Isolation and Partially Characterization of Bacteriocin Produced by Lactic Acid Bacteria Isolated from Barley Beer

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Twenty lactic acid bacteria (LAB) were isolated from barley beer, six of these beer isolates were observed to secrete the inhibitory compounds into the cell-free filtrate with optimal production occurring in the late exponential growth phase. The inhibitory spectra of these isolates included various Gram's positive and Gram's negative bacteria and a variety of beer-spoiling bacteria. Based on standard biochemical and microbiological tests, these isolates were tentatively identified as ***Lactococcus lactis subsp. lactis, Lactobacillus sakei, Lactobacillus brevis, Lactobacillus plantarum, Lactobacillus pentosus, Lactobacillus paracasei****. These inhibitory isolates were inactivated by treatment with proteolytic enzymes indication that the inhibitory compound was proteinaceous in nature, the proteinaceous nature and inactivation by catalase identified them as bacteriocins. These bacteriocins were partially characterized. Bacteriocins produced by these six isolates were active across a wide pH range, relatively insensitive to heat treatment. Stability of the secreted bacteriocins at varying pH values indicated that full activity was retained in all samples at pH 4-6 with a significant reduction in activity at pH 2-3 and pH 7-10.*

Key words: Lactic Acid Bacteria (LAB), Barley Beer, Bacteriocin.

1. INTRODUCTION

Lactic acid bacteria (LAB) are widely used as starter cultures and play an important role in food preservations and producing many of the characteristic flavour changes on the fermented products [1]. LAB also produce antimicrobial agents called bacteriocins, which may have a promising role to play in biopreservation [2,3,4]. Bacteriocins are low molecular weight proteins secreted by bacteria, which in low concentrations elicit a bacteriostatic or bactericidal effect, by a mechanism against which the producer cell protects itself by a corresponding immunity protein [5]. Bacteriocins and bacteriocin-producing strains of LAB have been the focus of extensive research in recent years due to their food-preserving potential [6]. Initial studies on bacteriocin production by LAB were focused on isolates associated with dairy products, although bacteriocinogenic activity has been discovered in bacterial strains from meat [7], silage and fermented vegetables [8], garlic and ginger root [9], sour dough [10], red wine [11] and malting & brewing environments [12,13]. Bacteriocins can be exploited to inhibit undesirable microorganisms in the fermentation of wine [11], beer [14], meat [15], vegetables [16] and dairy products [17,18]. The malted barley beer is produced with *Saccharomyces cerevisiae* and LAB naturally present in barley and maize. LAB can be found at most stages of malting and brewing, from the standing barley crop in the field to the finished

product beer [19]. They are assumed to be a harmless part of the natural malting and mashing microflora [20], although they may represent undesirable contaminants, during fermentation or in the finished product, where some LAB species cause beer spoilage. However, certain LAB exert a positive effect on the beer-manufacturing process through, among others, the inhibition of undesirable microorganisms (including beer spoiling bacteria) and wort bioacidification [21,22]. The objectives of this work were to screen the LAB component of the microflora for bacteriocin-producing LAB with the capacity to inhibit growth of beer spoilage and human pathogenic microorganisms and to characterize the antimicrobial producing beer isolates as well as the bacteriocin produced.

2. MATERIAL AND METHOD

Bacterial strains and growth media: Strains MSAS01, MSAS03, MSAS11, MSAS15, MSAS18 and MSAS20 were isolated from barley beer and identified as *Lactococcus lactis* subsp. *lactis*, *Lactobacillus sakei*, *Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus paracasei* respectively by using physiological and biochemical tests. All the lactic acid bacteria were grown in deMann Rogosa Sharpe Agar (MRS, HiMedia) and other pathogens were grown in nutrient agar medium and incubated at 30°C and 37°C respectively. The strains were stored at -20°C in MRS/nutrient broth containing 15% (v/v) glycerol. *Lactococcus lactis* subsp. *lactis* MTCC 3038 was used as indicator strain and it was procured from IMTECH Chandigarh, India.

