Encapsulation of *Zataria multiflora* Essential Oil in *Saccharomyces cerevisiae*: Sensory Evaluation and Antibacterial Activity in Commercial Soup

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ABSTRACT: *Nowadays rising consumer concern on the safety of synthetic chemical food preservatives is a reason for finding natural new antimicrobial agents, especially among the components of medicinal plants such as Essential Oils (EOs). However, most EOs are sensitive to oxygen, light, and temperature and can be easily degraded. Some EOs have strong taste, flavor, and affect the organoleptic characteristics of foods. Encapsulation can control these unpleasant characteristics. Using yeast cells as encapsulating agents and delivery systems for active ingredients has been widely investigated. Encapsulation in yeast cells has a wide range of advantages such as processes simplicity, commercial availability, low cost-high volume process, and needless of toxic solvents. In this study, the antibacterial activity of free and encapsulated Zataria multiflora Bioss. Essential Oil (ZEO) in Saccharomyces cerevisiae against Escherichia coli O157:H7 and Listeria monocytogenes as important foodborne pathogens were evaluated. The sensory evaluation of both forms of ZEO in a food model was also done. ZEO was successfully encapsulated into S. cerevisiae cells. Carvacrol and thymol contents in loaded yeasts*

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were determined. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of free and loaded ZEO were studied against Escherichia coli O157:H7 and Listeria monocytogenes; their antibacterial effects in the commercial chicken soup was investigated, and their sensory attributes in the commercial soup were evaluated as well. Our results showed significant decreases in the MIC and MBC values of ZEO in culture media after encapsulation; however, the antibacterial activity of ZEO in commercial chicken soup showed no significant differences after encapsulation ($P>0.05$). ZEO encapsulation improves its sensory score and hence, decreases its organoleptic effects in food ($P<0.01$). Considering acceptable sensorial scores of loaded ZEO in yeast cells, this method can practically be applied in food systems as natural biopreservation.

KEYWORDS: *Zataria multiflora Bioss.; essential oil; Saccharomyces cerevisiae; encapsulation; Escherichia coli O157:H7; Listeria monocytogenes*.

INTRODUCTION

The use of natural food preservatives has been extensively accepted by consumers, who are increasingly looking for healthier products [1-3]. Essential Oils (EOs) can be considered as good natural preservative options [4, 5]. EOs, extracted from plant components (buds, flowers, leaves, bark, twigs, seeds, herbs, wood, fruits, and roots) are aromatic volatile oily liquids that contain a combination of phenolic compounds, terpenoids, alcohols, aldehydes, and other important bioactive compounds. EOs often have antibacterial, antifungal, insecticidal, antioxidant, and anti-inflammatory characteristics, and have been generally recognized as safe (GRAS) natural substances, so they are useful for post-harvest preservation of crops and food products [6, 7]. Evaluation of the antimicrobial activity of some EO components on various microorganisms has shown that EOs are effective against almost all bacterial species, however, the mechanism of this antimicrobial activity is not completely clear yet [8-10].

Zataria multiflora Boiss. (Z. multiflora), belongs to the Lamiaceae family, a native plant of Iran, Pakistan, and Afghanistan. Z. multiflora, known in Iran as Avishan-e-Shirazi, is traditionally used for its antiseptic, anesthetic, and antispasmodic, analgesic, and anti-inflammatory effects. Phenolic compounds such as thymol and carvacrol are the major constituents in Z. multiflora Essential Oil (ZEO) give it antioxidant, antibacterial, and antifungal effects [11, 12].

Application of phytochemicals as food preservatives depends on the stability and bioactivity of their antimicrobial and antioxidant potencies, however, most EOs are sensitive to environmental factors (oxygen, light, and temperature) and have poor solubility in water. The encapsulation of EOs can protect them against environmental factors, improves their water solubility, controls their release, and increases their efficacy and bioavailability [13]. Some EOs have a strong aroma and taste that can be remedied by encapsulation [14]. Also, the encapsulation of EOs in very small amounts decreases their evaporation rate, promotes handling ability, and enhances dilution to reach a uniform distribution in the final product [15].

There are several technologies for encapsulation of polyphenols such as spray drying, coacervation, liposome entrapment, inclusion complexation, cocrystallization, nanoencapsulation, freeze-drying, yeast encapsulation, and emulsions [14, 16, 17].

