

Research Article



Isolation and Antimicrobial Spectrum of New Bacteriocin From *Lactobacillus rennanquilfy WHL 3*

Deshmukh P.V¹, Thorat P.R.²

¹Vidya Pratisthan's School of Biotechnology, Vidyarnagari, Baramati, Dist-Pune (MS), India.

²P.G. Dept. of Microbiology and Research Center, Shri Shivaji Mahavidyalaya Barshi, Dist-Solapur (MS), India.

*Corresponding author's E-mail: pradesh187@gmail.com

Accepted on: 27-07-2013; Finalized on: 30-09-2013.

ABSTRACT

Production of antimicrobial substances is often regarded a priori as an important trait in the context of bacterial fitness but also in terms of probiotic efficacy. Several probiotic bacteria produce a variety of antimicrobial compounds viz. short chain fatty acids, Hydrogen peroxide, Nitric oxide, Bacteriocins. Bacteriocins are peptide or protein complexes showing antibacterial activity against closely related species. These antimicrobial agents are gaining more and more attention as an alternative therapeutics not only in pharmaceutical but also as a preservative in food industries. In present study, bacteriocin production was carried out by *Lactobacillus rennanquilfy WHL 3* isolated from whey and identified by 16s rRNA sequence analysis. This newly isolated strain showed antibacterial activity against several Gram positive and Gram negative bacteria. To obtain maximum bacteriocin yield, medium was optimized with various fermentation conditions like carbon and nitrogen source, Growth factors, pH and incubation temperature. An isolate WHL 3 showed the maximum bacteriocin production in modified MRS medium containing 1% Glucose, 1% Peptone, and 0.8% Yeast extract. Maximum bacteriocin production was observed at 40°C temperature at pH 6.0. *Lactobacillus rennanquilfy WHL 3* is capable of producing bacteriocin at wide range of temperatures and hence it is an ideal strain for bacteriocin production for food and pharmaceutical industries.

Keywords: Antimicrobial activity, Bacteriocin, *Lactobacillus rennanquilfy WHL 3*, Probiotics.

INTRODUCTION

Probiotics are defined as, "live microorganisms which when consumed in adequate amounts, confer a health benefit on the host."¹ The majority of probiotics in use today include species of Lactic Acid Bacteria (LAB), including *Lactobacilli* as well as *Bifidobacterium*, non-pathogenic *Escherichia coli*, *Bacilli* and Yeasts such as *Saccharomyces boulardii*.

Several mechanisms of probiotic action have been described, the most common relating to their abilities to strengthen the intestinal barrier, to modulate the host immune system, and to produce antimicrobial substances.² Indeed, the production of antimicrobial substances is often regarded a priori as an important trait in the context of bacterial fitness but also in terms of probiotic efficacy. Several probiotic bacteria produce a variety of antimicrobial compounds viz. Short chain fatty acids, Hydrogen peroxide, Nitric oxide, Bacteriocins., that may enhance their ability to compete against other GI microbes and which could potentially inhibit pathogenic bacteria.^{3,4}

Bacteriocins are protein or protein complexes produced by bacteria and have antimicrobial activity against closely related species and various Gram positive and Gram negative bacteria including food spoilage bacteria and pathogens.⁵ Bacteriocins are in general cationic (i.e. they contain an excess of lysyl and arginyl residues) amphipathic molecules composed of 12 – 45 amino acids residues. They are usually unstructured in aqueous solution, but have the propensity to form a α - helical

structure when exposed to structure promoting solvents such as trifluoroethanol or when mixed with anionic phospholipid membranes.⁶

Several types of bacteriocins from food-associated lactic acid bacteria have been identified and characterized, of which nisin, diplococcin, acidophilin, bulgarican, helveticins, lactacins, lactolin and plantaracins are the important bacteriocins.^{7, 8} Nisin is the only bacteriocin commercially available.⁹ Other bacteriocins of *Lactobacilli* have been reported to be effective against closely related species of *Lactobacillus* and therefore considered as potential natural food preservative. However, studies relating to the antibacterial properties of these organisms are limited and not fully exploited for use.^{10,11}

The current work aimed to isolate bacteriocin producing Lactic Acid Bacteria from whey sample, Identification of strains by 16s rRNA sequencing method, production of bacteriocin and determination of antimicrobial activity against common pathogens.

