

Enhanced Biodegradation of Lindane Using Oil-in-Water Bio-Microemulsion Stabilized by Biosurfactant Produced by a New Yeast Strain, *Pseudozyma* VITJzN01

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Organochlorine pesticide residues continue to remain as a major environmental threat worldwide. Lindane is an organochlorine pesticide widely used as an acaricide in medicine and agriculture. In the present study, a new lindane-degrading yeast strain, *Pseudozyma* VITJzN01, was identified as a copious producer of glycolipid biosurfactant. The glycolipid structure and type were elucidated by FTIR, NMR spectroscopy, and GC-MS analysis. The surface activity and stability of the glycolipid was analyzed. The glycolipids, characterized as mannosylerythritol lipids (MELs), exhibited excellent surface active properties and the surface tension of water was reduced to 29 mN/m. The glycolipid was stable over a wide range of pH, temperature, and salinity, showing a very low CMC of 25 mg/l. Bio-microemulsion of olive oil-in-water (O/W) was prepared using the purified biosurfactant without addition of any synthetic cosurfactants, for lindane solubilization and enhanced degradation assay in liquid and soil slurry. The O/W bio-microemulsions enhanced the solubility of lindane up to 40-folds. Degradation of lindane (700 mg/l) by VITJzN01 in liquid medium amended with bio-microemulsions was found to be enhanced by 36% in 2 days, compared with degradation in 12 days in the absence of bio-microemulsions. Lindane-spiked soil slurry incubated with bio-microemulsions also showed 20–40% enhanced degradation compared with the treatment with glycolipids or yeast alone. This is the first report on lindane degradation by *Pseudozyma* sp., and application of bio-microemulsions for enhanced lindane degradation. MEL-stabilized bio-microemulsions can serve as a potential tool for enhanced remediation of diverse lindane-contaminated environments.

Keywords: Biodegradation, bio-microemulsion, glycolipids, lindane, *Pseudozyma* sp.

Introduction

The continuing increase in the application of pesticides for agricultural pest control and pharmaceuticals represents the major source of organochlorine pesticides (OCPs) in the environment. Soil contaminated with lindane (γ -HCH) is a major concern throughout the world because of its long persistence, high toxicity, and low aqueous solubility. Although the application has been banned or restricted in many countries, lindane residues still exist in the water and soil environments [33]. Bioremediation is established as a cheap and environmentally friendly technology to remove

toxic pollutants like lindane from the environment, in which microorganisms or their products are used to degrade and detoxify this chlorinated organic pollutant. However, the hydrophobicity and low aqueous solubility of lindane results in very slow and frequently unsatisfactory biodegradation. Therefore, a fundamental issue for the bioremediation processes is to overcome the limited accessibility of these hydrophobic pollutants for the microorganisms [34].

Use of surfactants is a promising method to improve the effectiveness of bioremediation of pesticides [9]. Biosurfactants are a group of amphipathic surface-active compounds produced

by living systems. They are made of different chemical structures involving phospholipids, glycolipids, lipopeptides, and polymeric surfactants [29]. Recently, biosurfactants are receiving increasing attention due to their unique properties such as (i) changing surface active phenomena, like lowering of surface and interfacial tensions, (ii) wetting and penetrating properties, (iii) hydrophilicity and hydrophobicity actions, (iv) metal sequestration, and (v) antimicrobial activity [15]. They can enhance the degradation by two ways. First, they increase the bioavailability for microorganisms. Second, they interact with the cell surface and allow the hydrophobic substances to attach more readily with microbial cells. Thus, addition of a biosurfactant is expected to enhance the pesticide degradation by mobilization, solubilization, or emulsification [23]. Recently, studies were performed to enhance the degradation of HCH isomers by bacterium *Sphingomonas* sp. NM05 through the addition of surfactants [17]. Microemulsions are transparent, thermodynamically stable, isotropic liquid mixtures of oil, water, and surfactant with or without a cosurfactant. The word bio-microemulsion is used to describe a microemulsion prepared with oil and biological surfactants, rather than synthetic surfactants. Recent advances in the field of environmental pollution monitoring have led to the application of microemulsions in bioremediation of hydrophobic pollutants. Microemulsions were used to enhance the bioavailability of OCPs and improve the bioremediation of soils contaminated with OCPs, including DDT and γ -HCH [36]. The microemulsions formed with non-ionic surfactants along with cosurfactants and plant oils for enhanced degradation of OCPs (DDT and γ -HCH) were described earlier [33–36]. So far, there is no report on the role of biosurfactants of yeast origin and O/W microemulsions formed with biosurfactants for enhancement of pesticide degradation.

Hence, in the present study, the yeast strain *Pseudozyma* sp. isolated from a pesticide-contaminated maize field was evaluated for its ability to produce biosurfactants and utilize lindane as the sole carbon and energy source. The biosurfactant, characterized as a glycolipid, mannosylerythritol lipid (MEL), was used to prepare stable O/W bio-microemulsions. The MELs exhibited extreme stability, impressive surfactant activity, and outstanding capacity to form bio-microemulsions for enhanced degradation of lindane. To the best of our knowledge, the present study is the first report that describes the potential of the yeast belonging to *Pseudozyma* sp. to degrade lindane. The application of the biosurfactant produced by this yeast in the formation of a bio-microemulsion of O/W for the

enhanced degradation of lindane at high concentration in both liquid medium and soil slurry is also a new approach.

Materials and Methods

Materials

Lindane was purchased from Sigma-Aldrich (USA), and chloroform and methanol (HPLC grade) were procured from SRL Chemicals India Ltd. All the media components were obtained from Hi-Media Chemicals (Mumbai, India) and used without further purification. MilliQ water was used for all the experiments.

