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Isolation, Characterization And Antibacterial Activity Of Bacteriocin Producing Bacteria From Dairy Effluents

Y Evangelin[#] , K Kasturi[&]

[#] *Research Scholar, Department of Bio-Technology, Acharya Nagarjuna University, Guntur-522510, Andhra Pradesh, India,*

[&] *Assistant Professor, Department of Bio-Technology, Acharya Nagarjuna University, Guntur - 522510, Andhra Pradesh, India,*

Corresponding Author: Y Evangelin

Abstract: The intent of study is to determine the antimicrobial activity of bacteria producing bacteriocin isolated from dairy effluents .Several bacterial isolates were picked from the samples by growing on selective media(MRS Agar media).Bacteriocin producing organisms were screened by agar spot assay test.Six isolates are able to produce bacteriocin whose antibacterial activity was analysed by Agar well diffusion assay test against indicator organisms which are pathogenic.Bacillus cereusATCC1178,Staphylococcus aureus MTCC87 were more sensitive to the isolates than Salmonella typhi MTCC734 and Pseudomonas aeruginosa MTCC424. The antibacterial protein bacteriocin was characterized based on different pHvalues(lactic acid production), catalase test. By biochemical and molecular characterization the best bacteriocin producing strain was identified as Paenibacillus lactis. Analysis of the 16S rRNA gene of the MSB2 type placed these isolate within the genus Paenibacillus, Moreover, over 99% similarity was observed to the 16S rDNA sequence of MB 1871 a strain isolated previously from raw and heat treated milk. This study reveals the possibility of isolating Bacteriocin strains from dairy effluents and using them to control pathogenic bacteria.

Key words: Dairy effluents , MRS Agar, Bacteriocins , Characterization,Antibacterial activity, Paenibacillus lactis.

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I. INTRODUCTION

During the last two decades due to enormous increase in milk production ,the number of dairy plants of medium and large size has increased for the efficient handling and processing of milk.The dairy industry in India on an average has reported to generate 6-10 litres of waste water per litre of milk proceesed depending on the process employed and product manufactured.This waste escalating disposal and pollution problems and represents a loss of valuable biomass and nutrients. However despite their pollution and hazardous aspects ,in many cases ,dairy processing wastes have a good potential of converting into useful products of higher value as byproduct or even as raw material for other industries. Most of the bacteria present in this dairy effluents are capable of producing a heterogenous array of molecules that may inhibitory either for themselves or for other bacteria (BaloguTV,et.al 2013)These molecules include toxins,primary metabolites, antibiotics and bacteriocins. Bacteriocins are ribosomally synthesized antimicrobial peptides that are active against other bacteria either of the same species (narrow spectrum)or across genera broad spectrum (MahrousH,MohamedA,2013) .Bacteriocins are produced by both gram positive and gram negative bacteria .In recent years bacteriocin producing bacteria have attracted significant attention because of their GRAS status and potential use as safe additives for food preservation .Nisin produced by Lactobacillus lactis,is the most thoroughly studied bacteriocin todate and has been applied as an additive to certain foods world wide.

In our study we have successfully attempted to isolate microbial strains from dairy effluents. Since bacteria isolated from dairy effluents generally regarded as safe(GRAS).T he organisms are assayed for its capability to produce bacteriocin and its inhibitory effect was checked against growth and proliferation of Salmonella typhi, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus cereus.The dilution at which the maximum activity of bacteriocin was determined , which would be attributed to be the therapeutic usage of probiotics against various microbial infections in humans.

II. MATERIALS AND METHODS :

Sample Collection: The primary clarifier effluent generated at Dairy industry near to Guntur was collected in sterile sampling vials. Two samples were maintained for further experiments.

Isolation of microbes : 1ml of the sample was serially diluted in 9 ml of sterile 0.8% saline . Dilutions up to 10^{-7} were achieved and plates corresponding 10^{-3},10^{-5} and 10^{-7} were plated on MRS Aggar medium(Deman,Rogosa and Sharpe Agar).The plates were incubated at room temperature for 24 hours and observed for colony forming units..Each distinct colony was isolated and plated separately as pure cultures. The pure cultures were maintained on MRS agar plate at 5^0c after visible growth on the plate. Subculturing is done at weekly intervalsThe isolates were designated as MSF1,MSF2,MSB1,MSB2,MSB3 ,MSB4.

