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# Synthesis and Characterization of Liposomes Derived from Oleaginous Yeast, *Candida tropicalis*

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**Abstract**

Liposomes are nano or microvesicles used as carrier molecules for the transport of hydrophilic and hydrophobic substances. Oleaginous yeast is one of the potential source for the production of liposomes and hence an attempt has been made to produce liposomes from the lipids of oleaginous yeast, isolated from cultivation soil. The candidate species of oleaginous yeast producing elevated quantity of lipid (3.9U/ml in 96 hrs) was identified by 18s rRNA sequencing as *Candida tropicalis*. The lipids and phospholipids extracted were confirmed using FTIR spectroscopy and were used for the synthesis of liposomes by solvent injection method. The size of the liposome (78.7nm) was determined by AFM analysis. The HPLC analysis revealed the presence of phosphatidylcholine (0.44%) and ethanolamine (0.15%). The encapsulation efficiency was between 30 – 50% as determined by UV-visible spectroscopy. Further research will throw light on the scope and applications of liposomes synthesized using *Candida tropicalis* in the biomedical and food industries.

**Keywords**: *Candida tropicalis*, Encapsulation, Liposome, Phospholipids, HPLC

## 1. Introduction

Yeasts are well-known diverse clusters of microorganisms exhibiting a high level of genetic variations within the same species. They have more biotechnological importance and finds a larger applications in various industries and a greater number of yeast species were identified and explored for their biotechnological potentials (Kieliszek *et al.,* 2017). The morphology of yeast cells facilitates single cell development and hence are better candidate for easy cultivation than fungi since the latter produces pellets or mycelia. Several species, such as *Lipomycesstarkeyi*, *Rhodosporidium toruloides*, *Candida curvata*or, *Rhodotorula* spp., *Trichosporon fermentans* and *Yarrowia lipolytica* were exploited for liposome production with cheese-whey, glucose, glucose syrups, xylose, hemicellulose hydrolysates, molasses, waste glycerol deriving from bio-diesel production, organic acids, etc. as substrata (Morata and Loira, 2017).

Oleaginous yeast is one of the suitable agent, which can produce and accrue high amount of lipid, for example selective strains under optimal conditions may accumulate about 20% to 76% lipid (Lamers *et al.,* 2016). Within cells, the lipids are found as granular forms and its content and fatty acid profile differs between species. The oleaginous yeast commonly produces myristic (C14:0), palmitic (C16:0), stearic (C18:0), palmitoleic (C16:1), oleic (C18:1), and linoleic (C18:2) acids (Chen and Wang, 2007; Carsanba *et al*., 2018). The microbial lipids are involved in the production of biodiesel (Sundarsingh *et al*., 2024), dietary supplements and infant nutrition (Beligon *et al*., 2016; Rodrigues *et al*., 2024). The oleo chemical production involves in the production of two classes of value added oleo chemicals, alkanes and fatty alcohol through free fatty acids derived pathways (Zhou *et al*., 2016). The liposomes produced from the lipids released by microorganisms (Bacteria, Archeae and Yeast) can be used for drug delivery in treating cancer and targeting biofilms (Makhlouf *et al*., 2023; Ameri *et al*., 2016). The phospholipids are the major components of liposomes among various lipid forms produced by yeasts. They are microscopic spherical structures with one or more phospholipid membranes that entraps aqueous component. Generally, liposome composition includes natural and/or synthetic phospholipids like Phosphatidylethanolamine, Phosphatidylglycerol, Phosphatidylcholline, Phosphatidylserine, Phosphatidylinositol. Among them Phosphatidylcholine and phosphatidylethanolamine constitute two major structural components of most biological membranes (Alavi *et al*., 2017).

Liposomes possess versatile properties such as target specificity, regulated release, biocompatibility and biodegradability, which makes them an efficient candidate for pharmaceutical applications (Liu *et al.,* 2022) and potential applications in various industries such as pharmaceutical, food and cosmetic industries. Liposomes serve as significant carrier of drugs and enhance the

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solidity and solubility of the drugs and aids in targeted drug delivery. Due to high encapsulation ability, they prevent degradation of encapsulated drugs due to pH, light and reduce tissue irritation (Alavi *et al*., 2017; Li *et al.,* 2015). Studies on the synthesis of liposomes from different sources of lipids and the characterization of liposomes have much focus in recent days due to their vast applications and biological importance. In this context, the present study aims to synthesize and characterize liposomes from the lipids derived from the oleaginous yeast isolated from soil samples under different vegetation.

