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Techno-commercial application of microencapsulated basil oil and thyme oil and their antibacterial activity against aquatic pathogens

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Abstract

A complete study was conducted through manufacturing of Microencapsulation of Basil Oil Powder (MEBOP) and Microencapsulation of Thyme Oil Powder (METOP) to evaluate the anti-bacterial activity against aquatic pathogens. An oil-in-water emulsion were prepared using gum with dextrin and the resultant matrix was spray dried with the average yield of 45% w/w of MEBOP and 50% w/w of METOP. Total oil content in the encapsulated powder in both were found to be 24.39% w/w of MEBOP 25.80% w/w of METOP respectively. Scanning Electron Microscopic (SEM) analysis was also done to confirm the encapsulation of oil and study possible structure of matrix. Microencapsulated powders were further subjected for antibacterial activity against aquaculture pathogens like *Vibrio harvey*, *Vibrio parahaemolyticus*, *Bacillus cereus*, *Aeromonas sp.*, *E. coli* and *Salmonella sp.* Results were compared against the respective oils at different concentrations. Phyto- constituents from Basil oil and Thyme oil like Thymol, Linalool, and Methyl chavicol were quantified using HPLC (High-Performance Liquid Chromatography) and GC (Gas Chromatography) which may be responsible for this activity. Furthermore, stress studies were conducted at 60 °C to understand the stability of Encapsulated oil powders to this and its commercial usage in formulations.

Keywords: Microencapsulated oil powder, anti-bacterial, aqua gut health, stress study

1. Introduction

Aquaculture industry is a growing sector at an annual growth rate of about 8.9% per annum. India being one of the largest producer and exporter of semi processed fish, shrimps, and prawns and to meet the growing consumer with regulatory demands, a natural alternative for antibiotics to control diseases and infections are required. A stable efficacious microencapsulation for essential oil has been thus designed for growth, gut health, and immune status within the stipulated time frame, helping farmers to achieve commendable income in commercial farms. Aquatic species like prawns and fishes are susceptible to several bacterial infections, mainly when it is in aquaria farms which leads to increased mortality rates and a decrease in productivity (Carine *et al.*, 2019) [6]. Plants naturally synthesize volatile oils which possess (Sonam Chouhan *et al.*, 2017) [15] strong antimicrobial activity against these bacterial, fungal, and viral infections. Meanwhile, in the food industry, they can help in maintaining quality characteristics and as a natural preservative for short-term storage.

Various chemical compounds have been reported (Alexandre Porte and Ronoel 2008) [1] from the essential oil of *Thymus vulgaris viz.*, p-cymene, γ -terpinene, thymol, geraniol, linalool, gamma-terpineol and carvacrol. Basil oil contains (Rajesh, 2014) [10] linalool (64.35%), 1, 8-cineole (12.28%), eugenol (3.21%), germacrene D (2.07%), terpineol (1.64%), cymene (1.03%). Essential oil derived from *Ocimum basilicum* associated with linalool showed (Kathirvel and Poonkodi 2016) [8] potent antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

As essential oils addition into aquatic feed formulation will not meet the requirement as per farmer's perspective, since it has strong odour and are unstable in nature. Thus, as an alternative to addition, application of the encapsulated plant-derived essential oil powder (Hammer *et al.*, 1999) [7] has been applied extensively in aquatic feed supplements to control microbial infections as safe, cost-effective, alternative to antibiotics.

An attempt has been made to encapsulate basil oil and thyme oil at commercial scale and to investigate their antibacterial activity against aquatic pathogens like *Vibrio harvey*, *Vibrio parahaemolyticus*, *Bacillus cereus*, *Aeromonas sp.*, *E. coli* and *Salmonella sp.* Further section mainly deals with antibacterial studies and its conclusion on usage.

2. Materials and Methods

2.1 Plant material

Basil oil and thyme oil was drawn from the raw material stores of Himalaya Wellness Company, Bangalore, India, and stored at room temperature, in an airtight container.

2.2 Chemicals

Mueller Hinton (MHA) was purchased from HI Media Chemicals, Mumbai, India. Authentic standards Thymol and Methyl chavicol used for HPLC were of analytical grade with purity ~ 99% and purchased from Sigma Chemicals, USA. Vanillin sulphuric acid, Toluene, ethyl acetate, Acetonitrile, and Acetic acid used for HPLC were of HPLC-grade and purchased from Fisher Scientific (USA).