MATERIALS AND METHODS

Chemicals and Media

Analytical grade chemicals were obtained from Qualigenes, Thomas baker and SD Fine India, while media were obtained from Hi-media, India.

Test Microorganisms

The test microorganisms viz. *Alcaligenes fecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*,



Bacillus coagulans, *Aspergillus bombycis* and *saccharomyces bayanus* were procured from local hospitals.

Isolation and Identification of Lactic Acid Bacteria

The whey sample was collected in aseptic condition from local dairy plant in Barshi and processed within five hours for further studies. MRS broth and agar were used for enrichment and isolation of culture of Lactic Acid Bacteria.¹² The sample was filtered through filter paper and 1ml sample was added into the 10ml MRS broth and incubated at 37°C for 24 hrs under Micro aerophilic condition. After incubation, loopful of culture was spreaded on sterile MRS agar plate and all plates were incubated micro aerobically at 37°C for 24 hrs.

The well isolated colonies were selected randomly and transferred in MRS broth. They were streaked on MRS agar to check the purity of the isolates and then stored in MRS soft agar (0.5%) overlaid with glycerol at -20°C.

Screening of bacteriocin producer

The selected lactic acid bacterial isolates were cultured in MRS broth and incubated at same above mentioned conditions for 20 hrs. Aliquots of cultures were spotted on the sterile MRS agar plates and incubated micro aerobically at 37°C or 24 hrs. After incubation plates were overlaid with soft nutrient agar inoculated with culture of *Bacillus coagulans* and incubated at 37°C for 24 hrs under aerobic condition.

Isolate showing maximum zone of growth inhibition of *Bacillus coagulans* was selected and designated as WHL 3 and used for further studies.

Bacteriocin production from selected isolate

Bacteriocin production from selected isolate were carried out in three different medium viz. MRS broth, BSM (Bacteriocin Screening Medium) and M17 Medium.¹³ The selected culture was inoculated into above mentioned medium and incubated micro aerobically at 37°C for 24 – 48 hrs. During fermentation, aliquots of samples were removed for Growth curve and protein estimation analysis and results were recorded.

The medium showing maximum optical density in growth curve analysis and protein concentration was selected and used for further optimization studies.

Optimization of some bacteriocin production conditions

Some optimal conditions for cultivation of selected isolate were investigated to obtain efficient or maximum bacteriocin production. Optimization was done by changing one parameter and keeping other parameter constant. The composition of the medium showing maximum optical density and protein concentration in above step was varied as follows.

Effect of Carbon and Nitrogen source

Different Carbon and Nitrogen sources viz. Glucose, Fructose, Lactose, sucrose and Nitrogen source viz.

Peptone, Triptone, Soyatone and Skimmed milk were used at 1% concentration.

Concentration of yeast extract

Yeast extract was applied as growth factor. The optimal concentration of yeast extract was also investigated. The concentration of Yeast extract viz. 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2% is added in medium containing optimum concentration of carbon and nitrogen source.

Initial pH of bacteriocin production medium

Initial pH of the optimized medium for maximum bacteriocin production was studied. The pH of medium was adjusted to 4 to 9 using 1N HCL and 1 N NaOH.

Production temperature

Suitable temperature for bacteriocin production was investigated. Various incubation temperatures such as 25, 30, 35, 40, 45, 50, 55 and 60°C were used for production studies.

Production of bacteriocin using optimum conditions

Bacteriocin production was performed in above optimized medium. 5ml of inoculum of WHL 3 isolate was inoculated in optimized medium and incubated micro aerobically at 37°C for 24 – 48 hrs. After fermentation broth was centrifuged at 12000rpm for 15 min. and supernatant was collected. pH of the supernatant was adjusted to 7.0 with 1N NaOH. Precipitation was done with Ammonium Sulphate at 40% and 70% saturation level at 4°C.

