



PRODUCTION, CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF A BIOEMULSIFIER PRODUCED BY *S.CEREVISIAE* UTILIZING EDIBLE OILS

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ABSTRACT

This study was designed to evaluate the ability of bioemulsifier to inhibit the growth of some pathogenic microorganisms. Commercially available *Saccharomyces cerevisiae* were tested for their ability to produce bioemulsifier. The production of bioemulsifier was detected by determination of emulsification index (E24%), qualitative drop-collapse test, emulsification activity (E.A) and Oil displacement. The results of bioemulsifier produced by *Saccharomyces cerevisiae* cultivated in Cooper and Paddock's broth containing different types of oils were: E24%= 66.6%, E.A= 184EU/ML and 1.8mm of oil displacement. Bioemulsifier extraction and solubilization was done by boiling (100° C) *S. cerevisiae* cells for 30 min in water, 0.067M phosphate buffer (PB) and 0.067M PB with 0.02M Na₂S₂O₅. The antimicrobial activity of extracted bioemulsifier was tested against some pathogenic microorganisms. Results revealed that bioemulsifier reduced the growth of bacteria (*Escherichia coli* and *Staphylococcus aureus*).

KEYWORDS: Bioemulsifier, Emulsification activity, Emulsification Index, Antimicrobial Activity.

INTRODUCTION

Bioemulsifier are a heterogeneous group of surface active molecules produced by microorganisms, which either adhere to cell surface or are excreted extracellularly in the growth medium. These molecules reduce surface tension in both aqueous solutions and hydrocarbon mixtures (Mulligan, 2005). Two closely related organisms that share the same species and same genus can produce different types of bioemulsifier isoforms with different physicochemical properties which help the microorganism to survive (Ron and Rosenberg 2001). The hydrophilic moiety is comprised of acid, peptide cations or anions, mono di or polysaccharides while hydrophobic moiety can be unsaturated or saturated chains of fatty acids (Maneerat and Dixit, 2007). Mannoprotein has been shown to be an effective bioemulsifier. The presence of hydrophilic mannose polymers covalently attached to the protein backbone provides the mannoprotein with the amphiphilic structure common to surface active agents and effective emulsifiers (Cameron *et al.*, 1988). In the yeast, *Saccharomyces cerevisiae*, the cell wall contains β (133)-D-glucan, β (136)-D-glucan, chitin, and mannoprotein (Cabib and Roberts, 1982). The polysaccharides appear to have a structural function, whereas the mannoprotein may act as "filler" and are important for the permeability of the cell wall (Zlotnik *et al.*, 1984).

β –glucan has attracted attention because of its bioactive and medicinal properties as immune stimulating (Vetvicka *et al.*, 2008), antimicrobial, anti-infective, anti viral, wound-healing (Bohn and BeMiller, 1995). β –glucan is known as a potent immunostimulant and has significant augmenting effects on the host defense system (Yadomae and Ohno, 1996). Besides, β –glucan is known to process antimicrobial and antitumor activities by enhancing host immune function, and activating macrophages, neutrophils and NK cells by binding to the β –glucan receptor on these cells (Bohn and BeMiller, 1995). β –glucan is a promising candidate as a immune – stimulatory agent for immune – compromised patients or those who are infected by multidrug-resistant bacteria (Yadomae and Ohno, 1996).

MATERIALS AND METHODS

Collection of Yeast

The commercially available yeast were collected from Departmental store. The samples were serially diluted (upto 10⁷) and 0.1ml from 10², 10⁴ and 10⁶ dilutions were spreaded on Yeast extract peptone dextrose agar using L-rod. The plates were incubated at 37°C for 48hours.

After incubation the colonies which showed typical morphology were selected. The pure strains of test organisms were obtained by streak plate method and

maintained on YPD slant for further studies (Deepa *et al.*, 2015).

Bioemulsifier Production from *Saccharomyces cerevisiae*:

Bioemulsifier production were carried out in 250 mL Erlenmeyer flasks containing 50 ml of Cooper and Paddock's medium. One ml of the seed culture was added and the flasks were incubated at ambient temperature with shaking for 4 days. Cells were harvested by centrifugation at 3000 rpm for 10 min. (Cameron *et al.*, 1988)

SCREENING OF BIOEMULSIFIER

Drop Collapse Test

Bio emulsifier production was screened using qualitative drop collapse test. Mineral oil was added to 96 well micro titrate plates. The plate was equilibrated for 1 hour at 37°C and 5ml of culture supernatant was added to the surface of the oil in it. The shape of drop on the oil surface was observed after one minute. Distilled water was used as negative control. (Suresh Chander *et al.*, 2004)

Oil Displacement Test

50ml of distilled water was added to the large petri-plate followed by 20µl of test oil. 10µl of culture supernatant was then added to the surface of oil. The diameter of the lysis was measured and compared with 1% sodium dodecyl sulfate. (Udayashree *et al.*, 2015).

