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Effect of Glycolipoprotein Biosurfactant from *Enterococcus faecium* on the Viability of Breast Cancer Cell Lines

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Abstract
Objectives: This study, was research for cytotoxicity of bio-surfactant created by *Enterococcus faecium* isolated from feces of Iraqi healthy breast-fed infants with age <6 months.
Methods: Cold acetone precipitation was used to the extraction of extracellular Glycolipoprotein biosurfactant and partially purify it. Biosurfactant was then evaluated against two the cell lines, a Breast cancer MCF-7 cell line and a human normal fibroblast cell line NHF), specifically for cell survival and proliferation.
Results: At all concentrations with varying percentage, the viability of the MCF-7 cancer cell line was shown to be reduced with the addition of biosurfactant.; maximum inhibition percentage was 74.2% at a 100 µg/ml concentration, which is lesser than 45.5% cytotoxicity Of NHF healthy fibroblasts cell line.
Conclusion: The findings of this study are highly encouraging in terms of the potential of Glycolipoprotein biosurfactants to treat cancer and encourage additional research with different cell lines.
Keywords: Anticancer effect, biosurfactant, *Enterococcus faecium*, FTIR

Introduction

Biosurfactants are microbial substances that are often synthesized by bacteria and released either extracellularly or on the surface of the cell.[1] Biosurfactants provide a wide range of advantages such as bioavailability, decreased toxicity, and efficacy in a variety of environmental conditions, such as temperature and pH,[2] Furthermore, biosurfactants outperform synthetic surfactants in terms of efficiency, selectivity, environmental friendliness, and stability.[3] Biosurfactant can be produced on several types according to the microorganism produced, such as: lipopeptides, glycolipids and glycolipoprotien are an alternative to traditional antibiotics against certain pathogens since they demonstrate potential antimicrobial and antiadhesive actions.[4] Additionally, biosurfactants may be effective as anticancer medications.[5] There have been reports of different kinds of biosurfactants being produced by bacterial genera including pseudomonas, *Lactobacillus, Bacillus, Rhodococcus, Acinetobacter, Arthrobacter* and *Enterococcus*.[6] *Enterococci* sp. are G+ve, facultative anaerobic coccus. are also occasionally discovered in the mouth and vagina. The most prevalent types of enterococci in humans are *Enterococcus faecalis* and *Enterococcus faecium*. Enterococci are commensals that colonize the digestive tract and take role in the immune system's regulation in both humans and animals.[7] Cancer is a complex illness with several challenges in therapy due to therapeutic effectiveness concerns and harming adverse effects for normal cells as described byAmendoeira et al.,[8] and Jarallah et al.,[9]. The identification as to novel cytotoxic substances is critical for the development of anticancer medicines. in order to identify a new safe natural cytotoxic compounds. current study presents the cytotoxicity of a novel glycolipoprotein bio-surfactant generated by a locally isolated *Enterococcus faecium* against MCF-7 cells.

Materials and Methods

Bacterial Isolation and Identification

Samples of feces have been collected from Iraqi healthy breast-fed infants with age <6 months, after delivery they were they were cultured on Bile Esculin with 24 hours at 37°C incubation condition. *Enterococci* and group D *streptococci* have the capacity to hydrolyze esculin when bile is present,[10] isolate of *Enterococcus faecium* was identified throughout microscopical, cultural and biochemical test according to Benson (2001),[11] and VITEK2 was used to confirm the results.

Screening Techniques of Biosurfactant

The screening of powerful biosurfactant producers may be done using a variety of techniques such as: Oil spreading method,[12] Surface tension measurement[13] and Emulsification index 24%.[14]

Extraction of Crude Biosurfactant from E. faecium

For *E. faecium* to produce crude biosurfactant, 20 ml of an overnight subculture were added to 1000 ml of culture broth, which was then incubated for 24 hours at 37°C in an anaerobic condition. A Millipore filter was used to remove the cells from the culture broth after it had been centrifuged at 4°C for 10 minutes at 12,000 rpm.

