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ANTI-MICROBIAL AND ANTI ADHESIVE PROPERTIES OF *LACTOBACILLUS* AND *BACILLUS SP*.

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ABSTRACT: The aim of this study was to determine the anti-microbial and anti-adhesive properties of a Lactobacillus and Bacillus, against several micro-organisms, including Gram-positive and Gram-negative bacteria. Biofilm producing micro- organisms were isolated from different samples. The isolated micro-organism were characterized and identified. The anti-microbial and anti-adhesive activities were determined. The bacteroicin production was quantified and was observed. The maximum bacteroicin production was observed among various isolated micoorganisms. The bacteroicin produced by the *Lactobacilli* and *Bacilli* showed antimicrobial activity against pathogenic micro-organisms (including *Pseudomonas, Escherichia col, Staphylococcus, Aspergillus and Salmonella typhi)*, the minimum inhibitory concentration (MIC) were achieved for bacteroicins the most. The antagonistic characteristics of bacteriocin were studied by agar well diffusion method. Furthermore, the micro- organism showed anti-adhesive activity against most of the micro-organisms evaluated. Biofilm production was observed and quantified among various biofilm producing isolated micro-organisms.

INTRODUCTION: Several bacteriocins from Gram positive bacteria are very effective, have broad inhibitory spectra and may be used as antimicrobial agents for various practical applications [1] Many of these lactic acid bacteria produce bacteriocins [2]. Bacteriocins are proteinaceous antibacterial compounds and exhibit bactericidal activity against species closely related to the producer strain [3]. Manybacteriocins are active against food-borne pathogens especially against Listeria monocytogenes [4]. Several types of bacteriocins from food-associated lactic acid bacteria have been identified and characterized, of which the important ones are nisin, diplococcin, acidophilin, bulgarican, helveticins, lactacins, and plantaricins [5]. Bacteriocin production seems to be aimed to compete against other bacteria which are present in the same ecological niche [6, 7, 8].

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Biosurfactants (BS) are amphiphilic compounds produced mostly by microbes on their cell surface, or secreted extracellularly and exhibit strong surface and emulsifying activities. They contain both hydrophobic and hydrophilic moieties that can reduce the surface or interfacial tension in liquids [9, 10]. Bio Surfactants are complex molecules that include glycolipids, rhamnolipids, lipopeptides, polysaccharide-protein complexes, phospholipids, fatty acids and neutral lipids [11, 12, 13]. Lactobacillus spp. are potent BS producing microorganism predominately found in the gastrointestinal microflora of human and animals. BS derived from lactic acid bacteria contributes to their high attributes of prevention of bacterial infections in the human body.

A number of studies reported the potential of lactobacilli as biosurfactant and bacteroicin producers and their significant role in public health [14, 15, 16]. Biofilm formation associated with severe problems once the biofilm formation has prolonged. Blocking and leakage due to biofilm formation affect the function of the device and need to be change regularly. Application of

biosurfactantto a surface modifies its hydrophobicity, interferes microbial adhesion and desorption processes; in that sense, the production of biosurfactant by probiotic bacteria in vivo can be considered as a defense against other colonizing food borne pathogens. Biosurfactant coating decreases the contact angle of silicone surface and it becomes hydrophilic [17, 18]. Lactic acid bacteria and Bacillus spp. impaired biofilm formations and studied and observed in present work.

MATERIALS AND METHODS:

Isolation and Morphological characterization of Microorganisms:

Isolation of *Lactobacillus, Bacillus, E.coli, Pseudomonas:* - *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. Bacillus* was isolated from soil samples and a pure culture of *Bacillus Subtilis* (IMTECH, CHD). *E.coli* was isolated from sewage sample. Presence of *E.coli* was tested using MPN method. *Pseudomonas* was isolated from soil samples. These organisms were subsequently grown and maintained in media which proved to be the most suitable for the growth (Kings Media).The screened organism was then characterized by using different tests.

The test applied include:

Gram staining, Endospore staining, Catalase activity, Motility test, Methyl red test, Indole test, Voges-Proskauer Test, Starch hydrolysis, Citrate test**.**

Preparation for Antimicrobial Products:

Determination of Bacteroicin production:

All the cultures were inoculated in their respective broths and incubated for 20 days. After incubation, cells were removed from the growth medium by centrifugation (10,000×g for 15 min, 4°C). The resultant supernatants are bacteriocins and can be stored at -20°C for further studies.

Estimation of bacteriocin Production:

A standard curve was prepared by taking BSA as a standard protein. The standard curve was prepared by taking the following amounts of water, BSA and Bradford reagent:

Standard curve for protein estimation Standard. Now after we have prepared a standard curve we check the OD of all the samples by putting them in volume.

