# Yeast Cell Wall Particle Encapsulation of Pro-Terpene Payloads

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## ABSTRACT

Terpenes are naturally occurring compounds produced by plants that have long been recognized for their insecticidal, antimicrobial, and antioxidant properties. However, applications of terpenes have been limited by their volatility and poor water solubility resulting in challenges to produce products without the use of surfactants or alcohols. We have developed methods using hollow, porous Yeast Cell Wall Particles (YCWPs) to efficiently encapsulate high levels of terpenes with stimuli-responsive control of terpene release using biodegradable pro-terpene compounds. Here we report an example of this approach using carvacrol. YCWP carvacrol and YCWP pro-carvacrol materials were synthesized, characterized for stability, controlled carvacrol release, and *in vitro* antibacterial properties.

***Keywords:*** yeast cell wall particles, terpenes, controlled release, pro-payload

## INTRODUCTION

Terpenes are a large class of naturally occurring organic compounds that constitute the primary components of essential oils obtained from plants. These essential oils, as well as other naturally occurring materials, such as marine and vegetable oils, are of great commercial interest due to their wide array of functional properties. Terpenes are used as flavors and fragrances in food and cosmetics, and are also used as active ingredients in insect repellents, biocides and in the pharmaceutical industry. Microencapsulation is a common technology to develop terpene formulations to enhance their chemical stability, shelf-life, functional activity, and the possibility to control terpene release. Microencapsulation techniques commonly employed in the preparation of terpene products include emulsification, spray drying, coacervation, freeze drying, *in situ* polymerization, extrusion, and fluidized bed coating. For comprehensive reviews on microencapsulation of terpenes and their commercial applications, the reader can refer to recent publications by Abbas *et al* [1], Ricke, *et al* [2], Durgesh *et al* [3], Van Vuong *et al* [4], and Wagh *et al* [5]. Despite their many attributes, the use of terpenes in some products is challenging due to their high volatility, poor water solubility, and susceptibility to degradation when exposed to air, heat, light and moisture.

We have developed methods using YCWPs to efficiently encapsulate high levels of terpenes. YCWPs are 3-5 $\mu$m hollow and porous microspheres purified from Baker's yeast (*Saccharomyces cerevisiae*) cell walls. We have used YCWPs for the encapsulation of a broad range of molecules for drug delivery and agricultural applications [6-9]. Our first-generation approach to load terpenes inside the hydrophobic YCWP cavity is based on passive diffusion of terpenes through the porous cell walls. Sustained terpene release from YCWPs is the reverse process and is a function of terpene water solubility. This approach has been successfully implemented to develop and commercialize a YCWP-terpene based fungicide for agricultural applications [10, 11].

Here, we report an improved approach to develop controlled release YCWP terpenes by encapsulating biodegradable pro-terpene compounds. Carvacrol, a phenolic terpene from oregano oil, which exhibits antimicrobial and anthelmintic properties was used to prepare a YCWP pro-carvacrol compound. The pro-carvacrol compound is (1) solid at room temperature (carvacrol is a volatile liquid at room temperature), (2) water insoluble, (3) stable at neutral pH, and (4) susceptible to chemical (pH) or enzymatic hydrolysis of a biodegradable linker providing for controlled carvacrol release.

## MATERIALS AND METHODS

### 1. Materials

YCWPs were purchased from Biospringer (Juno, WI). Carvacrol was procured from Penta Manufacturing (Livingston, NJ). All other materials were obtained from Fisher Scientific or Sigma Aldrich.

