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Stable Vesicle Production from Bacterial Total Lipid Extracts

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Abstract

The current study aims to produce stable liposomes from total lipid extracts from bacteria. Liposomes are the small vesicles that are made up of lipids. On their structural basis, they can be considered as simplified cell structure of cell membrane. Structure of liposomes depends on the pH of preparation buffer, method of preparation and the environmental condition in which they are prepared. Liposomes have importance in the field of medicines for diagnostic and therapeutic purposes. They mainly work as a vehicle for drug delivery. The objective of the current study was to make stable liposomes from two types of bacterial samples i.e., a Gram-positive and a Gram-negative strain. *E. coli* and *Bacillus sp*. were selected as representative of Gram-negative and Gram-positive bacteria, respectively. Lipid extraction was performed by various methods, out of which modified Bligh and Dyer method gave most effective results. Liposomes were prepared by extrusion and their stability and efficiency was tested by fluorescence spectrophotometer using OxanolVI. Our results showed that liposomes formed by lipids extracted from E. coli were more stable than the liposomes formed by lipids extracted from *Bacillus sp*.

Key words: liposomes, Lipids, diagnostic, therapeutic, purposes, fluorescence, *Bacillus sp*.

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1. INTRODUCTION

Liposomes are the vesicles that have concentric bilayer of phospholipids. They are formed instinctively in a polar or aqueous medium/solution. The word "liposome" arises from Greek word; 'lipos' called fats and 'soma' which means body/structure. They can be formed by cholesterol as well as some phospholipids which are non-toxic phospholipids [1].

The most common definition of the liposomes is "lipid vesicles enclosing aqueous space(s)". Simply, they can be called as the simplified form of cells. Their size ranges from 30 nm to few micrometres. They consist of one or more layers of phospholipid bilayer with polar head facing toward interior and exterior aqueous phases. However, it is not necessary that the lipids would agitate to a conventional bilayer structure because it depends on temperature, pH, environmental and methodology. and may form many kinds of colloidal particles. Due to these facts, the family of lipid micelles include various kinds of particles, which are colloidal, and some other structures with systematic classification. Their classification however based on their structure, preparation, and composition [2].

The first liposomes, which were described in 1965, had no surface modifications, but can have varied sizes, hardness, and biological properties by changing the lipid compositions. These liposomes are conventional, when filled with drug molecules, have been accepted and made available in markets (e.g., as Ambisome, Amphotec, and Abelcet) for the fungal infection's treatment. However, the work progress of these conventional liposomes is bounded by their bare lipid surface. These liposomes have the tendency to fuse with each other so that they can reduce their surface tension, which result in burden release over the time. In addition, proteins of serums can also attach to the unmodified/bare surface, which cause uptake by macrophages, which decreases the efficient delivery conventional liposomes to cellular targets and downgrade their circulation time in blood[3]. To overcome these downfalls, surface modification techniques were made in the late 1980s and early 1990s by attaching the surface of the conventional liposomes with many of inert polymeric molecules so that it can stabilize the liposomes and provide steric hindrance to the other binding molecules[3].

Although these techniques improved stability and increased circulation time, still, these stable liposomes lacked particularity for disease targets and control of timing for delivery. To overcome these downfalls, many engineering techniques, which modify many parts of the liposomes, have been added to improve their working. Recently, the use of inorganic/polymer nanoparticles for stabilization of liposomes has aroused as a trustworthy strategy to make liposomal carriers with hybrid nature[2].

Due to lipid content present in lipid micelles, the lipid characteristic of biodegradability is attributed to them. There are many types of both lipids and amphiphiles, which are used for the formation of liposomes[2].

The first products, which were very noticeable, were Doxil (Sequus) and DaunoXome (Gilead, Nexstar). Both are used as anti-cancer drugs, which were tested clinically, and results were positive which FDA (Food and Drug Administration) approved in 1990's [3].

The most important function of lipid micelles is their capability to encapsulate drugs of both types i.e., the lipophilic and hydrophilic. The reason, which makes them a good carrier, is that they enclose drugs and make them more stable and the increase in bioavailability when they are transferred to the body through liposomes. Liposomes also have a greater biodegradability rate inside the body, which make them safe as a carrier [4].

