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# Strategies to improve carotene entry into cells of *Yarrowia lipolytica* in a goal of encapsulation

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ABSTRACT

Yeasts are good materials for encapsulation of actives, but, as they are preformed capsules, they have to be loaded. Our goal was to load cells with a big and very hydrophobic molecule, β-carotene (LogP ≈ 15). In classical conditions, the entry of this compound into cells is negligible and we have worked on ways to favor the entry through the cell wall. Carotene was prepared at 0.3–3 g $L^{-1}$ in solvents to improve solubility and alter the cell wall structure. After a 3 h-incubation of cells with this solution, with the apolar hexane, the entry was increased to 95 μg $g^{-1}$ cell wet weight but the monopolar chloroform was much more efficient (220 μg $g^{-1}$ cell wet weight). However, increasing the carotene concentration in chloroform increased the adhesion of carotene on the cell wall (over 900 μg $g^{-1}$ cell wet weight) but did not increase the amount into the cell. The use of ultrasound (6-min-treatment at 50% and an output control of 4) as a green physical treatment increased significantly the result for β-carotene encapsulation (852 μg $g^{-1}$ cell wet weight).



## 1. Introduction

Encapsulation in yeast cells is a developing way of protecting actives. The utilization of yeast capsules is booming for flavor and fragrance compounds (Ciamponi et al., 2012; Dardelle et al., 2007; Normand et al., 2005; Pham-hoang, Voilley and Waché, 2016) but many studies take also an interest in the encapsulation of bigger bioactive compounds such as polyphenolic or carotenoid pigments (Sasaki et al., 2003) As yeast cells are preformed biocapsules, the step of loading is particularly important (Pham-Hoang et al., 2013). It is based on the passive diffusion of molecules through the yeast cell envelope. An active with a molecule weight up to 760 Dalton and appropriate lipid solubility can enter easily into the cell and be passively retained inside (De Nobel, Klis, Munnik, Priem, & van den Ende, 1990). According to the solubility–diffusion model (also known as the Meyer–Overton rule), which predicts membrane permeability from the molecule's oil–water partition coefficient (Missner and Pohl, 2009), hydrophobic molecules should enter easily into cell biocapsules. However, for polyphenolic and carotenoid molecules, loading yield (or encapsulation yield: EY%), varies between 5% for resveratrol (Shi et al., 2008) and 30% for curcumin (Paramera et al., 2011) whereas encapsulation of the highly hydrophobic β-carotene has not been successful yet. For small volatile molecules, although $LogP_{oct/wat}$ (i.e. Log of the partition coefficient of a molecule between octanol and water, a way to evaluate the hydrophobicity of a molecule) is not the only factor governing their entry (Pham-hoang, Voilley and Waché, 2016), their affinity for lipid phases of cells can make the encapsulation efficiency (EE%), reach almost 100%. Bigger molecules, despite their high partition coefficient, tend to crystallize and form supramolecular structures at rather low concentration. Solubility limitations appear to be the important parameter as supramolecular structures exceed the size limitation for loading.

Among polyphenols and carotenoids, the molecule exhibiting the highest LogP is β-carotene (LogP ≈ 15) (Yannai, 2003). However, although it exhibits an affinity for oil phases, carotene precipitates at concentrations which are very low, even in lipid phases (Cao Hoang and Waché, 2009; Ly et al., 2008; Waché, Bosser-DeRatuld, & Belin, 2006). As it is already a big molecule with a length longer than most classical cell membrane width, the fact that it

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readily organizes in supramolecular structures bigger than cell wall pore sizes makes it difficult to load into cells. Due to its physico-chemical properties, this molecule was a good model for our study. Moreover, it is not only a model but an important bioactive molecule. β-Carotene is a natural orange red colorant present in various fruits and vegetables such as carrot, tomato, pumpkin, orange, guava, mango, gac (*Momordica cochinchinensis*), palm kerns etc. which is not only a food color, but is also used for prevention and treatment of diseases (Burri, 1997). Many studies focusing on its functionalities showed that β-carotene plays an important role to reduce cancers and particularly breast cancer (Gandini et al., 2000; Riboli and Norat, 2003; Tamimi et al., 2005). It is believed that it can control and reduce destructive oxidative stress due to its chemical structure. This bicyclic molecule containing 11 conjugated double bonds exhibits ability to quench the singlet oxygen or free radicals so that it can protect cells from these damages (Schroeder and Johnson, 1995).

However, the use of β-carotene in the food industry is limited due to its instability when exposed to light, oxygen and temperature. Encapsulation of β-carotene is thus a way to improve the handling and solubilization of the molecule, to protect it and propose ways of controlled release (Cao-Hoang, Fougère and Waché, 2011; Cao-Hoang, Phan-Thi, Osorio-Puentes and Waché, 2011; Desobry et al., 1997). Various polymers such as maltodextrin, gum Arabic, etc. have been used as surrounding wall and the microcapsules were formed through a spray-drying or freeze-drying step (Krishnan et al., 2005; Raja et al., 1989). In this technique, the protection depends on the nature of the polymer material used. The yeast cell envelope contains a double barrier consisting of a wall and a semi-permeable membrane which has proven efficacy for the protection of intracellular components from the undesirable effect of light, oxygen, etc (Cid, Duran, del Rey, Snyder, Nombela and Sanchez, 1995). Moreover, studies have shown that the structure of yeast capsules can resist high temperature up to 256 °C (Paramera et al., 2011).