Biosurfactant Partial Purification

Cold acetone precipitation was used to partly purify the biosurfactant according to Choudhary et al.[15] Cold acetone was mixed in a 3:1 ratio to the crude biosurfactant solution, which was then allowed to stand at 4°C for 18 to 24 hours. After centrifuging at 10,000 rpm for 30 min., A pellet was collected. This pellet considered as partial purified biosurfactant. It was

then dispersed in sterile water after being further, allowed to evaporate to dryness to eliminate any remaining acetone.

Characterization of Partial Purified Biosurfactant

1-Chemical Analysis of Biosurfactant

Using D-glucose as a standard, the amount of carbohydrates in the biosurfactant was assessed using the phenol-sulfuric acid technique.[16] Bradford,[17] developed a method for determining protein concentration using bovine serum albumin as a standard, while Anschau[18] developed a method for estimating lipid content.

2-FT-IR

It is possible to identify the distinct components of mixtures with unknown compositions using Fourier transform infrared spectroscopy (FTIR), which is particularly useful for detecting different types of chemical bonding (functional groups), One milligramme of freeze-dried Bio-surfcatant was mixed with 100 mg of KBr and ground for 30 seconds at a pressure of 7500 kg to create transparent pellets that were then tested by spectrometry (FT/IR-4200, JASCO). 180 scans with a resolution of 4 cm^1 in the 550–4000 cm^1 range were used to capture all of the spectra. Background was provided by a KBr pellet which was then analysed using IR analytical software.[19]

3-Gas Chromatography Analysis

A number, kinds, and M.wt. of the components found in the isolated biosurfactant were determined by gas chromatography analysis. In the system (capillary column ZB-5MS), helium (He) will serve as the carrier gas at a flow rate of 2 ml/min. The injector and detector were each 230°C and 280°C in temperature. Before being subjected to GC-MS analysis, 0.1 g of a partially purified biosurfactant sample was diluted in methanol at a 100 g/ml concentration. Initially maintained at 80°C for 3 minutes, the column temperature was then raised to 280°C at a rate of 8°C/min and maintained for 10 minutes. Afterward, a 1 (1 sample was utilized with a 10:1 split ratio.[20]

Cytotoxicity Assay In-vitro

Two types of cell lines have been used: MCF-7 is a breast cancer cell line and Normal Human Fibroblast (NHF) cell line, which maintained according to Al-Shammari et al.[21] On 96-well plates, the MTT cell viability experiment was carried out to detect the cytotoxic effect.[22–24] These cells were grown in RPMI1640 culture media, to which (10%) heated inactivated fetal bovine serum (from Biowest, South America) was added at a humidified 37°C temperature, along with (5%) CO_2 [25]. To test the cytotoxic effect of *Enterococcus faecium* biosurfactant on cell growth and viability, the cell lines were cultured at 10^4 cell/well, after 24 h. or there was achieved confluent monolayer, biosurfactant was used to treat the cells. After 72 hours of treatment, the viability of the cells was assessed by removing the culture medium, adding 28 L of an MTT solution containing 2 mg/mL, and incubating the cells for 2 hours at 37°C. After discarding the MTT solution, the crystals in the wells were solubilized by adding 130 µL of DMSO, followed by incubation at 37°C for a 15-min with shaking.[26] The absorbency was measured with a microplate reader at 492 nm (test wavelength).

The assay was performed in triplicate. The cell growth inhibition rate (the percentage of cytotoxicity) It was estimated using the following equation.[27]

Cell growth % = (A1 / A0) x 100

A1: absorbance of treated cell A0 : Absorbance of non-treated cell

% Cytotoxicity = 100 – cell growth %

Results and Disscusion

Enterococcus faecium supernatant showed spreading on oil layer with a diameter 41 mm, this result indicated the *E. faecium* was able to produce biosurfactants and the isolate exhibiting the higher production (Figure 1). The diameter of the clearing zone formed by biosurfactant-containing solution has been shown to be directly proportional to the concentration of biosurfactant.[28] This technique is preferred to other screening methods since it is quick and simple to perform, demands a minimal amount of samples and no specialized equipment.[29] The development of a clear zone on the oil surface is a distinctive property of surfactants that, along with surface tension, serves as an indication of the ability of biosurfactant-producing bacteria.[30]

The isolate *E.faecium* showed the highest surface tension and Emulsification index 24% at 37°C with incubation time 48 hrs. (36 mN/m and 62% respectively).