Lipid micelles can be single lamellar or multilamellar depending on the content of lipids present, the surface charge of the lipids involved, size of lipids the method of preparation used. Formation of liposomes do not involve the addition of surfactants or emulsifiers [5]. The liposomal size may vary from 0.025µm to 2.5µm. The other factor, which is important in the liposomal classification, is the number of lipid bilayer present. The size a distinctive feature in determination of the half-life of the micelles while both size and the number of lipid bilayer influence encapsulation and amount of drug it traps in the centre. Liposomes are considered important because they have structure like the one the biological membranes thus help in study of the topology, photo physics, biochemistry and functioning of the membranes. In addition, the interaction of biological membranes can also be studied with the help of liposomes[5].

The industrial application of liposomes includes first the drug delivery system. Moreover, many other applications are present i.e., adjuvants of vaccination, enhance penetration of cosmetics working as their matrix, signal enhancer or signal carriers and, it acts as solubilizer for different kinds of ingredients [6].

Main aim of current study was the Preparation of stable liposomes from both Gram positive and Gram-negative bacteria and evaluation of stability of prepared liposomes.

2. MATERIALS AND METHODS

2.1 Samples

For the Gram-positive sample, strain of *Bacillus sp.* was used, while for the Gram-negative sample, strain of *E. coli* was used. Both the samples were taken from Zoology Department of GC University, Lahore.

2.2 LB Media

The media used for the streaking and culturing of the bacteria was LB media because this media has essential nutrients for almost every type of bacteria including the ones mentioned above in the samples.

2.3 LB Agar (250ml)

For the preparation of LB agar media (250 ml), first, 100 ml distilled water was added to an autoclaved flask of 300ml capacity. Into the water was added 2.5 g of tryptone, 1.25 g of yeast extract, 2.5 g of sodium chloride and finally 3.75 g of agar after carefully weighting over a weight balance. After the addition of all chemicals, raise the volume by adding distilled water up to 250 ml. After the mixing of all the ingredients, the mixture was autoclaved at 121 °C, 15 lb pressure for 15 min, and was poured into petri plates.

After the streaking of bacterial cultures, the petri plates were incubated at 37 °C for about 18 hrs.

2.4 LB Broth: (1000 ml)

For the preparation of LB media broth (1000 ml), first, 500 ml distilled water was added in an autoclaved blue capped bottle of 1000 ml capacity. Into the water was added 10 g of tryptone, 5 g of yeast extract and 10g of sodium chloride after weighting them carefully. After the addition of all chemicals, raise the volume by adding distilled water up to 1000 ml. After mixing all the ingredients, the mixture was autoclaved.

A single colony of bacteria was inoculated in 250 ml of media and was incubated at 37 °C for 18 hrs. Cells were harvested by centrifugation the following day.[7]

2.5 Lipid extraction

For the lipid extraction from the bacteria, 3 protocols were performed which are:

2.5.1 Bligh and Dyer method

For this protocol, following steps were followed:

The pellet was weighed which was obtained after the centrifugation by using a weighing balance. The dry weigh of the pellet was 0.2 g. A 3.75 ml mixture was made by adding chloroform and methanol in a ratio of 1:2 i.e., 1.25 ml of chloroform was taken and mixed in 2.5 ml of methanol. This mixture of chloroform and methanol was added to the pellet present in a falcon and mixed gently. After the pellet was dissolved in the organic mixture, the total mixture was transferred to a glass test tube. The mixture was vortexed for 5 minutes. 1.25 ml of chloroform was added to the mixture and the whole mixture was vortexed again for 5 minutes. 1.25 ml of distilled water was added to the mixture and whole mixture was again vortexed for 5 minutes. The glass test tube was covered with aluminum foil and transferred to centrifuge machine. The time was set for 5 minutes while the speed was set to 1000 rpm. Proper layers were not formed thus the mixture was again spun in the centrifuge for 5 minutes more at the speed of 1000 rpm for 5 minutes. Two distinct layers were obtained. Lower layer was recovered by using a Pasteur pipette. Lower layer contained lipids dissolved in chloroform[8].