Isolation, Cultivation, and Identification of Yeast Strain

The yeast strain used in the study was isolated from maize cultivation fields from Katpadi (12.98°N 79.13°E) by the enrichment method. Lindane (10 mg/l), an organochlorine pesticide with aqueous solubility of 7 mg/l, was used as the sole carbon and energy source to enrich the Mineral medium (MM) that contained (per liter) ammonium sulfate, 5 g; potassium dihydrogen phosphate, 1 g; dipotassium hydrogen phosphate, 2 g; magnesium sulfate, 0.5 g; sodium chloride, 0.1 g; manganese chloride, 0.01 g; ferrous sulfate, 0.01 g; and sodium molybdate, 0.01 g; at pH 7.2 ± 0.5. Enrichment was carried out for three times by increasing the concentration of lindane finally to 100 mg/l. The enrichment cultures were incubated at 28 ± 2°C for 7 days on a rotary shaker (100 rpm) each time. In addition, the lindane plate clearance assay was used to identify lindane-degrading yeast colonies. The obtained lindane-degrading yeast was named VITJzN01. A pure culture of the yeast was made by serial subculture. The strain was maintained in glycerol stock stored at –80°C. The working cultures were maintained at 4°C on yeast extract peptone dextrose (YEPD) agar slants with lindane (100 mg/l) and subcultured every fortnight.

The taxonomic identification of VITJzN01 was done by partial 18S rDNA, ITS1/2, and D1/D2 domain sequence analysis. Nuclear DNA extraction from the yeast was carried out by the method of Cheng and Jiang [4]. The partial rDNA sequences were amplified by PCR using the primer pair UL18F: 5'-TGTACACACCGCCCGTC-3' and UL28R: 5'-ATCGCCAGTTCTGCTTAC-3'. The BLASTN program was used for the similarity search of the rDNA sequences in the database available on the GenBank website (<http://www.ncbi.nlm.nih.gov/>). Multiple sequence alignments were made using the CLUSTAL X program and the sequences were deposited in GenBank under Accession No. JX454447. Phylogenetic and molecular evolutionary analyses were conducted by MEGA 5.1 software with the Kimura 2-parameter model and neighbour-joining algorithm [30].

Screening Methods for Biosurfactant Production

The screening of the yeast isolate for biosurfactant (BS) production was done following different methods, such as hemolytic assay, lipase production, drop collapse test, and oil displacement test [10].

Production and Purification of BS

To produce crude BS, seed culture was prepared by transferring the yeast colonies into the MM supplemented with 2.0% (w/v) dextrose. The culture was incubated at 30°C in a shaking incubator for 48 h. Additionally, 2% (v/v) of seed culture was transferred into 250 ml of basal medium, which was MM supplemented with 2% olive oil, and incubated at 30°C and 150 rpm for 7 days to obtain the highest cell and surfactant concentration. For isolation of the BS, the culture supernatant containing BS was separated from the cells by centrifugation at 15,000 ×g for 20 min. The BS was precipitated by adding an equal volume of ice-cold acetone into the cell-free supernatant and mixing thoroughly. The resulting white precipitate was collected by centrifugation (5,000 ×g for 15 min). The extraction procedure was repeated three times to get the maximum BS from the supernatant. The precipitated BS was dissolved in Milli-Q water and finally lyophilized to obtain crude BS [26]. The lyophilized BS was dissolved with chloroform, and then purified by silica-gel chromatography using chloroform–acetone as reported previously [12]. The purified fraction of BS was used for further experimentation.

The purified BS was analyzed using thin layer chromatography (TLC) with a solvent system containing chloroform: methanol: 7N ammonium hydroxide (65:15:2 (v/v/v)) [20]. Visualization was done by spraying 0.2% of anthrone reagent on the TLC plate and heating it at 110°C for 5 min.

Structural Characterization

Fourier-transform infrared spectroscopy. The preliminary characterization of the BS was carried out by TLC as mentioned above. Further characterization of the glycolipid surfactant was performed by means of IR affinity-1 FT-IR spectrophotometry (Shimadzu). The spectrum of the lyophilized glycolipid was recorded over a range of 4,000 to 400 cm⁻¹ at a resolution of 4 cm⁻¹.

Nuclear magnetic resonance analysis. The structure of the major component of the purified BS was characterized by ¹H and ¹³C nuclear magnetic resonance (NMR) with a Varian INOVA 400 (400 MHz) at 30°C using the CDCl₃ solution.

Gas chromatography-mass spectrometry. Acid degradation was performed by mixing the purified BS (10 mg) with 5% HCl-methanol reagent (1 ml) overnight at room temperature. After the reaction was quenched with water (1 ml), the methyl ester derivatives of the fatty acids were removed with *n*-hexane [21]. The fatty acid profile of the purified BS was analyzed by gas chromatography-mass spectrometry (GC-MS) (Perkin Elmer-Claruss 680) with the temperature programmed from 60°C (held for 2 min) to 300°C at 10°C/min (held for 6 min).

Physicochemical Characterization

Biochemical analysis of total carbohydrates, proteins, and lipids. The total carbohydrate, protein, and lipid contents of the BS were determined by the anthrone-sulfuric acid method [6], Folins-Ciocalteu method [16], and rhodamine 6G method [2], respectively.

Determination of Emulsification Activity

The ability of purified BS to emulsify various hydrocarbons was evaluated and compared with a chemical surfactant, sodium dodecyl sulfate (SDS; 10%). Briefly, 4 ml of the purified BS (1 mg/ml) or SDS and 4 ml of the tested hydrocarbons (diesel oil, olive oil, kerosene, hexane, benzene, and chloroform) were mixed well with vortexing for 5 min. The mixture was allowed to stand for 24 h. The emulsification activity was expressed as a percentage of the height of the emulsion layer (cm) divided by the total height of the mixture.

Surface Tension Measurement

The surface tension of the BS was measured using a travelling microscope (BESTO, Model TM 11) as described by Munguia and Smith [22] with minor modifications. Briefly, 5 ml of the BS taken in a glass test tube was maintained at a constant temperature of 30°C in a water bath. The surface tension was calculated based on the increase in the height of the liquid ascending through the capillary tube when inserted into the surfactant solution. Distilled water was used as a control. The surface tension was derived from the following formula:

$$\gamma = \frac{r h \delta g}{2}$$

where γ is the surface tension (mN/m); δ is the density (g/ml); g is the gravity (980 cm/s²); r is the capillary radius (0.05cm); and h is the height of the liquid column (cm). The critical micelle concentration (CMC) was determined from the intersection of the linear part of the curve drawn between the surface tension and concentration of BS.

Stability Analysis

The stability of the BS at extremes of environmental parameters like pH (4–12), temperature (20–120°C), and salinity (0–40% NaCl) was studied using emulsification activity (E_{24}) against olive oil. All the experiments were done in triplicates to avoid errors.

