International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 11 (2014) pp. 723-733**
http://www.ijcmas.com

Original Research Article

A Study on biosurfactant production in *Lactobacillus* and *Bacillus sp*

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A B S T R A C T

The aim of this study was to determine the antimicrobial properties and biosurfactant production by *Lactobacillus and Bacillus*, against several micro-organisms, including Gram-positive and Gram-negative bacteria. Biosurfactant production was determined. The bio surfactant production was optimized. The bacteroicin produced by the *Lactobacilli* and *Bacillus* showed antimicrobial activity against pathogenic micro-organisms (*Pseudomonas, Escherichia coli, Salmonella typhi, Staphylococcus* and *Aspergillus*), the minimum inhibitory concentration (MIC) were achieved for bacteriocins.

Keywords

Biosurfactant, *Bacillus*, *Lactobacillus*, Antagonism

Introduction

Biosurfactants are amphiphilic biological compounds produced extracellularly or as part of the cell membranes by a variety of yeast, bacteria and filamentous fungi (Anandaraj and Thivakaran, 2010; Ghayyomi Jazeh *et al.*, 2012) from various substances including sugars, oils and wastes (Jaysre *et al.*, 2011; Kigsley and Pekdemir, 2004). However, carbohydrates and vegetable oils are among the most widely used substrates for research on biosurfactant production by *Bacillus subtilis* strains. The amphiphiles that form micelles can be potentially used for surface chemical works are termed as surface active agents or surfactants. Soaps and detergents can be described as having similar characteristics as surfactants. The investigation has been made for its emulsification capacity as biopreservative in food. Production and extraction of biosurfactant was carried out using *Bacillus, Lactobacilli*, the parameter optimization for the better growth of the strain was carried out. The extraction and emulsification potency of the surfactant was performed in order to use as bioemulsifier in food (Priya and Usharani, 2009).

Biosurfactants are amphiphilic biological compounds produced extracellularly or as part of the cell membranes by a variety of yeast, bacteria and filamentous fungi from various substances including sugars, oils and wastes. Biosurfactants have recently become an important product of biotechnology for industrial and medical applications. Adsorption of biosurfactants to a substratum surface modifies its hydrophobicity,

interfering in the microbial adhesion and desorption processes in that sense, the release of biosurfactants by probiotic bacteria *in vivo* can be considered as a defence weapon against other colonizing strains in the urogenital and gastrointestinal tracts and on medical devices. Biosurfactants produced by *Lactobacilli,* in fact, have been shown to reduce adhesion of pathogenic micro-organisms to glass, silicone rubber, surgical implants and voice prostheses. A large variety of *Bacillus subtilis* strains produce lipopeptide biosurfactants which possess a high surfactant activity such as surface-active properties and antibacterial activity (Rashedi *et al.,* 2005). Surfactin is one of the most effective lipopeptide biosurfactants produced by *B. subtilis.* Bacteriocins are produced by bacteria and possess antibiotic properties. Bacteriocins are antimicrobial proteinaceous compounds that are inhibitory toward sensitive strains and are produced by both gram positive and gram negative bacteria (Thaniyavaran *et al.,* 2003).

Materials and Methods

Isolation and morphological characterization of microorganisms

Isolation of *Lactobacillus:* *Lactobacillus* was isolated from the samples collected from Bikaner, shiv Shakti and juice corner located in Abohar. Dilutions were plated into MRS (Man, Rogosa and Sharpe) agar to determine the best medium for the growth of *Lactobacillus.*

Isolation of *Bacillus subtilis:* *Bacillus subtilis* was isolated from soil samples. After incubation, individual colonies were selected and transferred into sterile broth medium. The screened organism was then characterized by using different tests. The test applied includes:

Gram staining, endospore staining, catalase activity, motility test, methyl red test, indole test, Voges-Proskauer test, starch hydrolysis and citrate test**.**

Biosurfactant production

Screening for biosurfactant production

Bio surfactant production from isolated bacteria was detected by using oil spreading technique and emulsification stability test in four different oils namely vegetable oil, kerosene, petrol and diesel.