Preparation of crude bacteriocin:MRS broth of primary inoculum was prepared in aliquots of 10ml and sterilized in an autoclave at 121^0c and 15psi. After incubation cells are removed by centrifugation at 10,000rpm for 10 min.The supernatant pH was adjusted to 6.5-7.0 with 1 N NaOH and filtered through 0.22 micro m membranes.

Identification of isolates: Bacterial identification was based on morphological, cultural, biochemical and molecular characterization. Gram reaction, production of catalase, lactic acid production test and growth on Demann Rogosa and Sharpe (MRS) broth as described by the Bergey's manual of Systemic Bacteriology (Kandler and Weiss1986) (ClausD,1986)

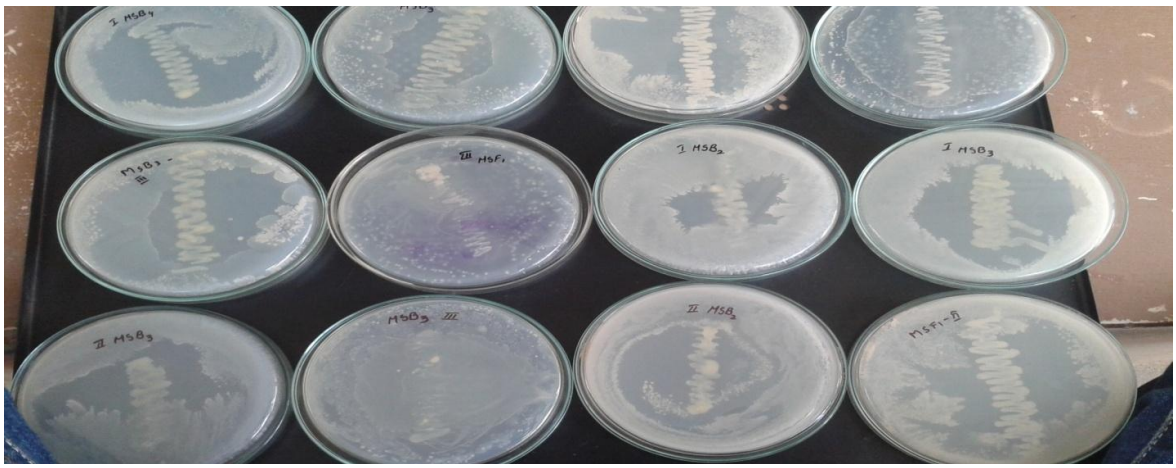

Fig.1 Streak Plate Cultures of different colonies

Colony morphology :The colonies of selected isolates were examined for type of growth, shape, elevation size pigmentation and consistency. MSB1 and MSB2 were gram stained.

Biochemical characterization :Selected isolates were characterized by testin g for IMViC test, starch hydrolysis, urease, hydrogen sulphide , catalase, gelatin liquefaction and nitrate reductase test and pure cultures were maintained at 2^0c in MRS broth with 10% glycerol and enriched in MRS broth incubated at 37^0 c for 24 hours.

Table 1: Biochemical characteristics of selected potential strains

S.no	Name of the test	Results	
		MSB1	MSB2
1	Indole test	-ve	-ve
2	Vogues proskaur test	+ve	-ve
3	Simmom citrate test	-ve	-ve
4	Starch Hydrolysis	+ve	+ve
5	Urease test	-ve	-ve
6	Methyl red test	-ve	-ve
7	Hydrogen sulphide test	+ve	-ve
8	catalase Test	+ve	+ve
9	Oxidase Test	-ve	+ve

10	Nitrate reductase test	**-ve**	**-ve**
11	Gelatin hydrolysis test	**-ve**	**-ve**

Fig.2 Starch Hydrolysis of Selected colonies

Detection of Antibacterial activity:

The Experimental strains *Salmonella typhi, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus cereus* were maintained in nutrient agar slants at 4^0c, 24 hours prior to the experiment cultures were thawed to room temperature . The inhibitory activity against target organisms were tested on Muller Hinton Agar medium by agar diffusion assay.(Kimura etal .1998) . These plates were inoculated with different concentrations of each target organism and plates were allowed to dry and sterile cork borer of diameter 7.0 mm was used to cut uniform wells in the agar plates(GirumT,et.al 2005)well was filled with different concentrations of filter sterilized supernatant obtained from culture grown in MSR medium.Incubate the plates at 37^0c for 24 hours. Inhibition zones around the wells arre measured and recorded. . The bacteriocin titre was determined by the serial two-fold dilution method previously described by Mayr-Harting et.al. (1972). Activity was defined as the reciprocal of the dilution after the last serial dilution giving a zone of inhibition and expressed as activity units (AU) per milliliter screening of bacterial isolates. The above process was repeated after optimization of medium components and physical parameters for enhanced bacteriocin production.