### 2. Methods

2.1 Synthesis of carvacrol-diacid (dicarvacrol-EDTA, DE): This compound was synthesized as previously described [12]. Briefly, carvacrol (18 mmol) and triethylamine (TEA, 64 mmol) were dissolved in 75 mL of anhydrous tetrahydrofuran (THF). Ethylenediaminetetraacetic (EDTA) dianhydride (9 mmol) was added slowly to the THF solution. The reaction mixture was stirred under nitrogen, at room temperature, overnight. The mixture was diluted in 500 mL of water and concentrated HCl was added to immediately acidify to pH 2. The precipitated product was filtered, washed with water, and dried under vacuum. Yield: 71%; off-white powder; $^1$H-NMR (DMSO-$d_6$, 500 MHz ): $\delta$ ppm, 12.3 (s, 2H, COOH); 7.2 (d, 2H, Ar-H); 7.0 (d, 2H, Ar-H); 6.9 (s, 2H, Ar-H); 3.9 (s, 4H, $CH_2$); 3.59 (s, 4H, $CH_2$); 2.89 (m, 2H, CH); 2.05 (s, 6H, $CH_3$); 1.18 (d, 12H, $CH_3$)

2.2 YCWP loading of carvacrol (Y-C): Dry YCWP was mixed with 0.5 $\mu$L water per mg YCWP. Then, carvacrol was absorbed into YCWPs by adding 1 mg carvacrol per mg YCWP and incubated at room temperature for 18-24 h. Y-C samples were stored at -20 °C.

2.3 YCWP loading of dicarvacrol-EDTA (Y-DE): Dry YCWP was mixed with 0.5 µL water per mg YCWP. Then, DE was absorbed into YCWPs by swelling the particles with a DE solution in DMSO (2.5 µL/mg YCWP). Y- DE was incubated at room temperature for 18-24 h to complete loading. The Y-DE was then lyophilized, and the loading process repeated until target concentrations of encapsulated DE were achieved. Y-DE samples were stored dry at -20 °C. Y-DE samples contain 1.85 mg DE per mg YCWP to yield 1 mg carvacrol/mg YCWP upon DE hydrolysis.

2.4 Carvacrol release from YCWP: Y-C and Y-DE samples were resuspended in phosphate buffer saline (PBS, pH 7) at a concentration of 1 mg YCWP/mL and incubated at 37 °C. Aliquots were collected at predetermined times, centrifuged and the supernatant collected to measure carvacrol release from the particles. Carvacrol was quantified by HPLC using a Waters Symmetry® C18 column (3.5 $\mu$m, 4.6x150 mm) with acetonitrile:water 70:30 as mobile phase, flow rate at 1 mL/min, injection volume of 10 $\mu$L, and carvacrol detection at 254 nm (Carvacrol retention time = 3.7 min)

2.5 Antimicrobial activity assay: Y-C and Y-DE samples were suspended in 100 $\mu$L of yeast peptone dextrose (YPD) growth medium and added to the first row (Row A) of a 96-well plate (all wells in the 96-well plate contain additional 100 $\mu$L YPD). Serial dilutions (1:1) were performed by transferring 100 $\mu$L from Row A to Row B, etc., and finally removing 100 $\mu$L from Row H. Diluted *Escherichia coli* cells (100 $\mu$L,$10^6$ cells/mL) were added to all wells of the plate. Initial (t = 0) and final (t=16 h, 37 °C) absorbance readings were taken at 650 nm. The minimum inhibitory concentration (MIC) was determined as the concentration that inhibits growth by more than 75%.

2.6 Simulated digestion assay: Y-C and Y-DE samples were resuspended in simulated gastric fluid (SGF) containing 3.2 mg pepsin/mL. The samples were incubated for 2 hours at 37 °C, samples centrifuged, and the supernatant was collected. The pellet was washed in PBS to neutralize residual SGF+pepsin and the PBS supernatant collected. The pellet was suspended in simulated intestinal fluid (SIF) containing 10 mg pancreatin/mL, incubated at 37 °C for 2 hours, and centrifuged to collect SIF supernatant. Carvacrol released from YCWPs was quantified in all supernatants by HPLC.