Oil spreading technique: 40 ml of distilled water were added to a large Petri dish (15 cm diameter) and 50 µl of oil were placed to the surface of water. Ten µl supernatant (from culture broth) was added to the surface of oil. Occurrence of clear zone was an indication of biosurfactant production. The diameter of clear zone on the oil surface was measured and compared to 10 µl of distilled water as negative control.

Extraction of Biosurfactants

Isolated colonies were inoculated into 50 ml suitable broth medium containing 0.5% (v/v) crude oil as the sole carbon and energy source. The broth cultures were incubated with shaking (150 rpm) for 7 days at 30 ± 2°C. The incubation periods were 48 h for NB and seven days for ISM at 30°C on a shaker operated at 150 rpm. At the end of the incubation periods, the cultures were centrifuged at 6000 rpm for 20 min for the removal of the cells. The cell free broth cultures (supernatants) were autoclaved at 121°C for 15 min and tested for the activity of the produced biosurfactants using oil displacement area (ODA) as described by [11, 12]

Checking the dry weight of biosurfactant produced

After 7 days incubation the bacterial cells were removed by centrifugation at 5000 rpm, 4^{o}C for 20 minutes. The supernatant was taken and the pH of the supernatant was adjusted to 2, using 1M H_2SO_4. Now add equal volume of chloroform: methanol (2:1). This mixture was shaken well and lift overnight for evaporation. White coloured sediments were obtained as a result i.e., the biosurfactant.

Now sterile plate was taken and its weight was measured. Now the sediment was poured on the plates. They were dried and their weight was measured. The dry weight was calculated using following formula:

Dry weight of biosurfactant = weight of the plate after drying - weight of empty plate.

Analysis and identification of biosurfactants

Biosurfactants were identified by TLC. Silica plates were prepared with silica gel and the crude biosurfactant was spotted on the plate along with the biosurfactants produced by us upon 7 days incubation. The biosurfactant was separated using the solvent chloroform: water: methanol in ratio 65: 24: 4.

- Ninhydrin reagent was sprayed to detect lipopeptide biosurfactant as red spots.
- Anthrone reagent was sprayed to detect glycolipid biosurfactant as yellow spots.

Determination of emulsification activity

Emulsification activity was measured using two methods: the measurement optical density at 540 nm and the measurement emulsion stability after 24 hour through calculated emulsification index (E24). In the first method, 2 ml samples of cell free supernatant were added to as screw-capped tubes containing 2 ml distilled water, and the solution mixed with 1 ml of a substrate (sunflower oil, olive oil, kerosene and kerosene mixed with 20% diesel). After a vigorous vortex for 2 min, the tubes were allowed to sit for 1 hour to separate aqueous and oil phase, before measuring the absorbance at 540 nm. Aqueous phase was removed carefully and OD at 540 nm was measured and compared with uninoculated broth used as negative control. Emulsification activity was defined as the measured optical density at 540 nm. Assays were carried out in triplicates. In the second method, 2 ml samples of cell free supernatant and 2 ml of oil were added to a screw cap tubes and vortex at high speed for 2 min. The mixtures were incubated at room temperature for 24 hours. The emulsification index (E24) was calculated by dividing the measured height of emulsion layer by the mixtures total height and multiplying by 100.The emulsification activities (E24) were estimated (Haddad *et al.,* 2009).

Stability of the biosurfactants

Checked the emulsification activity of all the cultures at different temperatures (4^{o}C, 22^{o}C, 37^{o}C), different pH (4, 6, 7, 8, 10), different NaCl concentrations (4%, 6%, 8%, 10%), different carbon sources (glucose, sucrose) and different nitrogen sources (yeast extract, urea). Thermostability, effect of salinity and pH were carried out (Haddad *et al.,* 2009).