After precipitation, broth was centrifuged at 15000 rpm for 20 min., precipitate was collected and stored in 0.2M Sodium Phosphate buffer (pH 6.9) and labeled as Crude Bacteriocin Preparation (CBP).¹⁴

Extraction of Bacteriocin

Chloroform – Methanol (2:1 v/v), was used for crude bacteriocin extraction. However produced precipitate at solvent – aqueous interphase was collected aseptically. Solvent was evaporated and precipitate was kept in buffer which was used for antimicrobial study.¹⁵

Antimicrobial activity of extracted bacteriocin

Agar well diffusion and paper disc methods were used to study antimicrobial activity of extracted bacteriocin. Agar well diffusion technique was performed as 0.1ml culture of test microorganisms is spreaded on sterile nutrient agar and wells were prepared. 100µl of extracted bacteriocin preparation (CBP) was added wells and plates were aerobically incubated at 37°C for 24 hrs.

Paper disc assay was done as, paper discs were prepared from CBP and 0.1 ml culture of test microorganism was spreaded on nutrient agar plate and disc was placed. Plates were aerobically incubated at 37°C for 24 hrs. After incubation zone of growth inhibition of test microorganisms were recorded.¹⁶



RESULTS

Isolation, Identification and Phylogenetic Analysis

Screening of bacteriocin producers were carried out by agar overlay method. In which the isolate showing maximum zone of growth inhibition of *Bacillus coagulans* was selected and used for production study (figure 1).

Lactic Acid Bacterial isolated designated as WHL 3 was selected as good candidate for bacteriocin production,

based on its ability to form clear and large zone of growth inhibition of *Bacillus coagulans*. Molecular identification was carried out of this isolate based on 16s rRNA gene sequencing. The phylogenetic tree was constructed by using Neighbour joining method by Kimura 2 parameter with 1000 replicas in MEGA 4.0 (Figure 2).

According to sequence similarities and multiple alignments, the isolate WHL 3 was found to be in close relation to *Lactobacillus rennanquilfy* WHL 3.