The yeast cell structure and its application in human nutrition make it an attractive and novel encapsulation vehicle in the food industry. The yeast membrane phospholipids act like liposome structures, so hydrophobic and also hydrophilic active food ingredients and pharmaceutical substances can be encapsulated in yeast cells [18]. The yeast cell wall consists of a beta-glucan network and a small amount of chitin with a mannoprotein layer, which makes it more useful compared to other carriers. The cell wall also allows molecules to diffuse easily [19].

The main advantage of the encapsulation in yeast cells is the simple and low-cost encapsulation process. The yeast cells are mixed with a solvent solution and stirred under controlled temperature during the encapsulation process. The non-loaded material will be washed out. The encapsulated yeast cells are then lyophilized at the end of the process. [18].

Among all of the yeast species, some of them attract more attentions including *Saccharomyces cerevisiae*, *Saccharomyces bayanus*, *Candida utilis*, *Kluyveromyces fragilis*, *Torulopsis lipofera*, *Endomyces vernalis*, and *Cryptococcus curvatus*. Among the microorganisms, baker's yeast (Saccharomyces cerevisiae) has emerged as a promising host for developing a new kind of drug delivery system[18].

To the best of our knowledge a few studies have reported ZEO encapsulation and its effects in food products. Besides, there is no reports on encapsulation of ZEO in yeast cells. Therefore, the present study is innovative in this aspect.

The aim of this study was to compare the antibacterial activity of free ZEO and its loaded form in *Saccharomyces cerevisiae* against *Escherichia coli* O157:H7 and *Listeria monocytogenes* as important foodborne pathogens and sensory evaluation of free and loaded forms of ZEO in a food model.

EXPERIMENTAL SECTION

Materials

Lyophilized *S. cerevisiae* (PTCC 5269) and *Listeria monocytogenes* (PTCC 1298) were purchased from the Industrial and Scientific Research Organization of Iran. *Escherichia coli* O157:H7 was a gift from microbial laboratory of School of Pharmacy (Mashhad, Iran). ZEO was purchased from the Iranian Institute of Research and Development in Chemical Industries, Karaj, Iran. Deionized water was purchased from Samen pharmaceutical company, Mashhad, Iran. Culture media were purchased from Himedia (India). Triphenyl tetrazolium chloride was purchased from Merck, Germany. The commercial chicken soup (ingredients: wheat flour, corn starch, vegetables, salt, whey powder, hydrogenated vegetable oil, milk powder, chicken meat extract, monosodium glutamate, spices, citric acid) was purchased from the Elite company in Iran.

METHODS

Cultivation of S. cerevisiae

YPD (yeast extract 1% w/v, bacteriological peptone 2% w/v, and glucose 2% w/v) was used as yeast cell (*S. cerevisiae* PTCC 5269) growth medium. Yeast cell suspension (1.5 mL) at a density of 108 CFU/mL was inoculate into 200 mL culture media and incubated in a shaker incubator at 37°C / 100 rpm for 11 h. The cultured cells were collected by centrifugation (3000 × g / 10 min), and washed five times with phosphate buffered saline (PBS). Finally, the cells were freeze-dried for later use [20, 21].

Encapsulation of ZEO in yeast cells

Two g ZEO, 1.5 g yeast cells, and 10 mL distilled water were mixed in a shaker incubator at 100 rpm / 40°C for 48 h. Then, the cells were centrifuged at 3000 × g for 10 min. After discarding the supernatant (oil and water), the cells were washed five times with PBS and then freeze-dried for 48 h [22, 23].

Calculation of carvacrol and thymol content in loaded yeast

In order to determine the carvacrol and thymol content in each milligram of loaded yeast, 20 mg of loaded yeast plus 2 mL deionized water and 8 mL ethanol were sonicated (Hielscher, Germany) in ice bath at 50 HZ for 15 seconds. The cell suspension was centrifuged at 3000 × g for 10 min, the supernatant lysate was filtered through a 0.45-micron membrane. The carvacrol and thymol content of the lysated yeasts was determined by HPLC method. The analysis was performed on a C18 column with acetonitrile: water (50:50) as mobile phase at a flow-rate of 1.0 mL/min, with UV/visible detection at 275 nm. The loading capacity was defined as [23]:

$$\%\ \text{loading capacity} = \frac{\text{concentration of released essential oil}}{\text{initial concentration of essential oil}} \times 100$$