2.3 Thin-layer chromatography

Thin-layer chromatography was performed on 10 × 10 cm HPTLC silica gel 60 F₂₅₄ plates (Merck, Germany). Toluene: Ethyl acetate in volume ratio 75:25 (% V/V) was used as a mobile phase. After the development of plates were scanned at 254 and 366 nm and characterised by spraying with vanillin-sulphuric acid and visualised at white light for identification.

2.4 Instrumentation

HPLC experiment was performed using a Shimadzu i-series LC2030 system equipped with a vacuum degasser, quaternary solvent mixing, auto-sampler, and diode array detector. UV spectra were collected across the range of 200–900 nm, extracting 275 nm for chromatograms. LC solution software was utilized for instrument control, data collection, and data processing. The column used was a Phenomenex Luna C₁₈ (4.6 × 250 mm, 5 µm). The gradient system of the mobile phase consisted of 0.2% v/v acetic acid in water (solution A) and 100% acetonitrile (solution B). The flow rate and column temperature were set to 1 mL/min and 40 °C, respectively.

GC was carried out using a Shimadzu GC-2014 with a capillary column ZB 5 (30 m × 0.25 mm i.d., 0.25 µm f.t.). The carrier gas used was N₂ at a flow rate of 1 ml/min. A flame ionization detector (FID) was used. The column temperature was programmed at 50 °C for 1 min and then heated to 265 °C. Injector temperature was 230 °C, detector temperature was 250 °C. GC solution software was utilized for instrument control, data collection, and data processing. The identification of thymol, linalool and methyl chavicol was primarily performed by comparison with the retention time of the respective standards.

2.5 Preparation of microencapsulation oil powder

Dextrin was dissolved in water and calculated amount of thyme essential oil and gum was added to the solution and homogenized at ambient temperature. This homogenized solution mixture was O/W (oil in water) emulsions which was further spray-dried. The dried powder was collected and stored at room temperature for further analysis. This was described as METOP. Similarly, Basil oil was also encapsulated with dextrin and gum using above mentioned

spray drying technique and resultant powder was described as MEBOP. These powders were further subjected to SEM, HPLC and other analytical techniques and antimicrobial studies.

2.5.1 Powder particle size and microcapsule surface analysis

Scanning Electron Microscope (EMITECH K 550 X) was used to analyse the surface morphology (Arana-Sanchez *et al.*, 2010) [2] and particle size distribution of the METOP and MEBOP. Microsphere microstructures images were taken at an accelerating voltage of 2.0 kV. The powder samples were directly sputtered on carbon tape-coated aluminium stubs with a brush. The gold coating of thickness was done using a sputter coater. After samples were viewed under SEM (Merlin VP Zeiss SEM) with SE 2 detector.

2.5.2 Total and surface oil determination

The total oil content of the microcapsules was determined by (Bae and Lee 2008) [3] distilling 5 g of encapsulated powder, dissolved in 150 mL distilled water for 3.0 hours in a Clevenger-type apparatus. Two mL of ethyl ether were added to extract the essential oil from the water phase. The solution was slowly brought to a boil and allowed to distil off. The resulting oil was collected in a pre-weighed beaker and the ethyl ether was allowed to evaporate at room temperatures for 24 h. The oil encapsulation retention (%) was calculated using the below equation,

$$\text{Oil retention} = \frac{\text{Oil weight}}{\text{Weight of the sample}} \times 100$$

2.5.3 Oil Encapsulation Efficiency (OEE)

Twenty mL of hexane were added to 2 g of powder and the mixture was stirred at 300 rpm for 10 min. The suspension was filtered (Bae and Lee 2008) [3] through a dried cellulose filter and was washed with 20 mL of hexane three times. The filter was kept in a desiccator under vacuum conditions to vaporize all residual solvent until obtaining constant weight, oil encapsulation efficiency (OEE) was calculated using the below equation,

$$\text{OEE (\%)} = \frac{\text{Total Oil} - \text{Surface oil}}{\text{Total oil}} \times 100$$