## 2. Materials and methods

### *2.1. Isolation of yeast from soil samples*

The top soil were collected from carrots, beans, coffee plantation areas and coffee processing areas in Kodaikanal, Tamil Nadu, India. These samples were kept in a pre-sterilized zip-lock cover and stored in a refrigerator. One gm soil sample was added to 50ml of Glycerol Enriched Medium (GEM) and incubated in shaker incubator 30ºC for 24 hrs at 120 rpm for enrichment of oleaginous yeast (Pan *et al*., 2009). 1 ml of enriched yeast culture was serially diluted from $10^{-1}$ to $10^{-5}$, 0.1 ml of the diluted samples were inoculated in GYEP medium by spread plate method. The plates were then incubated for 48 hrs at 30º C (Atlas, 2010).

### *2.2. Screening of yeast*

The morphology of the isolated yeast was studied by the wet mount technique. The Sudan red III (2 g in 100 ml of isopropanol) staining was done to qualitatively analyze the presence of lipids in the isolated yeast colonies. The yellow intracellular inclusions confirm the presence of lipids in the isolates (Jiru *et al*., 2016). Those isolates of yeasts which were confirmed for the presence of lipids were selected for further process. The selected yeast isolates were sub-cultured using GYEP for further use. The pre-culture of the yeast isolates was prepared by adding one loop of culture to 50 ml of GYEP broth and incubated for 48 hrs at 30ºC. 1 ml of pre-culture was transferred to 50 ml inoculation medium containing GYEP broth and incubated at 30ºC for 5 days in an incubator shaker.

### *2.3. Extraction of lipid from yeast sample*

Modified Bligh and Dyer method was adopted for extracting lipid from the yeast culture. In this method, centrifugation of 50 ml of yeast sample was carried out for 5 min at 5000 rpm. The pellet was washed two times with 50 ml of distilled water and to the washed pellet 10 ml of 4 M HCl was added. The mixture was incubated at 60ºC for 1-2 hrs for acid hydrolysis. After hydrolysis, the content was incubated at room temperature for 2-3 hrs with the addition of 20 ml of chloroform/methanol mixture (1:1). Then, the aqueous upper phase and organic lower phase of the mixture was separated by centrifugation at 2000 rpm for 5 min and the lipid in the lower phase was stored for further analysis (CastanhaI *et al*., 2013).

### *2.4. Estimation of wet biomass, glucose utilized and lipid from yeast*

The wet biomass of yeast was estimated at the various time periods of 24, 48, 72, 96 and 120 hrs. 5ml of culture was centrifuged at 5000 rpm for 5 min and the pellet obtained was weighed to know the wet biomass. The supernatant was used for estimation of concentration of glucose utilized by the biomass by Dinitro Salicylic Acid (DNS) method. In the estimation of lipid, 2 ml of concentrated sulfuric acid was added to the known volume of the sample and heated at 100ºC for 10min. The sample was allowed to cool for 5min in an ice bath and incubated for 15min at 37ºC with the addition of 5 ml of freshly prepared phospho-vanillin reagent was added to it and was incubated for 15min at 37ºC. The absorbance was read at 540nm in a UV spectrophotometer (Cheng *et al.,* 2011).