According to Kachrimanidou et al.,[31] after 32 hours, Limosilactobacillus fermentum ACA-DC 0183 showed the highest surface tension reductin (34.9 mN/m) among the Lactobacillus strains being studied.

Ghasemi et al.,[32] reported that the Biosurfactant produced by lactic acid bacterium *Pediococcus dextrinicus* SHU1593 is able to emulsify n-hexadecane (58%) and kerosene (56%) Figure 2.

According to Bhosale et al.,[33] acetone was investigated as a potential biosurfactant purification agent, and it is a quick and cost effective procedure that doesn't require specialist equipment.

In this study, chemical analysis of biosurfactant produced by *E. faecium* characterized as Glycolipoprotein and indicate

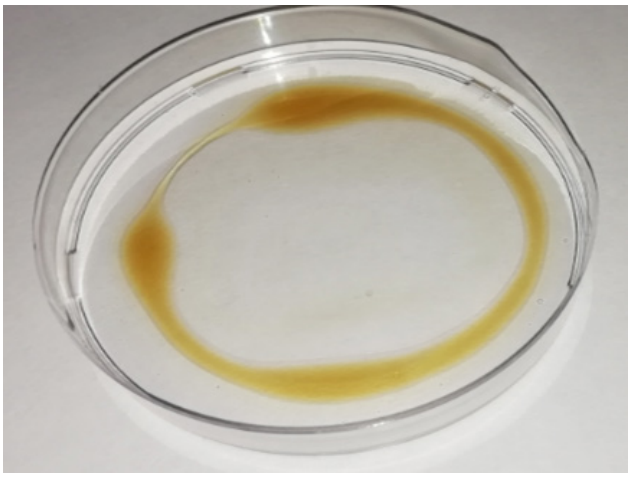

Fig. 1 **The spreading of partial biosurfactant produced by *E. faecium* on Oil surface.**

that the carbohydrate content of purified biosurfactant was 19.7% according to Dubois standard curve. According to Anschau standard curve the lipid content was 33% and the concentration of the protein was 28.5%, according to an analysis of the protein's absorbance using the Bradford standard curve. These results compared with Chaurasia et al.,[34] who reported that biosurfactant produced by *E. faecium* LM5 consisted of Lipid and peptides.

While Lara et al.,[34] mentioned that the biosurfactant produced by *Lactiplantibacillus plantarum* Tw226 consisted of protein as the main part (64%), then Lipids (21%), and carbohydrate (21%).

Hippolyte et al.,[36] demonstrated that the major component of biosurfactant produced by *Lactobacillus paracasei* subsp. tolerans was the protein part with ratio of (63%), carbohydrate (35%), and lipid (1%).

FTIR spectroscopy was used to characterise the molecular structure of the biosurfactant produced by *E. faecium* in MRS-Lactose broth. The signal in the band of 1064.71 cm^{-1} in the FTIR spectra Figure 3 revealed the existence of amine moieties in proteins, furthermore displayed a broad band at (3346 cm^{1}) this might be attributable to the polysaccharide O-H groups. This conclusion was consistent with Khademolhosseini et al.,[37] finding that polysaccharides of a biosurfactant made by Pseudomonas aeruginosa were present as shown by the presence of a -OH group at a wide peak of 3336 cm^{-1} and

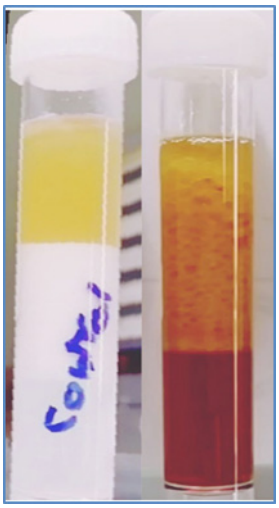

Fig. 2 **The emulsion form of *E. faecium* A: phosphate buffer B: *E. faecium* supernatant.**