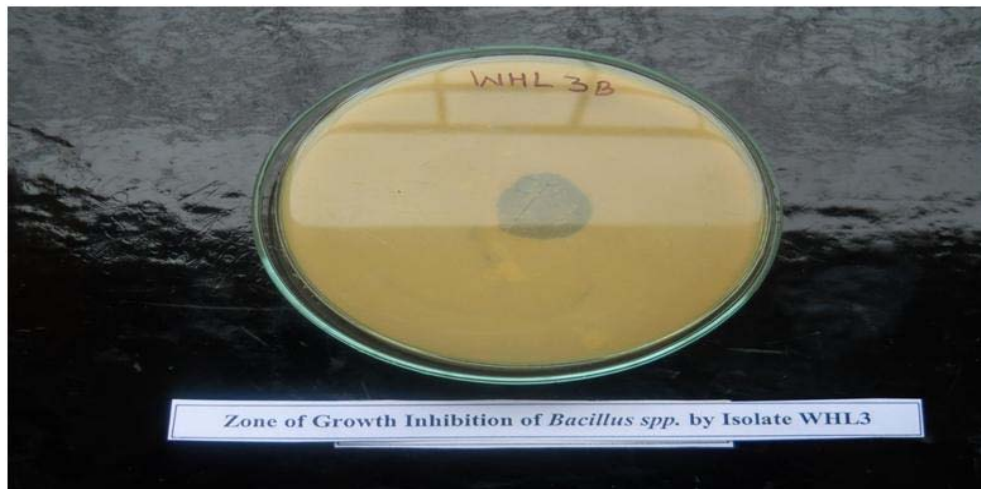


Figure 1: Zone of growth inhibition of *Bacillus coagulans* by WHL 3 isolate

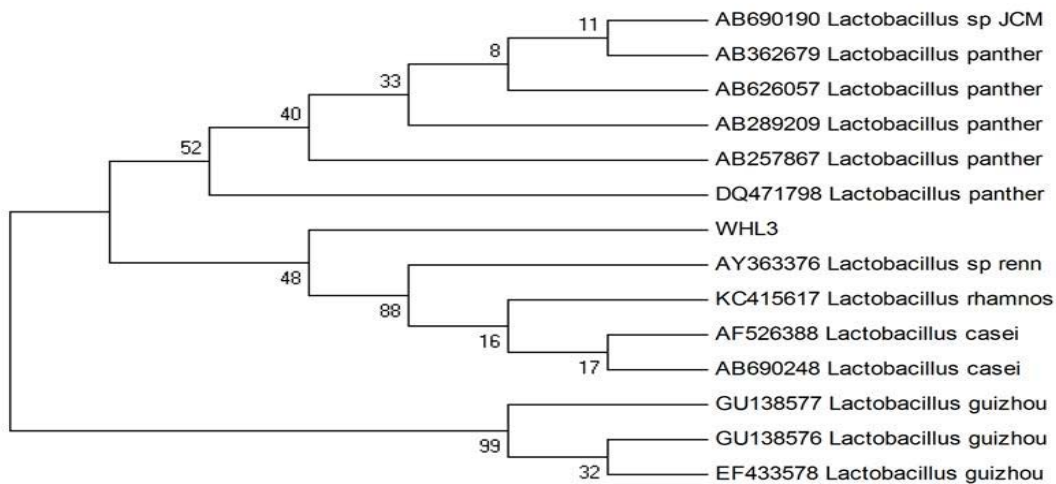


Figure 2: Phylogenetic tree of WHL 3 isolate

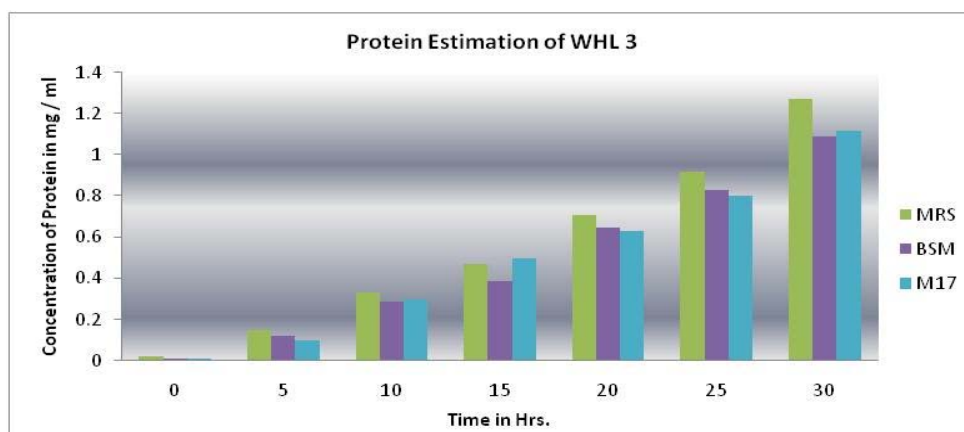


Figure 3: Estimation of protein in three different medium during fermentation by WHL 3 isolate