2.5.2 Folch method

For this protocol of lipid extraction, following steps were followed: The pellet was weighed which was obtained after centrifugation of bacterial culture by using a weighing balance. The dry weigh of the pellet was 0.16 g. Homogenization mixture was formed in such a way that the total volume of chloroform and methanol was 20X of the dry weigh of pellet while the ratio of chloroform to methanol was 2:1. So, for homogenization mixture, 6.4ml of chloroform was added to 3.2 ml of methanol. This homogenization

mixture was added to the falcon tube containing pellet and the mixture was agitated at room temperature. After agitation, the mixture was transferred to a glass test tube. Test tube was transferred to the centrifuge machine, and it was spun for 5 min at 1000 rpm speed. After centrifugation, the liquid phase was recovered. 0.9 % sodium chloride solution was prepared by adding 0.9 g of sodium chloride to 100ml of distilled water. The liquid phase recovered, was washed by 0.2 ml of 0.9 % of sodium chloride solution. Two layers were formed after washing. Lower layer was recovered, and upper layer was discarded. Lower layer had lipids dissolved [9].

2.5.3 Bligh and Dyer method (modified)

For this protocol, following steps were followed: Pellet obtained after centrifugation of culture media was weighed by using a weighing balance. Pellet weight was 0.2 g. 1% sodium chloride solution was needed in 100ml for the pellet weighing 1 g, so for 0.2 g, the volume required was calculated to be 20ml. 1% sodium chloride was prepared by adding 0.2 g sodium chloride in 20ml of distilled water. 1% sodium chloride solution was added to the falcon tube containing pellet and the pellet was re-suspended. The mixture was centrifuged for 5minutes at the speed of 2000rpm. Supernatant was discarded and the residue was freeze at -30C for 18hrs/ overnight. After 18hrs transfer the mixture to a glass test tube. The homogenization mixture was prepared by adding chloroform, methanol and distilled water in a ratio of 1:2:0.8 when the total volume is 114 ml when 100 ml of sodium chloride 1% solution was used. In this case when the sodium chloride solution was 20 ml, the total volume of the homogenization mixture was calculated to be 22.8 ml. For preparation of homogenization mixture, 6ml was chloroform was added to 12ml methanol and 3.8ml of distilled water. This homogenization mixture was added to the glass test tube containing the residue of re-suspended pellet. This whole mixture was allowed to stand at room temperature for 18 hrs. To make the final ratio of homogenization mixture to 1:1:0.9, 0.3 ml of distilled water was added and 0.6 ml of chloroform was added to the final mixture. Transfer the mixture to a separating funnel and separate the layers of the mixture [10].

2.6 Formation of thin film of lipids

After the whole protocol is followed for the extraction of lipids by Bligh and Dyer method [8,9]. In the last step, lower layer is retrieved which have lipids dissolved in chloroform but the lipids we need to form lipid micelles should be in their raw form not dissolved in an organic mixture, so to obtain raw lipids, the chloroform needs to be evaporated.

For the evaporation, there could be many methods used like air-drying or evaporation by heat but the problem in such methods is that the lipids are oxidized as soon as they come in contact with air. To overcome any such condition, the method used to evaporate the chloroform is evaporation by N_2 gas.

Evaporation was done by following steps: The mixture of lipids and chloroform after retrieval was transferred to a glass vial so that the surface area would be enlarged. The valve of the nitrogen gas tank was opened and the pressure was set minimal. The nitrogen gas was poured over the mixture at low pressure while rotating the vial between palms of hand to provide heat for evaporation. As the chloroform was evaporated, a thin film of lipids is observed at the bottom of vial. As soon as the thin film was evaporated, the vial was closed by lid tightly and kept at -80^{0}C to cease any more reactions of lipids [10].

2.7 Giant lipid vesicle preparation

Giant lipid vesicles were prepared by following method. The beaker was covered loosely with a para-film and the lipid film was pre-hydrated by water saturated nitrogen jet at a temperature of 45 ^{0}C for 15 minutes. The buffer which was to be encapsulated was preheated greater than the main phase transition temperature of the melting point of highest lipid which is greater than 10 ^{O}C. 0.2-0.45mm syringe filter was attached with a disposable plastic syringe. The rehydration buffer was added enough to immerse the pre-hydrated lipid thin film completely. The beaker was sealed with para-film tightly to avoid evaporation. After sealing, it was incubated at a temperature greater than the main phase transition temperature of the lipids, which is greater than 10 ^{0}C for several hours. In this incubation period, the swelling of lipid film was observed and vesicles were formed. The harvesting of lipid vesicles at this point was done using a Pasteur pipette. 50mg/ml of Oxonol VI was prepared. 150 µl were taken and the remaining stock solution was