Determination of antimicrobial activity of probiotics – test pathogens organisms by well diffusion method and over lay method.

Well Diffusion Method- The strains of pathogens were included in study (*E. coli, S. typhi, S. aureus, A. niger* and *P. aeruginosa*). Selective media were used to test the anti microbial activity against these pathogens. 0.1 ml dilution of each pathogen were tested by pour plate method, four holes were made by using sterile cork borer and then pure cultures were added and results were recorded for 3 days incubation with 24 hrs interval.

Results and Discussion

The following cultures were isolated from different milk samples, soil and sewage samples and were named accordingly and the names are as follows:
Lactobacilli, Bacilli, and *Pseudomonas* were isolated from different milk samples designated as L1, L2, L3, L4, L5, B1, B2, B3 and B4.

Morphological and biochemical characterization of microbial isolates

After isolation of the bacterial strains their morphological characteristics were checked to ensure that a specific strain was obtained. *Lactobacillus* and *Bacillus* are gram positive rod shaped bacteria. Results for catalase test, citrate utilization test, MR test and VP test were as shown in Table 1, 2 and 3.

Results for biosurfactant production

Biosurfactant production by oil spreading test: For Oil spreading test, the culture was centrifuged and supernatant was added in to oil containing plate. The biosurfactant producing organism would displace oil and form a clear zone in the centre of the plate which indicates the ability of isolated organism to displace the oil (Fig. 1) (Table 4 a, b).

Results for dry weight

Equal amount of supernatant of culture and chloroform: ethanol was kept overnight for evaporation and dry weight obtained are as shown in Table 5 and 6.

Results of emulsification

Effects of different pH, temperature, NaCl concentration, carbon source and nitrogen source on emulsification index are shown in Table 7, 8, 9, and 10 and Figure 2.

Results of antagonistic activity of bacteriocins

Antagonistic activity of bacteriocins is checked against pathogens. The pathogens are *E. coli, Aspergillus, P. aeruginosa, Staphylococcus* and *Salmonella typhi.* Antimicrobial activity of probiotics by well diffusion method was shown in Figure 3.

Conclusion and future research direction

The strains obtained were *Bacillus subtilis* and *Lactobacillus*. Out of the four strains of bacillus isolated from soil, only three produced biosurfactants. The strain B2 gave maximum diameter of zone formed by oil displacement method. The culture supernatant displaces oil by 2 cm. The oil displacement method was used for screening of biosurfactants. The *Lactobacillus* strain L2 also produced maximum biosurfactant.

The biosurfactant extracted was characterized using TLC. Upon checking the emulsification activity of the strains at different pH, temperature, NaCl concentrations, carbon source and nitrogen source it was observed that emulsification index in each case varied. The aim of this study was also to isolate *Bacillus* spp from

various sources and to screen their bacterocins as potential natural antibacterial agents for use against animal pathogens. The antagonistic activity of each bacteriocin was observed against the pathogens and significant zone of clearance was observed in case of L1 against staphylococcus and B2 against *E. coli*, *Aspergillus* and *Bacillus* spp and L4 against *Staphylococcus*.

Most of biosurfactant applications are in oil, food and cosmetic industry as well as in therapeutic agent. Biosurfactants are used to enhance oil recovery. Compared with chemical surfactants, they are very selective and required in small quantities and are effective under broad range of oil and reservoir conditions. Biosurfactants also have several applications in the food industry as food additives.