2.6 Antibacterial Activity

Evaluation of METOP and MEBOP by Agar Well Diffusion Method (Sarita *et al.*, 2019) [12]. To screen for antibacterial activity following culture strains of *Vibrio harvey*, *Vibrio parahaemolyticus*, *Bacillus cereus*, *Aeromonas sp.*, *E. coli*, and *Salmonella sp.* were used. About three to four isolated colonies of similar morphology were picked from the 18-24 h agar plate of pure cultures using a sterile loop and then inoculated individually into 4 mL peptone broth. The density/turbidity of the inoculum was adjusted to 0.5 McFarland turbidity standard, resulting in a suspension of 1.5 × 10⁸ CFU colony-forming units. Mueller Hinton agar was seeded with the test organisms and the plates were left to dry for five minutes. After drying, wells were made in the agar using a sterile cork borer measuring 9 mm in diameter. Different concentrations of METOP and MEBOP were dispensed into the labelled wells. The plates were then kept in the refrigerator for one hour for the extract to diffuse into the medium. The plates were then incubated at 37 °C for 24 h and

the zones of inhibition was measured in millimetres. Analysis was done in duplicates.

2.7 Stress studies

Monitoring of active components was performed in a stability chamber (Rohm Scientific Engineers, Ambala, India) capable of controlling temperature and relative humidity in the range of ± 2 °C and $\pm 5\%$, respectively. About 50g of MEBOP and METOP were packed in double-lined LDPE poly bags tied with a cable tag and placed in an HDPE bottle. Both the samples were placed in the stability chamber and set at 60 °C and 75% relative humidity. Samples were drawn for analysis at intervals fixed intervals of 15 and 30 days and stored at ambient temperature for physicochemical evaluation. Analysis of samples were performed in duplicates.

Statistical analysis

Values are in expressed in one-way ANOVA SPSS software. Data analyses was performed using. Experiments were run, with duplicate analysis in each of them.

3. Result and Discussion

Encapsulation using the spray-drying technology is highly preferred due to its economic advantages as compared to other drying methods. Encapsulation of essential oils using the spray drying technique is carried out to maintain its shelf life, efficacy trapping the bioactive compounds. The various matrix (Turchiuli *et al.*, 2014) [16] used to encapsulate bioactive components which include inulin, sodium caseinate, modified starch, natural gums, modified cellulose, gelatine, cyclodextrin, lecithin, and whey protein. In this study, maltodextrin was used as a matrix material because it is economical inert material, nutritious, heat stable (Bruna, 2018) [5], compatible to blend with various nutritional materials and other supplements and can be solubilized in aqueous media, encapsulation acts as a barrier against temperature, oxidation, humidity and pH.

To obtain a cost-effective microencapsulation powder maltodextrin was used, with an optimized solid content and spray dryer (Bruna *et al.*, 2018) [5] with controlled inlet and outlet temperature free flowing stabilized powder was obtained. The total solid percentage was adjusted 20% w/w and inlet/outlet temperature at 165°/90 °C and the feed flow rate was set 10ml/min before spray drying. Yield (%) obtained were 50% w/w for METOP and 45% w/w for MEBOP. Physicochemical parameters (Table 1) such as moisture content and bulk density were determined according to methods described in WHO guidelines. HPLC analysis was done for METOP and thymol was found to be 12.69% w/w and GC analysis report showed that the content of linalool at 0.30% and methyl chavicol at 0.29% w/w in the MEBOP. Surface oil content was quantified and found to be 2.78% w/w of METOP and 3.54% w/w of MEBOP, respectively. Oil Encapsulation Efficiency (OEE) was studied for the both the spray dried encapsulated oil powder was found to be 97.22% of METOP and 96.46% of MEBOP. During stress studies at 60 °C of both the powders, two major observations were noticed one being TLC pattern which was performed (Figure 2) and for pattern changes during 15th and 30th days' time intervals. It was confirmed that there were no pattern changes in the TLC fingerprint. Second observation was done for Total oil content (Table 1) of the encapsulated powders. It was found that the oil content was reduced to 23.5% in MEBOP and to 23.7% for METOP respectively at the end of

the Study period of 30 days calculated as percentage reduction from the oil content at initial stage. This also shows along with the un-altered TLC pattern and % of oil reduction during the stress study period, the volatile oils after microencapsulation are quite stable under stress conditions. Hence this study proof of concept for its commercial usage down line during handling and dosing in fish ponds by farmers of this encapsulated powders in large volumes.

