Optimization and partial characterization of bacteriocin produced by *Lactobacillus bulgaricus* -TLBFT06 isolated from *Dahi*

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Abstract: *Lactobacillus bulgaricus* is one of the predominant lactic acid bacteria of *dahi,* conferring technological and functional attributes. In the present study thirty *dahi* samples were investigated for bacteriocin producing *L. bulgaricus.* Fourteen different isolates were obtained and five were scrutinized for antibacterial activities against food born pathogens. Amongst, a strain TLB06FT was found to have a wide array of antibacterial activities against Gram positive and negative bacteria was selected for further characterization. Growth media optimization for this strain revealed maximum bacteriocin production on MRS media supplemented with glucose (2%), sodium chloride (1%), Tween-80 (0.5%) and yeast extract (1 %). In addition, optimization of growth conditions revealed maximum bacteriocin production at pH 5.5 and temperature of 30-37^{o}C. Bacteriocin showed thermo stability at 90^{o}C and remained highly active in the pH range of 3.5-7.5, inactive by protein catalyzing enzymes and showed no change in activity (800AUmL^{-1}) when treated with organic solvents and surfactants. The obtained bacteriocin was purified to 1600AU mL^{-1} by ammonium sulfate precipitation (80%) by using dialyzing tubing. In the same way, a single peak was obtained by RP-HPLC having antibacterial activity of 6400AU mL^{-1}. Thus, wild strains of *L. bulgaricus* have great potential for the production new and novel type of bacteriocins.

Keywords: *Lactobacillus bulgaricus*, bacteriocin, purification, characterization.

INTRODUCTION

Dahi, a traditional fermented milk product, is an essential diet component of the people in South Asian Countries. *Dahi* prepared locally by the fermentation of wild strains of lactic acid bacteria (LAB) (Dewan and Tamang, 2007). A Superior *dahi* must carries light paled to whitish color, slight sour to sweet taste, pleasant aroma and thick texture. It was believed for centuries that *dahi* provides remedy against the gastrointestinal problems (Harun-ur-Rashid *et al.,* 2007). This inherited character of *dahi* is attributed of LAB which are not only involved in the fermentation of milk, but also produced secondary metabolites like diacetyl, exopolysaccharides, hydrogen peroxide, reuterin, reutericyclin and antimicrobial substances or bacteriocin. These secondary metabolites confer unique nutritional characteristics to *dahi* and prolong storage life due to effective control against food spoilers and food borne pathogens (Zhu *et al*., 2000).

Amongst these metabolites, one group of pharmaceutical compounds known as bacteriocin that have great potential to be used as a food preservatives. These peptides have the antibacterial effect against the other microorganisms including harmful bacteria (Tagg *et al*., 1976). Their mode of action revealed that they cause cell wall porosity in targets bacteria thus inhibited the synthesis of their cell wall (Sullivan *et al.,* 2002). The production of these nutraceutical compounds is different from the antibiotics and generally their production is control by plasmids (Drider *et al*., 2006), which differentiate them from antibiotics the mode of action (Tannock *et al*., 1994). Bacterioicns are produced by all the genera of lactic acid bacteria, however, this characteristic is predominately associated with lactobacillus (Klaenhammer, 1993). Different categories of bacteriocins produced by this genus includeing acidophilin (Chumchalov *et al*., 2004), bulgarican (Simova *et al*., 2008), nisin (Chung *et al*., 1989), lactacins, diplococcin, plantaricins and helveticins, (Nettles and Barefoot, 1993), however, only few of them like nisin and acidophilin are comprehensively described (Leal-Sanchez *et al*., 2002).

Bulgarican is a bacteriocin produced by *L. bulgaricus,* first time reported by Balasubramanyam and Varadaraj (1998). Subsequently different properties were studied by Erdogrul and Erbilir (2006), Simova *et al.* (2008) and Tufail *et al*. (2011). A comprehensive study is required for determination of the role of various media constituents like Tween 20 and 80, glucose, NaCl and yeast extract on the bulgarican production. Therefore, in the present investigation roles of commonly used media constituents along with environmental conditions were optimized for maximum bacteriocin production. In addition, characterization and purification of bacteriocin produced

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by *L. bulgaricus*-TLABFT was carried out as a foremost investigation.