Preparation of O/W Bio-Microemulsion with the Purified BS and Solubilization Experiments

Preparation of the O/W microemulsion was done as described by Worakitkanchanakul *et al.* [31]. To prepare the O/W microemulsion, a stock solution of the BS was prepared in chloroform and distributed into test tubes (about 0.25 g per tube). The solvents were completely removed under vacuum. Next, olive oil was added to the test tubes containing BS to obtain the BS-OO (biosurfactant-olive oil) mixtures with various ratios (10:1; 20:1). The mixtures were then vigorously agitated using a vortex mixer and temperature-cycled several times between 25°C and 70°C until thoroughly mixed. Finally, an appropriate amount of MilliQ water (10–100 ml) was added at various concentrations to the BS-OO mixture and mixed well using an ultrasonicator.

An appropriate amount of lindane dissolved in methanol was added to a 100 ml Erlenmeyer flask, making the final concentration

to 700 mg/l (100-fold higher than its aqueous solubility) of lindane in each flask. The flasks were kept in laminar air flow in order to evaporate the solvent. Then, 10 ml of the BS or the O/W bio-microemulsion was added to each flask. The final volume solution in each flask was made up to 50 ml with MM. The flasks were kept at 25°C in shaking condition (250 rpm) for 96 h for equilibration. After equilibration, filtration of the medium was done to remove undissolved lindane, followed by extracting twice with an equal volume of hexane:acetone (1:1). A 1 ml aliquot of the extract was taken to determine the concentration of dissolved lindane.

Biodegradation Assay in Liquid Cultures and Soil Slurry

Even though the yeast strain VITJzN01 is a natural biosurfactant producer, the yield was less in the culture medium provided with lindane as a substrate. Therefore, to understand the role of BS in lindane solubilization and utilization by the yeast already formed and purified, BS was added into the culture medium during the biodegradation assays. Biodegradation experiments in liquid cultures were performed in 250 ml Erlenmeyer flasks, for 10 days at 30°C and 120 rpm. Lindane at a concentration of 700 mg/l in methanol was added to the bottom of sterile Erlenmeyer flasks. The methanol was allowed to evaporate under the laminar hood. Then, 10 ml of the prepared bio-microemulsion was added into the flasks and the volume made up to 50 ml with sterile MM. The yeast inoculum was added into the flasks (0.1 g/l) and incubated at the above-mentioned conditions. Simultaneously, three control flasks (i) without bio-microemulsions, (ii) with only purified BS and the yeast inoculums, and (iii) MM with bio-microemulsion but without the yeast inoculum were maintained. Samples were withdrawn at regular intervals to check the residual lindane in cultures by GC-MS analysis as described earlier [25]. The experiments were done in triplicates to obtain the best results.

Sandy clay loam soil that had a history of pesticide application for more than 20 years was collected from the maize cultivation fields for the soil slurry experiments. The soil was passed through a 1.0 mm sieve to obtain a uniform particle size. The soil characteristics were as follows: pH 6.15; total organic matter 1.18%; nitrate nitrogen 15.1p.m.; available phosphorus 40.6 ppm; and exchangeable potassium 59 ppm. The soil was sterilized for further experiments. For the enrichment of soil with lindane, 500 g of the soil was spiked with 20 ml of pesticide solution (100 mg/kg) in methanol. The mixture was stirred vigorously for homogeneous distribution of lindane in the soil. The homogenized soil was spread on a plastic tray and kept under a laminar hood for 2 h to evaporate any traces of methanol. The final samples were stored in Erlenmeyer flasks, under refrigeration. Soil slurry was prepared by adding 20 g of lindane-enriched soil to a 250 ml flask containing 40 ml of MM and 10 ml of bio-microemulsion. The slurry was incubated with *Pseudozyma* VITJzN01 for 30 days. Samples were withdrawn at regular intervals for the analysis of residual lindane, yeast growth, and chloride assay. Individual flasks (i) with soil slurry spiked with lindane, (ii) with soil slurry and yeast without bio-

microemulsion or BS, and (iii) soil slurry with yeast and BS without bio-microemulsion were maintained as controls.

Results and Discussion

Isolation and Identification of Strain VITJzN01

In the present study, a selective enrichment technique was used to isolate lindane-degrading yeast from the maize field. The yeast with a clearance zone around the colonies on the lindane spray plate was confirmed as a lindane-degrading yeast and named as VITJzN01. The yeast isolate VITJzN01 formed umbonate pinkish circular, opaque colonies with curled margin on YEPD agar within 48 h of incubation (Fig. 1A). Microscopic examination revealed long, oval shaped, budding yeasts that appeared parallel to one another (Fig. 1B). Phylogenetic analyses of the 18S rDNA, ITS, and the D1/D2 sequences of strain VITJzN01 and other related strains, as per neighbor-joining alignment, is presented in Fig. 1C. The strain VITJzN01 revealed close

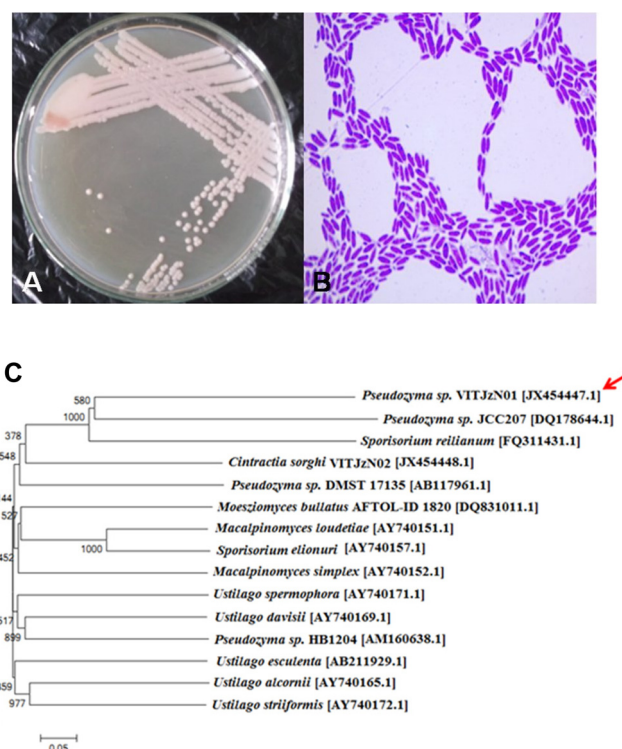


Fig. 1. Identification of the biosurfactant-producing yeast strain. (A) Colony morphology of *Pseudozyma* VITJzN01 on YEPDA plates after 48 h. (B) Microscopic view of the yeast under 100× objective of a bright field microscope. (C) Phylogenetic tree based on partial rDNA sequence showing the relationship of VITJzN01 with other closely related organisms. Accession numbers are provided in the parentheses. The scale bar represents 0.05 substitutions per nucleotide position.