## RESULTS AND DISCUSSION

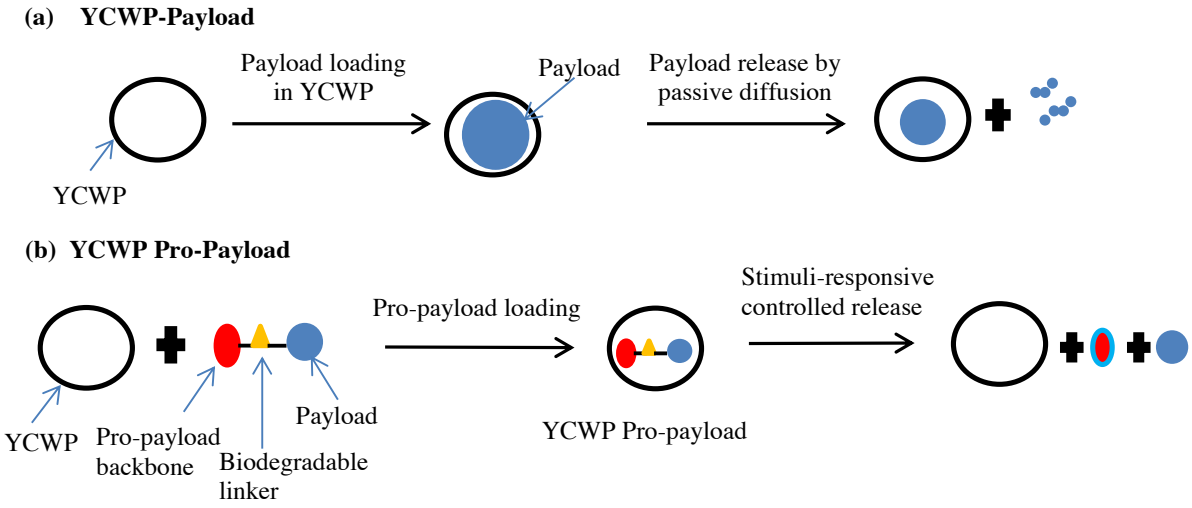
YCWPs are 3-5 $\mu$m, hollow and porous microparticles derived from Baker's yeast. The porous cell wall structure makes these particles excellent absorbent materials, and payloads can be loaded from aqueous and some organic solutions with high payload loading capacity in the large hollow cavity of the particles. Our first-generation approach for terpene encapsulation in YCWP (Y-Payload) is depicted in Figure 1a. This approach is based on the loading of terpenes inside the hydrophobic YCWP cavity by passive diffusion through the porous cell walls. High terpene loading capacity (up to 2:1 w/w payload:YCWP) is achieved with this method. Terpene release from YCWPs is based on passive diffusion out of the particles and is a function of terpene water solubility with complete terpene release in minutes to a few hours. This Y-Payload approach has been successfully implemented to develop and commercialize a YCWP-terpene based fungicide for agricultural applications [10, 11].

To better control terpene release we developed a new method to encapsulate terpene pro-payloads (Figure 1b). The pro-payloads are designed to be non-volatile solids at room temperature, water insoluble, but soluble in some organic solvents. The pro-payload contains a stimuli-responsive degradable linker for controlled payload release from YCWPs.

A pro-carvacrol compound (DE) was synthesized by ring opening condensation of EDTA dianhydride with carvacrol forming a carvacrol diacid with ester biodegradable bonds (Figure 2). DE is water insoluble, solid at room temperature, non-volatile, and highly soluble in DMSO. The kinetics of DE hydrolysis was monitored from carvacrol released into solution from a suspension of DE incubated at pH values from 1.5 to 10 as shown in Figure 3. The ester bonds of DE are susceptible to fast hydrolysis in basic pH and increased stability at lower pH values. More than 50% of DE was hydrolyzed within 30 minutes at pH 9 or 10, in comparison, it took 4 days at pH 7, 21 days at pH 5, and more than 60 days at pH 1.5 or 3 to reach 50% hydrolysis. The slow hydrolysis at neutral pH and acidic pH is desirable for YCWP pro-payload materials to improve formulation stability (pH 7) through the stomach to target gastrointestinal pathogens.

To characterize this new YCWP pro-payload approach, Y-C and Y-DE formulations at a 1:1 ratio of C:YCWP were tested for (1) kinetics of carvacrol release, (2) antimicrobial activity, and (3) stability during simulated digestion.