2.1. Screening of Antimicrobial Activity

Pure isolates of lactic acid bacteria were screened for antimicrobial activities against the indicator organisms *Lactococcus lactis* subsp. *lactis* MTCC 3038 by agar overlaid method and agar well diffusion test. For the agar overlaid method, 5 µl of overnight culture of each isolate of lactic acid bacteria was spotted onto the surface of MRS agar plates and incubated at 30°C for 24 h for development of colonies [23]. The developed colonies were overlaid with MRS soft agar inoculated with 1 ml of indicator organism at a level of 10^6 cfu/ml. Another set of MRS agar plates containing lactic acid bacteria colonies were overlaid with sterile MRS agar as control. The plates were incubated at 30°C for 24 hand checked for inhibition zones. The diameters of the inhibition zone were measured and recorded in millimeter. The zone of inhibition was scored on an abstract scale as follows: (-) no inhibition; (+) zone of inhibition 1-5 mm; (++) zone of inhibition 5-10 mm; (+++) zone of inhibition >10 mm [24]. For the agar well diffusion test, 1 ml of the indicator organism cultured in MRS broth at 30°C for 24 h was inoculated into 15 ml of semi-solid MRS agar, agitated and poured into sterile Petri dish. After solidifying, wells were cut into it using a sterile cork borer 4 mm in diameter. Fifty microlitre (50 µl) of supernatant fluid (centrifuged at 5000 rpm for 15 min) from the culture of the isolate under test for antimicrobial activities were added in the wells. The plates were kept in refrigerator for 4 h to ensure diffusion of the fluid into the agar [23]. The plates were incubated at 30°C for 24 h and examined for inhibition zones. The diameters of the inhibition zones were measured and recorded in millimeter.

2.2. Identification of the Lactic Acid Bacteria Isolates

The selected LAB isolates were characterized by the criteria based upon Carbohydrate fermentation profiles and biochemical characteristics, determined using API 50 CHL for

lactobacilli, pediococci and leuconostocs and API 20 STREP for lactococci, streptococci and enterococci (Biomerieux, France). The following standard tests were used for characterization of isolates: microscopic examination of cell morphology, physiological tests, biochemical test, cultural growth conditions and carbohydrate (sugar) fermentation profile. Identification was based on comparison of observed characteristics of isolates with those of lactic acid bacteria described in the Bergey's Manual of Determinative Bacteriology [25].

2.3. Bacteriocin Assay

The initial bacteriocin assay were performed using cell free filtrate (CFF) which was prepared from a bacterial culture from which cells have been removed after centrifugation at 15,000 xg for 30 min. at 4°C To eliminate growth inhibition caused by organic acid, the resulting CFF were adjusted to pH 6.5 with 1 M NaOH and filtered through 0.22 µm pore size membrane filters (Milipore TM). Antimicrobial activity secreted into liquid media was detected by agar-well diffusion method [26]. MRS soft agar (5ml) inoculated with 1% (v/v) of an indicator sensitive strain overnight culture was overlaid on an agar plate. After cooling, wells (6 mm diameter) were punched in the agar plates and filled with 50 µl of test samples. After incubation overnight, the antimicrobial activity was expressed as the diameter of the inhibition zones around the wells. Bacteriocin activity was assayed by two fold dilution of crude bacteriocin in terms of arbitrary unit (AU). Arbitrary unit was defined as the reciprocal of the highest dilution which showed a clear zone of inhibition [27].

2.4. Characterization of Bacteriocin

2.4.1. Effect of pH and heat treatment on bacteriocin activity

A 5-mL aliquot of partially purified bacteriocin was taken in test tubes and the pH values of the contents were adjusted to 2–10 individually, using either diluted NaOH or HCl (1 M NaOH or 1 M HCl solution). After allowing the samples to stand at room temperature for 2 h the activity was assayed as described earlier. Aliquots of bacteriocin-containing CFF obtained from beer LAB isolates were subjected to various temperature treatments and bacteriocin activity was quantified as outlined above and compared with that of the untreated samples.

