>2</sub>
1	1.0	15.0	20.0	23.32 ± 0.24	0.420 ± 0.00	1.26 ± 0.19	0.023 ± 0.00
2	1.0	15.0	30.0	11.86 ± 0.51	0.213 ± 0.01	1.02 ± 0.12	0.018 ± 0.00
3	1.0	45.0	20.0	32.92 ± 0.33	0.593 ± 0.01	1.33 ± 0.13	0.024 ± 0.00
4	1.0	45.0	30.0	7.74 ± 0.52	0.139 ± 0.01	1.72 ± 0.22	0.031 ± 0.00
5	3.0	15.0	20.0	8.60 ± 0.72	0.464 ± 0.04	1.76 ± 0.68	0.095 ± 0.04
6	3.0	15.0	30.0	11.31 ± 1.66	0.611 ± 0.09	1.73 ± 0.32	0.093 ± 0.02
7	3.0	45.0	20.0	3.99 ± 0.84	0.216 ± 0.05	3.12 ± 2.95	0.168 ± 0.16
8	3.0	45.0	30.0	13.50 ± 1.49	0.729 ± 0.08	2.74 ± 1.99	0.148 ± 0.11
9	0.32	30.0	25.0	32.28 ± 0.17	0.186 ± 0.00	3.16 ± 0.15	0.018 ± 0.00
10	3.68	30.0	25.0	6.73 ± 1.56	0.446 ± 0.10	0.41 ± 0.09	0.027 ± 0.01
11	2.0	4.8	25.0	10.12 ± 0.96	0.364 ± 0.03	0.53 ± 0.14	0.019 ± 0.01
12	2.0	55.2	25.0	12.22 ± 0.96	0.440 ± 0.03	0.62 ± 0.05	0.022 ± 0.00
13	2.0	30.0	16.6	3.14 ± 1.11	0.113 ± 0.04	13.73 ± 3.59	0.494 ± 0.13
14	2.0	30.0	33.4	6.24 ± 1.31	0.225 ± 0.05	10.47 ± 0.59	0.377 ± 0.02
15	2.0	30.0	25.0	33.30 ± 0.99	1.199 ± 0.04	0.65 ± 0.17	0.023 ± 0.01
16	2.0	30.0	25.0	28.38 ± 1.39	1.022 ± 0.05	0.79 ± 0.13	0.028 ± 0.00
17	2.0	30.0	25.0	24.81 ± 1.22	0.893 ± 0.04	0.69 ± 0.14	0.025 ± 0.01
18	2.0	30.0	25.0	29.02 ± 1.37	1.045 ± 0.05	0.51 ± 0.13	0.018 ± 0.00

