JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

Sharma et al. 2013 : 3 (2) 168-171

POTENTIAL OF *Lactococcus lactis* subsp. *lactis* MTCC 3041 AS A BIOPRESERVATIVE

Neha Sharma[1*], *Seema Garcha*[2], *Sunity Singh*[1]

Address(es): Neha Sharma
[1]Department of Biotechnology, Shoolini University,Bajhol, Post Office Sultanpur, Solan, HP-173212, India, Ph. No.-+919872420840.
[2]Department of Biotechnology, Punjabi University, Patiala.

*Corresponding author: neha1984micro@gmail.com, neha_sharma_pu@yahoo.co.in

ARTICLE INFO

Received 12. 3. 2013
Revised 5. 9. 2013
Accepted 23. 9. 2013
Published 1. 10. 2013

Regular article

OPEN ACCESS

ABSTRACT

Lactic acid bacteria especially in developing countries can be exploited against frequently occurring spoilage organisms of fresh fruits and vegetables in addition to pathogens. Keeping in views this antagonism imparted by bacteria *Lactococci*, the present study was taken and effectiveness of bacteriocin of *Lactococci* was also studied in preservatives and enzymes. Lactic acid bacteria *Lactococcus lactis* subs. *Lactis* MTCC 3041 was used as bacteriocin producer strain. Isolation of most frequently occurring spoilage organisms from spoiled Mango and Kinnow was done by microbiological procedures and were identified by microscopic studies as Isolate 1 and Isolate 2. It has limited use in processed salted food as no zone of inhibition was observed at and above 5% NaCl (w/v).0.3% (w/v) is the minimum concentration of KMS that provides stress to the microorganism for the production of bacteriocin. It is not suitable for food having sodium benzoate as preservative as with increase in concentration growth of *Lactococcus lactis* decreases. Presence of bacteriocin hinders the growth of the isolate 1 as fresh weight of the mycelium in test sample is 7.09% less than the control. Being non-pathogenic this organism can be safely used against spoilage organisms in addition to food borne pathogens.