2.4.2. Effect of enzymes on antibacterial activity

Proteolytic enzymes were used to determine if the inhibitory compounds produced by the LAB isolates were proteinaceous in nature and thus could be designated as bacteriocin-like inhibitory substances. Sensitivity to proteolytic enzymes was tested by treatment of each CFF containing bacteriocin activity with proteinase K, pronase E and catalase (Sigma) at a final concentration of 0.1 mg/ml.

2.4.3. Spectrum of activity

The activity spectrum of each bacteriocin producing LAB strain against a variety of available beer spoilage LAB and various Gram positive and negative human pathogenic strains was tested and quantified for both 2 µl cell spots of the producer cultures and

their CFF as described above. The cross sensitivity of eleven inhibitor-producing LAB was also determined using the same method. The sensitivity of the inhibitors produced by beer isolates to enzyme inactivation was examined to demonstrate whether growth inhibition of the indicator strain was due to bacteriocin-like inhibitors, hydrogen peroxide or some other, undefined compound

3. RESULT AND DISCUSSION

The objective of this study was to isolate and characterize barley beer derived LAB capable of producing antimicrobial compounds, which may be used as novel microbial controlling agents in the malting process or within an alcoholic beverage system. A total of 20 Lactic acid bacteria were isolated from barley beer and screened for their antagonistic activity against *Lactococcus lactis* subsp. *lactis* MTCC3038 (an indicator sensitive strain) using agar overlaid method as shown in Figure 1.

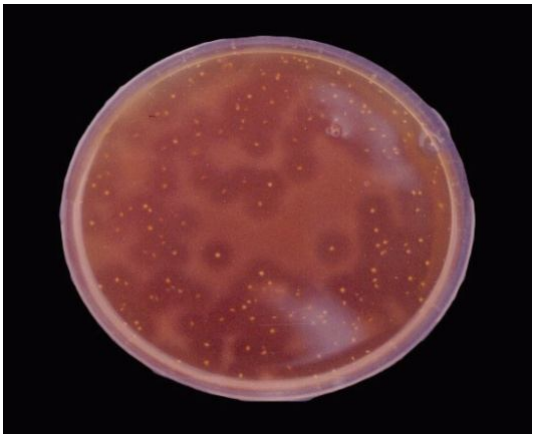

Fig. 1: Detection of bacteriocin production by agar overlaid method using bacteriocin sensitive strain, *Lactococcus lactis* subsp. *lactis* MTCC 3038.

Out of these 20 LABs only 6 strains showed antagonistic effect against the indicator strain. Growth inhibition of the indicator strain by cell-free filtrate (CFF) was taken as evidence that the inhibitory compound was released into the medium. These 6 producer strains were identified as: *Lactococcus lactis* subsp. *lactis* MSAS01, *Lactobacillus sakei* MSAS03, *Lactobacillus brevis* MSAS11, *Lactobacillus plantarum* MSAS15, *Lactobacillus pentosus* MSAS18 and *Lactobacillus paracasei* MSAS20, based on physiological and biochemical characterization. The antibacterial compounds produced by the isolated strains were inactivated on treatment with proteases which suggests that the produced active biomolecules are proteins. Overnight cultures of producer isolated strains were grown in MRS broth at 37°C for 48 h. After incubation, the broth were adjusted to pH 2.4 and the cells were removed by centrifugation at 5000 × g for 15 min. Supernatant was used as crude bacteriocin to evaluate antimicrobial activity using agar well diffusion method. An agar well diffusion method was used to assess the production of antimicrobial compounds by the selected isolates against beer spoilage strains and other human pathogens. The bacteriocin producers inhibited a range of beer spoilage LAB and human pathogens as tabulated in Table 1.

Table 1: Effect of partially purified bacteriocins produced by beer isolates against different beer spoilage and human pathogens by agar well diffusion method (zone of inhibition in mm).