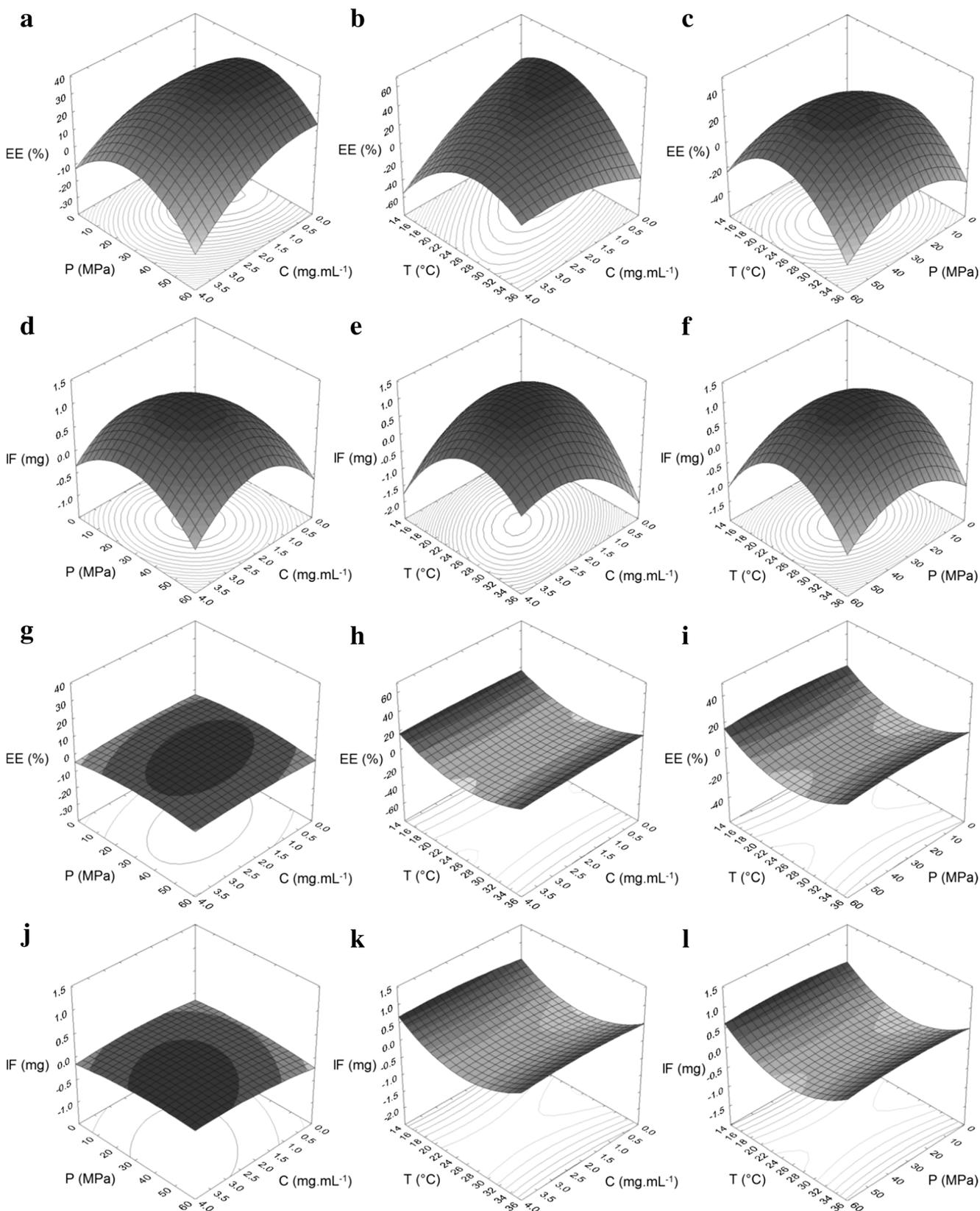
Values are mean of three replicates ± standard deviation ( $n = 3$ )

*C* fisetin concentration (mg mL<sup>-1</sup>), *P* dehydration osmotic pressure (MPa), *T* temperature (°C), *EE*<sub>1</sub> encapsulation efficiency quantified after direct extraction (%), *IF*<sub>1</sub> mass of internalized fisetin quantified after DE (mg), *EE*<sub>2</sub> encapsulation efficiency quantified after indirect extraction (%), *IF*<sub>2</sub> mass of internalized fisetin quantified after indirect extraction (mg)

**Table 2** Analysis of variance (ANOVA) of empirical models (CCD) for the encapsulation of fisetin via osmoporation using two quantification strategies (direct and indirect extraction)

Factor	df	EE <sub>1</sub>				IF <sub>1</sub>			
		SS	MS	<i>F</i> value	<i>p</i> value	SS	MS	<i>F</i> value	<i>p</i> value
Model	9	1800.20	200.02	7.80	0.0041	1.73	0.1926	7.82	0.0046
Residue	8	205.12	25.64			0.20	0.0246		
LoF	5	168.75	33.75	2.78	0.2144	0.15	0.0299	1.90	0.3166
PE	3	36.37	12.12			0.05	0.0157		
Total	17	2005.32				1.93			
<i>R</i> <sup>2</sup>		0.898				0.898			
	df	EE <sub>2</sub>				IF <sub>2</sub>			
		SS	MS	<i>F</i> value	<i>p</i> value	SS	MS	<i>F</i> value	<i>p</i> value
Model	9	179.81	19.98	3.73	<0.0001	0.2692	0.0299	6.01	<0.0001
Residue	8	42.83	5.35			0.0398	0.0050		
LoF	5	42.79	8.56	635.47	0.0001	0.0398	0.0080	450.47	0.0002
PE	3	0.04	0.01			0.0001	0.00002		
Total	17	222.64				0.3091			
<i>R</i> <sup>2</sup>		0.808				0.871			