Emulsification Assay

The cells were separated by centrifugation at 10,000 rpm for 15 min at 30°C. Three ml of the cell free culture broth was mixed with 0.5 ml of test oil, vortexed for 2min and incubated at 30°C for 1 hour for the phase separation. Aqueous phase was removed carefully and absorbance was recorded at 400 nm. Sterile LB broth was used as blank. Emulsification assay was expressed in terms of emulsification activity per ml (EU/ml).

Emulsification Index

The 24 hours grown culture of the two strains (*Pseudomonas fluorescens* and *bacillus subtilis*) was used for the emulsification index. The cells were separated by centrifugation at 10,000 rpm for 15min at 30°C. 2ml of the cell free culture broth was mixed with 2.0 ml of the test oil, vortexed for 2min and incubated for 24 hours. After 24 hours the emulsification layer was measured and percent emulsification index was calculated by the following equation. The E24 index is given as percentage of height of emulsifier layer divided by total height of the column multiplying by 100.(Letizia Fracchia *et al.*,2012).

Purification and Extraction of Bioemulsifier from *Saccharomyces cerevisiae*

The extraction and production was carried out at an optimized condition for 48 hours and the bacterial cells were removed by centrifugation at 10,000 rpm for 20

min under cooling condition. Bioemulsifier extraction and solubilization was done by boiling (100°C) *S. cerevisiae* cells for 30 min in water, 0.067M phosphate buffer (PB) and 0.067M PB with 0.02M Na₂S₂O₅. The cells were then centrifuged at 3,000 rpm for 10 min and the supernatant was assayed for emulsification activity. After extraction process, the precipitate was obtained. This precipitate was dissolved in maximum 3ml of chilled sterile distilled water and dialyzed extensively against sterile distilled water at room temperature for 48° C. Distilled water was changed after 12h. (Cameron *et al.*, 1988).

SDS-PAGE for Molecular Weight Determination

Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) was performed on a 5% stacking and a 12% separating gel according to the method of Laemmli (1970), to determine the molecular weight of purified protein. The molecular weight of the purified protein was determined in comparison to marker protein (standard protein marker, 14.4 - 116 kDa) after documentation.

ANTIBACTERIAL ACTIVITY OF BIOEMULSIFIER

Agar well diffusion technique (Perez *et al.*, 1990)

The extract was tested for antibacterial activity by standard agar well diffusion method against pathogenic bacteria *E.coli* and *S.aureus*. The pure culture of bacterial pathogens was sub cultured on nutrient broth. 20ml of nutrient agar were poured into the Petri plates. Wells of 6mm diameter were made on nutrient agar using gel puncture. Culture was swabbed uniformly using sterile cotton swabs, and then 50µl of 10% of bioemulsifier extract along with 0.004 µg/ml of tetracycline solution was loaded into the wells. After incubation at 37°C for 24 hours, the different levels of zone of inhibition were measured.

Minimum inhibition concentration (MIC) – Micro dilution (Kumarasamy *et al.*, 2002)

Minimum inhibition concentrations were done to determine the lowest concentration of the 10% extract of bioemulsifier with 0.004 of tetracycline µg/ml, where it can show the bactericidal and bacteriostatic effect. The test was performed in 96 well microtiter plates. Microtiter plate wells from each column in row 1 were marked and 100µl of 10% extract of bioemulsifier along with 0.004µg/ml tetracycline were added. 50µl of sterile distilled water was added to rows 2-12. Two fold serial dilutions were performed by transferring 50µl of solution from row 1 to row 2, using a multichannel pipette. This was repeated down the row 2 to 12. 50 µl of double strength nutrient broth and 20 µl of bacterial culture was added to all the wells in separate column, so the final concentration of the inoculum in all the wells. To prevent dehydration, the plates were covered with a plastic cover and then incubated at 37°C for overnight. The bacterial growth was determined after addition of 50µl of 2, 3, 5 Triphenyl tetrazodium chloride red (0.02mg/ml). The

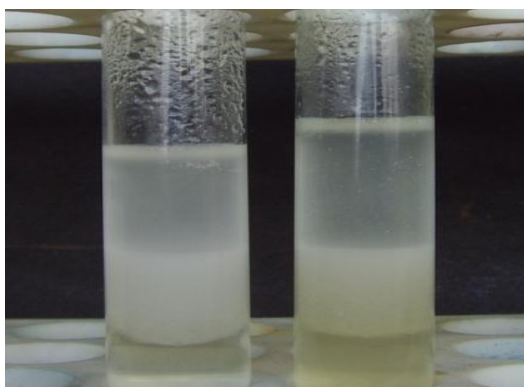
Minimum inhibition concentrations (MIC) of the isolates were taken as the lowest concentration of the antibiotic of which the bacterial tested did not show visible growth.