3ml Bradford reagent + 50µl sample + 150µl water. The O.D of supernatants were observed.

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, P.aeroginosa*.) 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 was made by using sterile cork borer and then pure cultures were added and results were recorded for 3 days incubation with 24 hrs interval .

Overlay method:

The strains of pathogens were included in study (*E.coli, S. typhi, S.aureus, A.niger, P.aeroginosa*.) 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 , incubated anaerobically for 4 days for growth , then an another layer of media having either pure culture and isolated culture was over laid over it , results were recorded after 4 days anaerobic incubation.

Quantification of biofilm with crystal violet assay:

After incubation for 24 hours, the cultured cells from the microtiter plate were disposed by quickly shaking the plate over a waste tray. The plate was washed once with dH2O and air-dried, then 125 µl of 0.1% crystal violet solution (Fisher, Cat. no. C581-25) was added to each well. The plate was stained for 10 minutes at room temperature. The crystal violet solution was removed and the plate was washed twice thoroughly with distilled water and allowed to air-dry completely. 125 µl of 95% alcohol was deposited to each well containing the solution and the plate was incubated at room

temperature for 10 minutes to allow the crystal violet stain to dissolve into the alcohol. The contents of the wells were then thoroughly mixed and the absorbance at 595nm was measured

RESULTS AND DISCUSSIONS:

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 samples designated as L1, L2, L3, L4, L5, B1, B2, B3, B4, P1, P2, P3, P4, P5, P6

Morphological Characterization of Microbial Isolates:

After isolation of the bacterial strains their morphological characterstics were checked to ensure that the specific strain was obtained as shown in the following table:

- *Lactobacillus* and *Bacillus*are gram positive rod shaped bacteria.
- *E.coli* and *Pseudomonas* are gram negative bacteria in morphology

TABLE 1: TABLE FOR MORPHOLOGICAL CHARACTERSTICS OF *BACILLUS, LACTOBACILLUS, E.COLI* AND *PSEUDOMONAS*

Characterstics Variable	*Bacillus*	*Lactobacillus*	*E.coli*	*Pseudomonas*
Colony size	Large disc like	Small and large	Small	Large
Surface	Flat and irregular	Smooth	Smooth	Irregular
Opacity	Opaque	Transluscent	Opaque	Opaque
Colour	Creamish off white	Creamish off white	Metallic green on EMB agar	Yellowish green
Motility	Motile	Non motile	Motile	Motile
Cell shape	Rod	Rod	Rod shape	Rods
Gram staining	Gram positive	Gram positive	Gram negative	Gram negative
Endosore staining	Endospores	Non endospores	Endospores	Non endospores