Keywords: Bacteriocin, preservative, lactic acid bacteria, *Lactococci*

INTRODUCTION

World production of vegetables amounted to 486 million tonnes, while that of fruits reached 392 million tonnes. Post-harvest fruit and vegetables losses are as high as 30 to 40% and even much higher in some developing countries (**Panhwar, 2006**). It is also a matter of concern that there is a wide gap between availability and the per capita income nutritional requirement of fruits. A recent survey shows that in India, about 50% of total fruits and vegetables produced annually are being lost due to poor post-harvest practices. Consequently, net per capita availability of fruits and vegetables is reduced (**Sudheer*et al*., 2007**). The low availability of quality fruits and vegetables is mainly due to considerably high post-harvest losses. 10-40% of our fresh fruits and vegetables are lost post-harvest due to poor handling, lack of infrastructure, preservation facilities and also microbial spoilage (**Panhwar, 2006**). A significant portion of losses of fruits and vegetables during post-harvest period is due to diseases caused by fungi and bacteria. The succulent nature of fruits and vegetables makes them easily invaded by these organisms. Besides attacking fresh fruits and vegetables, these organisms also cause damage to canned and processed products. Many serious post-harvest diseases occur rapidly and cause extensive break down of the commodity, sometimes spoiling the entire package. It is estimated that 36 % of the vegetable decay is caused by soft rot bacteria (**Sudheer *et al*., 2007**). Lactic acid bacteria (LAB) are gram positive, non –sporulating, acid tolerant bacteria and they produce specific proteinaceous substances, bacteriocin, that inhibits the growth of food borne pathogens (**Hilmi, 2010**). They are associated with nutritionally rich habitats. They can be found in the soil, water, manure, sewage, silage and other plant material. Some LAB are part of the microbiota on mucous membranes, such as the intestines, mouth and vagina of both humans and animals and may have a beneficial influence on these ecosystems. LAB can grow at temperatures from 5-45ºC and are tolerant to acidic conditions with most strains able to grow at pH 4.4 (**Holzapfel and Wood, 1998**). Bacteriocins are ribosomally synthesized, extracellularly released bioactive peptides or peptide complexes which have a bactericidal or bacteriostatic effect usually on closely related species (**Klaenhammer, 1993**).Commonly encountered spoilage microorganism of fresh fruits and vegetables are *Aspergillus, Saccharomyces, Staphylococcus* etc. Consumers have been consistently concerned about possible adverse health effects from the presence of chemical additives in their food. As a result, consumers are drawn to natural and "fresher" foods with no preservatives added. This perception, coupled with increasing demand for minimally processed foods with long shelf life and convenience, has stimulated research interest in finding natural but effective preservatives. Bacteriocins, produced by LAB, may be considered natural preservative or biopresevatives that fulfill these requirements. Bio preservation refers to the use of antagonistic microorganism or their metabolic products to inhibit or destroy undesired microorganisms in foods to enhance food safety and extend shelf life (**Schillinger *et al*., 1996**). It would also reduce post-harvest losses, control price rise and supplement the farmers' income. LAB and their bacteriocins have been consumed unintentionally for ages, laying down a long history of safe use. Bacteriocins have also been suggested as an alternative to antibiotic feeding and the use of bacteriocin producers able to colonize the gastrointestinal tract has successfully reduced the carriage of zoonotic pathogens (**Diez-Gonzalez, 2007; Calo-Mata *et al*., 2008; Line *et al*., 2008**). Nisin-producing dairy starters have been designed to specifically inhibit Staphylococcus aureus in acid-coagulated cheeses and *C. tyrobutyricum* in semi-hard cheeses (**Rilla *et al*., 2003; Rilla *et al*., 2004**). Nisin and pediocin PA-1 are bacteriocins licensed as food preservatives (**Simha *et al*., 2012**). Lactic acid bacteria especially in developing countries like India can be exploited against frequently occurring spoilage organisms in addition to pathogens. Keeping in views this antagonism imparted by bacteria *Lactococci*, the present study was undertaken with an objective to exploit Lactic Acid Bacteria for most frequently occurring spoilage organisms and effectiveness of this bacteriocin by a *Lactococci* was also studied in preservatives and enzymes.

MATERIAL AND METHODS

Microbial Cultures

- *Lactococcus lactis* subsp. *lactis* MTCC 3041- Bacteriocin producer strain
- *Lactococcus lactis* subsp. *lactis* MTCC 3038- Susceptible strain

Both the cultures were procured from MTCC, IMTECH, Sec- 39 A, Chandigarh and maintained by periodic sub culturing on the recommended MRS medium. Slants were incubated at 37ºC for 24 hr and stored under refrigeration conditions till further use.

Isolation of frequently encountered spoilage organisms

Samples of microbial spoiled Mango and Kinnow were collected aseptically and kept in sterile containers. 1 gm of these samples was suitably diluted and plated

on Rose Bengal Chloramphenicol Agar. The plates were incubated at 37ºC for 72 hr. Pure culture of most frequently encountered organism was preserved for use in the study. They were identified microscopically in the lab.

Assay of Bacteriocin

Preparation of inoculum of MTCC 3038

A 5 ml MRS media was inoculated with loop full of the 3038 culture. It was incubated at 37ºC overnight. This broth culture was further used for assay (**Choi *et al*., 2000**).

Preparation of bacteriocin

A 5 ml MRS media was inoculated with loop full of the 3041 culture. It was incubated at 37ºC overnight and used to inoculate 20 ml of the medium @ 1% (v/v) and further incubation was done at 37ºC for 24 hr. Then it was centrifuged at 10, 000 rpm for 15 min and pH was adjusted at 6.0 by using NaOH or glacial acetic acid (**Choi *et al*., 2000**).

Bacteriocin activity

It was done as per protocol given by **Tagg and Mcgiven (1971)**. Broth culture of susceptible strain was used for spreading (100 µl) and supernatant (50 µl) of producer strain was added in the wells. A control well containing distilled water was also run simultaneously. Plates were checked for presence or absence of zone of inhibition.