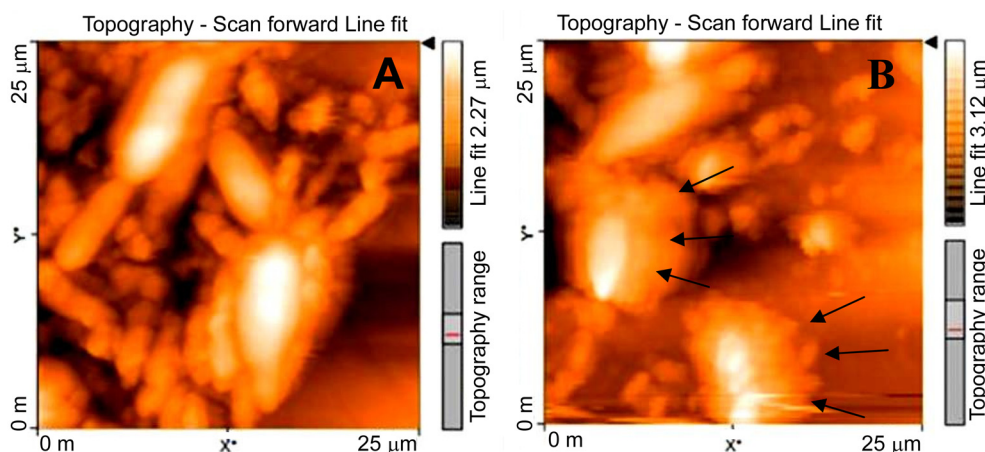


Fig. 2. Atomic force microscopic images of the biosurfactant-producing lindane degrader, *Pseudozyma* VITJzN01.

(A) Elongated budding yeast cell. (B) The yeast cell in the center surrounded by the biosurfactant on the extracellular matrix of the cell. The arrow mark denotes the produced biosurfactant around the yeast cell.

relatedness with soil basidiomycetes, belonging to class Ustilaginomycotina. The strain showed 99% identity and 58% coverage with strain *Pseudozyma* sp. JCC207 (Accession No. DQ178644.1). Therefore, it was identified and named as *Pseudozyma* sp. VITJzN01.

Screening of BS Production by VITJzN01

Preliminary identification of the BS production in *Pseudozyma* VITJzN01 was done by streaking on 5% blood agar. The strain produced a greenish zone of incomplete lysis (21 mm) around the colonies. Since the method alone is a poor indicator of biosurfactant production, the BS production was further confirmed by other tests, namely (a) clear halos of lipolysis on TBA (tributylin agar), (b) flat drops in the oil-coated microtiter plates, and (c) clearance zone of 415.6 mm² in the oil displacement test. The BS production was further visualized by AFM. Fig. 2 shows the budding yeast cell (A) and BS production and accumulation outside the yeast cells (B). As seen in the figure, the yeast cells have an elongated cell morphology. The ability of the yeast to produce BS in copious amount can be visualized from the AFM images. Youssef *et al.* [32] suggested the use of the drop collapse method as a primary method to detect BS producers, followed by the determination of the BS concentration using the oil spreading technique as a quick and easy protocol to screen and quantify BS production.

BS Production and Purification

The BS was produced from olive oil, by inoculating 2% (v/v) of the seed culture into the basal mineral medium and incubated at 30°C for 7 days. After the incubation

period, the surface tension of the basal medium was reduced from an initial value 68 mN/m to a final value of 38 mN/m, which was an indication of BS production in the medium. Similar observations were reported in the case of other BS-producing microorganisms [29]. This property of BS to reduce the surface tension of water clearly proves its potent surface-active nature.

The crude BS was extracted from the production medium using ice-cold acetone precipitation and gave a total yield of 8 g/l. The lyophilized crude BS was purified by silica gel column chromatography and visualized by TLC (data not shown). After charring the TLC plate at 110°C, brown spots were formed, which indicated the presence of glycolipids. Pure MEL compound (mixture of MEL a, b, and c) was used as a standard. Results showed that the BS from *Pseudozyma* had two fractions. The component that is less hydrophobic had an R_f value equal to that of MEL-b and the more hydrophobic component had R_f value equal to MEL-a. Therefore, the BS isolated from VITJzN01 might be a mixture of MELs similar to MEL-a and MEL-b.

Characterization of the BS from VITJzN01

The glycolipid nature of the BS was further confirmed by the IR spectra of the compound (data not shown). A sharp absorbance was seen with wave numbers 2,922.16 and 2,852.72 cm⁻¹. Absorbance in this region is caused as a result of C-H stretching vibrations, characteristic of carbon-containing compounds. Intramolecular hydrogen bonds also produce absorbance in this area. Other absorbance peaks were seen in the range of 1,737.86 and 1,724.36 cm⁻¹ corresponding to C-O-C vibrations in ester bonds in the

molecule. The presence of a peak at $1,649.14\text{ cm}^{-1}$ implies that the BS from *Pseudozyma* sp. had hydroxyl groups. Absorbance around the region $1,550\text{--}1,400$ signifies the presence of the C-H bond out of plane in the molecule. Adjacent to this peak was another broad intensity absorption peak at wave numbers $1,116.78\text{--}1,004.91\text{ cm}^{-1}$. Absorbance in these regions is because of the presence of C=O bonds and is caused by C=O stretching vibrations of the sugar component. Other significant peaks observed at $1,402\text{ cm}^{-1}$ together with 723.31 , $2,920$, and $2,852\text{ cm}^{-1}$ correspond to a long linear aliphatic chain, which is common in compounds with a lipid moiety. No absorption peaks corresponding to peptides were observed in the FTIR spectrum of BS. Thus, the glycolipid nature of the isolated and purified BS fraction was proved. The FTIR spectra of the compound were compared with the spectra of standard MEL glycolipid, and it also exhibited close similarity to the spectrum of glycolipids produced by other microorganisms [3, 8].