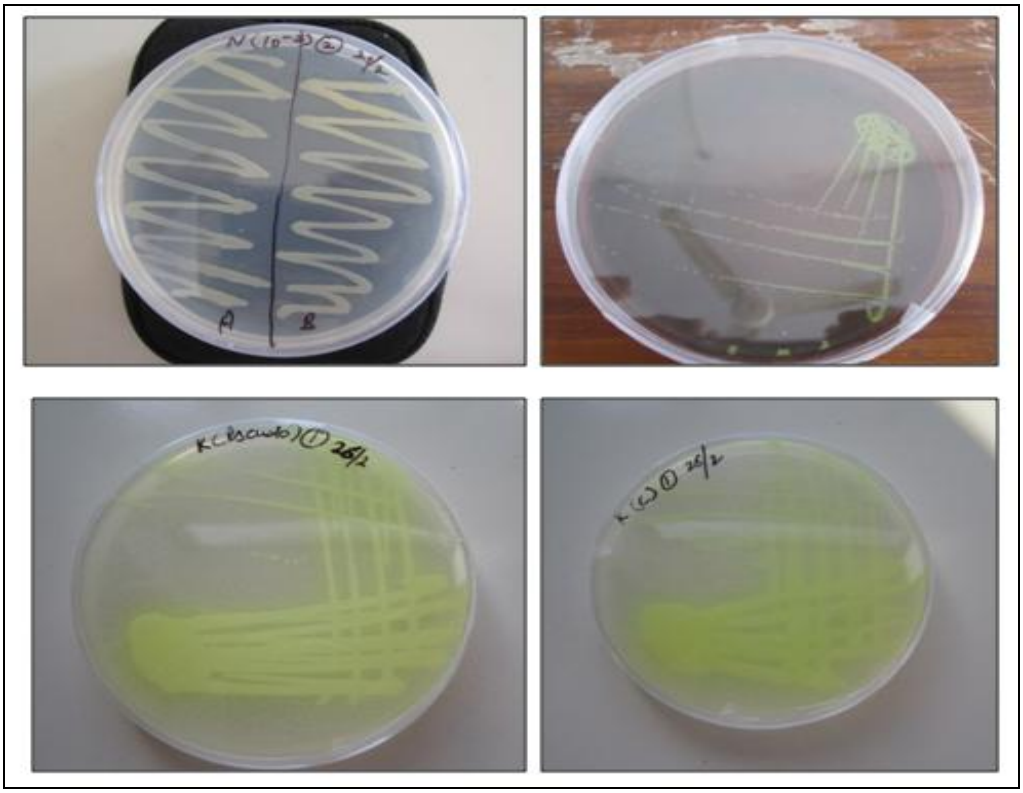

FIG. 1: ISOLATED CULTURE OF *BACILLUS* (I), *E.COLI* (II), *PSUEDOMONAS* (III) & (IV)

BIOCHEMICAL CHARACTERIZATION OF MIROBIAL ISOLATES- Results for the following table are shown in table 1.4: Catalase test, Citrate utilization test, MR test, VP test, IMVIC test , Indole test. For Bacillus *spp.*

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

Lactobacillus spp. Biochemical characterization of *Lactobacillus*

TABLE 3: RESULTS FOR BIOCHEMICAL CHARACTERIZATION

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%

For *E.coli:* Biochemical characterization of *E.coli*:

TABLE 4: RESULTS FOR BIOCHEMICAL CHARACTERIZATION OF *E.COLI*

Bacterial strain Characterstics	E1	E2
Catalase test	+ve	+ve
Indole test	+ve	+ve
Citrate test	+ve	+ve
MR test	+ve	+ve
VP test	-ve	-ve
NaCl concentration	Upto 4%	Upto 4%
Glucose	A	A
Sucrose	A+G	A
Fructose	A	A
Mannitol	A	A

A-acid productionG- gas production

Characterstics of *Pseudomonas* isolated from soilare as follows*:*

TABLE 5: BIOCHEMICAL CHARACTERISTICS OF *PSEUDOMONAS*

Strain characteristics	P1	P2	P3	P4	P5	P6
Catalase test	+ve	+ve	+ve	+ve	+ve	+ve
Indole test	-ve	-ve	-ve	-ve	-ve	-ve
MR test	-ve	+ve	-ve	-ve	-ve	-ve
VP test	-ve	-ve	-ve	-ve	-ve	-ve
Citrate test	-ve	-ve	+ve	+ve	-ve	+ve
NaCl concentration	Upto 6%	Upto 7%	Upto7%	Upto 7%	Upto 6%	Upto 6%

Glucose	+ve	+ve	+ve	+ve	+ve	+ve
Sucrose	+ve	+ve	+ve	+ve	+ve	+ve
Lactose	+ve	+ve	+ve	+ve	+ve	+ve
Fructose	+ve	+ve	+ve	+ve	-ve	+ve
Mannitol	+ve	+ve	-ve	-ve	+ve	-ve

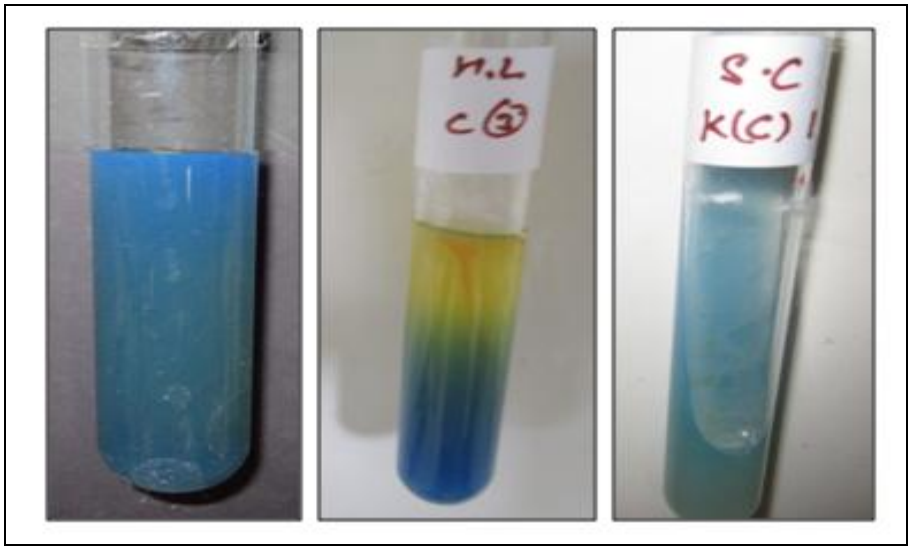

FIG. 2: IMAGES FOR MOTILITY AND CITRATE UTILIZATION [a) CONTROL b) HL POSITIVE c) CITRATE POSITIVE]

Results of Bacteriocin Produced:

Bacteroicin production was observed in both Bacilli and Lactobacilli. All the strains of bacilli did not produce the bacteroicin but all lactobacilli were capable of producing the same. Maximum production was observed in Lactobacillus B2 & L4

TABLE 6: BACTERIOCINS PRODUCED BY ISOLATED CULTURE

Bacterial cultures	Bacteriocin produced
Bacillus subtilis	0.26
B1	0.05
B2	0.3
B3	0
L1	0.24
L2	0.24
L3	0.21
L4	0.27
L5	0.23

Antagonistic activity of bacteriocins are checked against pathogens.