saved at 4 ^{0}C. 20 mg/ml of valinomycin was prepared in DMSO. 150 µl were taken while the remaining stock solution was saved at 4^{0}C. 1M KCl buffer was prepared. After the preparation of all the solutions, 2 ml KCl buffer was taken and mixed with 2 µl of Oxonol VI, 2µl of liposomes harvested and 2 µl of valinomycin. This mixture was transferred to fluorescence spectrophotometer when the excitation wavelength was set to 580 nm with the slit width of 200 nm while the emission wavelength was set to 660 nm with the width slit of 5 nm. For the reading of next solution, first the blank solution reading was measured and then the KCl buffer was added [11].

3. RESULTS AND DISCUSSIONS

3.1 Lipid extraction

There were 3 methods which were performed to extract lipids out from the bacterial pellet. These protocols are:

3.1.1 Bligh and Dyer method

Lipid extraction by Bligh and Dyer method was done by suspending bacterial culture in the organic solution of chloroform and methanol and further proceeding the protocol, lower layer formed after the final step was recovered. The lower layer consisted of chloroform and the lipids extracted from the bacterial pellet. The lower layer recovered was pale yellowish in color (Fig 1a).

3.1.2 Folch method

Second protocol performed to extract lipids out of bacteria was Folch method. This method is just like the method mentioned above, but in this method, after proceeding the protocol, in the final step 0.9% solution of NaCl was added instead which gave two distinct layers. The lower white coloured layer was recovered which contained lipids mixed in chloroform. (Fig 1b).

3.1.3 Bligh and Dyer method (modified)

Another protocol was also performed to extract lipids, which is a modification of Bligh and Dyer method. The organic solvent mixture used here also is the same as above methods i.e. chloroform and methanol. After the protocol was proceeded, in the final step, the layers were separated out by using a separating funnel. The lower layer contained lipids dissolved in chloroform. The layer obtained by this method was also yellowish in color like original Bligh and Dyer method (Fig 1c).

The layers obtained by all the three methods were then analyzed to choose the protocol that gave the best results. For the analyzation, thin film was made by all the 3 protocols and layer obtained by the modified Bligh and dyer method was selected for further experiments (Fig 1d).

3.2 Formation of thin film of lipids

For the formation of thin film of lipids, the layers obtained by the above-mentioned procedures were transferred into a glass vial and nitrogen gas was poured over them with constant heat provided with swirling the vial between palms of hands. As the organic solvent evaporated a monolayer thin film was obtained.

Thin film was made for both the sample bacteria i.e. *E. coli* and *Bacillus sp,* after the extraction of lipids by Bligh and Dyer method (Fig 1e).

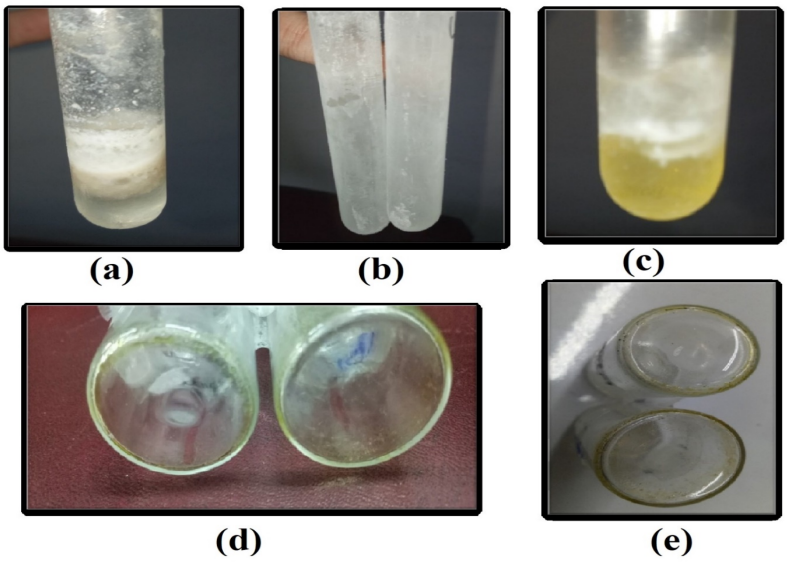

Fig.1. (a) Lower layer recovered by the Bligh and Dyer protocol. (b) Lower layer recovered by Folch method. (c) Lower layer recovered by Bligh and Dyer method (modified). (d) Monolayered thin film produced by Bligh and Dyer Method (e) Thin film obtained by *Bacillus sp* and *E. coli*

3.3 Giant lipid vesicle formation

Giant lipid vesicles were prepared by previously described method using working buffer (0.1mM KCl, 100 mM LiCl and 20 mM HEPES pH 7.5). Fig 2a shows a schematic diagram of prepared giant vesicles.