III. RESULTS:

Bacterial colonies from dairy effluent samples with typical characteristic namely pure white colonies small(2-3 diameter) with entire margins were picked from each plate and transferred to MRSbroth which was then subjected to classification on to the genera *Paenibacillus* based on morphological ,biochemical and molecular characterization(AshC;Priest 1993) .The culture supernatants obtained from six bacterial isolates of dairy effluent sample were tested for antibacterial activity against certain gram +ve and gram-ve bacteria.Bacteriocins obtained from the isolates showed inhibitory activity against *Salmonella typhi,Staphylococcus aureus,Pseudomonas aeruginosa* and *Bacillus cereus* . The resistant activity varied with each strain .The degree of inhibition was designated as very strong inhibition(15-18mm), strong inhibition(10-14mm), moderate inhibition(6-9mm) and no inhibition.MSB2 showed very strong activity against *Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus cereus* and strong inhibition (10-13mm). MSB3 inhibited *staphylococcus aureus and Bacillus cereus*, where as inhibition against salmonella,(RenataBromberg et.al 2004) . MSB4 showed moderate inhibition against *stapthylococcus aureus, bacillus cereus* and no activity against *salmonella typhi.*

IV. DISCUSSION

Dairy effluent samples were collected from different areas of Dairy, near to Guntur, for isolation of Bacteriocin producing Bacterial strains. The colonies from effluent sample are excepted to be litter higher. This is due to contamination from the animal, especially the exterior of the udder and the adjacent areas, bacteria found in manure,soil and water may enter from tested samples , out bacterial colones six isolates were isolated to draw consclusion about the bacteriocin producing Bacteria of producing dairy effluents samples.

The bacterial isolates were classified into the genera *Paenibacillus* based on their morphological and biochemical characters (sharpe 1929).some isolates werefound irregular,short,evencoccoid rodswith round tapered ends sometimes longer also (kandler et al ; 1938a).few strains were able to utilize cirate and are found be non-motile;calatase ,indole, MR-VP citrate negative(Kandler and weiss etal.1986) have classified Lacbacillus isolates from temperature regions according to their morphology ,physiology and molecular characteristies .Demanetal (1960) stated that lactobacilli are generally isolated on rich media such as MRS which is routinely used for most fermented food products.

Invitro assay was carried to characterize the antimicrobial potient of the culture supernatant to inhibit some pathogenic bacteria. Active supernatants of bacterial species were examined for bacteriocin production.(Lee,K.H 2001). Six isolates had inhibitory effects on sensitive bacteria including some common pathogenic bacteria. Among the bacteriocins tested bacteriocin from MSB2 ,MSB3 srtains had a broader host range . The inhibitory effect was assumed to be due to bacteriocin ,not H2O2 since there was no oxidizing effect on bacterial cells which will destroy the basic molecular structure of cell proteins and bacteriocin form the pores in the membrane of sensitive cells and deplete the transmembrane potential and the pH gradient , resulting in the leakage of cellular materials (chikindas etal.1993,MC Auliffe etal.2001).

These studies have shown the bacterial isolates are defensive and they have been labeled as exceptional bacteria as they have shown their constructive role on human pathogens by inhibiting the growth for which they are said to be the second immune system of the body.

Table2: ANTIBIOTIC SENSITIVITY SCREENING

Primary screening:

Indicator organisms:

1. *Salmonella typhi* MTCC 734
2. *Staphylococcus aureus* MTCC 87
3. *Bacillus cereus* ATCC – 11778

S.No	Organism code	I	II	III
1	MSF1	-Ve	+Ve	-Ve
2	MSF2	+Ve	+Ve	+Ve
3	MSB1	-Ve	+Ve	+Ve
4	MSB2	+Ve	+Ve	+Ve
5	MSB3	+Ve	_Ve	+Ve
6	MSB4	+Ve	_Ve	+Ve

Secondary screening:

Indicator Organisms:

1. *Salmonella typhi* MTCC 734
2. *Staphylococcus aureus* MTCC 87
3. *Pseudomonas aeruginosa* MTCC 424
4. *Bacillus cereus* ATCC – 11778

Test samples:

Antibiotic supernatant from broth of MSB2, MSB3, MSB4 isolates.