S. No.	Indicator strains/pathogens	MSAS01	MSAS 03	MSAS 11	MSAS15	MSAS 18	MSAS 20
1.	*L. brevis* MSASSPO 15	11	10	08	12	-	11
2.	*P. inopinatus* MSASSPO 18	14	12	14	11	12	11
3.	*L. backi* MSASSPO 23	12	10	12	10	08	08
4.	*L. casei* MTCC 1432	16	14	12	16	14	12
5.	*P. damnsous* MSASSPO 25	18	18	16	12	10	14
6.	*S. typhi* MTCC 734	13	12	12	10	10	10
7.	*S. aureus* MTCC 96	16	15	-	14	14	12
8.	*C. perfringens* MTCC 450	09	10	12	14	12	11
9.	*P aeruginosa* MTCC 2581	15	14	14	12	12	10
10.	*P. mirabilis* MTCC 425	12	12	10	10	08	12
11.	*K. pneumoniae* MTCC 530	08	14	12	12	08	-
12.	*E. coli* MTCC1687	07	-	12	12	08	08

Bacteriocin producers MSAS01, MSAS03, MSAS11 and MSAS15 had a relatively broad inhibitory spectra against the tested beer spoilers and human pathogens. The susceptibilities of Gram-positive and Gram-negative bacteria to growth inhibition by the crude bacteriocin of isolates showed inhibitory activity against *Salmonella typhi* MTCC 734, *Staphylococcus. aureus* MTCC 96, *Proteus mirabilis* MTCC 425, *Klebsiella pneumoniae* MTCC 530, *Pseudomonas. aeruginosa* MTCC 2581, *Escherichia coli* MTCC 1687,*Closrtidium perfringens* MTCC 450. The highest inhibitory activity was

against *Pediococcus damnosus* MSASSPO25 (18mm) followed by *Lactobacillus caesi* MTCC 1432 (16mm), both are beer spoilage pathogens. Same activity (16mm) was shown to *Staphylococcus aureus* MTCC 96 followed by *Pseudomonas aeruginosa* MTCC 2581(15mm) as shown in Figure 2.

Temperature and pH played an important role in cell growth as well as bacteriocin production. The stability of the secreted bacteriocins was tested at different temperatures and pH values. The bacteriocin activity from the beer isolates were tested at different temperatures and tabulated in Table 2. All bacteriocins were stable at -20°C in MRS for an extended period of time. Bacteriocin activity decreased after 4 weeks storage at 4°, 25°, 30°, 37° and 42°C and the inhibition zones were observed up to 100-121°C as shown in Table 2.