Table.1 Morphological characterization of cultures of *Bacillus* and *Lactobacillus*

Characterstics Variable	***Bacillus***	***Lactobacillus***
Colony size	Large disc like	Small and large
Surface	Flat and irregular	smooth
Opacity	Opaque	translucent
Colour	Creamy off white	Creamy off white
Motility	Motile	Non motile
Cell shape	Rod	Rod
Gram staining	Gram positive	Gram positive
Endosore staining	Endospores	No endospores

Table.2 Biochemical characterization of cultures of *Bacillus*

Bacterial strain Characterstics	B1	B2	B3
Catalase test	+ve	+ve	+ve
Indole test	-ve	-ve	-ve
MR test	+ve	+ve	+ve
VP test	-ve	-ve	-ve
NaCl concentration	Upto 10%	Upto 10%	Upto 10%
Glucose	-	-	-
Sucrose	A	A	A
Fructose	A+G	A	A
Mannitol	A	A	A

Table.3 Results for biochemical characterization for *Lactobacillus spp*

Bacterial strain Characterstics	L1	L2	L3	L4	L5
Catalase test	-ve	-ve	-ve	-ve	-ve
Indole test	-ve	-ve	-ve	-ve	-ve
Citrate test	-ve	-ve	-ve	-ve	-ve
MR test	+ve	+ve	+ve	+ve	+ve
VP test	-ve	-ve	-ve	-ve	-ve
NaCl concentration	Upto 8%	Upto 8%	Upto 4%	Upto 8%	Upto 8%

Table.4 Oil displacement

(a)

Bacillus strain	Biosurfactant production	Zone of clearance
B1	+ve	1cm diameter
B2	+ve	2cm diameter
B3	+ve	0.5cm diameter

(b)

Lactobacillus	Biosurfactant production	Zone of clearance
L1	+ve	1.5 cm
L2	+ve	2.0 cm
L3	+ve	1 cm
L4	+ve	1cm
L5	+ve	1.8 cm