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) tests

MIC tests were carried out according to *Sartoratto et al.* (2004), using a 96-well plate. The stock solutions of the free and loaded EO in lyophilized dead yeast cells as well as the dead yeast cells alone were diluted in brain heart infusion (BHI) broth media (Himedia). The EO was mixed with Tween 80 (6:4) and shaken until homogeneity. Further dilutions of free and loaded ZEO were made by sterile BHI broth to obtain concentrations in the range of 12/8 - 0/025 mg/mL. The first column of each plate contained no free or loaded EO and was defined as positive control. Gentamicin was used as

the reference control antibiotic. The inoculum of 10^5 CFU/mL was added to all wells and the plates were incubated at 37°C for 24 h. Antimicrobial activity was determined by adding 20 μL of 0.5% triphenyl tetrazolium chloride (TTC) aqueous solution (Merck, Germany). MIC was defined as the lowest concentration of free or loaded ZEO able to inhibit the visible growth, as indicated by TTC staining [24, 25]. To determine the MBC of free and loaded EOs, all wells without bacterial growth were cultured in BHI broth using a 96-well plate and incubated at 37°C for 24 h. The plates were checked for growth of bacteria using TTC staining. MBC was evaluated as the lowest free and loaded ZEO concentration at which no growth was observed in the plates [26].

Preparation of substrate

Commercial soup was prepared according to the manufacturer's instructions and aliquoted into graded glass containers and autoclaved at 121°C for 15 min [27]. Various concentrations of free and loaded ZEO (0.2, 0.4, 0.8, 1.6, 3.2 mg/mL) were added to each container except the controls.

Bacterial inoculation

Antibacterial activity of EO and its loaded form in yeast cells was investigated against *E. coli* O157:H7 and *L. monocytogenes* (PTCC 1298). To prepare the microbial suspension, each bacterial species was cultivated on nutrient agar at 37°C for 24 h, then a suspension of typical colonies was prepared in sterile distilled water and adjusted to 0.5 McFarland standard turbidity containing 1.5×10^8 CFU/mL, using spectrophotometry at 600 nm [28]. Then a suspension of 10^6 CFU/mL was prepared by dilution of the first suspension (1.5×10^8 CFU/mL) and inoculated into the soup to create density of 10^3 CFU in each mL soup.

The inoculated soup was refrigerated and bacterial count was performed at different time points (day 0, 1, 2, 4, 6, 8, 10, 12, and 14). The bacteria were enumerated by spread plating on BHI agar.

Sensory panel test

Sensory analysis of soup was performed in the sensory analysis laboratory of Department of Food Science, Faculty of Agriculture, Ferdowsi University of Mashhad on a group of 7 students of the faculty who were trained during the course of sensory analysis.

The sensory panelists were not informed about the experimental approach. The commercial soups containing different concentrations of free and loaded EO (0.2, 0.4, 0.8, and 1.6 mg/mL) were evaluated for appearance/color, taste/flavor, smell/odor, texture/mouth feel, and overall acceptability attributes on the basis of the 9-point hedonic scale (9=like extremely; 8=like very much; 7=like moderately; 6=like slightly; 5=neither like nor dislike; 4=dislike slightly; 3=dislike moderately; 2=dislike very much; 1=dislike extremely) by filling out a card [11].

Statistical analysis

Statistical analysis was carried out using SPSS, ver. 21 (SPSS, Inc. Chicago, IL, USA). Repeated measures analysis of variances followed by Dunnett's T3 post-hoc tests were used to compare bacterial counts (log scale) of groups during refrigeration period at the $P<0.05$ level. All sensory indices were described as median (minimum, maximum) for each group. Group sensory scores were compared using the Kruskal-Wallis Test. Pairwise comparison of experimental groups with the control group, and each group with the same pair of the microencapsulated group was performed using the Mann-Withney U test. Since this was multiple testing of the data the significance level was adjusted at $P<0.01$ [4].

RESULTS AND DISCUSSION

Quantitative determination of carvacrol and thymol in loaded yeast

There were 473.52 and 336.90 ppm (mg/Liter) carvacrol and thymol in 20 mg loaded yeast, so carvacrol and thymol contents in each mg of loaded yeast were 0.24 and 0.17 mg, respectively. The loading capacity of carvacrol and thymol were 30.88% and 44.53%, respectively.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) tests

Both free and encapsulated ZEO showed proper antibacterial activity against *E. coli* O157:H7 and *L. monocytogenes* in culture media. However, the lyophilized dead cells alone showed no antibacterial effects. The MIC values of free ZEO against *E. coli* and *L. monocytogenes* were 0.4 and 0.2 mg/mL, respectively.