The NMR data of BS produced by VITJzN01 are summarized in Tables 1 and 2. The ^1H NMR spectrum of BS

Table 1. ^1H NMR data for BS produced by *Pseudozyma* VITJzN01 (CDCl_3 400 MHz).

Functional group	Chemical shift δ (ppm)
D-Mannose (Sugar)	
H-1'	4.78 s
H-2'	5.51 d
H-3'	5.08 dd
H-4'	5.26 dd
H-5'	3.72 m
H-6'	4.23 m
meso-Erythritol (Alcohol)	
H-1	3.72 m
H-2	3.72 m
H-3	3.72 m
H-4a	3.98 dd
H-4b	4.02 dd
Acetyl	
-CH ₃ (C-6')	2.12 s
(C-4')	2.04 s
(C-2')	2.18 s
Acyl (Fatty acid)	
-CH ₃	91 t
-(CH ₂) _n -	1.30 b
-CO-CH ₂ -(C-3')	2.38 t
-CO-CH ₂ -(C-2')	2.28 t

(s-singlet, d-doublet, dd-double double, t-triplet, m-multiplet, b-broad)

signified a typical glycolipid structure (Table 1). The chemical shifts of sugar alcohol corresponded with the pyranosylmannose and erythritol similar to the observation reported previously [13]. The ^1H spectrum showed that acetyl groups were linked to C-6', C-4', and C-2' positions. On the other hand, the ^{13}C spectrum indicated the presence of acetyl carbon at C-2' and C-4', as shown in Table 2. The total number of carbons was calculated as 10 in the sugar moiety. The presence of a long-chain aliphatic fatty acid group was evident in the ^{13}C spectrum. Similar chemical shifts were also observed in the NMR analysis of glycolipid MEL-a produced by *Pseudozyma rugulosa* NBRC 10877 [21]. Glycolipid producers isolated from sugarcane also showed

Table 2. ^{13}C NMR data of the BS produced by *Pseudozyma* VITJzN01 in CDCl_3 .

Functional group	Chemical shift δ (ppm)
D-Mannose (Sugar)	
C-1'	99.2
C-2'	70.5
C-3'	71.6
C-4'	65.3
C-5'	74.9
C-6'	63.5
meso-Erythritol (Alcohol)	
C-1	64.7
C-2	71.8
C-3	72.4
C-4	72.5
Acetyl (Fatty acid)	
-C=O (C-4')	171.5
(C-2')	171.8
-CH ₃ (C-4')	20.6
(C-2')	20.6
Acyl	
-CH ₃ (C-3')	14
(C-2')	14
-(CH ₂) _n -	22.3-31.6
-CH=CH-	124.5-132.1
-CH=CH-CH ₂	26
-C=O (C-3')	172.3
(C-2')	174.7
-CO-CH ₂ -(C-3')	34.4
(C-2')	35.4
-CO-CH ₂ -CH ₂ -(C-3')	26.8
(C-2')	27.1

Table 3. Fatty acid composition of BS produced by *Pseudozyma* sp. VITJzN01 from olive oil.

Fatty acid type	Composition (%)
C8:0	2.404
C10:0	5.485
C12:0	2.561
C14:0	14.832
C15:0	2.733
C16:0	47.039
C17:0	7.172
C17:1	5.328
C19:1	4.485

similar profiles for NMR chemical shifts [19]. Based on these results, it was confirmed that the BS produced by the isolated yeast *Pseudozyma* sp. VITJzN01 was a mixture of MELs.

To characterize the hydrophobic moiety in the BS, we investigated the fatty acid profile of these glycolipids. The fatty acid composition of the BS is presented in Table 3. Interestingly, unlike the already reported MELs, the major fatty acids in BS produced by *Pseudozyma* sp. VITJzN01 were C16:0 hexadecanoic acid (47%) and C14:1 tetradecanoic acid (14%). Previous reports on MEL described C8:0 and C12:1 to be the major components of the fatty acid tail [7, 21]. The presence of long-chain fatty acids contributes to the greater hydrophobicity and thus higher emulsification activity for the BS. From the above-mentioned structural analyses, the BS produced by *Pseudozyma* sp. VITJzN01 was identified to be a mixture made of two components, 4-O-[(4',6'-di-O-acetyl-2',3'-di-O-alka(e)nonyl)- β -D-mannopyranosyl]-

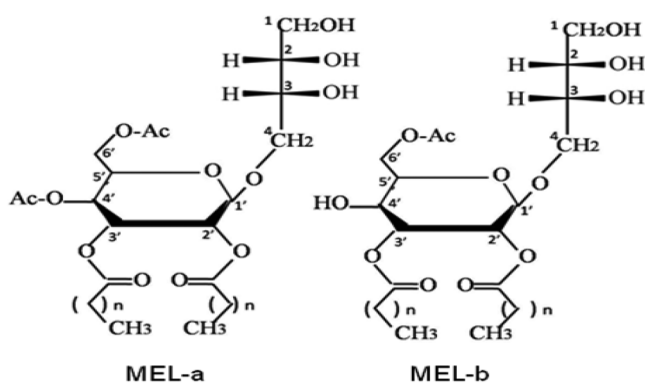
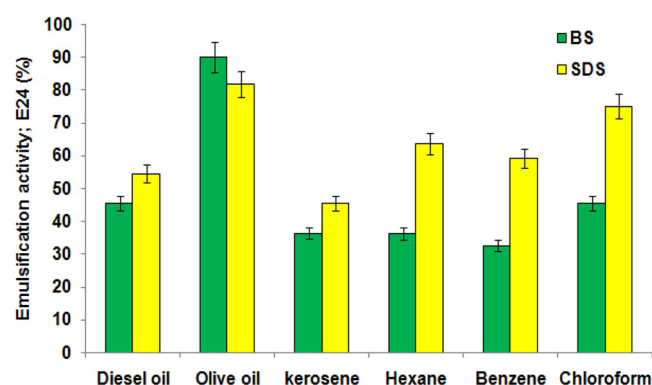
D-erythritol and 4-O-[(6'-O-acetyl-2',3'-di-O-alka(e)nonyl-4'-hydroxy)- β -D-mannopyranosyl]-D-erythritol; namely MEL-a and MEL-b as presented in Fig. 3.