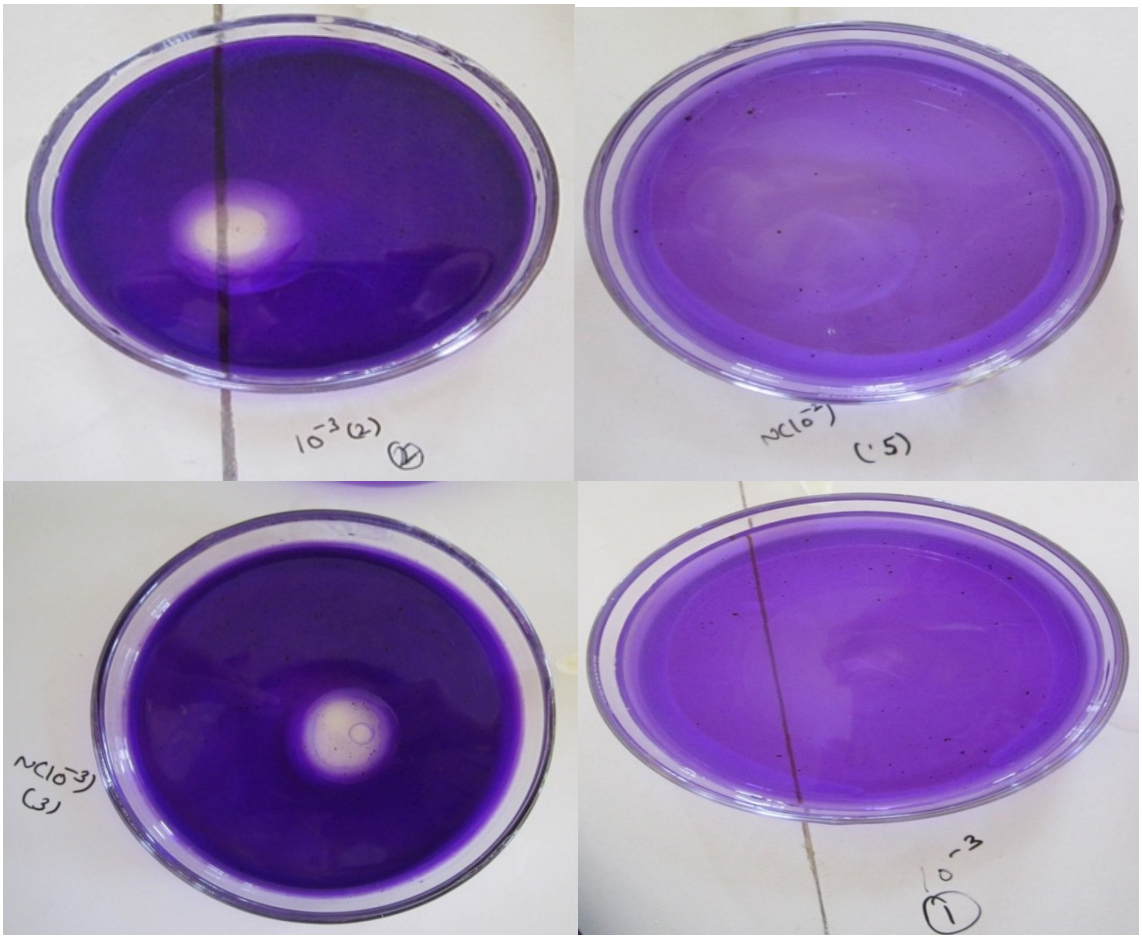

Fig.1 Biosurfactant positive cultures show oil dispersion

Table.5 Results of dry weight for *Bacillus*

Bacterial strain	Dry weight
B1	0.125g
B2	0.105g
B3	0.14g

Table.6 Results of dry weight of *Lactobacillus*

Lactobacillus strain	Dry weight
L1	0.21g
L2	0.213g
L3	0.31g
L4	0.109g
L5	0.195g

Table.7 Emulsification index for *Bacillus* at different pH

(a)

Bacterial cultures	pH- 4	pH-6	pH-7	pH-8	pH-10
B1	47.8%	11.1%	5%	50%	52.3%
B2	50%	25%	10%	20%	45%
B3	55%	17.3%	6.6%	22.7%	58%

(b)

Lactobacillus culture	pH-4	pH-6	pH-7	pH-8	pH-10
L1	25%	38.8%	42.1%	45%	50%
L2	50%	27%	0%	5%	40%
L3	38.8%	30%	0%	4.5%	30%
L4	11.7%	23.8%	85%	5%	5%
L5	25%	25%	10%	5%	5%

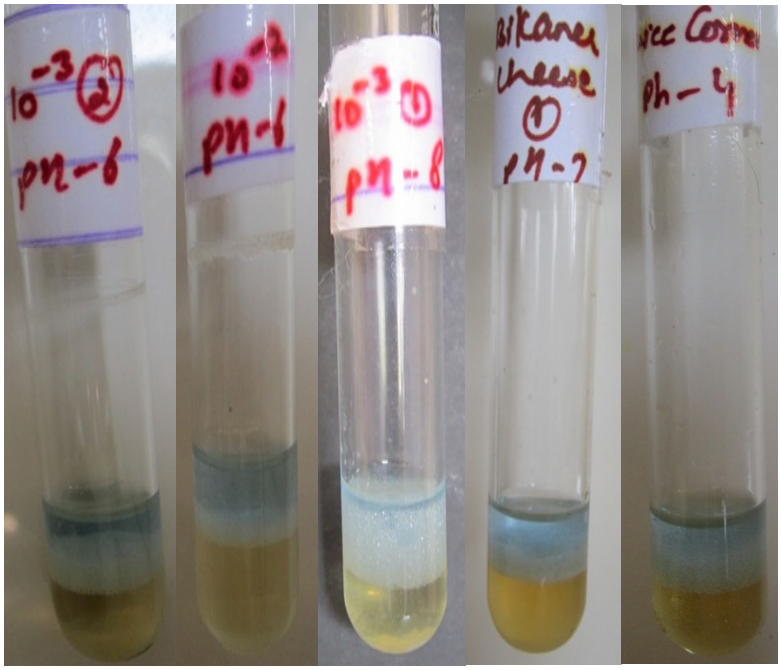

Fig.2 Emulsification at different pH

Table.8 Emulsification of diff. cultures at diff. temperature

Bacillus culture	AT 4^0C	AT 22^0C	AT 37^0C
B1	0%	5%	10%
B2	0%	5%	10%
B3	0%	5%	40%
L1	35%	25%	10%
L2	45%	25%	5%
L3	20%	25%	55%
L4	40%	40%	35%
L5	35%	20%	15%