Table 1: Results of MIC and MBC tests of free and encapsulated ZEO in culture media.

		E. coli	L. monocytogenes
Free ZEO	MIC (mg/mL)	0.4 (0.2 carvacrol)	0.2 (0.1 carvacrol)
	MBC (mg/mL)	0.4 (0.2 carvacrol)	0.2 (0.1 carvacrol)
Encapsulated ZEO	MIC (mg/mL)	0.4 (0.1 carvacrol)	0.2 (0.05 carvacrol)
	MBC (mg/mL)	0.4 (0.1 carvacrol)	0.4 (0.05 carvacrol)

The same value was considered as MBC level for free EO. The MIC values for loaded yeast against *E. coli* and *L. monocytogenes* were also 0.4 and 0.2 mg/mL, respectively. The same value was considered as MBC level for loaded yeast against *E. coli*, but the MBC value of loaded yeast against *L. monocytogenes* increased to 0.4 (Table 1).

Many researchers have previously reported the antimicrobial activity of ZEO against different food borne microorganisms.

The MIC value for ZEO against *L. monocytogenes* has been reported as 0.625 mg/mL [11, 29], 0.008 mg/mL [30], and 0.0095 mg/mL [31] by previous studies. The MIC value for ZEO against *E. coli* also has been reported as 1.25 mg/mL [29] and 0.25 mg/mL [32]. The MBC of ZEO against *L. monocytogenes* has been reported to be over 0.512 mg/mL [30].

The MIC and MBC values in our study for ZEO are within the range with previous reports. The wide range of MIC values by various studies can be attributed to the differences in plant source, test methods, bacterial strains, genetic constitution, and harvest season as well as the components of applied ZEO [28, 33]. Our findings confirms that encapsulation of EOs results in expectedly regular antibacterial effects compared to other reported non-encapsulated EOs, while providing all the aforementioned benefits of encapsulation.

ZEO composition was determined by gas chromatography (GC). Carvacrol with a 51.55% share was the major component, so there were 0.20 and 0.10 mg carvacrol in 0.4 and 0.2 mg free ZEO, respectively, while, carvacrol contents in 0.4 and 0.2 mg of loaded yeast were 0.1 and 0.05 mg, respectively. It means that each loaded yeast group had half the carvacrol in comparison with the same free EO group, but, had the same antibacterial effect. Therefore, the antibacterial activity of EOs in culture media increases in encapsulated form. This is in line with the previous reports that the antimicrobial properties of EOs may increase after encapsulation [15, 34-37]. The MIC and MBC of liposomal, nanoliposomal, and nanoemulsion encapsulated forms of antimicrobial agents have been reported to be lower compared to their free form [15, 34, 36], suggesting that encapsulation enhances transport mechanisms through the cell membrane of the target microorganisms. The delivery system generally acts as a reservoir for antimicrobial compounds, providing a steady concentration in the aqueous bulk phase over an extended period of time. This is chiefly important because the solubility of EOs in water is low, which would limit the total content of antimicrobial compound that can be loaded in a finite aqueous system and would thus limit the time frame of action, due to the rapid consumption or degradation of the bioactive molecules [15]. Despite the reported antibiotic efficacy of S. cervisiae and the extracted ß-glucans, the lyophilized dead yeast in our setting showed no bactericidal or bacteriostatic effects. The antibacterial effects of S. cervisiae has been attributed to the live yeast toxins in a previous study [38-40]. Also, yeast ß-glucans may probably be inactivated through binding to cell proteins. A previous study has shown that physical mixture of the active component, berberine, with freeze-dried yeast has shown the same effect of pure berberine [19]. This result is in accordance with our results.

Antibacterial activity in food model

The bacteriological tests on inoculated commercial chicken soup showed no significant differences between the free and loaded forms of ZEO. But, Increasing the ZEO concentration (free and loaded forms in yeast cells), decreased the logarithmic number of both bacteria ($P<0.05$). This finding is in agreement with the findings of other studies [41, 42].