### *2.5. Molecular identification of yeast*

The potentially important yeast strain was selected and the culture was inoculated on PD broth and incubated at 30ºC for 24 hrs. Twenty-four hours old yeast culture was used for DNA extraction by silica column-based method (Santos *et al*., 2010). The intergenic spacer regions (ITS) of DNA was amplified using the primers ITS1 (5'TCCGTAGGTGAACCTGCGG'3) and ITS4 (5'TCCTCCGCTTATTGATATGC'3) adopting the procedure described by Williams *et al.,* (1995). The optimum PCR cycle conditions, a reaction volume of 25 µL containing 12 µL of 2X Master mix1.5 µL of Forward prime (ITS1) and Reverse primer (ITS2), 5µL of deionized water and 5µL of isolated DNA as template were used. The reaction mixtures were subjected to 30 cycles performed in a thermocycler (Eppendorf, USA) of the following incubation: Denaturation at 94ºC for 5 min, initial denaturation at 94 ºC for 30 sec, annealing 50 ºC for 60 sec, and extension for 72 ºC for 1 min and final extension at 72 ºC for 10 min (Ali and Latif, 2016). The PCR products were purified and sequenced using an ABI 3730xl sequencer (Applied Biosystems). The sequence was analyzed with the NCBI BLAST online tool used to confirm the organism, for getting accession number, the sequence was sent to GenBank. The query sequences and 19 related sequences from the GenBank (NCBI) database were aligned and phylogenetic analyses were done using MEGA 11 version. The phylogenetic relationship of the Oleaginous Yeast strain is displayed in a distance based Neighbor-Joining tree.

### *2.6. Separation of phospholipid*

The extracted lipids were dissolved in 30ml of hexane, the flask was kept in an ice bath, and to this 60ml of cold acetone was added with continuous stirring. The phospholipids get precipitated, and the supernatant was discarded. Following precipitation of phospholipid, the supernatant was discarded. Further, ice cold acetone was used to wash the precipitate (Gladkowsi *et al.,* 2012)

### *2.7. FTIR conformation of lipid and phospholipids*

The sample was subjected to FTIR spectroscopic analysis (IR-Affinity 1S, Shimadzu). The lipid and phospholipids samples were dissolved in methanol for analysis. Then the sample was subjected to FTIR analysis in the range of 4,000–400 $cm^{-1}$ in FTIR spectroscopy with 1 $cm^{-1}$ resolution of (Ami *et al.,* 2014).

### *2.8. Synthesis of liposome*

Solvent injection method involves injection of methanol-lipid solutions into warmed aqueous phases (1M Tris buffer or water). The methanol vaporizes upon contacting the aqueous phase, and the dispersed lipid forms liposomes (Laouini, 2012).

### 2.9. Liposome characterization

#### 2.9.1. Size analysis

Atomic force microscopy (AFM) was used to measure the size of newly synthesized liposomes. This sample was mounted as a thin film on a coverslip to analyse its size according to Laouini, 2011. The topography analysis was done at 50µm.

#### 2.9.2. Phospholipids analysis

Phospholipids separations were carried out using a HPLC system (Agilent- infinity 1260, Agilent Technologies, India). Spherical silica particles of 5 µm bearing 80 Å pores packed in stainless-steel Beckman Ultra sphere SI 250 mm × 4.6 mm ID analytical column was used for the separation of phospholipids. Acetonitrile methanol 85% phosphoric acid (100:10:1.8, v/v/v) was applied for the isocratic separation of phospholipids, with 1.5ml/min, 75ba and 25°C as flow rate, pressure and temperature of the mobile phase respectively. The detector wavelength was fixed as 205 nm for getting the eluting peaks with greater sensitivity. Prior to pumping, the mobile solvent was subjected to ultrasonic bath for degassing. The phospholipid sample in the chloroform-methanol mixture was dried under a stream of nitrogen gas. The residue obtained was dissolved in n-hexane and 2-propanol (3:1, v/v) solvent mixture for analysis at 205nm with retention time of 20 mins.

#### 2.9.3. Efficiency of entrapment

The liposomal formulations were centrifuged at 11000rpm for 30 min to segregate entrapped and free drugs. The supernatant after appropriate dilution was analyzed by UV-visible spectrophotometer (UV-1800, Shimadzu) to determine the quantity of free drugs. The entrapped drugs in the liposome was also determined by UV-visible spectrophotometer after redispersal of the pellet in chloroform. The procedure described by Srilatha *et al*. (2021) was adopted to calculate the encapsulation efficiency.