Particle size and microsphere structures of the spray-dried powder of (METOP and MEBOP) were determined using a Scanning Electron Microscope (SEM) and it is confirmed (Figure 1) that the core material of basil oil and thyme oil was successfully encapsulated by maltodextrin. The particle size of the METOP was observed 32.44 ± 22 μm and 31.78 ± 45 μm of MEBOP. Smaller oil droplets with microcapsule sizes are desirable (Bruna *et al.*, 2018) [5] for the various formulation not only in animal feed supplements but also in functional foods, pharmaceuticals and nutraceuticals. Larger sizes result in poor encapsulation efficiency and there is a chance of breakup during the atomization and drying processes. In this study, MEBOP has a more uniform particle distribution when compared to METOP. According to (Nameer Khairullah Mohammed *et al.*, 2020) [9] particle size and uniformity and quality of microcapsules surface highly depend upon viscosity or total solid concentration in the emulsion medium. Also, METOP has a heterogenous capsule structure with a strong stress tolerance capacity than MEBOP.

Commercial products are monitored for stability at an accelerated temperature and humidity, which helps us to arrive at its shelf life at real time. Stress studies are normally carried out at high temperatures & humidity with a shorter period. The stress studies (Table 1) performed at higher temperature and humidity reflects that the material was stable when it was analysed for compounds Thymol (Figure 3), methyl chavicol (Figure 4) and linalool.

A study conducted by (Youngseok *et al.*, 2020) [17] antibacterial activity with the extracted essential oils of *Abies halophila*, *Pinus thunbergii*, *Pinus parviflora*, *Tsuga sieboldii* and *Pinus rigitaeda* against fish pathogenic species like *Edwardsiella tarda*, *Photobacterium damsela*, *Streptococcus parauberis*, and *Lactococcus garvieae* and proved that *Abies halophila* and *Pinus thunbergii* showed strong antibacterial activity against *Edwardsiellatarda* and *Photobacterium damsela*. Shehata *et al.*, 2013 [14] investigated the effect of essential oil of ginger, black cumin, thyme, clove and watercress on the performance of Nile tilapia (*Oreochromis niloticus*) fingerlings fish and evaluated the antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Listeria monocytogenes*, *Lactococcus lactis*, *Bacillus cereus*, etc., and this study proved the addition of thyme oil 0.25% to fish diet reduces feed consumption ratio (FCR) and improves body weight gain, blood parameters like protein, albumin, aspartate aminotransferase, and alanine aminotransferase. Similarly, (Salgado-Nava *et al.*, 2020) [11] prepared microencapsulated Mexican oregano essential oil powder and established antibacterial activity against *Penicillium expansum*.

MEBOP and METOP were investigated to evaluate their antibacterial activity against aqua pathogenic bacteria strain *Vibrio harvey*, *Vibrio parahaemolyticus*, *Bacillus cereus*, *Aeromonas sp.*, *E. coli*, and *Salmonella sp.* using the disc diffusion method. The zone of inhibition of MEBOP and METOP obtained by the agar dilution method are shown in Table 2. The results revealed that MEBOP and METOP were

potentially effective in inhibiting microbial growth of aquatic pathogenic bacteria with minimal sample concentration levels. The minimum inhibitory concentration (MIC) of MEBOP for *Vibrio parahaemolyticus* and *Vibrio harvey* is 50 mg/ml and for *Bacillus cereus*, *Aeromonas sp*, *E. coli*, and *Salmonella sp* is 80mg/ml. MIC of METOP for *Vibrio parahaemolyticus* and *Vibrio Harvey* and *Aeromonas*, *E. coli* and *Salmonella sp*. is 10 mg/ml and *Bacillus cereus* is 100mg/ml. The encapsulated oils MIC against as such essential oils used for encapsulation, this helps us to arrive at equivalent dose of powder as mentioned in Table 2. This antibacterial activity may have been attributed to thymol and methyl chavicol and linalool present in the MEBOP and METOP. According to (Boruga *et al.*, 2014) [4] the antimicrobial activity depends on their chemical constituents in the essential oil thyme and basil. Mainly presence of phenolic compounds (thymol) and terpene hydrocarbons (γ -terpinene), methyl chavicol (Rajesh 2014) [10] and linalool

respectively. The purpose of this study was to investigate the possibility of substituting antibiotics used in aquatic farms to treat bacterial infections (Figure 5). Encapsulated thyme and basil oil powder showed potent activity against the aquatic pathogens and can be used as a natural antibiotic substance (Youngseok *et al.*, 2020) [17] to compensate for side effects such as the emergence of resistant strains and to control environmental hazards disadvantage of using chemically produced antibiotics. Not only in the antibacterial activity but also these oils through powder form are also known to improve the microbial gut flora of fishes (Seden *et al.*, 2009) [13] and shrimps and support immunity. Basil oil and Thyme oil also exhibit antioxidant activity to reduce the oxidative stress and improve overall wellness productivity (Carine *et al.*, 2019) [6] and life span of aquatic animals. This prophylactic and curative properties of MEBOP and METOP makes it a candidate for developing agents in aquaculture aids in commercial yields and economical impact.