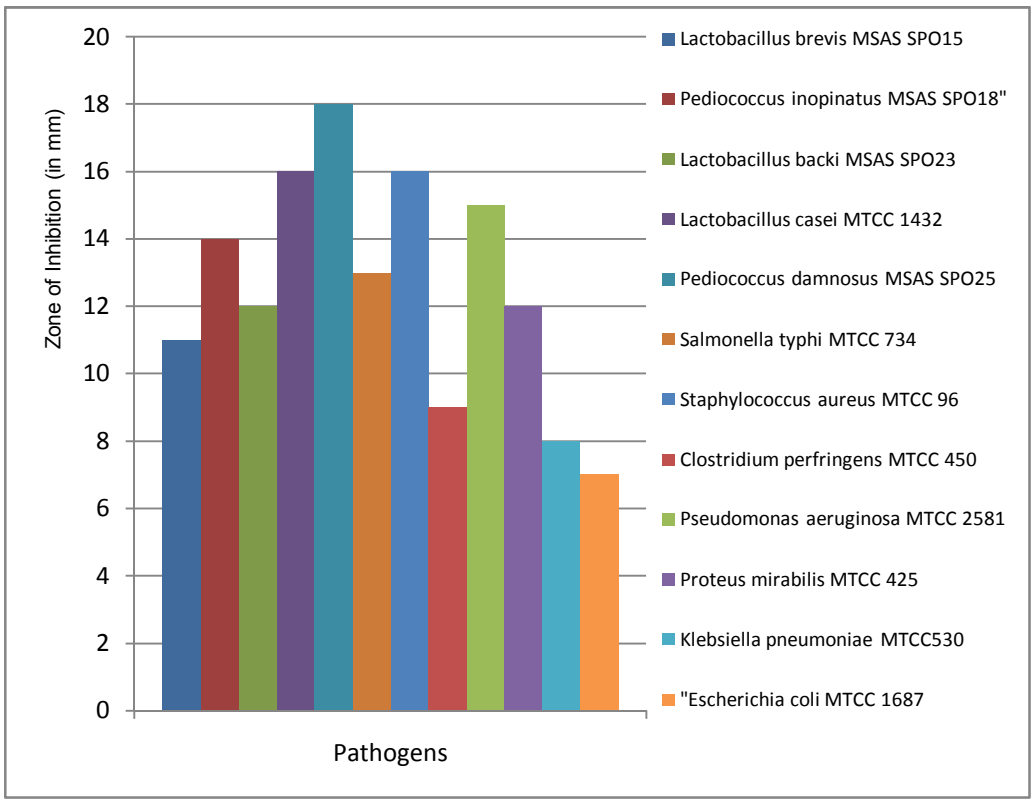

Fig. 2: Antibacterial activity of bacteriocin produced by *L. lactis* subsp. *lactis* MSAS01 isolated from beer, against different pathogens.

Stability of the secreted bacteriocins at varying pH values indicated that full activity was retained in all samples at pH 4-6 with a significant reduction in activity at pH 2-3 and pH 7-10 as shown in Table 3. Bacteriocins produced by isolates MSAS01, and MSAS15 displayed maximum activity over the widest pH range (pH 4-8) with bacteriocin activity reduced by 50% at pH 2-3 and pH 9-10.

Our results indicate that the LAB isolates have a wide activity range against beer spoilage LAB. This is important as alcoholic drinks are subject to spoilage by these LAB which under certain conditions can proliferate and cause rope or slime formation, or give an off-odour due to diacetyl production. The addition of bacteriocins in a pure form is not allowed in the production of German beers due to the Purity Law [28]. However, it may be possible to introduce bacteriocins as a natural consequence of biological acidification of mash or wort by bacteriocin-producing LAB. In conclusion, we have shown the presence of bacteriocin in barley beer derived LAB, this characteristic makes them interesting for their application in brewing and will be the focus of future research.

Table 2: Effect of temperature and storage treatment on activity of bacteriocins against *Lactococcus lactis* subsp. *lactis* MTCC 3038.

Heat treatment	Inhibitory activity of bacteriocins (AU/ml)					
	MSAS01	**MSAS03**	**MSAS11**	**MSAS15**	**MSAS18**	**MSAS20**
-20°C X 30 days	25600	6400	12800	12800	6400	6400
4°C X 30 days	12800	6400	6400	12800	3200	3200
25°C X 30 days	6400	3200	1600	1600	1600	1600
30°C X 30 days	3200	1600	1600	1600	1600	800
37°C X 30 days	3200	1600	800	1600	800	800
42°C X 30 days	800	400	400	800	400	400
80°C X 1 h	6400	3200	3200	1600	1600	1600
90°C X 1 h	6400	3200	3200	1600	1600	1600
100°C X 1 h	6400	-	800	800	800	400
115°C X 1 h	1600	-	400	400	-	-
121°C X 1 h	400	200	200	200	-	-

Table 3: Effect of different pH on activity of bacteriocins against *Lactococcus lactis* subsp. *lactis* MTCC 3038.

pH	Inhibitory activity of bacteriocins (AU/ml)					
	MSAS01	**MSAS03**	**MSAS11**	**MSAS15**	**MSAS18**	**MSAS20**
2	12800	6400	6400	12800	6400	3200
3	12800	6400	6400	12800	6400	6400
4	25600	12800	25600	25600	25600	25600
5	25600	25600	25600	25600	25600	25600
6	25600	25600	25600	25600	25600	25600
7	25600	25600	25600	25600	25600	12800
8	25600	12800	12800	25600	12800	12800
9	12800	6400	6400	12800	6400	6400
10	12800	6400	3200	12800	3200	6400

REFERENCES

[1] E. Caplice and G.F. Fitzgerald; "Food fermentations: role of microorganisms in food production and preservation", Int. J. Food Microbiol., Vol. 50, pp.131-149,1999.

