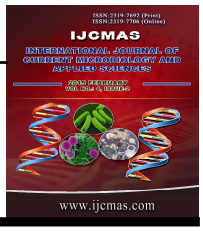
Int.J.Curr.Microbiol.App.Sci (2015) 4(2): 88-96

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 2 (2015) pp. 88-96**
http://www.ijcmas.com



Original Research Article

Determination of biopreservative effects of bacteriocins isolated from lactic acid producing bacteria against food spoiling fungi

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Keywords

Biopreservative, lactic acid producing bacteria, *L. acidophilus, L. plantarum, L. delbrueckii*

A B S T R A C T

Food spoilage is the main threat to food industry which makes the food unfit for human consumption. Many species of fungi are responsible for food spoilage. Since the last century, the concept that bacteriocin extracted from one microorganism can be used against another microorganism has been known, so the aim of this study was the application of bacteriocins as biopreservative. Lactic acid bacteria were isolated from yogurt samples. Four species of lactic acid bacteria *L. acidophilus*, *L. plantarum*, *L. delbrueckii* and *L. bulgaricus* were isolated and their bacteriocins were extracted. By using ammonium precipitation method bacteriocin was extracted from culture broth. Food spoiling fungi were isolated from bread, orange, rice and lemon. Antifungal activity of extracted bactreriocins was determined at 2-12 pH range, storage conditions (-20^{o} C, 4^{o} C and 37^{o} C for 30 days) and heat temperatures (60^{o} C, 100^{o} C and 120^{o} C for 15 min) by agar well diffusion method against *Aspergillus niger*, *Mucor* and *Penicillium.* Isolated fungi were sensitive to all bacteriocins extracted from lactic acid bacteria but bacteriocin extracted from *Lacobacillus acidophilus* showed maximum zone of inhibition. Maximum activity of all lactobacilli species were recorded in 4-8 pH range and at -20 ^{o}C storage temperature.It was concluded that comparatively better antifungal activity was exhibited by bacteriocin from *L. acidophilus.* This bacteriocin after purification and characterization can be used as biopreservative for the preservation of food.

Introduction

Due to alarming increase in population, there is a great pressure on the production of food to such a level at which it can fulfill the standard requirement of good life. There are great hurdles in the way to achieve this goal. One of them is the spoilage of food due to some bacteria and fungus (Ayanda *et al.*, 2013).

Many industries are facing food losses due to fungi and bacteria which deteriorate the food. These microorganisms produce mycotoxins which are health hazardous and cause food poisoning in humans (Ajimal *et al.*, 2011).

Since the beginning of life fermentation, low

or high temperature, acidity, use of stabilizer, thermal pasteurization, sterilization and freezing like techniques has been use to control the food spoilage but the excessive use of these technique change the flavour, taste and texture of food (Blake *et al*., 1995; Leistner, 1994).

Lactic acid bacteria (LAB) made it possible for human to increase the shelf life of food and food products by using antimicrobial activity of LAB without damaging food contents (Soomro *at al.,* 2002). Since the last century, the concept of microbial interference, the antagonism displayed by one microorganism towards another, has been known (Holzapfel *et al*., 1995; Jay, 1996).Different compounds such as diacetyl, organic acids, hydrogen peroxide and proteins (bacteriocins) are produced during lactic fermentation and used against food spoiling microorganisms (Anderssen *et al*., 1998; Zhennai, 2000).

Lactic acid bacteria are usually found in food products, also known as probiotics and produce bacteriocins which are proteinaceous compounds (Ray, 1992). Bacteriocins are manufactured by LAB as by-products that make them as attractive natural preservatives. Bacteriocins are released peptides or proteins of lower weight that have bactericidal or bacteriostatic effect (Chen and Hoover, 2003; Cotter *et al*., 2005).

Bacteriocins are degraded in the digestive system by the proteolytic enzymes and appear to be non-toxic and non-antigenic to human as well as animals. Bacteriocins have many applications especially in food industry. These properties have made it possible to use them for food preservation and to increase the shelf life of food products.