Demonstration of Chemical nature of bacteriocin

It was done as described by Kim et al., 2000 (**2000**). Stock solution of Proteinase K was made in 50 mM phosphate buffer @ 1mg/ml (pH 7.0). 5 ml of supernatant was prepared. Enzyme and supernatant was incubated for 2 hr at 37ºC and then kept in boiling water for 3 min to denature the enzyme. Sensitivity of bacteriocin to enzyme was checked by well diffusion method.

Effect of preservatives

Sodium Chloride

Overnight culture of producer strain was used to inoculate MRS broth tubes having different concentrations of NaCl namely 2.0, 3.0, 4.0, 5.0 (% w/v) @ 2% (v/v) and OD was recorded at 600 nm after every 3 hr to trace the growth. Sensitivity of bacteriocin against MTCC 3038 was also checked by well diffusion method as described above. The zone of inhibition was measured.

Sodium Benzoate

Overnight culture of MTCC 3041 was used to inoculate MRS broth tubes having different concentrations of Sodium Benzoate namely 0.1, 0.2, 0.3, 0.4, 0.5 (% w/v) @ 1% (v/v) OD was recorded at 600 nm after every 3 hr to trace the growth. Sensitivity of bacteriocin against MTCC 3038 was also checked by well diffusion method as described above. The zone of inhibition was measured.

Potassium Meta Bisulphite (KMS)

Overnight culture of MTCC 3041 was used to inoculate MRS broth tubes having different concentrations of KMS namely 0.1, 0.2, 0.3, 0.4, 0.5 (% w/v) @ 1% (v/v). Activity of bacteriocin was checked by well diffusion method and zone of inhibition was measured.

Activity of Bacteriocin against isolated spoilage organisms

Yeast

Cell suspension of isolated yeast culture was prepared in sterile saline blank. Sensitivity of yeast towards bacteriocin was checked by well diffusion method as described above in which instead of MTCC 3038 this cell suspension (100 µl) was used for spreading on MRS agar plates ,supernatant (50 µl) was added in the wells and plates were observed for zone of inhibition.

Mold

Potato Dextrose Broth was used to grow isolated fungus culture. 50 ml of the selective medium was inoculated with mold isolate. 10 ml of supernatant was added to it and incubation at 37ºC for 72 hr was done. A control flask without supernatant was also run. After 72 hr of incubation freshweight of the mold was recorded to ascertain whether the bacteriocin produced by MTCC 3041 is effective against spoilage mold.

RESULTS AND DISCUSSION

Isolation of most frequently encountered spoilage organisms

Most frequently encountered spoilage microorganisms were isolated from spoiled Mango and Kinnow. Pure culture was made. They were microscopically viewed for identification purposes. Fungus (Isolate 1) was viewed in 10% KOH (w/v) under 40X. As shown in Figure 1, this fungus belongs to class –*Ascomycetes*, Order- *Eurotiales*. It is a species of *Aspergillus*. It is most commonly encountered fungus present in air and is also naturally present on fruits and vegetables. Moreover so because of the specialized structure called 'asci'. Conidiophores were nonseptate and conidia were borne in chain. Conidia were of green colour (**Pelczar *et al*., 2005**). Wet mount of yeast (Isolate 2) was made and viewed under 40X as shown in Figure 2. On the basis of colony characters this isolate was identified as yeast which was further supported by microscopic observation. It was elliptical and length varied between 2-2.5µ whereas width varied between 4-5µ.

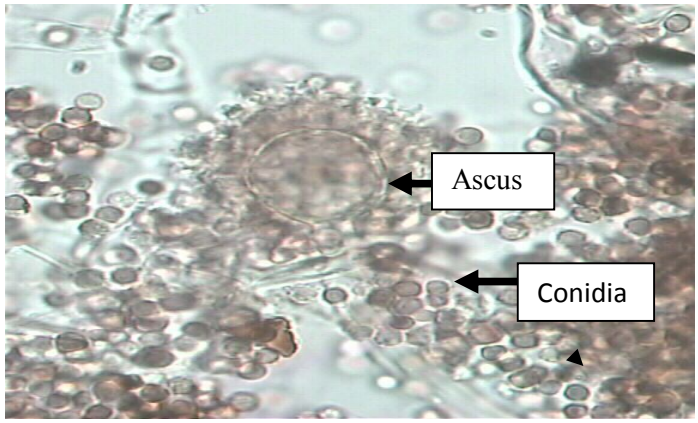

Figure 1 Isolate 1 as viewed under 40 x. Presence of asci and conidia indicates it to be a sp. of *Aspergillus*