[2] L.D. Vuyst, and E.J. Vandamme; "Lactic Acid Bacteria and Bacteriocins: Their Practical Importance. In: Bacteriocins of Lactic Acid Bacteria", Microbiol. Genetics and Application, De Vuyst, L. and E.J. Vandamme (Eds.). Chapman and Hall Ltd., Glasgow, pp. 1-12, 1994. http://www.academicjournals.org/ajb/htm

[3] J.C. Piard and M.J. Desmazeaud; "Inhibiting factors produced by lactic acid bacteria.2. Bacteriocins and other antibacterial substances", Lait, Vol. 72(2), pp.113-142, 1992. http://www.ncbi.nlm.nih.gov

[4] K. Nithya, D. Senbagam, B. Senthilkumar, N. Udhayashree and R. Gurusamy; "Characterization of bacteriocin producing lactic acid bacteria and its application as a food preservative", African J. Microbiol. Res., Vol. 6(6), pp. 1138-1146, 2012.

[5] B.G. Contreras, L.D. Vuyst, B. Devreese, K. Busanyova, J. Raymaeckers, F. Bosman, E. Sablon, and E.J. Vandamme; "Isolation, purification and amino acid sequence of lactobin A, one of the two bacteriocins produced by Lactobacillus amylovorus LMG P-13139", Appl. Environ. Microbiol., Vol. 63(1), pp.13-20, 1997.

[6] J.B. Luchansky; "Overview on applications for bacteriocin producing lactic acid bacteria and their bacteriocins", Antonie van Leeuwenhoek, Vol. 76(1-4), pp. 335, 1999.http://www.ncbi.nlm.nih.gov

[7] U. Schillinger, and F.K. Lücke; "Antibacterial activity of Lactobacillus sake isolated from meat", Appl. Environ. Microbiol, Vol. 55(8), pp.1901–1906, 1989.

[8] S. Ohmomo, S. Murata, N. Katayama, S Nitisinprasart, M. Kobayashi, T. Nakajima, M. Yajima and K. Nakanish; "Purification and some characteristics of enterocin ON-157, a bacteriocin produced by Enterococcus faecium NIAI 157", J. Appl. Microbiol., Vol. 88(1), pp.81–89, 2000. http://www.ncbi.nlm.nih.gov/pubmed/10735246

[9] M.E. Janes, R. Nannapaneni and M.G. Johnson; "Identification and characterization of two bacteriocin-producing bacteria isolated from garlic and ginger root", J. Food Prot., Vol. 62(8), pp. 899–904, 1999.

[10] A.G. Larsen, F.K. Vogensen, and J. Josephsen; "Antimicrobial activity of lactic acid bacteria isolated from sour doughs: purification and characterization of bavaricin A, a bacteriocin produced by Lactobacillus bavaricus MI401", J. Appl. Bacteriol., Vol. 75(2), pp. 113–122, 1993. http://www.ncbi.nlm.nih.gov/pubmed/8407671

[11] L. Navarro, M. Zarazaga, J. Saenz, F. Ruiz-Larrea and C. Torres; "Bacteriocin production by lactic acid bacteria isolated from Rioja red wines", J. Appl. Microbiol., Vol. 88(1), pp. 44–51, 2000. http://onlinelibrary.wiley.com/doi /pdf

[12] A. O'Mahony, T. O'Sullivan, Y. Walsh, A. Vaughan, M. Maher, G.F. Fitzgerald and D. V. Sinderen; "Characterization of antimicrobial producing lactic acid bacteria from malted barley", J. Inst. Brew., Vol. 106(6), pp. 403–410, 2000.