First, YCWP samples were incubated in PBS (pH 7) at 37 °C and carvacrol released from YCWP was quantified by HPLC. The results in Figure 4 clearly show the greater pH 7 stability of Y-DE compared to Y-C. The Y-C control sample rapidly released carvacrol in water. Y-DE resuspended at the same concentration showed a small burst release (<20%) followed by slow hydrolysis and release of carvacrol over 16 days to achieve complete carvacrol release. Nile red dye was used to stain carvacrol for microscopy imaging. The pictures in Figure 4 provide visual evidence that both YCWP samples are fully loaded with carvacrol at the start of the release assay, but only the Y-DE remains fully loaded after 24 h incubation in PBS.




**YCWP Pro-payload properties**

- High payload capacity (up to 2:1 w/w payload:YCWP)
- Pro-payloads are solid (non-volatile) at room temperature and water insoluble (stable YCWP pro-payload core)
- Pro-payload hydrolysis via a biodegradable linker leads to payload (water soluble) release from YCWP

Figure 1: Schematics of (a) diffusion-controlled terpene loading in YCWP and terpene release and (b) pro-terpene payload loading in YCWP and terpene stimuli-response controlled release

Next, we evaluated the antimicrobial activity of both YCWP formulations to demonstrate that Y-DE retains the biological activity of carvacrol. Table 1 shows the Minimum Inhibitory Concentration (MIC) of YCWP samples and controls. Empty YCWPs have no antimicrobial effect on *E. coli*, and both positive control Y-C and free carvacrol samples show the same MIC activity. The slightly enhanced MIC for Y-DE is due to the additional antimicrobial effect of EDTA generated during hydrolysis of the pro-carvacrol compound (data not shown).

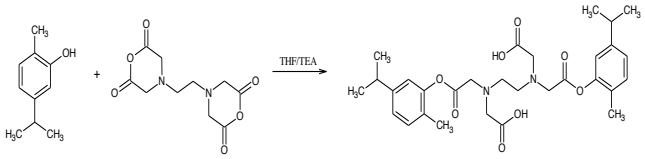



Figure 2: Synthesis of pro-carvacrol compound

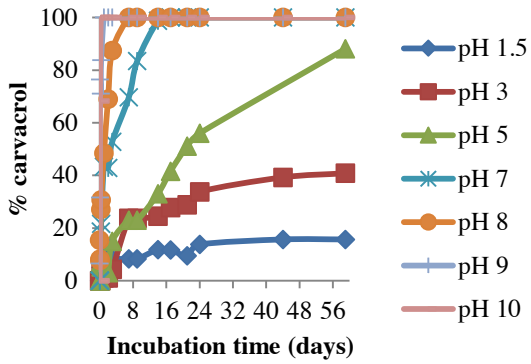



Figure 3: Kinetics of DE hydrolysis to generate free carvacrol at 37 °C

YCWP samples were next evaluated for stability during simulated digestion (Figure 5a). Samples were first incubated in SGF containing pepsin followed by incubation in SIF with pancreatin. Y-C releases carvacrol primarily in SGF due to carvacrol diffusion out of YCWPs. Y-DE is stable in SGF due to its water insolubility and low ester bond hydrolysis rate at pH<2. Carvacrol is partially released from Y-DE in SIF due to the presence of lipases in pancreatin that hydrolyze ester linkages. Up to 25% carvacrol is released during the first hour incubation in SIF with pancreatin, and addition of more enzyme increased carvacrol release as seen in Figure 5b.