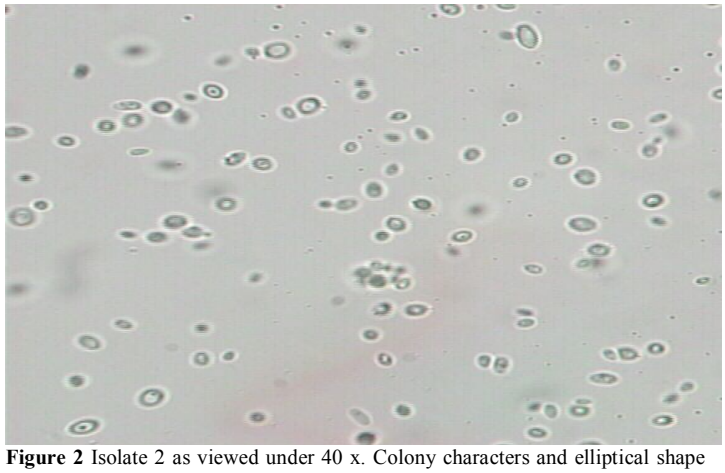

Figure 2 Isolate 2 as viewed under 40 x. Colony characters and elliptical shape indicates it to be belonging to yeast

Growth Curve

Figure 3 represents the growth curve and different growth phases of the bacteriocin producing strain. From the O.Ds it is clear that 6-21hr is the early exponential phase of bacterial growth in which bacteria is actively dividing. On the other hand 21-28 hrs of growth are late exponential phase of growth of bacteria and 28-37 hr of growth is stationary phase and then steep decline phase starts. In addition to expected reasons decline in growth is due to the absorption of bacteriocin on the cell of producing strain in the stationary phase or due to proteolytic degradation (**Lejeune*et al*., 1998**; **Parente and Ricciardi, 1999**).

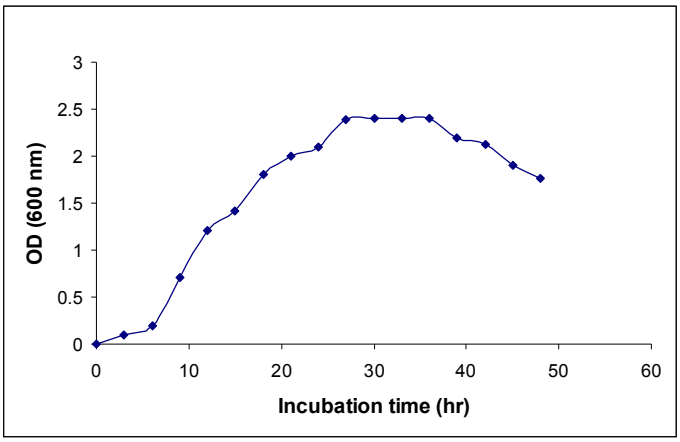

Figure 3 Growth curve for *L. lactis* subsp. *lactis* MTCC 3041

Demonstration of proteinaceous nature of bacteriocin

Proteinase K was treated against bacteriocin by the method given by **Kim *et al*** (**2000**). The antibacterial activity was completely eliminated when treated with Proteinase K. this result strongly suggests that the antibacterial compound is composed of proteins (**Franz *et al*., 2007**; **Yamamoto *et al*., 2007**).

Effect of preservatives

Bacteriocin can also be used in combination with other preservatives to check whether it is effective in their presence. For this three preservatives were chosen- NaCl, Sodium Benzoate and KMS.