The antagonism was observedagainst E.coli, *Aspergillus, P.aeruginosa, Staphylococcus, Salmonella typhi.* The anatagonistic activity was observed maximum in Bacillus i.e B2, rest all showed antagonistic activity.

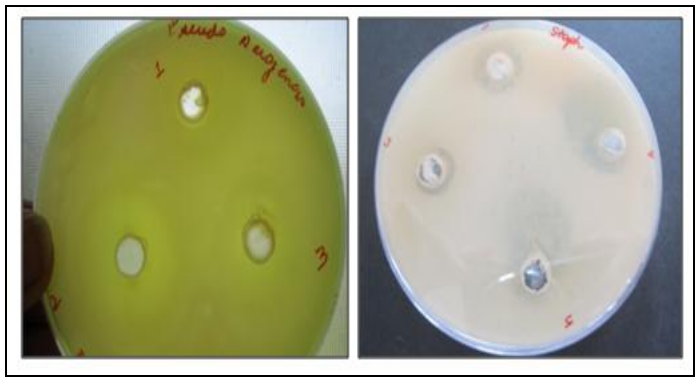

FIG.3: SHOWING THE ANTIMICROBIAL ACTIVITY OF THE BACTEROICINS AGAINST TEST PATHOGENS

Results of Antagonistic Activity of Bacteriocins

TABLE 7: ZONE OF INHIBITION (MM)

Pathogen / Bacteriocin	*E.coli*	*Aspergillus*	*P.aeruginosa*	*Staphylococcus*	*Salmonella Typhi*
L1	9mm	3 mm	0 mm	15 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	10mm
L5	10 mm	6 mm	4 mm	12 mm	0 mm
B1	5mm	9mm	10mm	6mm	10mm
B2	24mm	21mm	12mm	20mm	18mm
B4	1mm	10mm	6mm	3mm	3mm
Bacillus subtilis	4mm	25mm	5mm	7mm	2mm

Results of Quantitative Method of Biofilm Produced:

The biofilm production by pseudomonas was observed. Seven strains of pseudomonas were isolated and all showed biofilm production but they varied in amount. Maximum biofilm production was in P6.

TABLE 8: BIOFILM PRODUCED BY ISOLATED CULTURES

***Pseudomonas* strain**	**O.D (biofilm produced)**
P1	0.250
P2	0.130
P3	0.430
P4	0.450
P5	0.380
P6	0.590
P7	0.290

FIG.4: RINGS INDICATE BIOFILM FORMATION

Results of Biofilm Inhibition in the Presence of Bacteriocins:

When the biofilm producing organisms was grown in the presence of bacteriocins, it was observed that biofilm production was comparativelyless in some cases**. L2** was overall very effective against each strain.

TABLE 9: EFFECTS OF BACTERIOCINS AGAINST THE BIOFILM PRODUCTIONS.

	P1	**P2**	**P3**	**P4**	**P5**	**P6**
Without bacteriocin/ control	**.31**	**.43**	**.33**	**.26**	**.35**	**.27**
L1	0.50	0.30	0.26	0.35	0.20	0.29
L2	0.26	0.25	0.26	0.29	0.21	0.34
L3	0.25	0.24	0.28	0.24	0.22	0.40
L4	0.22	0.19	0.29	0.34	0.26	0.38
L5	0.17	0.31	0.24	0.32	0.32	0.37
B1	0.30	0.38	0.34	0.33	0.44	0.40
B2	0.38	0.43	0.26	0.37	0.37	0.35
B3	0.29	0.32	0.31	0.30	0.40	0.26
B4	0.37	0.32	0.24	0.44	0.29	0.33

CONCLUSION: The strains obtained were *Bacillus subtilis* and *Lactobacillus*. The supernatant of strain L4 produced maximum amount of bacteriocins. The aims of this study were to isolate antimicrobial-producing *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 maximum in case of B2 against all test pathogens and L1 against *Staphylococcus* and B4 against *Psuedomonas.* Upon checking the biofilm production it was observed that maximum amount of biofilm was produced by strains P6 and P3. Maximum biofilm inhibition by bacteriocins was observed in case of P3 in the presence of bacteriocin produced by L1, L2, L3, L5, B2 and B4.

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