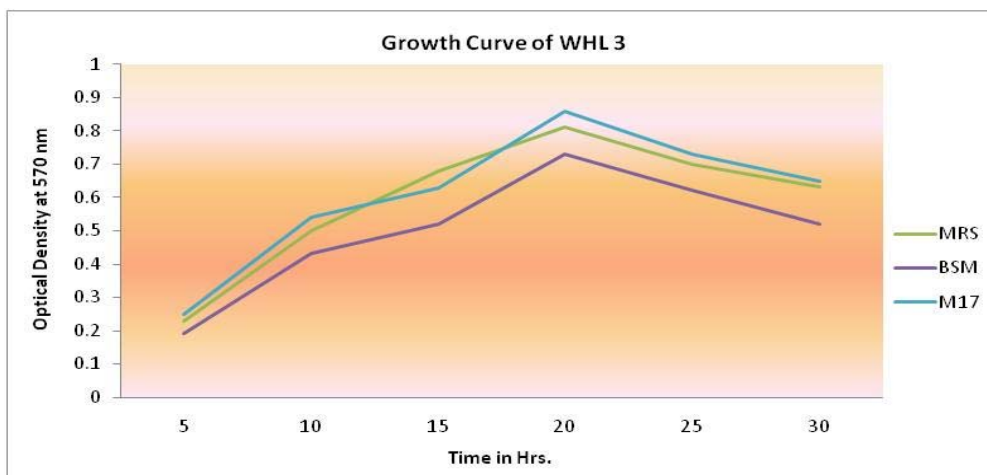


Figure 4: Growth curve of WHL 3 isolate in three different media

Production of bacteriocin before optimization

Bacteriocin production was carried out in three different medium viz. MRS broth, BSM and M17 broth. The inoculum of selected WHL 3 isolate was added and incubated micro aerobically for 24 – 48 hrs during incubation, protein estimation and growth curve was studied at time intervals (fig.3& 4). Among them MRS is the best medium for bacteriocin production by *Lactobacillus rennanquilfy* WHL 3, as it shows maximum protein concentration. But maximum bacteriocin production was achieved in modified MRS medium.

Optimization of production conditions

To obtain maximum bacteriocin yield, production conditions including Carbon and Nitrogen source, Growth factors, Initial pH and Incubation temperature were investigated and results were showed in fig. (5 to 9). From that result it was observed that maximum bacteriocin production was achieved at 1% Glucose, 1% Peptone and 0.8% Yeast extract. And less bacteriocin production was observed in Sucrose, arabinose, Soyatone and skimmed milk containing medium.

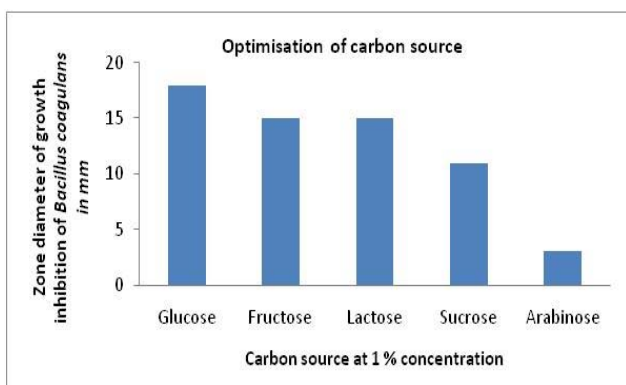


Figure 5: Optimization of Carbon source at 1 % concentration

Production of Bacteriocin in optimized medium

Optimized medium is prepared on the basis of above component optimized results. This newly modified medium is used for bacteriocin production from WHL3

isolate. After incubation broth was processed as explained earlier and extracted bacteriocin preparation was used for antimicrobial activity.