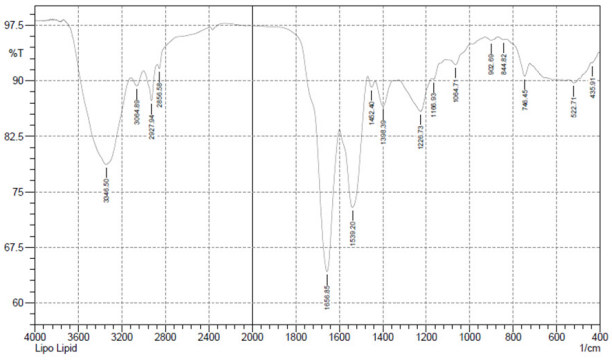

Fig. 3 **FT-IR of partially purified biosurfactant produced by *Enterococcus faecium*.**

then another band at (2927 cm^{-1}). This might be due to the C-H (CH3) group, Kanakdande et al.,[38] also were showed a band found at 2958 and 2927 cm^{-1} indicated C–H (CH3) and (CH2) stretching, which most likely associated with the lipopeptide portion of the molecule of Staphylococcus nepalensis (KY024500) biosurfactant. The (1656 cm^{-1}), 1539 cm^{-1}, and 1452 cm^{-1} peaks in the FT-IR spectra (figure) may be identified to the C=O, N-H, and C-N, respectively. According to Satpute et al.,[39] the slight peak at 2851 cm^{1} indicates the presence of a C-H bond. Both the peak at 1666 cm^{1} and the peak at 1550 cm 1 indicate the existence of -NH stretching, which both indicate the presence of proteins.

By using GC-MS, the existence of fatty acids in the biosurfactant produced by *Enterococcus faecium* ES7 was identified. As shown that biosurfactant comprises a of a percentage (65.41%) of Palmatic acid (C16H32O2), Heneicosane (C21H44), Benzoic acid (C7H6O2), 4-n-Hexylthionic s,s-dioxid (CC11H22O2S), Pentacosane (C25H52), Ethyltridecanoate (C15H30O2), Nonadecane (C19H40), hexadecanoic acid methyl ester (1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine C17H34O2) and octadecanoic acid methyl ester (C19H38O2). In addition, several other fatty acids have a lesser ratio. A recent study[40] also reported that Hexadecanoic acid, Octadecadienoic acid, Octadecenoic acid, Methyl stearate, Eicosanoic acid were the major components for the biosurfactant isolated from Brevibacterium casei, Sen et al.,[41] observed the gas chromatography and mass spectroscopy details of the fatty acid was 11hydroxyundecanoic acid (C11:0), tridecanoic acid (C13:0), pentadecanoic acid (C15:0), 16-hydroxyhexadecanoic acid (C16:0), 9,12-octadecadienoic acid (Z,Z)- (C18:2), and 17-hydroxyoctadecanoic acid (C18:0).

Cytotoxicity of Biosurfactant

Biosurfactant's cytotoxicity was assessed against (MCF-7) breast cancer cell line and (NHF) Normal human derived adipose tissue cell line. Five concentration of Biosurfactant were tested against MCF-7 and compared with the impact of biosurfactant on the NHF cell lines. Maximum cytotoxicity was recorded with the MCF-7 cell line at a dose of 100 g/ml, followed by a 72 hour incubation period. This resulted in a 74.2% reduction in cell viability. On the other hand, the cytotoxicity of the NHF cells was only 45.5%.

In this current study we tested Glycolipoprotein biosurfactant produced by *E. faecium* as breast cancer therapy. The findings clearly show that increasing biosurfactant dosages causes higher levels of growth cell inhibition, the cytotoxicity rate against the studied MCF-7 cell line as shown in Figure 3.