3.4 Liposomes analysis

The stability of prepared liposomes were analyzed by using fluorescence spectrometer at excitation wavelength of 580nm. Table 1 showing reduction potential of liposomes prepared by the two samples i.e. *E. coli* and *Bacillus sp*. respectively. Fig 2b shows the comparison of effect of increasing K+, - valinomycin inward diffusion on the liposomes.

Table 1. Analysis of giant vesicles prepared by lipids extracted from *E. coli and Bacillus sp.*

Lipids extracted from *E. coli*		
|K+|(inner) mM	|K+|(Outer) mM	E nerst mV
0.1	0.1	0
0.1	0.2	18.1
0.1	0.5	42.02
0.1	1	60.12
0.1	2	78.22
0.1	5	102.14
0.1	10	120.24
0.1	25	144.16
0.1	50	162.26
0.1	100	180.36
0.1	150	190.94
0.1	220	200
Lipid extracted from *Bacillus sp*		
0.1	0.1	0
0.1	0.2	18.1
0.1	0.5	42.02
0.1	1	60.12
0.1	2	78.22

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0.1	5	102.24
0.1	10	120.24
0.1	25	144.16
0.1	50	162.26
0.1	100	180.36
0.1	150	190.94
0.1	220	200

Liposomes are very important and are used in many fields. It acts as a model of biological membrane due to similar structure and can be used to study the transporting behavior of living biological membranes for different types of materials. Membrane proteins can be reconstituted using liposomes. Another important use of liposomes is in the field of biochemistry where the study of micro-photosynthesis is done by their use [11].

In the field of medicines, liposomes use can be seen in both, the diagnostic field as well as the therapeutic field. Drugs delivered by liposomes prove to be less toxic towards human body reducing the side effects of medicines [1].

The most known and actively ascending field for liposomal use is the use of lipid micelles as the drug carrier. Liposomes are more stable, less reactive with body cells, specified in their working and more biodegradability.