*R*<sup>2</sup> correlation coefficient, *F* value test for comparing model variance with error variance (residue), *p* value defines the significance of the variables *df* degrees of freedom, ANOVA for *EE*<sub>1</sub> encapsulation efficiency quantified after direct extraction (%), *IF*<sub>1</sub> mass of internalized fisetin quantified after DE (mg), *EE*<sub>2</sub> encapsulation efficiency quantified after indirect extraction (%), *IF*<sub>2</sub> mass of internalized fisetin quantified after indirect extraction (mg), *SS* sum of square, *MS* mean square, *LoF* lack of fit, *PE* pure error



that cells did not have the capacity to internalize enough fisetin content present in solution. However, the fisetin

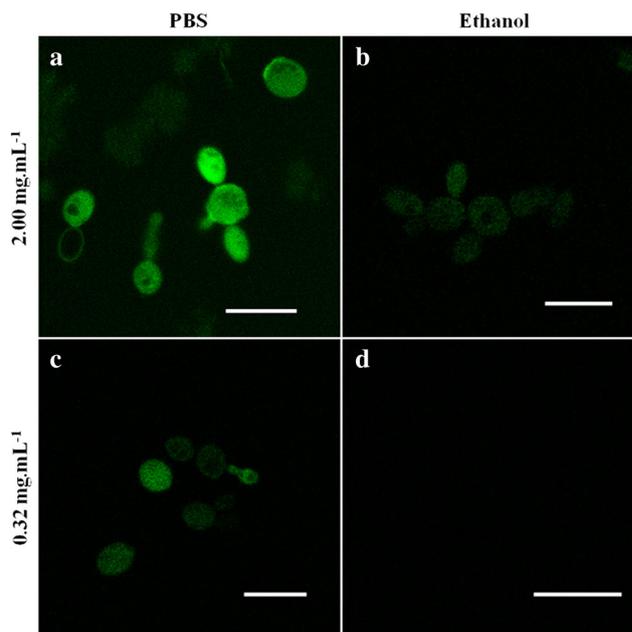
concentration does not affect the EE quantified by IE method, since response surface results show no

◀ **Fig. 1** 3D surface plots of models for EE and IF responses for the encapsulation of fisetin via osmoporation using two quantification strategies (direct and indirect extraction). EE and IF for the DE method (a–f). EE and IF for the IE method (g–l). RS of dehydration osmotic pressure  $\times$  initial fisetin at  $T = 25^\circ\text{C}$  (a, d, g, and j), RS of temperature  $\times$  initial fisetin at  $P = 30\text{ MPa}$  (b, e, h, and k) and RS of temperature  $\times$  dehydration osmotic pressure at  $C = 2.00\text{ mg mL}^{-1}$  (c, f, i, and l). For each response surface, the *black/dark gray area* represents the optimal results

maximum slope and the optimal area extends throughout the study range between  $0.32$  and  $3.68\text{ mg mL}^{-1}$  (Fig. 1g, h). For the IF response, both quantification methods show that IF content is affected by fisetin concentration. Optimized values can be observed in the center of RS for DE method (Fig. 1d) and in  $C \geq 2.00\text{ mg mL}^{-1}$  for the IE method (Fig. 1j). The dehydration osmotic pressure level significantly affected the EE and IF responses when DE method was used. The optimal results were observed at  $P = 30\text{ MPa}$  (Fig. 1a, c, d, f). In the IE method, the EE optimum values were also observed in the center of RS with  $P = 30.0\text{ MPa}$  (Fig. 1g) and to IF response  $P \geq 30.0\text{ MPa}$  (Fig. 1j). When DE method was used, the optimal temperature was observed at  $25.0^\circ\text{C}$  (Fig. 1b, c, e, f). However, the IE method showed different EE and IF responses to temperature changes. The optimal results were observed on the extreme conditions, i.e.,  $T \leq 16.6^\circ\text{C}$  and  $T \geq 33.4^\circ\text{C}$  (Fig. 1h, i, k, l).