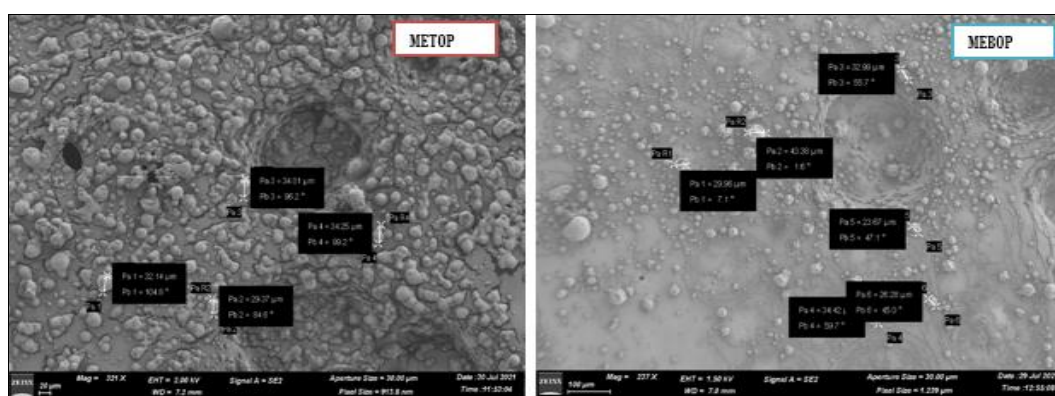


Fig 1: Scanning Electron Microscope Micrograph of METOP & MEBOP

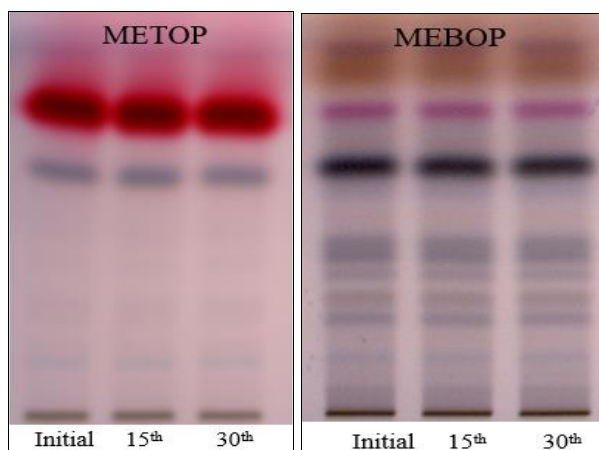


Fig 2: Thin-layer chromatography of METOP & MEBOP

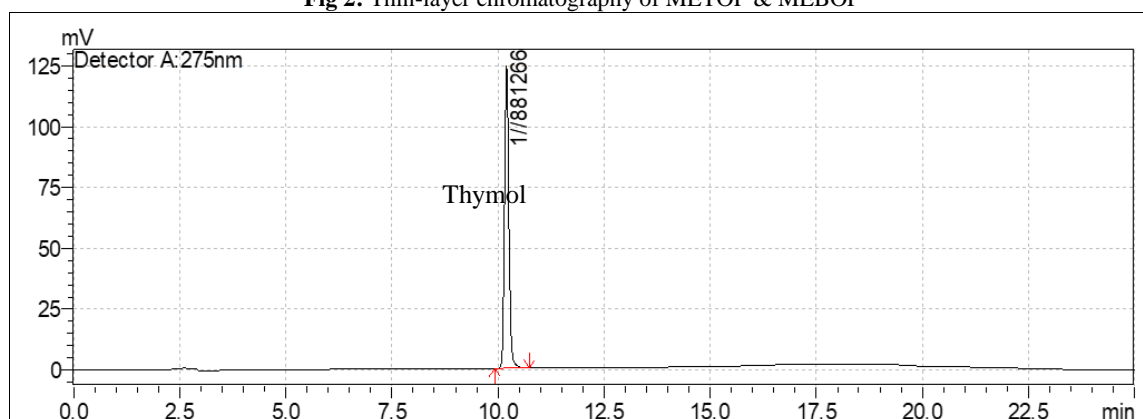