Basidiomycetous yeast belonging to *Pseudozyma* sp. are well-known producers of MEL glycolipids from various substrates like *n*-alkanes [12] and soybean oil [14, 21]. A strain of *Candida* sp. SY16 was also reported to produce glycolipid homologous to MEL-b [11]. In the present study, potential lindane degrader *Pseudozyma* VITJzN01 produced a mixture of MELs from olive oil as substrate.

Physicochemical Characterization of BS

Biochemical analysis. The purified BS was analyzed for total carbohydrates, lipids, and proteins. The BS gave positive reactions for sugars with anthrone and for lipids with rhodamine reagent. A negative reaction was observed with Folin's reagent indicating the absence of proteins in the BS. The chemical composition of BS was determined as $34.47 \pm 0.5\%$ carbohydrates and $65.89 \pm 0.5\%$ lipids, reconfirming the glycolipid nature of the BS.

Emulsification activity, critical micelle concentration, and stability analysis. The BS produced by *Pseudozyma* VITJzN01 demonstrated stable emulsions with a number of hydrocarbons (Fig. 4). The E_{24} of the BS was compared with the E_{24} of chemical surfactant SDS. Among the tested hydrocarbons, olive oil ($E_{24} = 90\%$) was proved as the best substrate for emulsification in the case of the BS. In all other hydrocarbons, better emulsification was obtained with SDS. The lowest emulsification by the BS was observed when benzene was used as the substrate ($E_{24} = 32\%$). The glycolipid MEL-B produced by *Pseudozyma antartica* also showed a similar ability to emulsify soybean oil better than other hydrocarbons such as tetradecane, hexadecane, and

**Fig. 3.** Presumed structures of biosurfactants produced by *Pseudozyma* sp. VITJzN01 from olive oil ($n = 8\sim 16$).**Fig. 4.** Emulsification activity (E_{24}) of the purified biosurfactants produced by VITJzN01 compared with SDS. The error bars represent the mean \pm standard deviation.

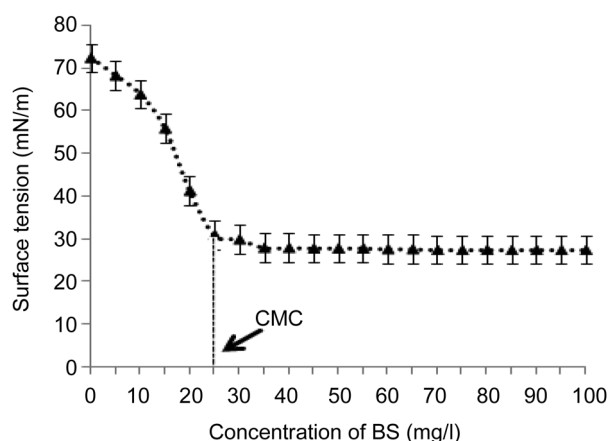


Fig. 5. The surface tension versus concentration of biosurfactant produced by VITJzN01 plot showing the CMC value.

cyclohexane [12]. The results showed the ability of the newly produced BS by the yeast *Pseudozyma* VITJzN01 to emulsify a wide range of aliphatic and aromatic hydrocarbons, and hence its applicability in oil recovery and bioremediation of oil-contaminated soil can be suggested.

The solubility of surfactants within the aqueous phase is defined by CMC and is used to measure the efficiency of the BS. The lower the value of CMC, the lower is the amount required to reduce the surface tension of water and hence indicating its higher efficiency [29]. In the present study, the BS produced by *Pseudozyma* VITJzN01 had a CMC of 25 mg/l when the surface tension of water was 29 mN/m (Fig. 5). This is a very small value when compared with the chemical surfactants such as SDS (6.8 mM) [18] as well as with other surfactants such as flavolipid produced by *Flavobacterium* (300 mg/l) [1] and crude biosurfactant produced by *Pseudomonas* SP4 (200 mg/l) [24]. Thus, the potential of the BS produced by *Pseudozyma* VITJzN01 for industrial and environmental application is depicted.

The stability of the BS at various pH, temperature, and salt concentration values was experimented using the emulsification activity (Fig. 6). The emulsification activity was retained over a pH range of 6–12 and it was highest around neutral pH. The lower activity in the low acid pH may be due to ionization of the functional group of the BS and precipitation. The BS showed excellent emulsification activity over a wide temperature range of 20–120°C, with a slight decrease in the E_{24} value at 120°C. The BS produced by VITJzN01 also showed stable emulsification even at a high salt concentration of 35%. This property of BS to show surface activity at high salt concentrations justifies its utility in marine environments and other hypersaline

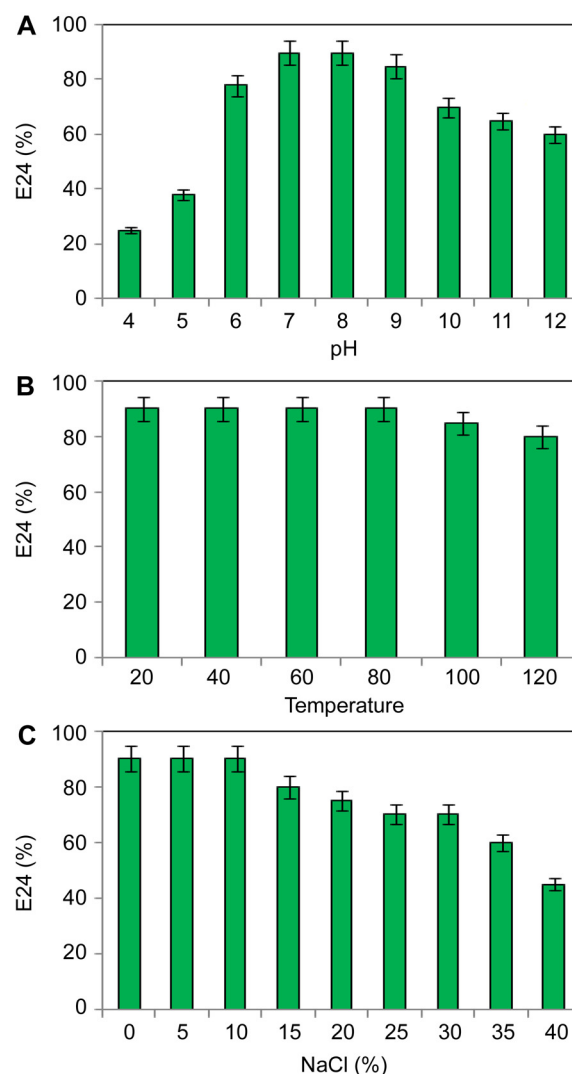


Fig. 6. The effects of (A) pH, (B) temperature, and (C) NaCl concentration on the emulsification activity of the biosurfactant produced by VITJzN01.