Test	*Salmonella typhi*	*Staph. aureus*	*P.aeruginosa*	*Bacillus cereus*
MSB2	Positive	Positive	Positive	Positive
MSB3	Positive	Negative	Negative	Positive
MSB4	Positive	Negative	Negative	Positive

Table 3: Antibiotic Estimation Activity

Primary Estimation:

S. No	Organism	Inhibition zone		
		50µl	**100µl**	**250µl**
1	MSF1	0.5mm	0.7mm	1.0mm
2	MSF2	0.7mm	0.9mm	1.2mm
3	MSB1	3.3mm	4.1mm	5.3mm
4	MSB2	2.9mm	3.7mm	5.1mm
5	MSB3	1.1mm	1.9mm	2.2mm
6	MSB4	1.7mm	3.7mm	4.9mm

Secondary estimation:

Test	**Concentration mg/ml**	***Salmonella typhi***	***Staph. aureus***	***P.aeruginosa***	***Bacillus cereus***
		Zone in mm			
MSB2	0.1	---	8	---	3
	0.5	---	10	---	6
	1.0	11	15	13	13
MSB3	0.1	---	9	---	---
	0.5	---	11	---	5
	1.0	---	16	---	11
MSB4	0.1	---	---	---	---
	0.5	---	2	---	4
	1.0	---	9	---	11