#### Intracellular fisetin location after PBS or ethanol washings

Fluorescence confocal microscopy was performed to examine the intracellular location of fisetin and the solvent effect. All cell samples were previously dehydrated using water-glycerol solution at  $30.0\text{ MPa}$ . After 60 min, cells were rehydrated in *iso*-osmotic conditions and the fisetin was immediately added, followed by PBS or ethanol washing. The first condition was chosen based on optimal results of EE and IF quantified after DE, where the fisetin concentration was  $2.00\text{ mg mL}^{-1}$  and  $0.01\text{ M}$  PBS washing. We observed that yeast cells were uniformly filled with fisetin (Fig. 2a). In some yeast cells, the fisetin seems to be distributed only on cell wall or plasma membrane. Probably, these cells have not gone through sufficient structural changes to allow internalization of fisetin or the osmoporation was excessive, causing plasma membrane rupture and leakage of intracellular fisetin content. The encapsulation was also performed with fisetin concentration of  $0.32\text{ mg mL}^{-1}$  and PBS washing. In this condition, significant reduction of fisetin emission signal inside the yeast cells was observed (Fig. 2c). Yeast cells treated

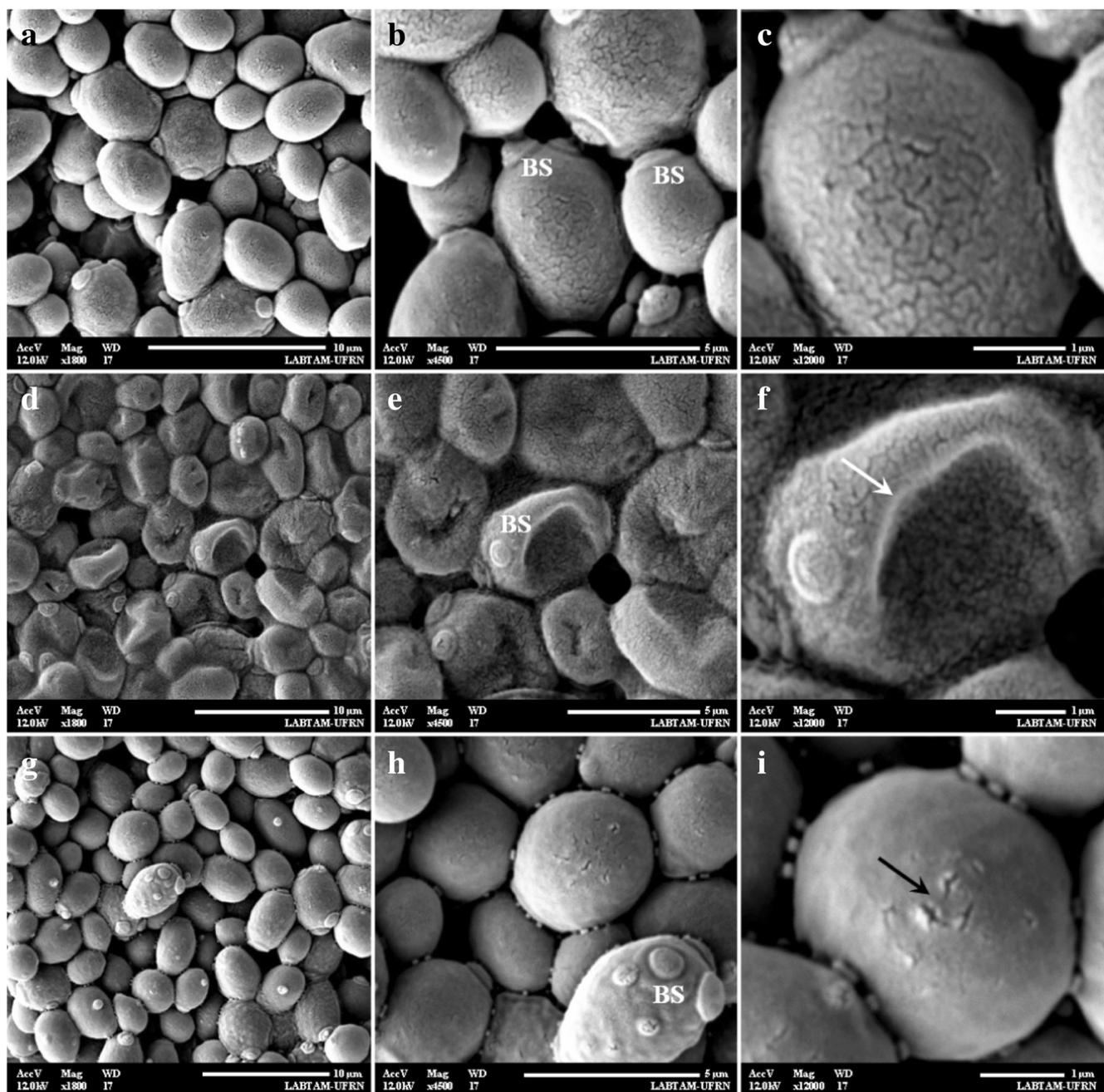


**Fig. 2** Observations of the intracellular fisetin after osmoporation and the effect of the solvent used in the washing steps. Yeast cells were treated with water-glycerol solutions at  $30.0\text{ MPa}$  and  $25.0^\circ\text{C}$  for 60 min and washed three times with PBS or ethanol. Representative images were acquired with fluorescence confocal microscopy. Fisetin final concentration:  $2.00\text{ mg mL}^{-1}$  (a and b) or  $0.32\text{ mg mL}^{-1}$  (c and d). Cells washed with PBS (a and c) or ethanol (b and d). Bar scale:  $10\text{ }\mu\text{m}$

at the same conditions ( $C = 2.00\text{ mg mL}^{-1}$ ) were washed three times with ethanol (Fig. 2b), and lower intracellular fisetin content was observed when compared with the cells washed with PBS. The negative effect of ethanol was most pronounced when fisetin concentration was reduced to  $C = 0.32\text{ mg mL}^{-1}$  (Fig. 2d), since the internalized fisetin content in these cells was almost null.

#### Observations of high-resolution cell surface during osmoporation

The hyperosmotic stress environment could lead to modifications on yeast encapsulating structure, i.e., cell wall and plasma membrane. Thus, scanning electron microscopy (SEM) was performed to observe yeast cell surface changes during osmoporation. Yeast cells in *iso*-osmotic conditions were observed after 60 min at  $1.4\text{ MPa}$  (Fig. 3a–c). Budding scars (BS) and natural roughness of cell wall surface were observed on yeast cell surface. Then, cells were transferred to glycerol solution at  $30\text{ MPa}$  (Fig. 3d–f). Cells were randomly deformed due to the increase in external osmotic pressure (white arrow), and the cell wall roughness persisted on this stage. The yeast *S. cerevisiae* was able to recover all its cell volume during the rehydration (Fig. 3g–i). We also observed that yeast cell surface was smoothed after rehydration probably due to proteins, glucans or polysaccharides reorganization.



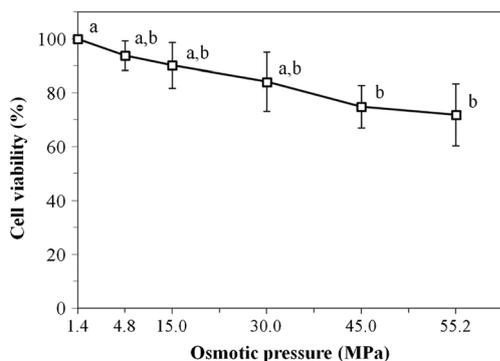
**Fig. 3** Representative images of yeast *S. cerevisiae* during osmoporation. Microphotographs were acquired by SEM in different stages of osmoporation. Cells in *iso*-osmotic water-glycerol solution at 1.4 MPa (a–c); dehydrated at 30.0 MPa for 60 min leading to reduction of cellular

volume (white arrow) (d–f) and rehydrated with the *iso*-osmotic solution (g–i). Stretch marks on cell surface (black arrow). BS budding scars. Bar scales: (a, d, and g) 10 μm, (b, e, and h) 5 μm, and (c, f, and i) 1 μm