The successful development of nicin as safe natural biopreservative increased the interest in the field of bacteriocin research (Deraz *et al*., 2005; Deegan *et al*., 2006) due to its antimicrobial property and stability in different conditions such as temperature and pH (Nwuche, 2013).

Considering these qualities this study is based on the aim to extract the bacteriocins from lactic acid bacteria isolated from locally produced yogurt and their use to check their biopreservative effect against food spoiling fungi to fulfill the demand of consumer for safe food.

Materials and Methods

Collection and isolation

50 samples of locally produced yogurt were poured separately in the plates of de-Man-Rogosa-Sharp (MRS) agar. Poured plates were incubated at 37 °C for 20-72 hours in anaerobic conditions. After incubation different colonies were appeared on poured plates. Colonies were got separated on morphological basis. These colonies were further conformed on biochemical basis. These morphological and chemical properties were parallel to the properties described in Bergey's Manual of systematic bacteriology (Krieg, 1984).

Bacteriocin extraction

Culture media having lactic acid bacterial strains were taken out from incubator and used for further processing. For the extraction of bacteriocin a cell free supernatant was obtained by centrifugation method (8000 rpm for 25 min, at 4 °C). Cell free supernatant was taken and pH 6.8 was adjusted by adding phosphate buffered saline (PBS) in the cell free solution to exclude the antibacterial effect of organic

acids. The obtained cell free supernatant was then precipitated with 40% solution of ammonium sulphate. The mixture was stirred for 90 minutes at 4 °C and then centrifuged at 15000 rpm for 45 minutes at 4 °C. The precipitates were collected in PBS and then further used in well diffusion assay to check the antimicrobial activity (Savadogo *et al* ., 2004; Yang *et al* ., 1992)

Characteristics of bacteriocin

Bacteriocin was confirmed through proteolytic enzymes (protease, proteinase K, trypsin, and lipase).Stability of isolated bacteriocin was checked at pH range (2-12), at different heat temperatures (60° C, 100° C and 120° C for 15 min) as well as at different storage temperatures (-20° C, 4° C and 37° C) for 30 days. All the bacteriocin producing isolates were challenged against each other to check the cross sensitivity.

Food spoiling fungus

To check the effect of *Lactobacilli* against different food spoiling fungus *Aspergillus niger*, *Mucor* and *Penicillium* were isolated from different sources in Sabouraud Dextrose Agar (Steven, 1981)

Antifungal activity:

Wells loaded with bacteriocin were made in Mueller-Hinton agar to check their effect against food spoiling *Aspergillus niger*, *Mucor* and *Penicillium* fungus. 10 ml soft agar containing 1ml of inoculums of mould was then poured onto the agar plates. After loading the wells with bacteriocin these fungus plates were incubated at 30 °C. for 24 hours (Lind *et al.*, 2005). Diameter of zone of inhibitions were measured as recommended by Rammelsberg and Radler, (1990).

Statistical analysis

Each single value was obtained after getting the average of three readings. Results were expressed after apply the Standard Error (Mean ± SE) and analysis of variance (ANOVA) under completely randomized design. Different alphabetic show the significant values of means that are ($P < 0.05$).

Results and Discussion

Out of 50 samples 30 samples were isolated as lactic acid bacteria on morphological and microbiological bases. These lactic acid bacteria were rod, coccobacilli and cocci shaped. Characteristically colonies of Lactic acid bacteria were convex, white to creamy in colour and smooth in texture. Biochemical characteristics of these isolated colonies were Gram positive, Indole negative, acid fast negative, oxidase negative and catalase negative. *Lactobacillus fermentum, L. acidophilus, L. plantarum, L. delbrueckii and L. bulgaricus* were conformed on the basis of sugar fermentation tests (Axellsson *et al* ., 1993; Krieg, 1984).

Food spoiling fungi were identified on the basis of macroscopic and microscopic characteristics. Isolate A was identified as Aspergillus *niger*, isolate B was identified as Mucor and isolate C was identified as Penicillium.