Effect of NaCl on growth and production of bacteriocin

MTCC 3041 was inoculated @ 1% (v/v) in MRS broth tubes containing NaCl at different concentration namely 2, 3, 4, 5 (% w/v) activity was checked by well diffusion method. As shown in table 1 at 2% (w/v) NaCl zone of inhibition is 197 mm. As concentration increase up to 4%, the zone of inhibition increases up to 307 mm. No zone of inhibition is observed at 5% (w/v). It is probably due to lowered water activity and salt stress which enhances bacteriocin production. Corresponding growth curve were also plotted (Figure 4). Duration of log phase decreased as NaCl concentration increases compared to growth curve at optimum condition. The extent of growth decreases with increase in salt concentration from 2.0% to 5.0% (w/v). The higher concentration of NaCl decreased the rate of growth of *L. lactis* subsp. *lactis* and bacteriocin production (**Patricia *et al*., 1999**). It can tolerate low concentration of NaCl and can survive in broth with 4% NaCl (**Parente *et al*., 1994**).

Table 1 Effect of NaCl on production of bacteriocin

S.No.	Conc. (% w/v)	IZD (mm)
1	2	197
2	3	202
3	4	307
4	5	-

Legend: Conc. - Concentration, IZD- Inhibition zone of diameter, mm-milimeter

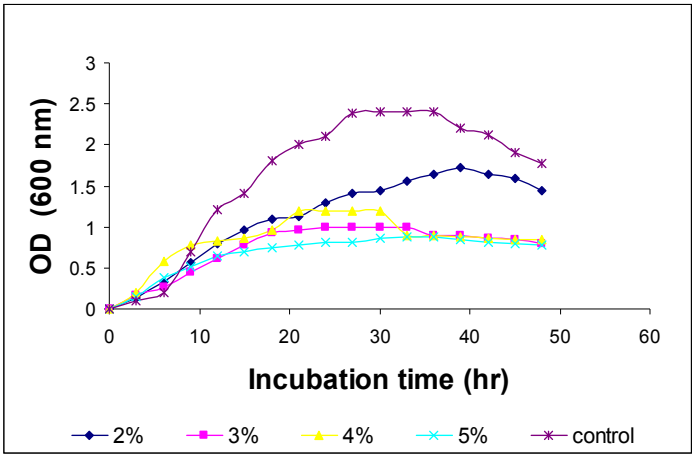

Figure 4 Effect of NaCl on growth of *Lactococcus lactis subsp. lacti* MTCC 3041

Effect of Sodium Benzoate on growth of *Lactococcus lactis* subsp. *lactis* MTCC 3041

MTCC 3041 was inoculated @ 1% (v/v) in MRS broth tubes containing Sodium Benzoate at different concentration namely 0.1, 0.2, 0.3, 0.4, 0.5 (%w/v). Activity was checked by well diffusion. As seen in Figure 5, there is decrease in growth with increase in concentration of sodium benzoate from 0.1% to 0.5% when compared with control. At 0.5% (w/v) concentration there is a marked decrease in multiplication of bacteria. Low biomass translates into less bacteriocin production. The duration of exponential phase decreases gradually and thus affects bacteriocin production. Sodium Benzoate presents a good inhibition effect against yeasts and molds, worse on butyric bacteria and hardly effects on lactobacilli (**Waldemar *et al*., 2002**).

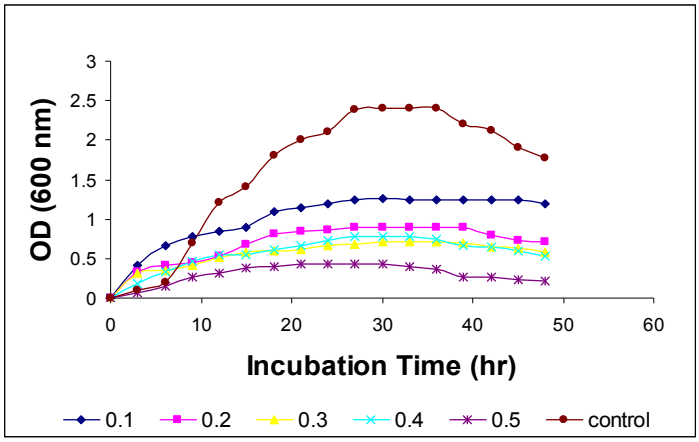

Figure 5 Effect of sodium benzoate on growth of *Lactococcus lactis subsp. lactis* MTCC 3041

Effect of KMS on production of bacteriocin

L. lactis subsp. *lactis* MTCC 3041 was inoculated @ 1% (v/v) in MRS broth tubes containing KMS at different concentration namely 0.1, 0.2 and 0.3 (% w/v). Activity was checked by well diffusion. As shown in table 2 at 0.1 and 0.2% (w/v) no zone of inhibition is formed. At 0.3% (w/v) zone of inhibition of 190 mm is formed. This is probably the minimum threshold concentration that provides stress to this microorganism and triggers bacteriocin production. It implies that this bacterium can be exploited as a bio preservative in foods which contain KMS as an additive.