The findings of the viability testing and the morphological observations demonstrated the high degree of selectivity of the glycolipoprotein biosurfactant from *E. faecium* Since just cancer cells dissociated from the biosurfactant-treated wells, but normal NHF cells did not. Similar findings from other studies for the selective use of Biosurfactant against cancer cells while having little or minimal impact on normal cells.[42] The total number of cells was found to have significantly decreased, most likely as a result of the presence of a detergent-like action that disrupts cell membranes, as in this case when applying biosurfactant.[43]

Even though the NHF cell line was treated with the biosurfactant shown in (Figure 4), conditions that stimulated a more significant rise in cell viability without membrane

damage evident by morphological visualization were exposure durations to the biosurfactant that did not affect cell viability, suggesting the biosurfactant's safety toward NHF cells Figure 5 and 6.

Many research on various cancer cell lines were conducted by inducing of apoptotic cell death. Early research conducted by Christova et al.,[44] showed that rhamnolipid biosurfactant suppressed the growth of BV-173 pre-B human leukemia cells.

Biosurfactants have been found to have anti-cancer properties. As an instance, Rahimi et al.,[45] in their study, it was discovered that isolated glycolipid biosurfactant from Pseudomonas aeroginosa stimulated cell death in breast cancer cells, and that the contact with the cell membrane was causing the most noticeable impact on the viability of the cells.

Thakur et al.,[46] reported that MCF-7 cells treated with rhamnolipids floated after losing contact with neighboring cells and becoming detached from the surfaces. The fact that these morphological alterations are signs of apoptotic cells led researchers to hypothesize that rhamnolipids could be able inhibit MCF-7 from proliferating.

Christova et al.,[47] established that A biosurfactant called Trehalolipid produced by *Nocardia farcinica* strain BN26 had antiproliferative action against human cancer cells, which subsequently resulted in inhibited viability of these cancer cells (BV-173, HL-60, KE-37, HL-60/DOX, and JMSU-1).

Recently, Waghmode et al.,[48] discovered that the biosurfactant produced from *Planococcus maritimus* (SAMP MCC 3013) that exhibited cytotoxic effect against the malignant Hela, HCT and MCF7, cell lines with Ic50 values of 41.41, 42.79 and 31.23 respectively.

According to Zhao et al.,[49] investigation, the lipopeptide under study at concentration of 100 µM totally kill (K562) cells by forming reactive oxygen species (ROS) in (K562) cells, this inhibits Bcl-2 expression, promotes the release of Cytochrome C, and ultimately results in apoptosis.

Wang et al.,[50] simply researched and reported the biosurfactant's mechanisms. Surfactant suppressed activity of the cyclin b1/p34Cdc2 and G2specific-kinase and caused p53 tumor suppressor accumulating and the cyclin kinase inhibitor p21waf1/cip1. These results indicate that biosurfactant induced the G2/M arrest of MCF7 cells by controlling the cell cycle factors in those cells. They also showed that surfactin promotes death in Hep-G2 cells via Reactive oxygen species-endoplasmic reticulum stress-Ca_2 extracellular signal-regulated protein kinase pathways.