RESULT AND DISCUSSION

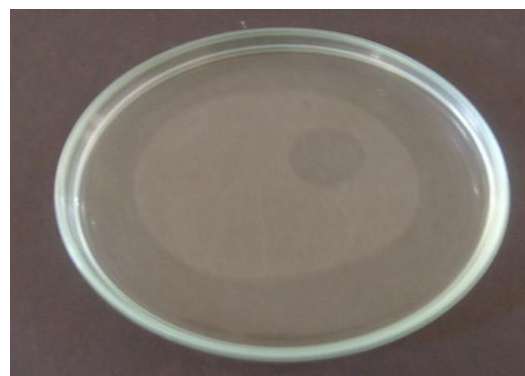
An initial screening of bioemulsifier producing isolates were grown in Yeast peptone dextrose broth supplemented with oils and then tested for qualitative drop collapse. Only one yeast isolates were showed good bioemulsifier activity and was selected as potent strain and used for further study.

Screening of Bioemulsifier

Bioemulsifier production is growth associated. The yeast strain of *Saccharomyces cerevisiae* produced bioemulsifier when it was grown in Yeast extract peptone dextrose broth containing oil. The highest emulsification index (E24%) value on the coconut oil (66.60%) followed by olive oil (63.66%). The maximum bioemulsifier was produced from *Saccharomyces cerevisiae* in the presence of coconut oil and olive oil, and had an E.A for both oils as 184 EU/ml and 123 EU/ml. The results of drop collapse in microtitre plate for *Saccharomyces cerevisiae* showed a large spread in coconut oil for the droplet in compares with others oils this indicates that production of bioemulsifier reduced the surface tension leading to droplet collapsing. The oil displacement method for Bioemulsifier produced by *Saccharomyces cerevisiae*. These isolates were exhibited oil displacement activity. Maximum oil displacement was obtained in coconut oil (1.8mm) followed by olive oil (1.3mm). Javed Maniyar *et al.*, 2011 revealed that the Bioemulsifier production from *Streptomyces* sp.S22 was induced by sunflower oil and their bioemulsifier activity increased from 145 EU/ml to 320 EU/ml. Mbawala and Mouafo, 2012 showed that the eight strains of *Lactobacillus* spp. have showed a positive result in emulsification test. The highest potential of emulsifying activity (% E24) of 56.80 was obtained from the fermentation broth of the TM1 isolate. Jyotsna and Dhirendra, 2014 observed that the *P. fluorescens* showed highest oil displacement for soybean oil (2.5mm) followed by 2mm for mustard oil, 1.5mm for olive oil and followed by 0.7mm for sunflower oil and coconut oil.

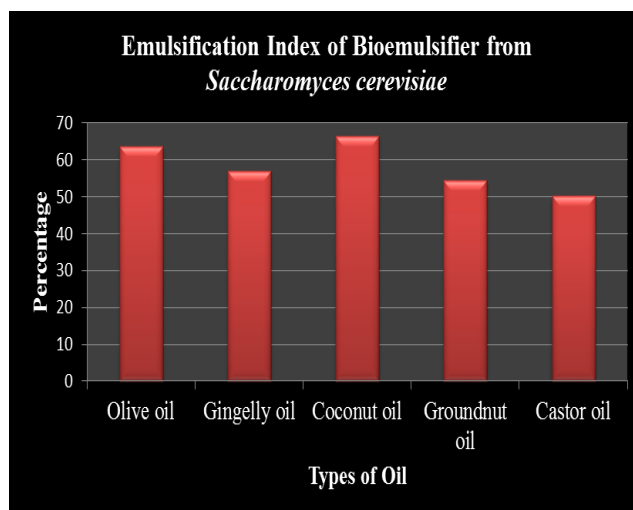


Emulsification Index



Oil displacement

Figure - 1 Screening Methods of Bioemulsifier Produced by *Saccharomyces cerevisiae*.



Extraction of Bioemulsifier

The extraction of *Saccharomyces cerevisiae* using $\text{Na}_2\text{S}_2\text{O}_5$ is a reducing agent known to increase bioemulsifier yield and prevent oxidation. Heat extraction solubilizes the mannoproteins in the outer layer of the cell wall. After extraction of component can be further purified by using dialysis method.