Table 2 Effect of KMS on production of bacteriocin

S.No.	Conc. (%w/v)	IZD (mm)
1	0.1	-
2	0.2	-
3	0.3	190

Legend: Conc. - Concentration, IZD- Inhibition zone of diameter, mm-millimeter

Control of spoilage isolates using bacteriocin

Cell suspension of isolate 2 (yeast) was made in sterile saline blank. Well were made in MRS agar poured petriplates. These plates were spread with the cell suspension and wells were filled with supernatant of MTCC 3041 and efficacy was checked by well diffusion. It is a qualitative test done with a view to widen susceptible spectrum. The yeast strain isolated is not affected to any appreciable level by the bacteriocin.Isolate 1 (mold) was inoculated in 50 ml of potato dextrose broth and 10 ml of supernatant was added to it. Incubation was done at 37ºC for 72 hr. Fresh weight of control and test sample was compared. As shown in table 3, the fresh weight of control mycelium is 2.95 gm. Percentage decrease of 7.09% is observed. Presence of bacteriocin hinders the growth of this mold. It can therefore be exploited for control of spoilage molds of fresh fruits and vegetables.

Table 3 Effect of bacteriocin on spoilage molds

S. No.	Sample	Fresh wt. (g)
1	Control	2.95
2	Bacteriocin and isolate	2.76

CONCLUSION

As more and more bacteriocins, producers that are LAB (probiotics) are being isolated and characterized, usually from food environments, the potential for their use increases. Bacteriocin should not be seen as a primary means of food preservation, rather, they contribute to the 'hurdle' approach to food preservation and safety whereby a number of barriers, both intrinsic and extrinsic, act as

hurdles for microorganism to overcome in the food. Being non-pathogenic this organisms can be very safely used. Their antimicrobial spectrum should be studied to extend their use against food spoilage organisms in addition to food borne pathogens.

Acknowledgements: The authors are grateful to Punjabi University, Department Of Biotechnology, Patiala, Punjab, India for support and institutional facilities.

Conflicts of interest: None

REFERENCES

CALO-MATA, P., ARLINDO, S., BOEHME, K., de Miguel, T., Pascoal, A., BARROS-VELAZQUEZ, J. 2008.Current applications and future trends of lactic acid bacteria and their bacteriocins for the biopreservationof aquatic food products.*Food and Bioprocess Technology*, 1, 43-63.

CHOI, HJ., CHEIGH, CL., KIM, SB., PYON, YR. 2000. Production of a nisin like bacteriocin by *Lactococcus lactis* subsp. *lactis* A164 isolated from Khimchi. *J ApplMicrobiol*, 88, 563-571.

DIEZ-GONZALEZ, F.2007.Use of bacteriocin in livestock. InM.A. Riley & O. Gillor *Research and applications in bacteriocins,* 117-129. Norfolk Horizon. Bioscience.

FRANZ, MAP., TOIL, MD., HOLY, VA.,SCHILLINGER, U., HOLZAPFEL, WH. 2007. Production of nisin like bacteriocin by *L. lactis* strain isolated from vegetables. *J Basic Microbiol,* 37(3), 187-196.

Global market review: Potential for horticultural exports from India. 2009. *Agri export advantage*, 11 (4).

HILMI, HTA. Lactic acid bacteria and their antimicrobial peptides: Induction, detection, partial characterization, and their potential applications [PhD Thesis].2010. University of Helsinki, Finland.

HOLZAPFEL, WH., WOOD, BJB. 1998. The genera of lactic acid bacteria .1st edition. Blackie Academic and Professional, London.