### Impact of osmoporation on yeast cell viability

The general purpose of osmoporation is to improve internalization or diffusion of molecules inside the cells. However, this process can affect cell structure and viability. In other words, the maintenance of cell viability and integrity is important to stabilize the active ingredient inside the yeast, as the cell envelope acts as a preformed capsule and the maintenance of cell viability is strongly linked to fisetin

encapsulation efficiency inside cells. In order to assess possible impacts of structural modifications to cells during osmoporation, assays regarding the cell viability were performed. Indeed, it was observed that osmoporation did not significantly affect ( $p < 0.05$ ) yeast viability when dehydration is performed up to 30.0 MPa, with 84 % of viable cells after this treatment (Fig. 4). Our results showed decreased yeast cell viability ( $p < 0.05$ ) for treatments performed at 45.0 and



**Fig. 4** Evolution of the viability of yeast cells during different osmotic pressures. Yeast cells were maintained for 60 min at 1.4 (control), 4.8, 15.0, 30.0, 45.0, or 55.2 MPa and then rapidly rehydrated (1.4 MPa). Cell viability was performed by the CFU method. Mean  $\pm$  standard deviation ( $n = 3$ ). Different letters (a, b) indicate significant differences by Tukey HSD post hoc test ( $p < 0.05$ )

55.2 MPa, with 75 and 72 % of viable cells, respectively (Fig. 4), when compared to control samples at 1.4 MPa.

## Discussion

### Effects of fisetin concentration, dehydration osmotic pressure, and temperature

The observations obtained during quantification suggest that in order to get the highest EE, each process requires a specific concentration of the active ingredient to be encapsulated (Fig. 1). In other words, in vivo encapsulation systems, such as yeast cells, do not support an excessive concentration gradient of the active ingredient. These results confirm the previous observations of Paramera et al. (2011b) which presented the encapsulation of the hydrophobic colorant curcumin using the yeast cell plasmolysis method and observed that low curcumin concentrations led to higher EE. Ciamponi et al. (2012) also reported that increasing limonene (hydrophobic) concentration from 5 to 30 % (w/w) do not translate into better EE using *S. cerevisiae* as encapsulation matrix. In addition, metabolic and structural changes may occur when the cells get exposed to high concentrations of certain substances. For example, the addition of oxidants (diamide or calcium) in the medium leads to an increased pore formation and higher pore size in *S. cerevisiae* membranes (Souza Pereira and Geibel 1999). Modifications to the contact solution osmolarity induce irreversible disruption of the yeast cell envelope and lead to cell death (Simonin et al. 2007). In the case of IF responses (Fig. 1d, j), it is noteworthy that the highest IF content must not necessarily correlate to the highest EE, since the IF response is dependent on the amount of initially added fisetin and volume of cell suspension, while EE is calculated as the ratio between the mass of encapsulated fisetin and the initially

added mass of fisetin (Eqs. 3, 4, and 5). Paramera et al. (2011a) observed similar results with 35 % of yield for curcumin encapsulation using *S. cerevisiae*. Thus, in encapsulation processes, it is important to find an adequate balance between EE and IF (or yield) of the active ingredient in order to achieve the best experimental conditions for the bioprocess. The best experimental results in this study were observed at  $C = 2.00 \text{ mg mL}^{-1}$ .