SDS PAGE Gel electrophoresis

The purified enzyme was resolved on a SDS-PAGE (5% stacking and 12% separating gel) found to be a homogenous monomeric protein as evident by a single band corresponding to 58 kDa for *S.cerevisia* on SDS-PAGE relative to the standard molecular weight markers. Matthew Ilori and Dan-Israel Amund, 2001 reported that the purified bioemulsifier of *P. aeruginosa* subjected to a SDS PAGE. A single SDS - PAGE band was obtained from the purified protein complex preparation. The apparent molecular weight was estimated as 14,322 Da.

Antibacterial activity of Bioemulsifier

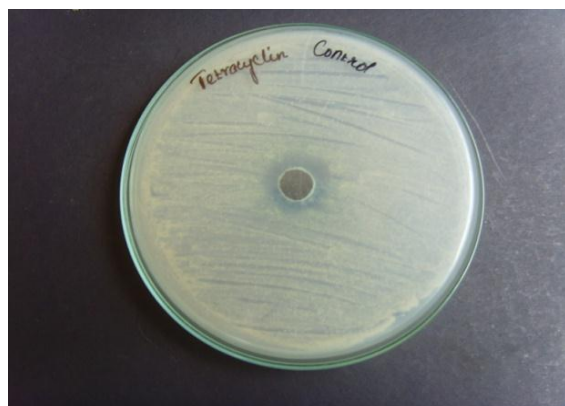
Agar well Diffusion Method

The antibacterial activity of 10% of extract of bioemulsifier with 0.004µg/ml of tetracycline were investigated by agar well diffusion method against *Escherchia coli* and *Staphylococcus aureus* pathogen. The maximum zone of inhibition were obtained in

bioemulsifier from *Saccharomyces cerevisiae* produce the zone of (25mm) against *Escherichia coli*. The minimum zone of inhibition were obtained in bioemulsifier from *Saccharomyces cerevisiae* produce (12mm) against *Staphylococcus aureus* while compared to the control of tetracycline produce the zone of (15mm, 07mm) , so hereby concluded that the maximum zone were obtained in synergistic study were shown in (Table -2) . Abalos et al., 2001 reported that the rhamnolipid mixture obtained from *P. aeruginosa* showed inhibitory activity against the bacteria (*Escherichia coli*, *Micrococcus luteus* and *Alcaligenes faecalis*, *Serratia marcescens*, *Mycobacterium phlei* and *Staphylococcus epidermidis*) and fungi (*Aspergillus niger*, *Chaetomium globosum*, *Enicillium crysogenum*, *Aureobasidium pullulans*, *Botrytis cinerea* and *Rhizoctonia solani* in various concentrations. The present study also clearly demonstrated the antimicrobial effect of both the bacterial and yeast strains such as, *Pseudomonas aeruginosa* and *Saccharomyces cerevisiae* against several microbial pathogens. It is suggested to use this compounds.



Antibacterial activity of Bioemulsifier



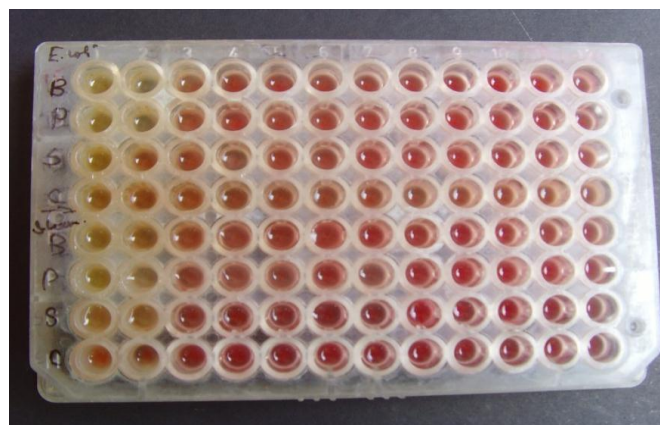
Antibacterial activity of Antibiotic

Table: 2 Synergistic Effect of Extract of bioemulsifier with Tetracycline

S.No	Isolates	Zone of Inhibition in mm	
		<i>S.cerevisiae</i>	Tetracycline
1	<i>E.coli</i>	25	15
2	<i>S.aureus</i>	13	07

Minimum Inhibitory Concentration

Best minimum inhibitory concentration was recorded in bioemulsifier from *Saccharomyces cerevisiae* (25µg/ml) and control of tetracycline (50µl) concentration against the *Escherichia.coli* and *Staphylococcus. aureus*. Xiangyang Liu et al., 2012 showed that C14-surfactin and C15-surfactin had synergistic antifungal activities with KTC against *Candida albicans* at 12.5 mg/mL and 6.25 mg/mL, respectively. Based on these MIC values, the FICIs of C14- surfactin and C15-surfactin were less than 0.4 and 0.3 (all of them were less than 0.5), respectively, so the combination of surfactins and KTC were synergistic.



Minimum Inhibitory Concentration of Bioemulsifier

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