## 3. Results and Discussion

### 3.1. Isolation of Oleaginous yeasts and its screening

The collected soil samples were enriched with glycerol enriched medium and grown GYEP medium, which facilitates the oleaginous yeast to accumulate lipids as it is the sole carbon source and a lipid analogue, (Papanikolaou and Aggelis, 2011); and four oleaginous yeast isolates, named as OY1, OY2, OY3 and OY4, were obtained. Similar to the present isolation, Pan *et al*., 2009, Amaretti *et al*., 2010 have obtained oleaginous yeasts from different natural ecosystems like soil, water and flower surfaces. Many studies reported that the carbon excess and nitrogen-limited medium are suitable for the isolation of oleaginous yeasts (Schulze *et al*., 2014). The use of GYEP medium in the present experiment as a carbon excess and nitrogen-limited medium, resulted in the isolation of oleaginous yeasts. Glucose, as simple sugar, can be readily utilized by the yeast isolates than other sugars such as xylose. The xylose will not be completely utilized by the isolates, as reported by Pan *et al.* (2009). The morphologically different isolates OY1, OY2, OY3 and OY4 were identified as spherical, round, elongated rods and disc-shaped cells by wet mount technique. The isolates OY1, OY2, OY3 and OY4 were screened for the presence of lipids using the Sudan red III dye. The yellow intracellular inclusions were observed in the isolates. The Sudan red III dye stains the lipid yellow (Jiru *et al*., 2016). The yellow intracellular inclusions confirmed the presence of lipids in the yeast isolates. All four isolates OY1, OY2, OY3 and OY4 showed a positive result and they were selected for the estimation process.

### 3.2. Estimation of biomass, utilization of glucose and production of lipid

The biomass, glucose utilization and lipid production by the isolates were estimated at different time intervals viz., 24, 48, 72, 96 and 120 hrs (Table 1). The strain OY3 showed increased glucose utilization increase in biomass. After the exhaustion of nutrients, the biomass decreased and the lipid accumulation increased up to 3.9 U/ml at 96 hrs of incubation, whereas the other isolates OY1, OY2, OY4 have not utilized glucose completely, which lead to poor lipid accumulation. Hu *et al.* (2011) reported that the oleaginous yeast utilized glucose and xylose and yielded 0.17g and 0.15g of lipid/g of glucose and of xylose, respectively.

**Table 1**. Estimation of biomass, glucose and lipid

| Incubation time (hrs) | Isolate No. | Biomass (U/ml) | Glucose (U/ml) | Lipid (U/ml) |
|---|---|---|---|---|
| 24 | OY-1 | 0.11 | 4.3 | 0.31 |
| | OY-2 | 0.18 | 1.7 | 0.62 |
| | OY-3 | 0.06 | 13.01 | 0.2 |
| | OY-4 | 0.1 | 13.02 | 0.06 |
| 48 | OY-1 | 0.12 | 1.8 | 0.02 |
| | OY-2 | 0.14 | 1.6 | 0.21 |
| | OY-3 | 0.13 | 3.21 | 0.21 |
| | OY-4 | 0.11 | 3.14 | 0.1 |
| 72 | OY-1 | 0.41 | 1.93 | 0.59 |
| | OY-2 | 0.49 | 1.92 | 0.56 |
| | OY-3 | 0.58 | 1.87 | 0.96 |
| | OY-4 | 0.53 | 2.44 | 1.06 |
| 96 | OY-1 | 0.46 | 2.91 | 1.33 |
| | OY-2 | 0.43 | 4.27 | 2.36 |
| | OY-3 | 0.52 | 1.57 | 3.9 |
| | OY-4 | 0.53 | 2.7 | 3.2 |
| 120 | OY-1 | 0.41 | 2.94 | 0.75 |
| | OY-2 | 0.36 | 2.11 | 1.06 |
| | OY-3 | 0.43 | 2.86 | 2.97 |
| | OY-4 | 0.37 | 2.85 | 3.07 |

*Glucose standard, Y= 4.061x-0.094, $R^2$= 0.957
*Lipid standard, Y=3.58x-0.019, $R^2$= 0.954

### *3.3. Molecular identification by 18s rRNA sequencing:*

The NCBI BLAST similarity search results showed 98% of identity and the sequence was submitted in GenBank (Accession number: MH424455). The OY3 was identified as *Candida tropicalis* by 18s rRNA sequencing using the ITS1 and ITS4 primers. Further, the sequences obtained from this study and that of closely related sequences from GenBank are displayed in Figure 1.