The addition of free and loaded ZEO at concentrations of 0.2, 0.4, 0.8, 1.6, and 3.2 mg/mL caused a significant

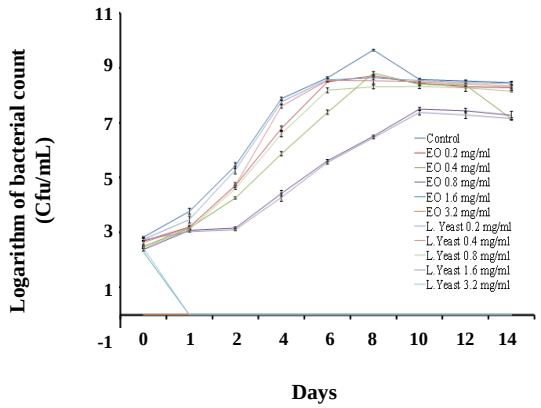

Fig. 1: Bacterial counts of inoculated E. coli at different time points in commercial soup with different concentrations of free and loaded ZEO. (ZEO=Zataria multiflora Bioss. essential oil; EO=free essential oil; L. yeast=loaded yeast).

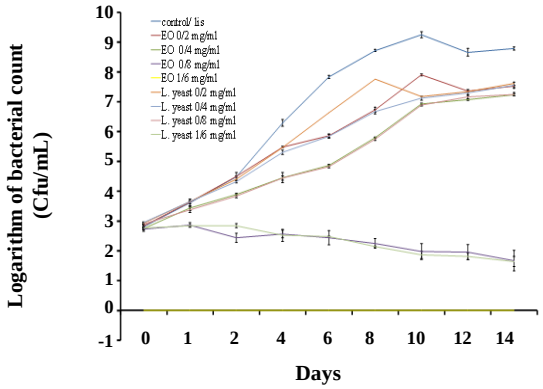

Fig. 2: Bacterial count of L. monocytogenes at different time points in commercial soup with different concentrations of free and loaded ZEO. (ZEO=Zataria multiflora Bioss. essential oil; EO=free essential oil;L. yeast= loaded yeast).

reduction (P<0.05) in the growth rate of *E. coli* O157:H7 in commercial soup at refrigeration temperatures (Fig. 1).

E. coli counts in all groups containing free or loaded EO were significantly (P<0.001) lower than that of the control group except for the group containing 0.2 mg/mL loaded yeast. Our results showed that free and loaded EO groups with the same carvacrol content, had the same antibacterial effects, so contrary to increased antibacterial effect of the encapsulated form in culture media, the antibacterial effect of encapsulated form in commercial soup had no significant differences with the same group of free EO (Table 2).

Addition of free and loaded ZEO at concentrations of 0.2, 0.4, 0.8, and 1.6 mg/mL caused a significant reduction (P<0.05) in the growth rate of *L. monocytogenes* in commercial soup at refrigeration temperatures (Fig. 2).

All free and yeast loaded EO groups had significantly (P<0.001) lower bacterial counts (*L. monocytogenes*) compared with the control group (group with no free or loaded EO). Our results showed that free and loaded EO groups with the same carvacrol contents had equal antibacterial effects, so, contrary to increased antibacterial effect of encapsulated form in culture media, the antibacterial effect of encapsulated form in commercial soup had no significant differences with the same group of free EO (Table 3).

Although encapsulation of both clove EOs (CEOs) by chitosan (CS)–Myristic acid (MA) nanogel [37] and Rosmarinus officinalis EOs (REOs) by chitosan (CS)-benzoic acid (BA) nanogel [35] have been shown to be more effective in controlling Salmonella population on beef compared to their free forms, our results showed no significant change in the antibacterial effect of ZEO in encapsulated compared to free form in soup. This inconsistency can be due to the different methodologies used for encapsulation of active ingredients. Also, food models applied for investigation of antimicrobial activity of encapsulated EOs were different.

Sensory panel test

The results of sensory assessment of commercial soup are shown in Fig. 3. The control sample had the highest scores in all sensory attributes. Ignoring the control sample, the score for 0.2 mg/mL loaded yeast was higher than other samples. The sample containing 1.6 mg/mL of free EO had the lowest score. The scores of taste/flavor, smell/ odor, texture/ mouthfeel, and overall acceptability of the sample containing 0.4 mg/mL loaded yeast (containing 0.1 carvacrol) were significantly (P<0.01) higher than those of the sample with 0.2 mg/mL free EO (containing 0.1 carvacrol). In the sample containing 0.8 mg/mL loaded yeast (containing 0.2 carvacrol) the scores were significantly (P<0.01) higher than in the sample containing 0.4 mg/mL of free EO (containing 0.2 carvacrol). Likewise, in the sample containing 1.6 mg/mL loaded yeast (containing 0.4 carvacrol) they were significantly (P<0.01) higher than in the sample containing 0.8 mg/mL of free EO (containing 0.4 carvacrol). This aspect highlights the slow and gradual

Table 2: Comparison of antibacterial (E. coli) activity of ZEO and its loaded form in commercial soup according to carvacrol content in free and loaded ZEO.