Table.9 Emulsification of different cultures at different NaCl conc

NaCl conc. Culture	4% NaCl	6%NaCl	8%NaCl	10%NaCl
B1	0%	0%	0%	0%
B2	25%	11.1%	0%	0%
B3	9%	6.6%	0%	0%
Bacillus subtilis	45%	11.1%	5%	0%
L1	29.4%	5%	0%	0%
L2	55.5%	33.3%	5%	0%
L3	5%	5%	0%	0%
L4	10.5%	5%	0%	0%
L5	0%	0%	0%	0%

Table.10 Emulsification for cultures of *Bacillus and Lactobacillus* at diff. carbon and nitrogen source

(a)

Bacillus culture	Glucose	Sucrose	Urea	Yeast extract
B1	16.6%	15.3%	41.6%	6.6%
B2	46.6%	20%	15.3%	7.6%
B3	50%	30.7%	10%	10%
Bacillus subtilis	7.6%	62.5%	66.6%	40%

(b)

Lactobacillus culture	Glucose	Sucrose	Urea	Yeast extract
L1	30%	15%	20%	0%
L2	46.6%	15%	44.4%	47%
L3	18.2%	42.2%	49%	17.2%
L4	16.6%	26.6%	21.4%	.27%
L5	25%	25%	42.8%	66.6%

Table.11 Antagonistic activity of isolated lactobacillus and bacillus against test pathogens

Pathogen isolates	*E.coli*	*Aspergillus*	*P.aeruginosa*	*Staphylococcus*	*Salmonella typhi*
L1	9mm	3 mm	0 mm	21 mm	5mm
L2	10mm	12mm	5mm	10mm	0 mm
L3	9mm	12 mm	0 mm	15 mm	2cm
L4	0 mm	0 mm	4 mm	2 mm	15mm
L5	10 mm	6 mm	4 mm	12 mm	0 mm
B1	5mm	9mm	10mm	6mm	10mm
B2	24mm	21mm	12mm	2cm	13mm
B3	1mm	10mm	6mm	3mm	3mm
Bacillus subtilis	4mm	20mm	5mm	7mm	2mm

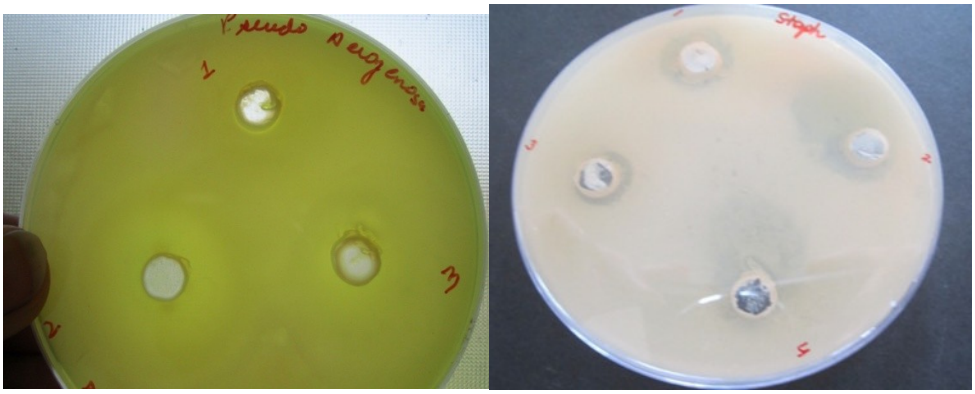

Fig.3 Zone of clearance showing antimicrobial activity

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