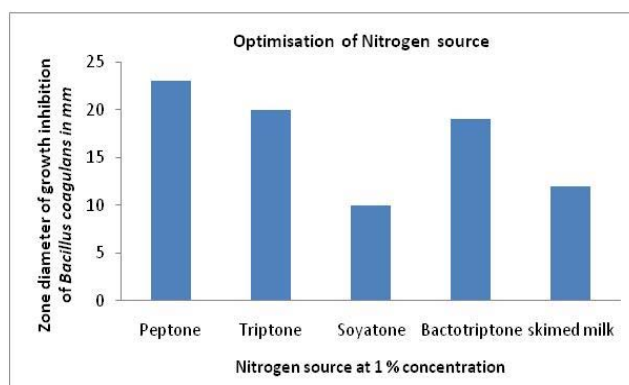


Figure 6: Optimization of Nitrogen source at 1% concentration

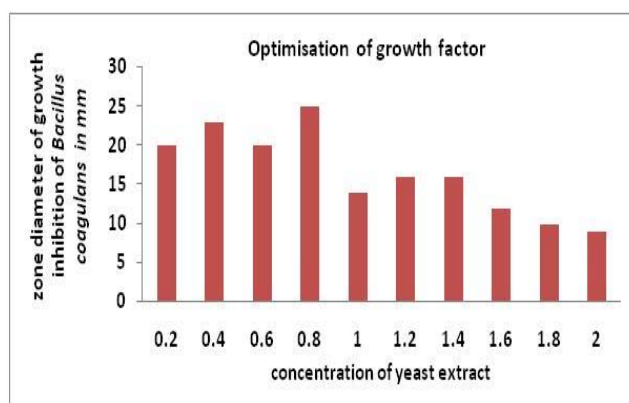


Figure 7: Optimization of Growth factor (Yeast Extract) at various concentrations

Antimicrobial spectrum of *Lactobacillus rennanquilfy* WHL 3

Crude bacteriocin preparation obtained from isolated WHL 3 strain was tested for antimicrobial activity against test microorganisms viz. *Alcaligenes fecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Pseudomonas aeuroginosa*, *Staphylococcus epidermidis*, *Bacillus coagulans*, *Aspergillus bombycis* and

saccharomyces bayanus by agar well diffusion method. The antibiogram of bacteriocin from isolated WHL 3 strain against test microorganisms were shown in Table 1.

Table 1: Antibiogram of *Lactobacillus rennanquilly* WHL 3 against test microorganisms

Name of test Microorganisms	Zone diameter of growth inhibition of test Microorganisms in mm.
<i>Alcaligenes fecalis</i>	20
<i>Escherichia coli</i>	24
<i>Klebsiella pneumoniae</i>	23
<i>Proteus vulgaris</i>	20
<i>Pseudomonas aeruginosa</i>	18
<i>Staphylococcus epidermidis</i>	26
<i>Bacillus coagulans</i>	21
<i>Aspergillus bombycis</i>	17
<i>saccharomyces bayanus</i>	16

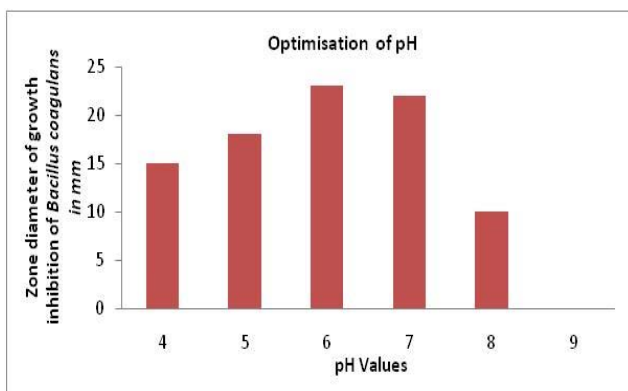


Figure 8: Optimization of pH

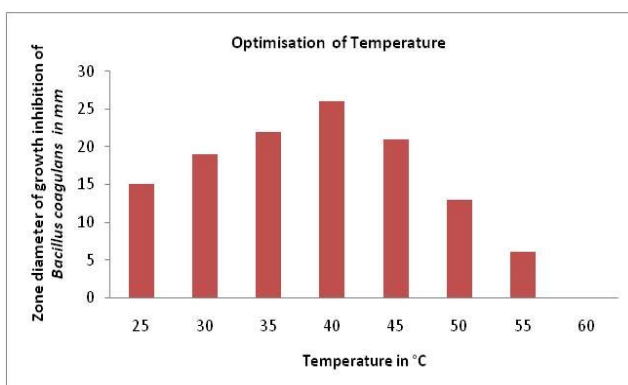


Figure 9: Optimization of incubation Temperature in °C

DISCUSSION

Lactic acid bacteria play important roles in food fermentation, and have also been shown to exert a variety of positive health and nutritional effects.¹⁷ Previous research results showed that bacteriocin from *Lactobacillus* spp. inhibited growth of pathogenic bacteria.