Lactobacillus acidophilus showed maximum zone of inhibition against *Penicillium* (31.00±4.00 a), *Aspergillus* (28.50±3.50 abc), and *Mucor* (28.00± 1.00 a-d) at 4, 8, 6 pH respectively. The minimum zone of inhibition against *Penicillium* (19.50±0.50 gh), *Aspergillus* (15.00±0.00 hi), and *Mucor* (14.00± 1.00 i) were appeared at 12 pH (Table 2). *Penicillium*, *Aspergillus* and

Mucor were more sensitive to *lactobacillus Plantarum* at 4,4,8 pH (Table 3). *Lactobacillus delbrueckii* was effective in 4-8 pH range. In 10-12 pH range its antimicrobial activity decrease abruptly. At 10 and 12 pH its activity was zero against *Penicillum* and *Aspergillus* respectively (Table 4).The minimum inhibitory activity of *Lactobacillus bulgaricus* against fungus was appeared at 12 pH (Table 5).

Antimicrobial activity of bacteriocins extracted from all the *lactobacilli* was observed even after heating it at 60^{o} C, 100^{o} C and 120^{o} C for 15 min but its ability to inhibit the bacterial growth decreased along the increase in heat temperature. *Lactobacillus bulgaricus* could not inhibit the growth of *Mucor* at 120^{o} C (Table 2,3,4,5).

Bacteriocins activity was satisfactory against fungus at -20^{o} C, 4^{o} C and 37^{o} C storage temperatures / 30 days. The percentage of effectiveness reduced more at 37 ^{o}C as compare to 4 ^{o}C storage temperature (Table 2,3,4,5).

In recent years fungus resistance to antifungal and other food preservatives has come into view (Yoneyama and Katsumata, 2006). This thing emphasizes us to use bacteriocin as new antifungal agent as well as biopreservative (Kumar and Schweiser, 2005; Fisher *et al.*, 2005).

The purpose of this study was to extract bacteriocins from locally isolated lactic acid bacteria and to screen them as potential candidate for alternative antifungal agents against food spoiling fungi. Lactic acid bacteria were isolated from the samples of yogurt. These lactic acid bacteria were characterized and antifungal activity of extracted bacteriocins were evaluated.

Lactic acid bacteria occur naturally in several raw materials but mostly they found in milk and milk products (Azadina et al.,2009; Forouhandeh et al., 2010). So lactic acid bacteria were isolated from locally produce yogurt.

Bacteriocins were extracted through precipitation method, with the use of ammonium sulphate, from enrichment culture of lactic acid bacteria in MRS broth. Yang *et al.*, (1992) performed this technique in his study and preferred it because of high yield and less time consumption.

Bacteriocins extracted from purified *lactobacilli* isolates were proteinaceous in nature and had low molecular weight. According to Abada (2008) lower molecular weight is the unique property of bacteriocin among all biopreservatives. The proteinaceous nature of the bacteriocin was conformed through proteolytic enzymes. There were no zones of inhibition after proteolytic treatment of bacteriocin. Our results about bacteriocin nature were in good agreement with Malini and Savitha, (2012).

Inhibitory activity of bacteriocins was checked by agar well diffusion method and zones of inhibition were measured. Different area of zones of inhibition of fungus was according to their degree of sensitivity to lactobacilli isolates. All the isolates of *lactobacilli* showed different zones of inhibition against *Aspergillus niger, Mucor* and *Penicillium* at different pH, heat temperatures as well as storage temperature. These variations in zones of inhibitions may be due to different characteristics of fungal species (presence or absence of receiving sites or immunoprotien) as well as specific activity of bacteriocin used.

On the basis of our study results all the isolates of *lactobacilli* showed great

antimicrobial activity in 4-10 pH range. As pH turned alkaline the antimicrobial activity of *lactobacilli* decrease sharply. All the isolates showed minimum zones of inhibition on 12 pH while *Lactobacillus delbrueckii* showed no zone of inhibition against *Aspergillus* (Table 4). Our results were similar to the results of Gerez *et al*., (2009) and Todorov *et al.,* (2006).