conditions. The tolerance of BSs to a wide range of pH, temperature, and NaCl was widely studied [5, 28].

O/W Bio-Microemulsion Preparation and Lindane Biodegradation Assays

Solubilization of lindane in O/W bio-microemulsion.

The BS and olive oil were mixed in different ratios to produce O/W bio-microemulsions with high energy sonication and it was found that both components were completely miscible at all ratios. This observation denotes the good hydrophobic property of the BS. Water was added into the BS-OO mixtures to obtain O/W bio-microemulsions. It was interesting to note that the addition of water up to 50 ml

did not affect the single-phase transparent look of the BS-OO mixture, which might be due to the formation of bio-microemulsions. On further dilution of the BS-OO mixture with water, turbidity was found, which indicated the solubilization equilibrium. The ideal ratio of BS-OO for bio-microemulsion formation was recorded as 10:1.

In the present study, lindane at a concentration 100-fold higher than its aqueous solubility was added into flasks along with O/W bio-microemulsion. Better solubilization of lindane was observed when the BS-OO ratio was 10:1 rather than 20:1. The role of oil phase to form bio-microemulsions that are able to solubilize lindane efficiently was well depicted. The solubilization of lindane was calculated based on the GC quantification of the residual lindane. The residual analysis of dissolved lindane was 40-fold higher (≈ 280 mg/l) than the aqueous solubility of lindane (7 mg/l) when BS-OO was 10:1. Worakitkanchanakul *et al.* [31] demonstrated the ability of glycolipid MEL produced from *Pseudozyma* sp. to form water-in-oil bio-microemulsions without using any cosurfactant [31]. Manickam *et al.* [17] reported that the aqueous solubility of lindane was enhanced 11-fold by the addition of 40–60 $\mu\text{g/ml}$ concentration of biosurfactant sophorolipid in a culture of *Sphingomonas* NM05. Sharma *et al.* [27] reported that BS produced by *Pseudomonas aeruginosa* WH-2 improved aqueous phase partitioning of HCH-muck. Our study showed that MELs from *Pseudozyma* formed stable bio-microemulsion with olive oil and water without the use of any cosurfactant. The bio-microemulsion was used for solubilization of lindane 100-folds above its aqueous solubility limit. Therefore, the present work proves the increased efficiency of O/W bio-microemulsions in the solubilization of lindane compared with the BS or chemical surfactants alone.

Degradation of Lindane in Liquid Cultures by *Pseudozyma* VITJzN01 in the Presence of O/W Bio-Microemulsions

Fig. 7A shows the effect of O/W bio-microemulsions and BS on the biodegradation of lindane by VITJzN01. After 12 days of incubation, almost complete removal of lindane (100%) was achieved in the absence of BS as well as O/W bio-microemulsions (MM+L+Y). The results confirmed the ability of the yeast strain to utilize lindane as the sole carbon and energy source in MM even though the degradation efficiency is low. However, in the presence of BS (MM+BS+Y), there was enhancement of degradation (*i.e.*, 100% removal in 10 days). The degradation rate was much higher in the presence of O/W bio-microemulsions (MM+L+ME+Y). An enhanced degradation of 36% was noted on day 2 and resulted in complete removal of lindane on the 6th day of

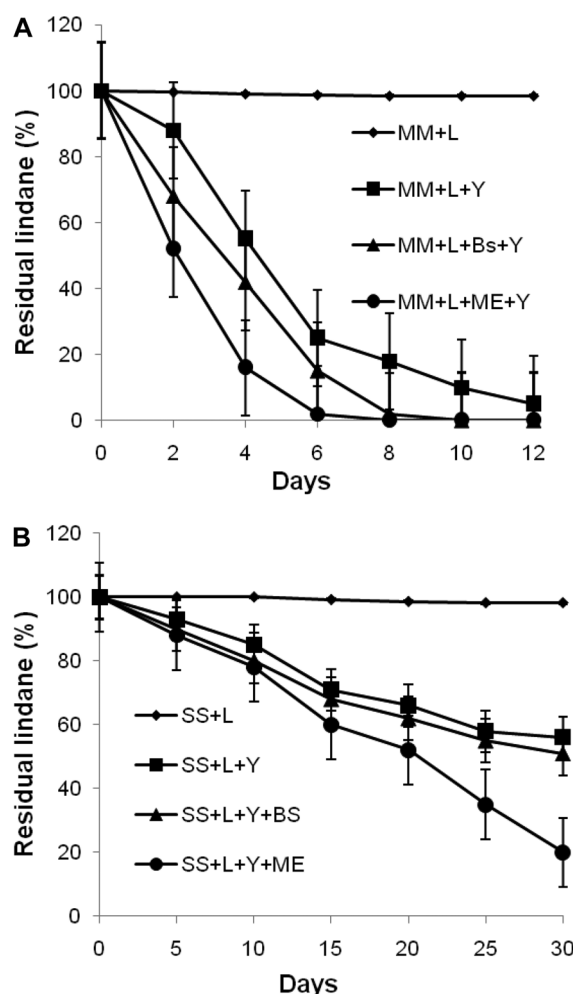


Fig. 7. Degradation of lindane by VITJzN01 under various treatments.