Fig 3: HPLC analysis of Thymol

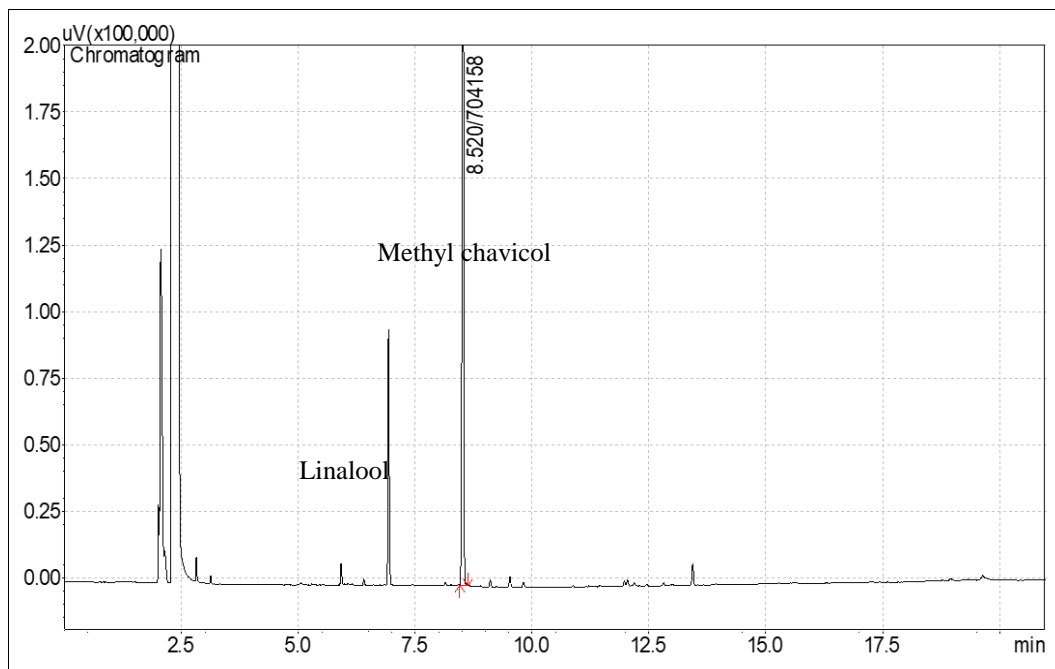


Fig 4: GC analysis of Methyl chavicol and linalool

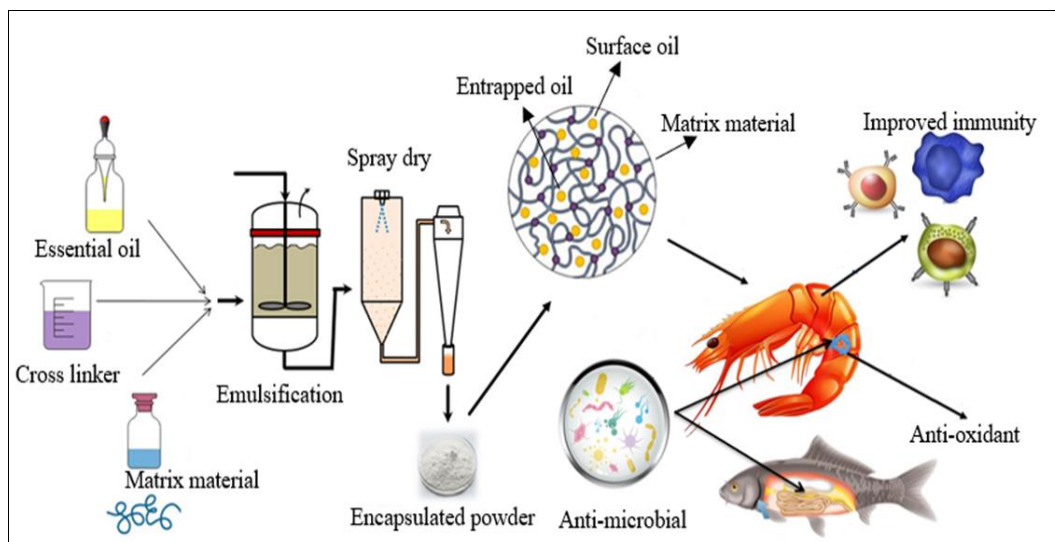


Fig 5: Schematic diagram of the preparation of microencapsulated oil powder for aquatic Health