In our study extracted bacteriocin were stable after heating at 60 ^{o}C , 100 ^{o}C , 120 ^{o}C for 15 minutes. But its efficacy reduced at 100 ^{o}C as compare to 60 ^{o}C and at 120 ^{o}C as compare to 100 ^{o}C. Rowaida *et al.*, (2009) ; Mojgani and Amirinia, (2007) stated that it's a good property of bacteriocin that it remains effective even at 121 ^{o}C for 15 minutes. Due to this property it remains effective during many food safety processes like pasteurization.

Bacteriocin extracted during this study showed effectiveness after storing at -20 ^{o}C , 4 ^{o}C and 37 ^{o}C for 30 days. Antifungal activity was remained same after storing at -20 ^{o}C for 30 days but the potential to inhibit the growth of fungus reduced little bit after storing at 37 ^{o}C for 30 days. Retention of antifungal activity of lactic acid bacteria was higher when bacteriocins were stored at colder temperatures as compared to warmer temperatures (Rowaida *et al.,* 2009 ; Bizani and Brandelli, 2002 ; Malini and Savitha, 2012).

As well as the cross activity of bacteriocins among different Lactobacilli species is concerned, Rodriguez, (1996) and Stevens *et al.,* (1991) proved that Nisin had an inhibitory effect against a wide range of food spoiling microorganisms including fungus but it was useless against its taxonomically relate bacteria. So, when the cross activity of extracted bacteriocins were evaluated among different bacteriocin producing lactobacilli, no significant zones of inhibitions were found.

Characteristics of bacteriocin make it valuable among antifungal substances. Use of bacteriocin as biopreservative is safe in food additives. It has the potential to protect the food and inhibit the growth of spoiling agents along with limiting the risk of rancidity. Bacteriocin is pH, heat and storage temperature dependent. So, due to these qualities bacteriocins produced by LAB is the best option as biopreservative for the preservation of food at commercial level

Table.1 Morphological Characteristics.

Isolates	Macroscopic	Microscopic
Isolate A	initially white, quickly becoming black, Reverse was pale yellow	Hyphae were septate , hyaline, Conidia were brown to black
Isolate B	Fluffy appearance, resembles cotton candy,white initially and becomes grayish brown , reverse was whit	Nonseptate hyphae, Sporangiophores were short
Isolate C	flat, filamentous, velvety, woolly	septate hyaline hyphae, simple or branched conidiophores

Table.2 Effect of *L. acidophilus* against food spoiling fungus at different conditions

L. Acidophilus	***Mucor***	***Aspergillus***	***Penicillium***
pH			
2	22.50± 2.50 efg	19.50±0.50 gh	20.00±0.00 fgh
4	25.00± 0.00 b-f	20.00±0.00 fgh	31.00±4.00 a
6	28.00± 1.00 a-d	23.50±1.50 c-g	27.50±2.50 a-e
8	20.00± 0.00 fgh	28.50±3.50 abc	23.00±2.00 d-g
10	17.00± 2.00 hi	27.50±2.50 a-e	29.00±1.00 ab
12	14.00± 1.00 i	15.00±0.00 hi	19.50±0.50 gh
Heat Temp/15min			
60 ^{o}C	22.50±1.50 ab	27.50±0.50 a	22.50±2.50 ab
100 ^{o}C	19.50±0.50 bc	22.50±2.50 ab	20.00±0.00 bc
120 ^{o}C	18.50±1.50 bc	20.50±1.50 bc	17.00±2.00 c
Storage Temp			
-20 ^{o}C	34.50±0.50 a	32.50±2.50 a	20.00±0.00 c
4 ^{o}C	25.50±1.50 b	30.0±0.00 ab	18.50±1.50 c
37 ^{o}C	18.50±1.50 c	27.50±2.50 b	16.00±1.00 c

Table.3 Effect of *L. plantarum* against food spoiling fungus at different conditions