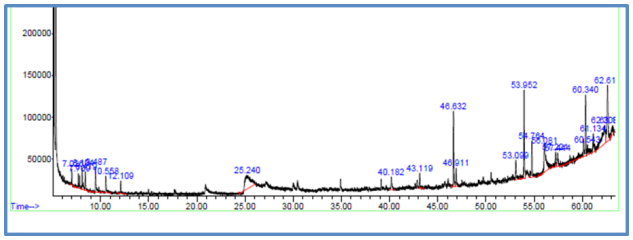

Fig. 4 **Gas Chromatography of biosurfactant from *Enterococcus faecium*.**

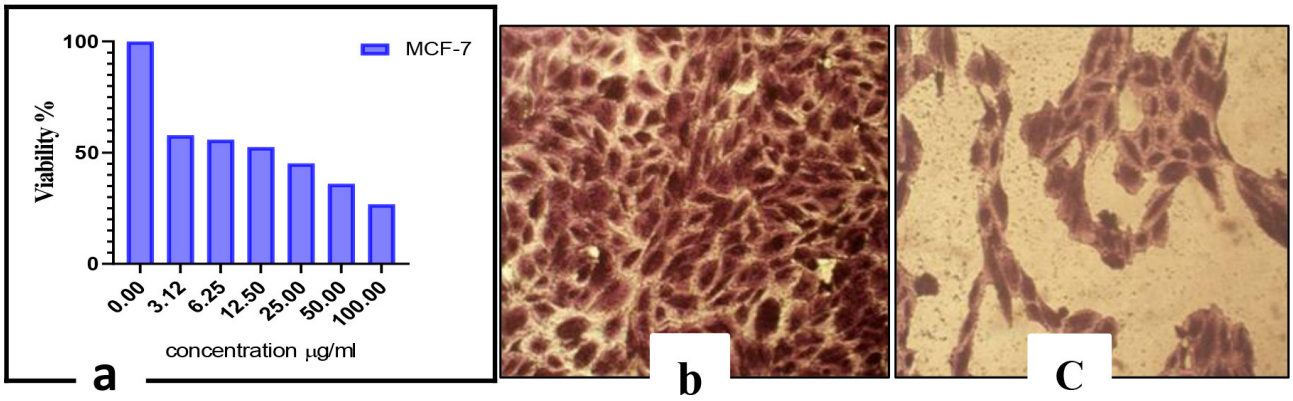

Fig. 5 **Biosurfactant treated MCF-7human breast cancer. They contain progesterone, estrogen receptors positive cell line, demonstrating good responsiveness to therapy in comparing to control non-treated cells. (a): the cytotoxicity assay, (b): Control non-treated cells under an inverted microscope, (c): the treated cells with biosurfactant under an inverted microscope.**

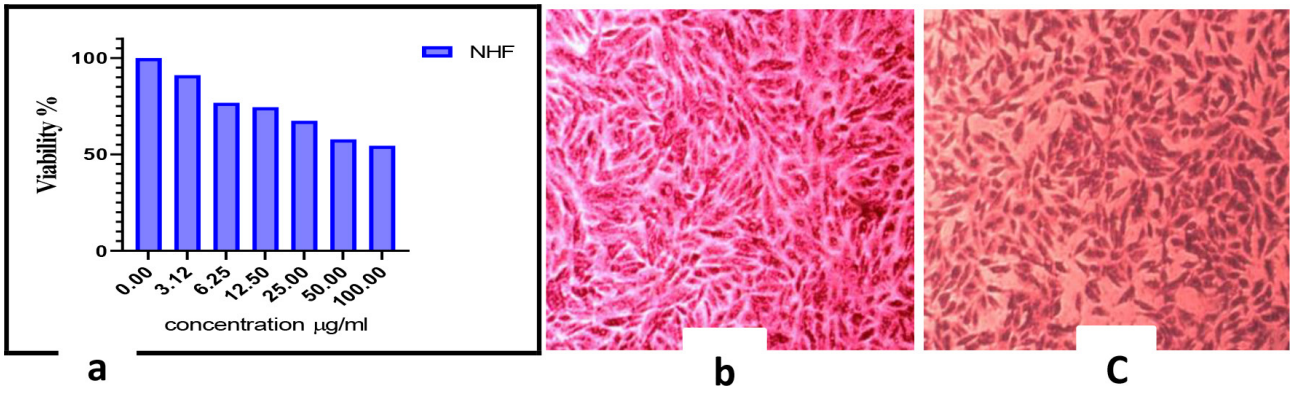

Fig. 6 **Normal human derived adipose tissue (NHF) cells treated with biosurfactant in vitro. (a): cytotoxicity assay, (b): the control cells under an inverted microscope.**

Conclusion

The Glycolipopeptide biosurfactant produced by *Enterococcus faecium* had anticancer effect against breast cancer cell line *in-vitro* and this study are highly encouraging in terms of biosurfactants' potential to cytotoxic for cancer cell lines and potentially for other medical applications; nevertheless, more investigation into their methods of action is needed. ■

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