Table 4(a): Catalase Test

S.NO	+Ve	-Ve
1	MSB1	MSB2
2	MSF1	MSB3
3	MSF2	MSB4

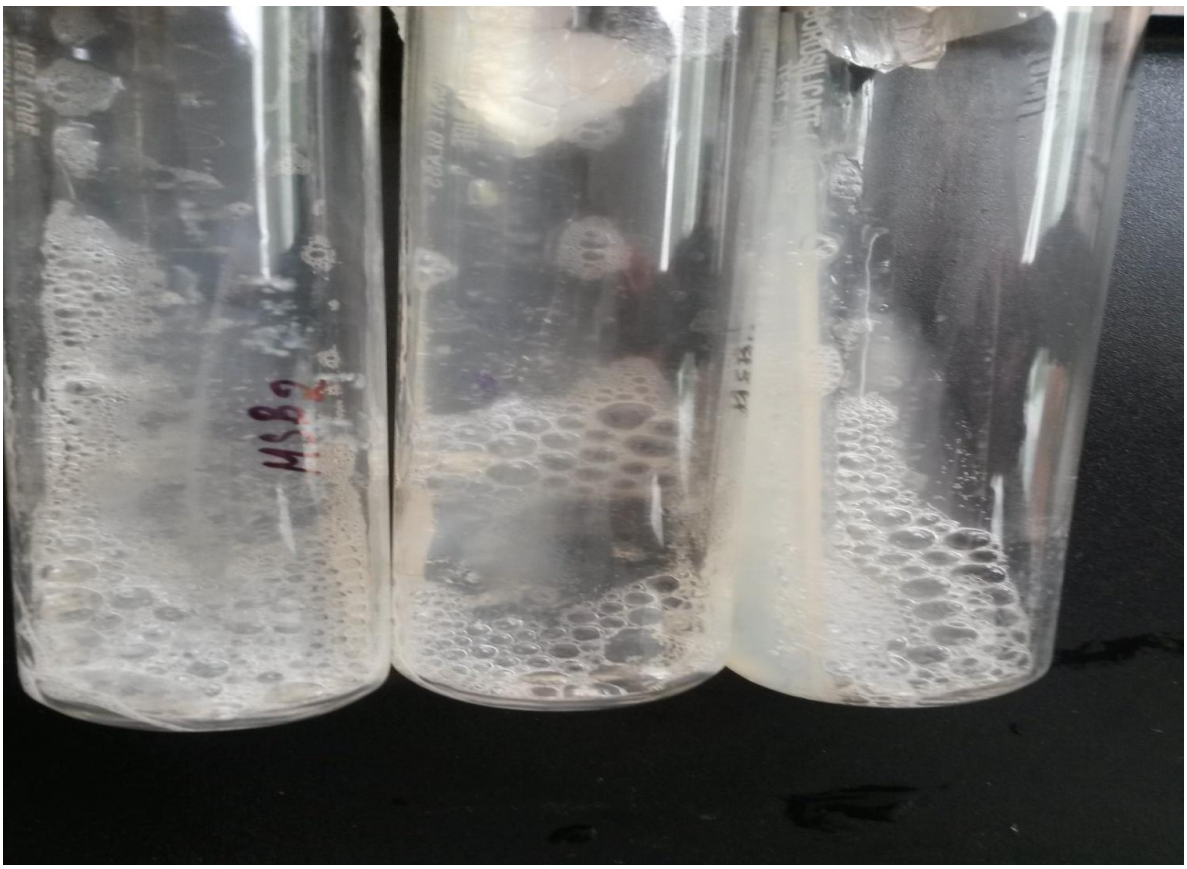

Fig.3 Catalase Test

Table 4(b): Lactic acid production test

S.no	**Organism Isolated**	**Observed pH**
1	MSF1	3-4
2	MSB3	3-4
3	MSF2	4-5
4	MSB1	4-5
5	MSB2	4-5
6	MSB4	4-5

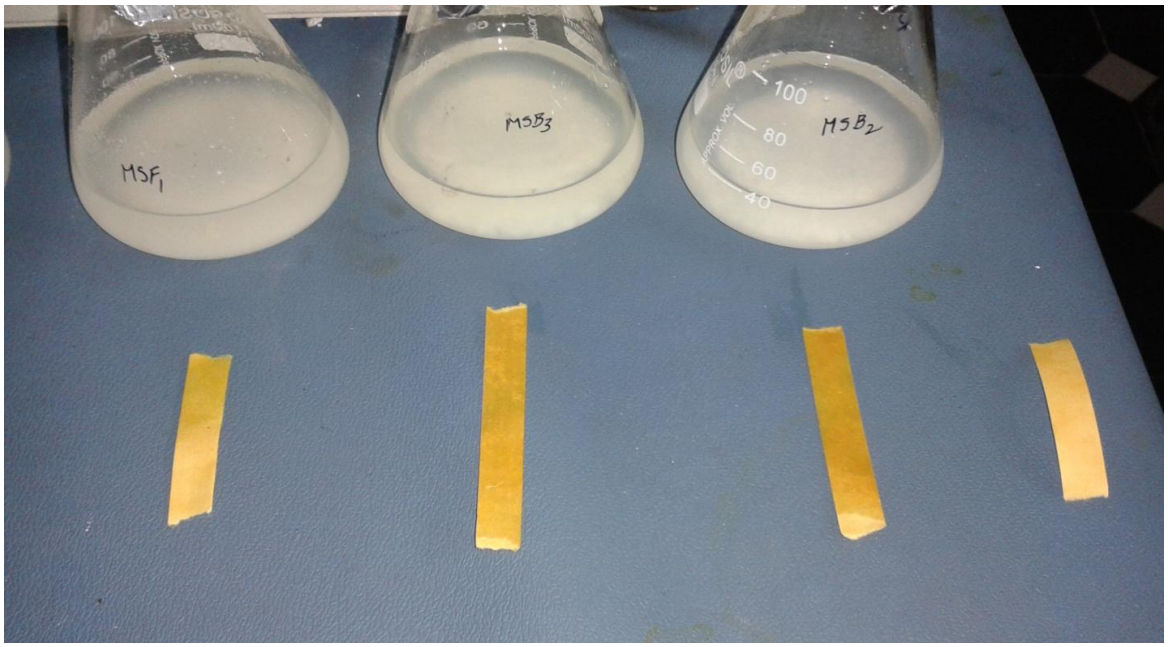

Fig.4 Lactic Acid Production

V. CONCLUSION:

In this the microbiota from dairy effluent is efficient in inhibiting the pathogenic microorganisms and will act as a barrier by developing its antimicrobial activities in the host defense system. Bacteriocin activity was found more in MSB2 andMSB3.It was observed that after optimization of medium components and physical parameters, bacteriocin production was enhanced. Best bacteriocin producing bacterial strain MSB2 was identified as Paenibacillus lactis. The inhibitory spectrum of the bacteriocin has potential application as a biopreservative in food industry.

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