L. Plantarum	***Mucor***	***Aspergillus***	***Penicillium***
pH			
2	20.00±2.00 b-e	18.00±2.00 e	20.00±0.00 b-e
4	22.50±1.50 bc	20.50±1.50 bcd	23.50±0.50 ab
6	21.00±1.00 bcd	19.50±0.50 b-e	20.00±1.00 b-e
8	27.50±2.50 a	18.50±1.50 cde	18.50±1.50 de
10	17.50±2.50 def	18.50±1.50 cde	19.50±0.50 b-e
12	18.00±1.00 de	16.00±1.00 ef	13.50±0.50 f
Heat Temp/15min			
60 ^{o}C	25.00±1.00 cd	40.00±0.00 a	31.00±1.00 b
100 ^{o}C	21.50±0.50 de	27.50±1.50 bc	25.00±1.00 cd
120 ^{o}C	19.50±0.50 e	27.50±2.50 bc	23.00±1.00 de
Storage Temp			
-20 ^{o}C	25.00±1.00 bcd	40.00±0.00 a	30.00±2.00 b
4 ^{o}C	22.50±1.50 cd	27.50±2.50 bc	22.50±0.50 cd
37 ^{o}C	15.50±1.50 e	27.50±2.50 bc	20.00±1.00 de

Table.4 Effect of *L. delbrueckii* against food spoiling fungus at different conditions

L. delbrueckii	***Mucor***	***Aspergillus***	***Penicillium***
pH			
2	0.00±0.00 l	17.50±1.50 d-g	13.50±1.50 fgh
4	11.00±1.00 hii	12.00±2.00 ghi	15.00±0.00 e-h
6	21.50±1.50 bcd	27.50±1.50 a	26.00±1.00 ab
8	20.00±0.00 cde	30.00±3.00 a	25.00±0.00 abc
10	18.50±0.50 def	15.50±0.50 e-h	0±0.00
12	16.00±2.00 d-h	0±0.00	6.50±3.50 ijk
Heat Temp/15min			
60 ^{o}C	27.50±1.50 a	22.50±0.50 b	19.50±0.50 bcd
100 ^{o}C	21.00±1.00 bc	21.50±1.50 b	17.50±1.50 cd
120 ^{o}C	20.00±1.00 bc	20.00±0.00 bc	16.00±2.00 d
Storage Temp			
-20 ^{o}C	23.50±1.50 a	20.00±0.00 ab	18.50±1.50 bc
4 ^{o}C	16.00±1.00 bcd	17.50±2.50 bcd	17.50±1.50 bcd
37 ^{o}C	14.50±1.50 cd	17.50±0.50 bcd	14.00±0.00 d

Table.5 Effect of *L. bulgaricus* against food spoiling fungus at different conditions

L. bulgaricus	***Mucor***	***Aspergillus***	***Penicillium***
pH			
2	0.00±0.00	8.50±1.00 j	7.05±1.50 jk
4	12.50±2.50 efg	13.00±2.50ef	10.01±0.00 hi
6	15.00±3.00 bcd	17.50±1.05 bc	20.10±1.05 ab
8	12.00±2.00 efg	20.00±3.05 a	15.00±0.01cde
10	10.50±1.50 gh	13.50±0.50 ef	7.10±0.00 kl
12	7.50±2.50 jk	7.0±0.15 li	6.70±3.50 lm
Heat Temp/15min			
60 ^{o}C	13.00±1.00 de	18.50±0.05 ab	20.00±0.50 a
100 ^{o}C	12.50±1.50 de	16.01±1.00 bc	17.50±1.05 bc
120 ^{o}C	0±0.00	11.05±0.50 e	13.00±1.01 d
Storage Temp			
-20 ^{o}C	12.50±2.50 cd	16.00±0.00 a	15.50±1.05 b
4 ^{o}C	11.00±1.00 de	12.00±2.51 cd	13.05±1.15 c
37 ^{o}C	10.00±2.00 d	09.10±0.01 ef	09.01±0.10 ef

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