During osmotic dehydration, the cells lose a part of their intracellular volume due to osmotic water outflow and solubilized components outside the cell (Gervais and Beney 2001). In addition, rehydration allows large quantities of water to enter inside the cell, along with other solutes in solution, due to modifications on the cell permeability (Pedrini et al. 2014; Schaber et al. 2010). These phenomena could explain the increase of EE and IF reported in this study (Fig. 1). Moreover, submitting yeast cells to osmotic pressure greater than 30 MPa could lead to irreversible destabilization of the plasma membrane and consequent reduction of cell viability (Fig. 4, between 25 and 30 % of cells) (Dupont et al. 2010; Simonin et al. 2007). This may cause the outflow of intracellular content and could explain the reduction of EE observed at  $P > 30.0$  MPa. Also, osmotic pressure below 30.0 MPa does not affect the natural barriers of yeast envelope that block fisetin to go through membranes and get in the cell (Pedrini et al. 2014). These findings suggest the existence of a permeability adaptive mechanism of yeast membranes caused by the osmotic pressure of the medium. In fact, it has been shown that the *S. cerevisiae* permeability is directly affected by osmotic pressure changes of the surrounding medium, which may allow passive diffusion of solubilized components in solution (Gervais et al. 1992).

Eukaryotic cells, such as yeasts, have a steady response to increased temperature (Jenkins 2003). The yeast membrane structure and function could be affected by sublethal temperatures  $>25.0$  °C and lead to transient permeability. This permeability increase could be due to the activity of  $\text{H}^+$ -ATPase enzyme and can be catalyzed by protons flow on the plasma membrane. In certain conditions, this flow of protons dissipates the driving force of protons resulting from heat stress, leading to membrane permeability (Coote et al. 1994). These observations could explain the increase of EE at 25 °C for DE method (Fig. 1b, c, e, f). In the case of IE method, we have observed two distinct optimal results at extreme temperatures (Fig. 1h, i, k, l). It is inferred that low temperatures ( $\leq 16.6$  °C) might exert a protective effect on fluorescence fisetin, leading to a higher intensity emission peak in LC. The protective effect of some antioxidants with similar characteristics to fisetin under low temperatures (below 20 °C) is supported by previous research results (Kechinski et al. 2010; Naczak and Shahidi 2004). On the other hand, the higher temperature ( $T \geq 33.4$  °C) could have caused more intense osmoporation effect to the cells, leading to an increased number and/or

higher pore size in the yeast membrane, allowing the entry of larger fisetin quantities during rehydration into the cell. Indeed, maintaining yeast cells at temperatures above 40 °C seems to provide less protection against the membrane permeability increase in the first 5 min of heating (Panaretou and Piper 1992). At this temperature range, the yeast protection envelope could avoid fisetin degradation. This structure has proven to be capable of supporting temperatures up to 246 °C, while maintaining the integrity of the internalized cell substances (Normand et al. 2005).

### Solvent washing effect and confirmation of fisetin internalization

In this study, both quantification strategies showed significant differences in terms of EE and IF. These observations suggest that ethanol used in washing stages by IE method contributed to the observed differences. Overall, it was demonstrated that EE and IF play a role on the encapsulation efficiency. Our results showed that the amount of internalized fisetin was much lower when the concentration was reduced from 2.00 mg mL<sup>-1</sup> (Fig. 2a) to 0.32 mg mL<sup>-1</sup> (Fig. 2c) and PBS washing. The most evident reduction was observed for cells washed with ethanol at the same experimental conditions (Fig. 2b, d). This proves that ethanol washings affect the encapsulated fisetin content. This finding could be explained by fisetin intracellular solubilization (nonpolar) or structural damages caused to the yeast envelope, which would lead to fisetin leakage (Davey and Hexley 2011; Weber and de Bont 1996). These observations are in agreement with preliminary quantitative results. Indeed, the ethanol could cause many negative effects for encapsulation processes, including dissolution of the encapsulated substance and yeast intracellular compartment destabilization, both important phenomena for the transport and protection of active ingredients (Weber and de Bont 1996). In fact, these observations could be useful for the development of validation strategies for active ingredient extraction in applications of yeast capsules in alcoholic systems such as beverages, pharmaceuticals, and cosmetics.