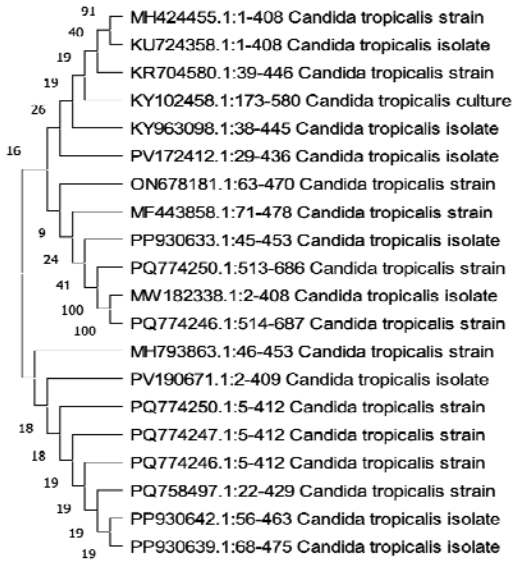


**Figure 1**. Phylogenetic tree of the isolate OY-3 (MH424455 *Candida tropicalis)*

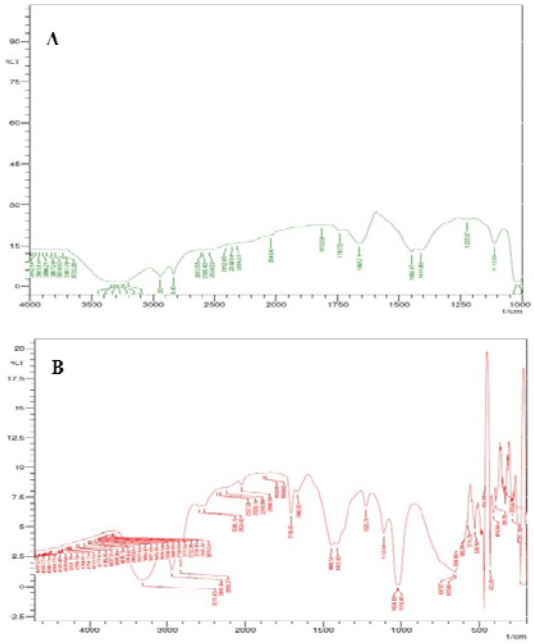


**Figure 2.** FTIR spectra of lipids (A) and phospholipids (B).

### *3.4. Liposome characterization:*

#### *3.4.1. .Size analysis*

The size of the liposome of *Candida tropicalis* at 50µm topography was about 78.7nm (Figure 3). The liposomes synthesized by solvent injection method had an encapsulation efficiency of 20-40%, with the vesicles measuring up to 50-100nm. The encapsulation efficiency of the liposomes is based on their size, which makes them more suitable for pharmaceutical applications (Nsairat *et al*., 2022).