In present study, we isolated the Lactic Acid Bacteria designated WHL 3 from whey samples and identified by 16s rRNA gene sequencing and phylogenetic tree. From

phylogeny tree it was showed that isolate WHL 3 shows 48% similarities with *Lactobacillus rennanquilly*.

Bacteriocin production is frequently regulated by pH, incubation temperature, Growth factors, carbon and nitrogen source.¹⁸ In specific cases, higher bacteriocin production levels have been recorded at suboptimal conditions.¹⁹ In our present investigation, we observed that isolate WHL 3 grows well and produce maximum protein in MRS medium, to obtain good yield of bacteriocin, the MRS medium was modified on the basis of results obtained in optimization studies. In optimization study we observe the maximum zone of growth inhibition of test microorganisms at 1% glucose, 1% peptone, 0.8% yeast extract, pH 6.0 and at temperature 40°C.

The above results correlates with results of Svetoslav (2005), in that he studied the production and optimization of bacteriocin from *Enterococcus faecium* ST 311LD.²⁰ Our results were also similar to the results of Bing Han (2011), in that he studied optimization for bacteriocin production by *Lactobacillus plantarum* YJG.²¹ During present investigation, we studied the antimicrobial activity of bacteriocin produced from WHL 3 isolate, and found that it shows considerable zone of growth inhibition of test microorganisms listed above. From that results it was clear that all test microorganisms used were sensitive to bacteriocin from WHL 3. This result shows similarity to the results of Asma Ansari (2012), in that they studied production and antimicrobial spectrum of bacteriocin from *Bacillus subtilis* KIBGE IB – 17.²²

CONCLUSION

Finally from this present study it was concluded that, the isolate *Lactobacillus rennanquilly* WHL 3 shows good growth and bacteriocin production in above modified MRS medium. And from antimicrobial spectrum of WHL 3 it was concluded that all the test microorganisms used in study were sensitive to bacteriocin from WHL 3.

REFERENCES

1. Pineiro M, Stanton C, Probiotic bacteria: legislative framework – requirements to evidence basis, Journal of Nutrition, 137, 2007, 8505-8535.
2. Corr SC, Hill C, Gahan CGM, understanding the mechanism by which probiotics inhibit gastrointestinal pathogens, Adv. Food Nutr. Res., 56, 2009, 1-15.
3. Atassi F, Servin AL, Individual and co-operative roles of lactic acid and hydrogen peroxide in the killing activity of enteric strain *Lactobacillus johnsonii* NCC 933 and vaginal strain *Lactobacillus gasseri* KS 120 against enteric uropathogenic and vaginosis – associated pathogens, FEMS Microbiology Letters, 304, 2010, 29-38.
4. Chenoll EA, Novel probiotic *Bifidobacterium bifidum* CECT 7366 strain active against the pathogenic bacteria *Helicobacter pylori*, Applied Environmental Microbiology, 77, 2011, 1335-1343.