(A) Lindane degradation efficiency in liquid culture [MM+L, lindane mineral medium; MM+L+Y, lindane medium with yeast; MM+L+BS+Y, lindane medium with BS and yeast; MM+L+ME+Y, lindane medium with bio-microemulsions and yeast]. (B) Residual lindane in soil slurry [SS+L, lindane soil slurry; SS+L+Y, lindane soil slurry with yeast; SS+L+BS, lindane soil slurry with BS; SS+L+Y+ME, lindane soil slurry with yeast and bio-microemulsions). The value given is the mean of triplicate experiments together with the standard deviation.

incubation. The lindane degradation was apparently negligible in the controls (MM+L) after 12 days. The addition of BS as well as O/W bio-microemulsions increased the solubility and thus the substrate availability to the yeast strains. For both treatments, nearly 50–80% degradation was observed in 4 days. Similar enhancement of degradation of other pesticides had been reported earlier in the presence of microemulsions made from synthetic surfactants and cosurfactants [33, 34].

Effect of O/W Bio-Microemulsions on the Biodegradation of Lindane in Soil Slurry

Degradation of lindane in soil slurry by *Pseudozyma* VITJzN01 with or without O/W bio-microemulsion is shown in Fig. 7B. The residual lindane estimated in soil slurry was found to be higher than in liquid cultures, as degradation was observed only after 5 days. During the 30 days incubation, the strain VITJzN01 could only degrade less than half (60% residual lindane) of the initial concentration, when no bio-microemulsion was added into the system (SS+L+Y). The lower degradation rate in the absence of solubilizing agent clearly indicated that crystalline lindane coated on the sand and soil particles was unavailable to be degraded by VITJzN01. Consequently, there was a need for the accession of the proper solubilizing agent, which played a significant role in promoting the degradation. When BS was used as a solubilizing agent along with the yeast inoculum (SS+L+Y+BS), 50% lindane degradation was observed, whereas when O/W bio-microemulsion (10 ml, 10:1; BS: O-O) was used as a solubilizing agent to amend the soil slurry containing yeast *Pseudozyma* VITJzN01 (SS+L+Y+ME), the residual lindane in the soil slurry as recorded in the GC chromatogram was less than 20%. After 30 days of incubation, the lindane degradation in soil slurry was negligible in the absence of yeast and O/W bio-microemulsion. In the soil slurry experiments, when yeast was added into the soil, notable degradation of lindane was observed. This confirmed the ability of the yeast as a

potential degrader of lindane in soil. The uptake of lindane by the yeast cells might be possibly due to the involvement of extracellular and intracellular enzymes. This is a natural process but slow in occurrence. The uptake of bio-microemulsions containing lindane was confirmed from the GC chromatogram of the residues extracted from the liquid culture and soil slurry, which showed no peak corresponding to the BS or olive oil used. Consequently, our reported work confirms that addition of O/W bio-microemulsion can enhance the lindane degradation ability of the yeast, which may be interpreted as an eco-friendly approach.

Zheng *et al.* [34] reported the increase of degradation of DDT at high concentration with the use of microemulsions formed with non-ionic surfactants, linseed oil, and co-surfactant in the case of fungi *Phenarochaete chrysosporium*. A schematic representation of the present study is presented in Fig. 8. In our study, bio-microemulsions of olive oil and water formed by BS without addition of any cosurfactant enhanced the solubilization and degradation of lindane at high concentration by *Pseudozyma* VITJzN01 in both liquid culture and soil slurry.

As a conclusion, in the present study, the yeast strain *Pseudozyma* sp. VITJzN01 was isolated from maize fields with the ability to utilize lindane as the sole carbon and energy source. Even though yeasts belonging to genus *Pseudozyma* were previously noted for their glycolipid production, this is the first report on its ability to degrade a

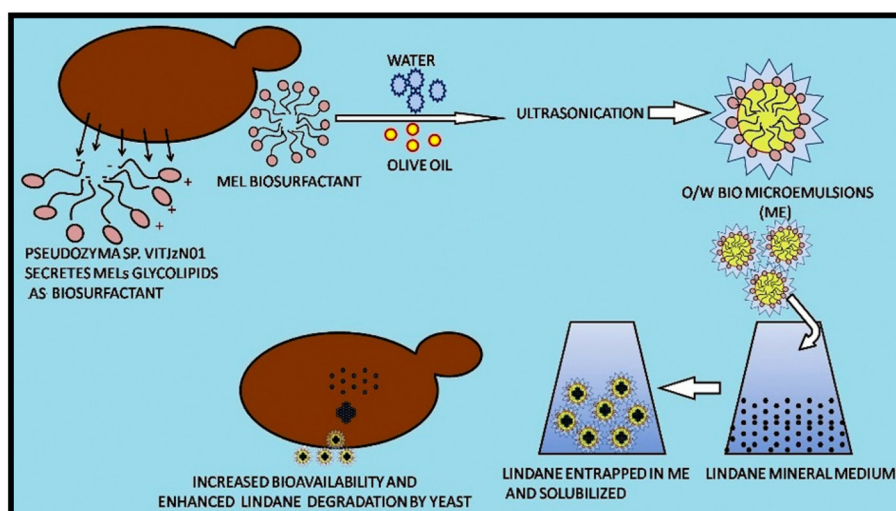


Fig. 8. A schematic representation of the theme of the present study.

The yeast *Pseudozyma* sp. VITJzN01 produces biosurfactant mannosylerythritol lipids (MELs) with hydrophilic (+) and hydrophobic (-) moieties. O/W bio-microemulsions (ME) are formed with MEL, olive oil, and water by ultrasonication. The O/W ME is used to solubilize lindane in mineral medium for increased bioavailability and degradation by the yeast in the liquid medium.

harmful pesticide such as lindane. As studies on the potential of indigenous yeast strains to degrade lindane and other chlorinated pesticides are scanty, our findings encourage exploring the crucial role of these organisms in biogeochemical cycling and environmental clean-up. The yeast produced MEL glycolipids as biosurfactants when grown on olive oil. O/W bio-microemulsions formed with the MEL effectively enhanced the degradation of lindane at high concentration by VITJzN01, increasing the solubility and its bioavailability. Previously, non-ionic surfactants along with cosurfactants were commonly employed for microemulsion-mediated soil remediation. Application of MEL-stabilized O/W bio-microemulsions without cosurfactant is definitely a novel and eco-friendly approach over the use of conventional surfactant micelles to enhance the bioremediation of lindane in soil and aqueous environments.

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