### Osmotic dehydration and cell functionality

In this work, we observed that cells have the capacity to restore their volume after rehydration (Fig. 3f, i). The yeast cell wall is an elastic structure that allows the cell to contract or expand when submitted to hyper- or hyposmotic solutions. This elasticity is conferred mainly to the three-dimensional  $\beta$ -1,3-glucans network (Lipke and Ovalle 1998). Dupont et al. (2011) reported that *S. cerevisiae* (wild type) is able to completely restore cell volume (40 % reduced from its initial volume) when osmotic pressure was reduced from 30 to 1.4 MPa. It was also observed the presence of some stretch marks on cell surface after rehydration (Fig. 3i, black arrow).

From the data obtained during the quantification and confocal microscopy, it is believed that these stretch marks do not contribute negatively to the integrity of the encapsulating structure of the yeast cell. Indeed, the high percentage of viable cells during osmotic dehydration up to 30 MPa (Fig. 4) suggests that osmoporation do not strongly affect the cell structure, i.e., plasma membrane and cell wall, and functionality. Dupont et al. (2010) have observed similar results with 83.5 % of viable cells after the osmotic dehydration at 30 MPa. Beney et al. (2001) have reported that *S. cerevisiae* viability is strongly linked to the osmotic pressure of the medium.

Our presented data shows that the gradient osmotic pressure which cells are submitted can cause reduction of cell viability/integrity (up to 16 % at 30 MPa). It may induce cell disruption and leakage of intracellular content, but it does not jeopardize the use of osmoporation technique on encapsulation processes. However, it should be considered in case of an industrial process where the maintenance of maximum cell viability is strongly necessary.

### The cell osmoporation improves the fisetin encapsulation

The use of yeast cells as protective matrices for bioactive substances is an issue that has been investigated over the last 40 years (Pham-Hoang et al. 2013). Even considering the benefits of higher stability, performance, efficiency, simplicity, and safety provided by yeast biocapsules, the key step for scale-up is the presence of natural yeast cell envelope barriers that complicate the internalization of the active ingredient into the cell (Nobel and Barnett 1991). In this context, our results have shown that cell osmoporation promotes the increase of encapsulation efficiency and internalized fisetin content. This enhanced encapsulation fisetin process might be based on increasing porosity of the yeast cell envelope during osmoporation, which allows the entry of large quantities of fisetin into the cell during rehydration. The increase in cell transient porosity caused by osmotic pressure changes in the medium has been reported in the literature. For example, Laroche et al. (2001) reported that yeast cell survival is related to the occurrence of membrane permeabilization during dehydration and rehydration performed with water-glycerol solutions. Dupont et al. (2010) reported that yeast cells have an adaptive response (structure and functionality) as a function of osmotic pressure of the medium. The authors observed that the osmotic shock performed at 30 MPa did not induce loss of plasma membrane integrity, while osmotic pressure at 110 or 166 MPa caused irreversible internalization of plasma membrane and cell death. Pedrini et al. (2014) observed an instantaneous internalization of a fluorescent polysaccharide in *S. cerevisiae* cells during osmoporation, with an improved 10-fold cell staining for cells dehydrated at 30 MPa and rehydrated, in comparison with cells not submitted to this treatment.

In summary, this work has shown that osmoporation significantly increases the EE and IF. The choice of the quantification strategy also influenced the responses, the direct extraction method being more effective, reliable, and reproducible. The empirical models for this method presented desirable degrees of reliability and reproducibility, and they can be used to predict values within the study range. Observations by fluorescence confocal microscopy showed that fisetin was uniformly internalized into yeast cells and the ethanol washing steps negatively affected the EE and IF content, probably due to structural cell damages. These observations were consistent with the numerical values found by quantification. Using scanning electron microscopy and cell viability, it was observed that cell integrity was maintained during the osmoporation process. These findings suggest that yeast cells under osmotic stress could develop transitory permeability to molecules dissolved in their environment, regardless of their hydrophobic nature. These results provide the basis for the development of new protection and vectorization technologies for sensible biomolecules using *in vivo* systems.

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#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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