We will be investigating different scaffolds for pro-carvacrol and other pro-terpene compounds to control release for different oral applications (*e.g.*, intestinal infectious diseases).

| Sample | MIC 75% (average n=4) ($\mu$g carvacrol/mL) |
|---|---|
| Empty YCWP | Not active |
| YCWP + EDTA | 3125 ± 35 |
| Carvacrol | 625 ± 0 |
| YCWP Carvacrol | 625 ± 0 |
| YCWP Dicarvacrol-EDTA | 459 ± 133 |

Table 1: Antimicrobial activity of YCWP samples on *E. coli*

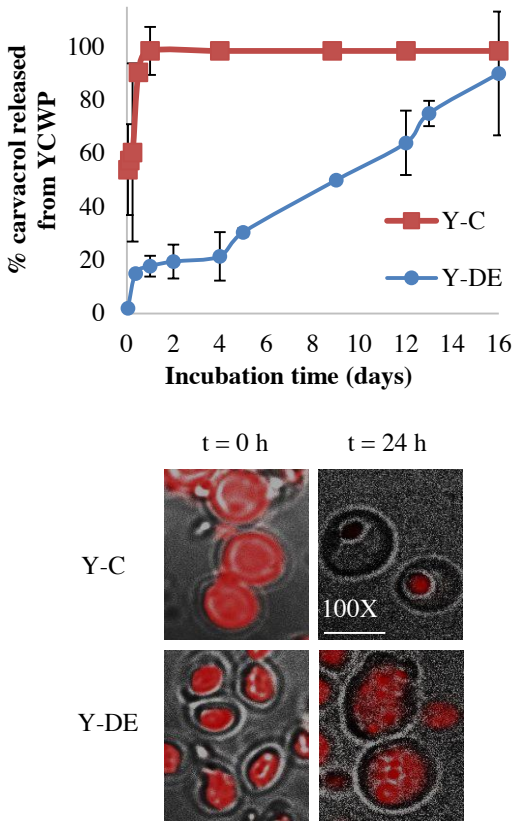



Figure 4: Kinetics of carvacrol release from YCWP in PBS (pH 7) showing stability of Y-DE, and micrographs of YCWP samples collected at t =0 and t = 24 h

## CONCLUSIONS

A new method to use Yeast Cell Wall Particles for terpene encapsulation has been developed. YCWPs containing a pro-carvacrol compound exhibit the same loading capacity as YCWP-Carvacrol, but with additional benefits of improved stability and control over carvacrol release due to a biodegradable linker in the pro-carvacrol structure that is susceptible to pH and esterase induced hydrolysis. This approach will be investigated for development of YCWP pro-terpene compositions aimed for oral drug delivery efforts.

## ACKNOWLEDGEMENTS

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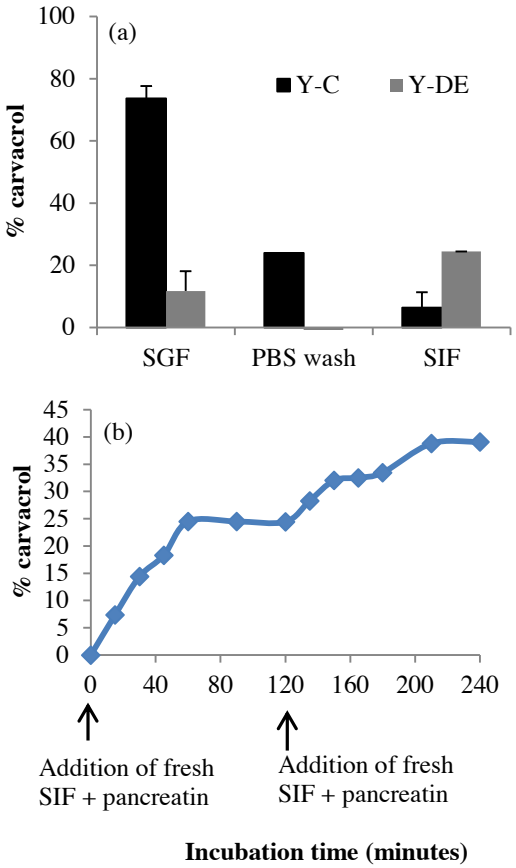


Figure 5: (a) Percentage carvacrol released from YCWP samples during simulated digestion, and (b) kinetics of carvacrol release from Y-DE in SIF

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