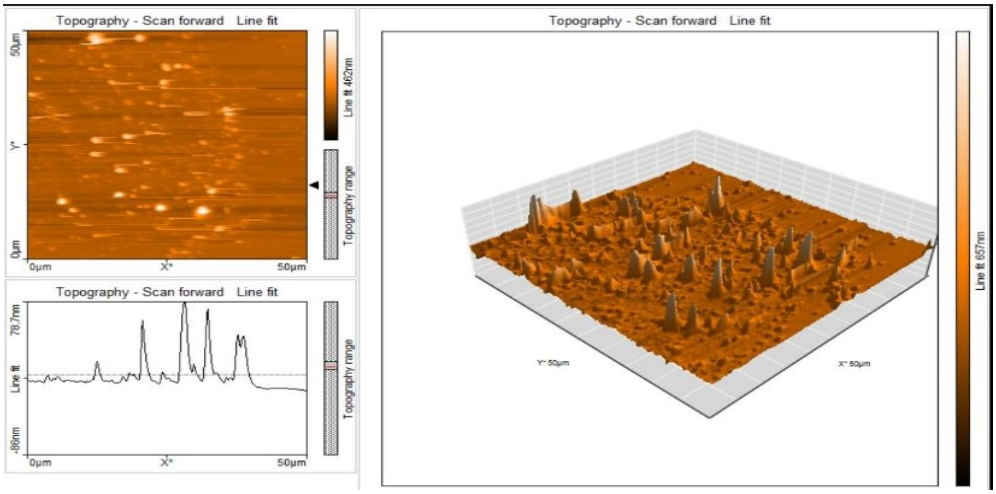


**Figure 3.** Liposome topography of *Candida tropicalis*

#### *3.4.2. Phospholipids analysis*

The peaks obtained at a retention time of 20 min indicated the presence of phospholipids. The phosphatidylcholine and phosphatidylethanolamine present in the samples were 0.44% and 0.15% indicated by the area of the peak obtained. The chromatogram developed by HPLC analysis indicates the presence of phosphatidylcholine (0.44%) and ethanolamine (0.15%) through the presence of peak at the retention time of 10.720 and 11.847 minutes. The traces of other lipids such as phosphatidylguanine (Rt 9.187 mins), phosphatidylinositol (Rt14.493 mins), phosphatidic acid (Rt 17.147 mins) were also found (Figure 4). The traces of other lipids may be due to improper temperature

conditions maintained during precipitation procedures (Aisha *et al*., 2014).

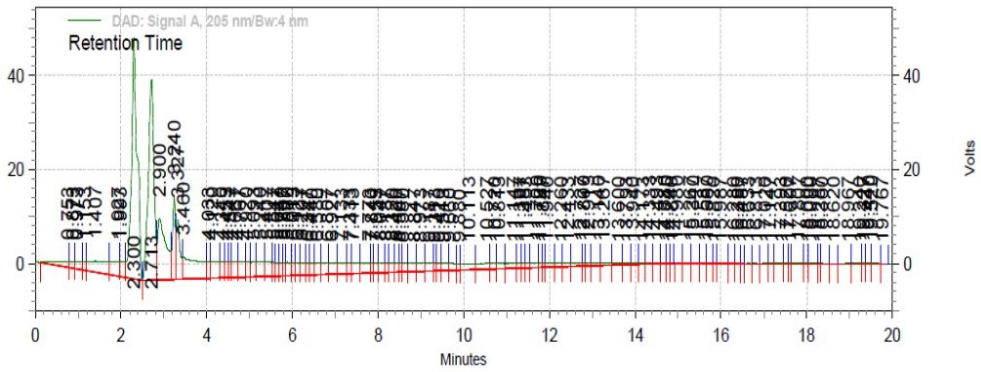


**Figure 4**. HPLC chromatogram of phospholipids

### *3.4.3. Encapsulation Efficiency*

The PDE for the yeast liposome was determined by UV-visible spectroscopy using the drug ciprofloxacin at 600nm and the results are given in Table 2. The PDE for the liposome was 40.9% for ciprofloxacin. The encapsulation efficiency of liposomes synthesized by solvent injection method ranges up to 30-50%. The use of solvents like ethanol may increase the efficiency up to 49% (Ong *et al*., 2016). The study by Fan *et al.* (2008) reveals that the encapsulation efficiency of liposomes ranges from 20-40% when synthesized by ethanol injection method which is in corroboration with our results.

**Table 2**. Determination of PDE using UV-Vis spectroscopy

| CONTENT | OD VALUE (nm) | CONCENTRATION (U/ml) |
|---|---|---|
| Pellet | 0.009 | 0.000153 |
| Supernatant | 0.013 | 0.000221 |

*Ciprofloxacine standard, Y=0.017*0.000, $R^2$=0.964

### *3.4.4. Lipid and phospholipids confirmation by FTIR*

The lipid and phospholipids were confirmed by the presence of functional groups using FTIR. The lipid and phospholipids extracted were confirmed by the appearance of peaks at the wave numbers of 1200-1000$cm^{-1}$ and 1740$cm^{-1}$ respectively (Figure 2). The peaks related to C-H stretching vibrations, C = O and C-O-C stretching in esters, $CH_2$, C-O-C bending and $CH_2$ rocking (Fan *et al*., 2008) were used to identify lipid content of the fungal biomass.

## 4. Conclusion

Liposomes are amphibilic carriers with high biocompatibility, degradability and low immunogenicity and allow modifications with diverse functional properties. These properties authorize them as potential agent for various clinical and pharmaceutical applications especially in drug delivery. The present study emphasizes *Candida tropicalis* as a prospective candidate for the production of liposomes.

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