In this study, we aim to microencapsulate β-carotene into yeast cells. This molecule is rather bigger and more hydrophobic than all the compounds encapsulated in yeast to date. The objective was thus to facilitate the entry into the cell by solubilizing the molecule to avoid any macromolecular structure that would exceed the pore envelope size and by altering the envelope structure to make the entry of molecules easier. Two strategies were followed, the first one was based on the use of an organic solvent and the second on the use of ultrasounds. Yeast cells loaded with β-carotene were analyzed qualitatively using confocal laser scanning microscopy. Then, assessment of the β-carotene content and quality was carried out using the UV–Vis spectrophotometric method. Moreover, the antioxidant capacity was evaluated through a TEAC (Trolox Equivalent Antioxidant Capacity) test to confirm the efficacy of the encapsulation process.

## 2. Experimental conditions

### 2.1. Materials

β-carotene with a purity of 97% was purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). All of the solvents used in this study were purchased from Sigma Aldrich.

### 2.2. Methods

#### 2.2.1. Yeast cells and culture conditions

The strain *Yarrowia lipolytica* W29 (ATCC20460; CLIB89) was cultured in yeast extract peptone dextrose agar (YPDA) medium (yeast extract 10 g $L^{-1}$, peptone 20 g $L^{-1}$, glucose 20 g $L^{-1}$, agar 15 g $L^{-1}$) for 48 h and used to inoculate the YPO liquid medium (yeast extract 10 g $L^{-1}$, peptone 20 g $L^{-1}$, methyl oleate 5 g $L^{-1}$, Tween 80 0.2 g $L^{-1}$) and grown at 27 °C, 140 rpm in a baffled Erlenmeyer flask. After 24 h, cells in the mid-logarithm growth phase were harvested and washed several times with water.

#### 2.2.2. Solvent-mediated encapsulation process

In the first step of this study, the encapsulation process was carried out using solvent to facilitate the loading of β-carotene inside the cell. β-Carotene was dissolved in chloroform, ethanol, acetone or hexane to a concentration of 0.3, 1, 2 or 3 g $L^{-1}$. Ten milliliter of stock solution were then mixed with 50 mL of washed cells at a concentration of $OD_{600} = 10$ (optical density corresponding to 2.4 $10^8$ cells/ml), giving β-carotene final concentrations in working solutions of 0.05; 0.17; 0.33; 0.5 g $L^{-1}$, respectively. After the incubation for 3 h at 27 °C and 140 rpm in baffled Erlenmeyer flask, loaded yeasts were harvested after washing cycles with water. In order to remove the unbounded β-carotene, loaded yeasts were washed once with decane and then with water. The β-carotene-containing cells were placed in a freezer at −78 °C for 24 h. After complete freezing, the frozen cells were lyophilized for 20 h using a freeze dryer (Heto PowerDry PL6000, Thermo Scientific, France) with a condenser temperature of −55 °C and a pressure of 4 hPa. The resulted capsules were then stored in closed glass vials at room temperature and without light until further experiment.

#### 2.2.3. Ultrasound-mediated encapsulation process

The ultrasound process was carried out with a Sonics & Materials Inc. (Danbury, USA) Vibra Cell processor (250 W, 20 kHz) at 50% dutycycle and an output control of 4, with a 3 mm stepped microtip. The first sonication cycle was made to solubilize β-carotene powder in olive oil (1 g $L^{-1}$) giving a clear orange solution within 2 min of this treatment. Yeast cells were pretreated by immersion in protective agent solutions (glycerol 15% or trehalose 0.37%) for 20 min. The ready-for-encapsulation cellular suspension was then added to the β-carotene-olive oil solution. The mixture was sonicated for 6 min prior to be incubated for 3 h at 27 °C and 140 rpm in baffled Erlenmeyer flask for encapsulation. The following steps are alike the process described in section 2.2.2.

#### 2.2.4. Confocal laser scanning microscope

The confocal laser scanning microscopy Eclipse TE 2000E, Nikon (Japan) with He/Ar laser multiraires system was used to confirm the presence of β-carotene inside the cells. Molecule-liposoluble-specific probe nile Red (9-diethylamino-5H-benzo[alpha]phenoxazine-5-one) was used to stain β-carotene and lipid bodies. The nile red stock solution was prepared in ethanol at a concentration of 1 mg $mL^{-1}$ and used to stain the cells at a work concentration of 1 μg $mL^{-1}$. For a better visualization of the samples, yeast cells were marked with Calcofluor white. This cell wall-specific fluorochrome dye was used from a stock solution of 1 mg $mL^{-1}$ in distilled water and added to a final concentration of 1 μg $mL^{-1}$. The fluorescence excitation wavelengths used were 408 nm and 488 nm for Calcofluor white and Nile red, respectively. Sample images were displayed and taken using software EZ-C1 version 3.80.

#### 2.2.5. Extraction from yeast capsules and determination of β-carotene content and of the supramolecular structure

Prior to extraction, loaded yeasts were suspended in solution of acetone/ethanol (1:1 v/v) and lysed using Disruption cell system at 2.2 kBar. The suspension was then mixed with hexane to the volume ratio of hexane/acetone/ethanol of 50:25:25 for the better extraction follow by 30 min of incubation. The supernatant was obtained after centrifugation at 4000 rpm for 10 min. The organic layer was separated and passed through the Shimadzu UV1650 PC