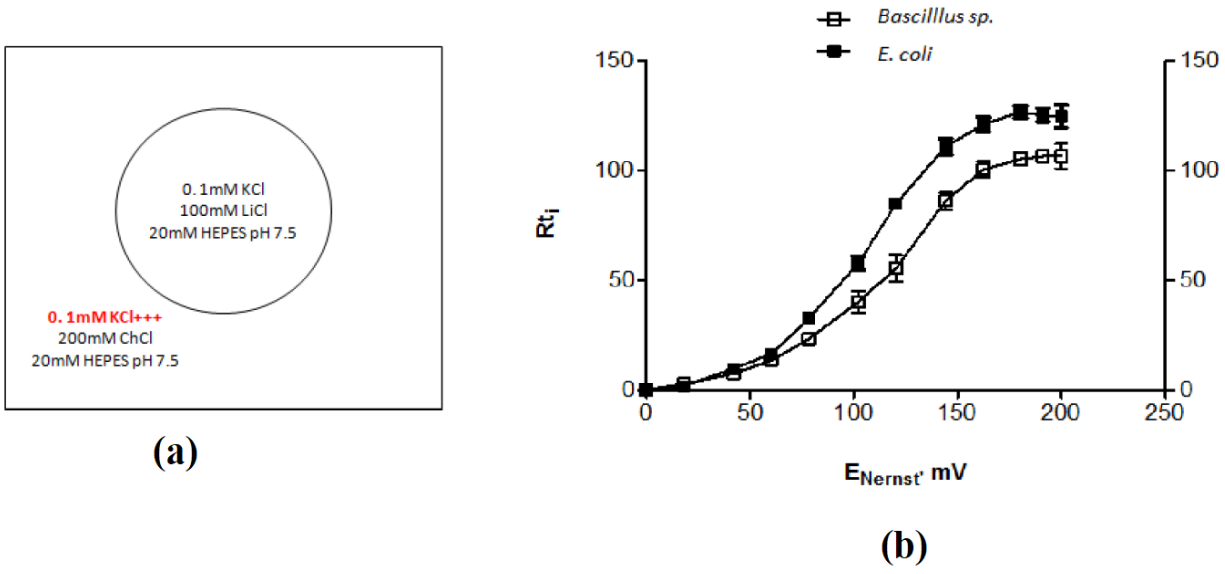

Fig.2. (a) A schematic representation of giant lipid vesicles (b) Effect of increasing K+ valinomycin inward diffusion gradient on Rt ratio of Oxinol VI.

The recent field for the liposomal use is the macrophage activation in the body. When liposomes enter the body, they are engulfed, and the liposomes will activate the macrophages and produce an immune response [13].

The objective of the study was to prepare giant liposomes from lipids of Gram-positive and Gram-negative bacteria and check their stability. To achieve this goal, *E. coli* was used as a representative of Gram-negative and *Bacillus sp* as a representative of Gram-positive bacteria. The lipid extraction was done by 3 very basic protocols in which the organic solutions were added to the bacterial pellet along with distilled water which in the end produced layers one of which had been known to dissolve the lipid content. There were three

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protocol which were followed: (1) Bligh and Dyer method, (2) Folch method and (3) Bligh and Dyer method (modified).

From all the above-mentioned protocols, a layer of chloroform mixed with lipids was obtained, but when the concentration of lipid content was checked, it was observed that more concentration of lipids was extracted out of bacterial membrane when Bligh and Dyer method was applied. Also, when the chloroform was evaporated to make a thin film of lipids, a monolayered lipid thin film was obtained by Bligh and Dyer method while the thin films obtained by the next two protocols mentioned above did not gave a monolayered thin film which is important for the formation of stable liposomes which were required. Based on these observations, this protocol was proceeded for further experimental trials of extracting lipids [1].

After the recovery of lipids dissolved in chloroform was to evaporate chloroform in such a way that a monolayered thin film is left behind. To evaporate chloroform 3 approaches were considered i.e. (a) by heat, (b) through rotary evaporator (c) by nitrogen gas.

If mixture was exposed to heat through water bath, chloroform would evaporate but the lipids left behind will be exposed to air. When air would encounter the lipids, oxygen in the air oxidize the lipid film and will make them unable to make liposomes.

For the second approach the rotary evaporator was considered but it also cannot omit the air contact with lipids. To overcome this shortcoming, liposome making buffer could be added before evaporation and liposomes could be made but those liposomes would not be stable because of contact of buffer with chloroform (which was not evaporated) and exposure to heat from the evaporator [14].

The third approach was to evaporate chloroform by nitrogen gas. This approach is most suitable for extraction of unoxidized and stable lipids for the formation of liposomes because, with this method, air contact is omitted completely. Nitrogen gas is denser than air and is non-reactive so, when nitrogen is poured over the mixture, air present in the vial comes out and due to its non-reactive nature, it doesn't react with lipids and give stable lipids. Due to these factors, the evaporation of chloroform was done by this approach.

The thin layer formation worked as a test or analysis for choosing the more efficient protocol for the lipid extraction. From the Bligh and Dyer method a monolayer of thin film was obtained which determined it as the best protocol to be followed, thus for the extraction of lipids this method was followed in the proceeding attempts.

Liposomes were prepared by extrusion method and these liposomes were further subjected to fluorescence spectrophotometric analysis by using Oxonol VI dye, developed by Needham *et al.,* 1998. Oxonol VI dye is widely used for measuring membrane potential of cells [14]. When Oxonol VI is added to the media, it binds strongly with the vesicles present with the cell membrane. Because of this binding, both the emission spectrum and absorption spectrum move towards the longer wavelength, which can be measured. Another important feature of Oxonol dye is that its anions are more permeable in the cell membrane. It has been observed that when an internal positive charge is present, this dye tends to move towards the positive charge and thus the dye move from inside of the vesicle and more towards the membrane itself that make their attachment stronger and thus the spectral change will also increase [16].