Table 1: Stress study of METOP & MEBOP

MEBOP				
No	Parameters	Initial at 25 °C ⁺	15 th days at 60 °C ⁺	30 th days at 60 °C ⁺
1	Description	Off white powder	Off white powder	Off white powder
2	LOD (%) w/w	3.97±23	2.87±09	2.51±17
3	pH (1% solution) w/v	7.47±01	6.11±02	5.23±55
5	Linalool (%) w/w	0.30±78	0.31±23	0.31±04
6	Methyl chavicol (%) w/w	0.29±11	0.23±19	0.26±06
7	Volatile oil (% v/w)	24.39±01	19.71±14	19.67±14
8	TLC finger print	Complies	Complies	Complies
METOP				
No	Parameters	Initial at 25 °C ⁺	15 th days at 60 °C ⁺	30 th days at 60 °C ⁺
1	Description	Off white powder	Off white powder	Off white powder
2	LOD (%) w/w	3.96±01	1.51±45	1.68±15
3	pH (1% solution) w/v	5.87±28	5.90±12	5.93±23
4	Thymol (%) w/w	12.69±74	9.20±01	9.33±10
5	Volatile oil (% v/w)	25.80±55	19.74±08	19.60±12
6	TLC fingerprint	Complies	Complies	Complies

Note: Values are mean ± SD of two parallel measurements;

Table 2: Antibacterial study of METOP & MEBOP

No	Organism	Thyme oil	Basil oil	METOP					MEBOP					Tetra cyclin
		20 mg/ml	20 mg/ml	100 mg/ml	50 mg/ml	25 mg/ml	20 mg/ml	10 mg/ml	125 mg/ml	100 mg/ml	80 mg/ml	50 mg/ml	25 mg/ml	1 mg/ml
Zone of inhibition in mm														
1	<i>Vibrio harveyi</i>	37.3±31	25.6±23	40.7±85	17.9±07	14.8±45	13.3±21	12.3±14	12.1±54	11.3±45	11.2±14	11.2±63	N/Z	28.1±01
2	<i>V. parahaemolyticus</i>	30.3±15	22.7±16	41.5±06	33.1±59	19.2±21	17.4±78	14.5±34	17.9±46	14.8±26	15.5±62	11.7±25	N/Z	12.7±54
3	<i>Bacillus cereus</i>	21.3±33	15.9±99	28.5±09	N/Z	N/Z	N/Z	N/Z	13.8±81	13.4±71	10.4±01	N/Z	N/Z	42.9±17
4	<i>Aeromonas</i>	33±61	25.8±25	40.9±01	37.5±08	15.5±44	15±30	12.3±78	21.3±20	19.9±19	18.2±45	N/Z	N/Z	34.3±34
5	<i>E. coli</i>	22.7±84	16.4±11	28.6±36	19.2±01	13.2±31	12.9±01	10.8±34	12.5±34	12.2±61	12.3±34	N/Z	N/Z	32±11
6	<i>Salmonella sp.</i>	19±71	15.7±06	31.2±06	15.7±06	12.4±01	11.2±28	11.2±98	11.9±01	11±93	10.6±93	N/Z	N/Z	30.9±77

Note: Values are mean ± SD of two parallel measurements; N/Z-No zone of inhibition; METOP-Micro encapsulated thyme oil powder; MEBOP-Micro Encapsulated Basil oil powder; +-Relative humidity at 75%

4. Conclusions

Microencapsulated thyme oil and basil oil powders processing was successfully demonstrated using the spray drying technique. Antibacterial activity was established against *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Bacillus cereus*, *Aeromonas sp.*, *E. coli*, and *Salmonella sp.* and was found to have potent antibacterial activity at the concentration level 100mg/ml of METOP and 80 mg/ml of MEBOP. Quick stress study was performed to evaluate volatile oil degradation found to be stable with Relative humidity at 75%. Microencapsulated oil structure was confirmed by SEM analysis. Encapsulated powders of both basil and thyme oil can be used along with feed supplements against the complications in commercial Aquatic farms for controlling bacterial infections.

Declaration of conflicting interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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