5. Cleveland J, Montville TJ, Nes IF, Chikindas ML, Bacteriocins safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology*, 71, 2001, 1-20.
6. Gert NM, Wil NK, Arnold JM, Driessen, Bacteriocins: Mechanism of membrane insertion and pore formation, *Antonie Van Leeuwenhoek*, 76, 1999, 185-198.
7. Aly EA, Characterization of a Bacteriocin – like inhibitory substances produced by *Lactobacillus plantarum* isolated from Egyptian Home – made Yogurt, *Science - Asia*, 33, 2007, 313-319.
8. Nettles CG, Barefoot SG, Biochemical and genetic characterization of bacteriocins of food – associated lactic acid bacteria, *Journal of Food Protection*, 56, 1993, 338-356.
9. Balasubramanyam BV, Varadaraj MC, Cultural conditions for the production of bacteriocin by a native isolate of *Lactobacillus delbrueckii ssp. Bulgaricus CFR – 2028* in milk medium, *Journal Of Applied Microbiology*, 84, 1998, 97-102.
10. Abdel – Bar N, Harris ND, Rill RL, Purification and properties of an antimicrobial substance produced by *Lactobacillus bulgaricus*, *Journal of Food Science*, 52, 1987, 411-415.
11. Reddy GC, Shahani KM, Friend BA, Chandan RC, Natural antibiotic activity of *Lactobacillus acidophilus* and *bulgaricus* production and partial purification of *Bulgaricus* cultured, *J. Dairy Products*, 8, 1984, 15-19.
12. De Mann, Rogosa JC, Sharpe ME, A Medium for the cultivation of Lactobacilli, *Journal of Applied Bacteriology*, 23, 1960, 130-135.
13. Jamriangrit P, Characterization and purification of a bacteriocin produced by *Lactobacillus plantarum PMU 33* strain from fermented fish products, (Dissertation), Mahidol University, 2004, ISBN 974 – 04 – 4663 – 9.
14. Navarro L, Zarazaga M, Saenz J, Ruiz- Larrea F, Torres C, Bacteriocin production by lactic acid bacteria isolated from Roja red wines, *Journal of Applied Microbiology*, 88, 2000, 44-51.
15. Deshmukh PV, Thorat PR, Isolation and Extraction of Bacteriocins produced by Lactic acid bacteria isolated from raw milk, *Indian streams research journal*, 3(3), 2013, 1-7.
16. Sansit J, Detection of bacteriocins from starch utilizing and lactic acid producing bacteria (Dissertation), Suranaree University of Technology, (2004), ISBN – 974 – 533 – 377 – 8.
17. Gilliland SE, Health and nutritional benefits from Lactic acid bacteria, *FEMS Microbiology Reviews*, 87, 1990, 175-178.
18. Sri Usmiati, Tri Marwati, Selection and optimization process of bacteriocin production from *Lactobacillus sp*, *Indonesian Journal of Agriculture*, 2(2), 2009, 82 – 92.
19. Parente E, Ricciardi A, Addario G, Influence of pH on growth and bacteriocins production by *Lactococcus lactis subsp. Lactis 140 VWC* during batch fermentation. *Applications of Microbiology and Biotechnology*, 41, 1994, 388-394.
20. Svetoslav D, Todorov, Leon MT, Dicks, Optimization of bacteriocin ST 311LD production by *Enterococcus faecium ST 311LD* isolated from spoiled black olives, *Journal of Microbiology*, 43(4), 2005, 370-374.
21. Bing H, ZhanqiaoT, Baosheng L, Qingshan M, Rijun Z, Optimization of bacteriocin production by *Lactobacillus plantarum YJG* isolated from mucosa of the gut of healthy chickens, *African Journal of Microbiology Research*, 5(10), 2011, 1147-1155.
22. Ansari A, Aman A, Siddiqui NN, Iqbal S, Shah AQ, Bacteriocin (BAC – IB 17): Screening, isolation and production from *Bacillus subtilis KIBGE IB – 17*, *Pakistan Journal of Pharmaceutical Sciences*, 25 (1), 2012, 195-201.

Source of Support: Nil, **Conflict of Interest:** None.

Corresponding Author's Biography:



Mr. Pravin Graduated and Post graduated from Shivaji University, India. At post graduation level taken specialization in Probiotics, Bacteriocin and Antimicrobial Peptides. Working as Assistant Professor at Vidya Pratisthans College of Agricultural Biotechnology, Baramati, India.



Dr. P.R. Thorat is Graduated and Post Graduated from Shivaji University, India. He is having 25 years of teaching experience and 15 years of research experience at Shivaji University and Solapur University, India. He handled multiple Major and Minor Projects of UGC, and also guiding post graduate and PhD students. His area of specialization is Environmental microbiology and Medical Microbiology.