In the experiment to measure the stability of liposomes, the valinomycin (with K+) when added as a buffer to make liposomes, the internal positive charge of the liposomes increased making them more attractant towards the Oxonol dye. When Oxonol dye was added, it binded to the membrane of the liposomes. The more will be the K+ the more will be the influx of the Oxonol dye and the more will be the stability of the liposomes [17].

After the addition of dye, the activity of the dye was checked through fluorescence spectrophotometer. When the Oxonol dye was activated, it produced an increased spectral emission producing a fluorescence, which was measured by the spectrophotometer [18].

4. CONCLUSIONS

In conclusion, our results showed that, best method for lipid extraction is Bligh and Dyer method and best way to make a thin lipid film is by drying by using Nitrogen gas. Also, the results show that liposomes produced by Gram negative strain i.e., *E. coli*, are more stable as compared to the liposomes produced by Gram positive strain i.e., *Bacillus sp*.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Ameri A, Moghimipour E, Ramezani Z, Kargar M, Hashemitabar M, Saremy S, Handali S. Formulation of a new generation of liposomes from bacterial and archeal lipids. Tropical journal of pharmaceutical research: TJPR. 2016;15(2):215.
2. Miere F, Fritea L, Cavalu S, Vicas SI. Formulation, characterisation and advantages of using liposomes in multiple therapies. Pharmacophore. 2020;11:1–12.
3. Xing H, Hwang K, Lu Y. Recent developments of liposomes as nanocarriers for theranostic applications. Theranostics. 2016;6(9):1336–1352.
4. Has C, Sunthar P. A comprehensive review on recent preparation techniques of liposomes. Journal of liposome research. 2020;30(4):336–365.
5. Patil YP, Jadhav S. Novel methods for liposome preparation. Chemistry and physics of lipids. 2014;1;177:8-18.
6. Barenholz Y. Liposome application: problems and prospects. Current opinion in colloid & interface science. 2001;6(1):66–77.
7. Lessard JC. Growth media for E. coli. Methods in enzymology. 2013;533:181–189.
8. Gorgich M, Mata TM, Martins AA, Branco-Vieira M, Caetano NS. Comparison of different lipid extraction procedures applied to three microalgal species. Energy reports. 2020;6:477–482.
9. Modiri S, Zahiri HS, Noghabi KA. Evaluation of bacterial lipid production: Quantitative and qualitative measurements: Tips and guidelines. Methods in molecular biology (Clifton, N.J.). 2019;1995:395–403.
10. Zhu TF, Budin I, Szostak JW. Preparation of fatty acid or phospholipid vesicles by thin-film rehydration. Methods in enzymology. 2013;533:267–274
11. Lewis T, Nichols PD, McMeekin TA. Evaluation of extraction methods for recovery of fatty acids from lipid-producing microheterotrophs. Journal of microbiological methods. 2000;43(2):107–116.
12. Li J, Wang X, Zhang T, Wang C, Huang Z, Luo X, Deng Y. A review on phospholipids and their main applications in drug delivery systems. Asian journal of pharmaceutical sciences. 2015;10(2):81–98.
13. Lasic DD, Barenholz Y. Handbook of nonmedical applications of liposomes: Theory and basic sciences. London, England: CRC Press; 2020.
14. Sihorkar V, Vyas SP. Potential of polysaccharide anchored liposomes in drug delivery, targeting and immunization. Journal of Pharmaceutical Sciences. Sci. 2001;1;4(2):138-58.
15. Szoka F, Olson F, Heath T, Vail W, Mayhew E, Papahadjopoulos D. Preparation of unilamellar liposomes of intermediate size (0.1–0.2 µm) by a combination of reverse phase evaporation and extrusion through polycarbonate membranes. Biochimica et Biophysica Acta (BBA)-Biomembranes. 1980;1;601:559-71.
16. Needham T. Visual complex analysis. Oxford University Press; 1998.
17. Wang T, Deng Y, Geng Y, Gao Z, Zou J, Wang Z. Preparation of submicron unilamellar liposomes by freeze-drying double emulsions. Biochimica et Biophysica Acta (BBA)-Biomembranes.. 2006;1;1758(2):222-31.

18. Apell H-J, Bersch B. Oxonol VI as an optical indicator for membrane potentials in lipid vesicles. Biochimica et biophysica acta. Biomembranes. 1987;903(3):480–494.