KIM, CH., JI, GE., AHN, CE. 2000. Purification and molecular characterization of a bacteriocin from *Pediococcus* sp. KCA 1303-10 isolated from fermented flat fish. *Food SciBiotechnol*, 9(4), 270-276.

KLAENHAMMER, TR. 1993. Genetics of bacteriocins produced by lactic acid bacteria FEMS. *Microbiol Rev,* 12, 39-85.

LEJEUNE, R., CALLEWAERT, R., CRABBE, K. 1998. Modelling the growth and bacteriocin productions by *Lactobacillus amylovorus* DCE 471 in latch cultivation. *J ApplMicrobiol*, 84, 159-168.

LINE, JE., SVETOCH, EA., ERUSLANOV, BV., PERELYGIN, VV., MITSEVICH, EV., MITSEVICH, IP.,LEVCHUK,VP.,

SVETOCH,OE., SEAL,BS., SIRAGUSA,GR., STERN,NJ. 2008. Isolation and purification of enterocin E-760 with broad

antimicrobial activity against gram- positive and gram-negative bacteria. *Antimicrobial Agents and Chemotheraphy*,52, 1094-1100.

PANHWAR, F. 2006. Postharvest technology of fruits and vegetables. http://www.eco-web.com/editorial/060529.html.

PARENTE, E., RICCIARDI, A. 1999. Production, recovery and purification of bacteriocins from lactic acid bacteria. *Appl Microbiol Biotechnol,* 52, 628-38.

PARENTE, E., RICCIARDI, A., ADDARIO, G. 1994. Influence of pH on growth and bacteriocin production by *Lactococcus lactis* subsp. *lactis* 140 NWC during batch fermentation. *Appl Microbiol Biotechnol,* 41(4), 288-294.

PATRICIA, U., HAMELIN, J., JEAN-PAUL, LEP., BLANCO, C. 1999. Influence of osmolarity and the presence of a cosmoprotectant on *Lactococcus lactis* growth and bacteriocin in production. *J Appl Microbiol* ,65 (1), 291-293.

PELCZAR, MJ., CHAN, ECS., KRIEG, NR. 2005. Microbial agents of disease: Bacteria. *Microbiology*, 5th edition.

RILLA, N., MARTÍNEZ, B., DELGADO, T., & RODRÍGUEZ, A. 2003. Inhibition of *Clostridium tyrobutyricumin*Vidiago cheese

by *Lactococcus lactis* ssp. *lactis* IPLA 729, a nisin Z producer. *InternationalJournal ofFood Microbiology*,85, 23-33.

RILLA, N., MARTÍNEZ, B., RODRÍGUEZ, A. 2004. Inhibition of a methicillin-resistant *Staphylococcus aureus* strain in Afuega'l

Pitu cheese by the nisin Z-producing strain *Lactococcus lactis subsp. lactis* IPLA 729.*Journal of Food Protection*,67, 928-933.

SCHILLINGER, U., GEISEN, R., HOZAPFEL, WH. 1996. Potential of antagonistic microorganisms and bacteriocins for the biological preservation of foods. *Trends Food SciTechnol* , 7,158-64.

SIMHA, BV., SOOD, SK., KUMARIYA, R., GARSA, AK. 2012. Simple and rapid purification of pediocin PA-1 from *Pediococcus*

pentosaceous NCDC 273 suitable for industrial application.*Microbiological Research* .http://dx.doi.org/10.1016/j.micres.2012.01.001.

SUDHEER, KP,INDIRA, V. 2007. Post harvest technology of horticultural crops. *Horticulture Science*,7.

TAGG, JR., MCGIVEN, AR. 1971. Assay system for bacteriocins. *Appl Microbiol*, 21(5), 943.

WALDEMAR, D., KINGA, RK. KATARZYNA, C., DAGMARA, M. 2002. The influence of sodium benzoate on microflora of low salt herring . *J Polish Agri Uni*, 5(2).

YAMAMOTO, Y., IOGAWA, Y., SHIMOSAKA, M., OKAZAKI, M. 2003. Purification and characterization of a novel bacteriocin produced by *Enterococcus faecalis* strain RJ-11. *App and Env Microbiol*, 69, 5746-5753.