Free essential oil (mg/mL of soup) (log bacterial count (mean ± standard error))	Carvacrol content (mg/mL of soup)	Yeast loaded essential oil (mg/mL of soup) (log bacterial count (mean ± standard error))	Carvacrol content (mg/mL of soup)	Bacterial count comparison
0.2 (6.63 ± 0.009)	0.1	0.4 (6.72 ± 0.0186)	0.1	no Significant differences (P= 0 .111)
0.4 (6.21 ± 0.015)	0.2	0.8 (6.44 ± 0.051)	0.2	no Significant differences (P= 0.256)
0.8 (5.26 ± 0.020)	0.4	1.6 (5.22 ± 0.040)	0.4	no Significant differences (P=0.999)
1.6 (0.25 ± 0.002)	0.8	3.2 (0.27 ± 0.001)	0.8	no Significant differences (P=0.139)

Table 3: Comparison of antibacterial (L. monocytogenes) activity of ZEO and its loaded form in commercial soup according to carvacrol content in free and loaded ZEO.

Free essential oil (mg/mL of soup) (log bacterial count (mean ± standard error))	Carvacrol content (mg/mL of soup)	Yeast loaded essential oil (mg/mL of soup) (log bacterial count (mean ± standard error))	Carvacrol content (mg/mL of soup)	Bacterial count comparison
0.2 (5.76 ± 0.004)	0.1	0.4 (5.63 ± 0.045)	0.1	no Significant differences (P= 0.524)
0.4 (5.16 ± 0.025)	0.2	0.8 (5.16 ± 0.008)	0.2	no Significant differences (P= 1.000)
0.8 (2.32 ± 0.168)	0.4	1.6 (2.32 ± 0.016)	0.4	no Significant differences (P=1.000)

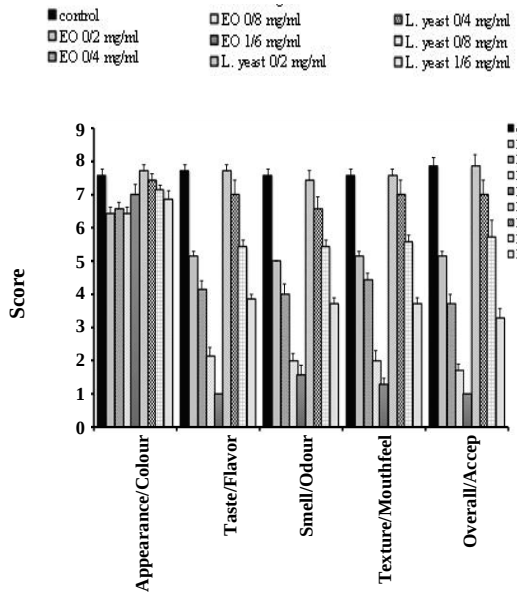

Fig. 3: Sensory evaluation of free and loaded essential oil in commercial soup. (EO=free essential oil; L. yeast= loaded yeast).

release of essential oil encapsulated in yeast cells during the test time. This result indicates that encapsulation of ZEO in yeast cells modifies the organoleptic properties of ZEO in foods.

Our results confirms the results of previous researchers. Dima et al. (2014) mentioned that coriander and allspice EOs have strong taste and smell that may repel the consumer. Encapsulation in β-cyclodextrin can be used to reduce the consumer rejection. This can also increase the shelf-life [43]. Microencapsulating the cold pressed flaxseed oil by spray drying, using an emulsion containing modified starch, has resulted in higher sensory evaluation scores [44]. All in all, high dosage of essential oils in foods has low sensory acceptability. This is an important point that limits their direct use in food products; therefore, encapsulation is a suitable method to overcome these problems.

CONCLUSIONS

Our findings showed that ZEO encapsulation significantly enhances its antibacterial efficacy against pathogenic bacteria in culture media; improves sensory properties of EO, and decreased its organoleptic effects in food. Hence, yeast-cell-based encapsulation can mask the flavor of EOs and be applied to food and food ingredients.

Further studies are suggested with various encapsulation methods for other active nutritional ingredients.

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