[13] A. Vaughan, V.G.H. Eijsink, T.F. O'Sullivan, K. O'Hanlon, and D. van Sinderen; "An analysis of bacteriocins produced by lactic acid bacteria isolated from malted barley", J. Appl. Microbiol., Vol. 91, pp. 131–138, 2001.

[14] K. Ogden, and R.S. Tubb; “Inhibition of beer spoilage lactic acid bacteria by nisin”, J. Inst. Brew., Vol. 91(6), pp. 390–392, 1985. http://chemport.cas.org.

[15] L. Saucier, A. Poon and M.E. Stiles; “Induction of bacteriocin in Carnobacterium piscicola LV17”, J. Appl. Bacteriol., Vol. 78(6), pp. 684–690, 1995.

[16] M.A. Daeschel, and H.P. Fleming; “Selection of lactic acid bacteria for used in vegetable fermentations”, Food Microbiol., Vol. 1, pp.303–313, 1984.

[17] D. Casla, T. Requena and R. Gomèz; “Antimicrobial activity of lactic acid bacteria isolated from goat’s milk and artisanal cheeses: characteristics of a bacteriocin produced by Lactobacillus curvatus IFPL 105”, J. Appl. Bacteriol., Vol. 81(1), pp. 35–41, 1996.

[18] R.P. Ross, M. Galvin, O. McAuliffe, S.M. Morgan, M.P. Ryan, D.P. Twomey, W.J. Meaney and C. Hill; “Developing applications for lactococcal bacteriocins”, Antonie van Leeuwenhoek, 1999, Vol. 76, pp. 337–346, 1999.

[19] K. Sakamoto, and W.N. Konings; “Beer spoilage bacteria and hop resistance”, Int. J. Food Prot., Vol. 89(2-3), pp. 105–124, 2003.

[20] T. F. O’Sullivan, Y. Walsh, A. O’Mahony, G.F. Fitzgerald, and D. van Sinderen; “A Comparative study of malthouse and brewhouse microflora”, J. Inst. Brew., Vol. 105 (1), pp. 55–61, 1999.

[21] D. Lewis; “Biological mash and wort acidification”, The New Brewer, Vol. 15, pp. 36–45, 1998.

[22] A. Vaughan, S. Rouse and D.V. Sinderen; “Investigating the Antimicrobial Efficacy of a Lactococcal Bacteriocin for the Development of Microbiologically Stable Beer”, J. Inst. Brew, Vol. 110(3), pp.181–188, 2004.

[23] L. Gonzalez, H. Sandoval, N. Sacristan, J.M. Castro, J.M. Frensno and M.E. Tornadijo; “Identification of lactic acid bacteria isolated from Genestoso cheese throughout ripening and study of their antimicrobial activity”, J. Food Control, Vol. 18(6), pp. 716-722, 2007.

[24] D.J. Hartnett, A. Vaughan and D. Van Sinderen; “Antimicrobial producing lactic acid bacteria from raw barley and Sorghum”, J. Institute Brewing, Vol. 108(2), pp. 169-177, 2002.

[25] J.G. Holt, N.R. Krieg, P.H.A. Sneathm, J.T. Staley, and S.T. Williams; “Bergey’s Manual of Determinative Bacteriology”, 9th edn. Baltimore(MD): Williams and Williams, 1994.

[26] J. R. Tagg and A.R. McGiven; “Assay system for bacteriocin”, Appl. Micrbiol.,Vol. 21(5), pp. 943, 1971.

[27] R.Yang, M.C. Johnson and B. Ray; “Novel method to extract large amounts of bacteriocin from lactic acid bacteria”, Appl. Environ. Microbiol., Vol. 58(10), pp. 3355-3359, 1992.

[28] M.A. Franchi, A.A.L. Tribst, and M. Cristianini; “The effect of antimicrobials and bacteriocins on beer spoilage microorganisms”, Int. Food. Res. J., Vol. 19(2), pp. 783-786, 2012.