MATERIALS AND METHODS

Bacterial strains and growth media

Producer strains were isolated from *dahi* and selective media of MRS (deMan Regosa and Sharp, Oxide England) additionally supplemented with 2% glucose was used for activation and sub culturing. Initially, morphological and biochemical characterization of all isolates of *L. bulgaricus* was carried out according to procedures described by Cappucccino and Sherman (1996). Later on five isolates were scrutinized and their identification was further confirmed by the amplification of 16S rRNA region by selective primers using sequence of :Bulgfor (5`-TCAAAGATTCCTTCGGGATG-`3) and :Bulgrev (5`-TACGCATCATTGCCTTGGTA-`3) and by API 50 CHL system (BioMerieux, France).

Indicator strains for antibacterial assay were *Listeria monocytogenes*-ATCC 19115, (grown on Listeria enrichment broth base, CM0863 oxoid, Hampshire England), *Staphylococcus aureus*-ATCC 6538, (culture on Staphylococcus Medium No110, Hampshire, England), *Pseudomonas aeruginosa* ATCC 25923 and *Escherichia coli*-ATCC25922 (culture on Brain Heart Infusion (BHI) medium Oxoid Ltd, Baingstoke, United Kingdom). *Enterococcus faecalis* AR 03 (culture on M-17 supplemented by 5% NaCl), *Clostridium butyricum* (RCM), *Salmonella typhimurium.* The strain was originated from the American Type Culture Collection (Manassas, Va., USA) and donated for this study by Department of Pathology, Pakistan Institute of Medical Science (PIMS). The stocks of all were maintained in 20 % (v/v) glycerol and stored at -80^{o}C.

Screening of L. bulgaricus for antibacterial activities and bacteriocin bio assay

All isolates were screened for antibacterial activities by a paper disc method as reported by Havoorver and Harlander (1993) and then arbitrary unit (AU) for the activity of the bacteriocin was determined according to procedures of Batdorj *et al*. (2006). Bacteriocin containing supernatant was serially diluted in phosphate buffer (20mM) and 10µL of each dilution was applied by a well diffusion method for determination of minimum inhibition concentration. The titre was defined as the reciprocal of last dilution that inhibited the growth of indictor strain and expressed as AU mL^{-1} and calculated by equation = (2^{n} x 1000) 10µL^{-1}.

Optimization of bacteriocin production

A. Optimization of growth media for bacteriocin production by TLB06FT

Since bacteriocin production is a growth-linked phenomenon, TLB06FT was allowed to grow on various preparations of growth media for the selection of best combination. The preparation included MRS as basic growth medium along with these constituents; sodium chloride, glucose tryptone, Tween 80 and yeast extracts.

B. Optimization of growth condition of bacteriocin production by TLB06FT

Growth kinetics of the selected strain of TLB06FT was carried out for the determination of best-suited growth temperature and pH. Different preparations of growth media were made and adjusted to the initial pH of (2.5 to 10.5). Each medium was inoculated with 1% overnight-activated culture of TLB06FT and incubated at 37^{o}C for 24 hours (Yildirim and Johnson, 1998).

Characterization of bacteriocin production

A. Acid and heat tolerance of bacteriocin produced by TLB06FT

The bacteriocin was subjected to different level of pH (2.5 to 10.5) and temperature (60 to 120^{o}C) to check its stability under stress conditions. Residual activity was determined by procedure as described by Oh *et al*. (2000).

B. Effect of enzymes, organic solvents and surfactants on bacteriocin produced by TLB06FT

To determine the effect of different enzymes in the activity of bacteriocin, cell free supernatant was allowed to grow in the presence of 1mg mL^{-1} of each of the following enzymes; Proteinase k, Lipase, α-amylase and trypsin (Invanova *et al*., 2000). In the same way cell free supernatant was incubated with respective organic solvent (1%) including β-mercaptoethanol (50mM), acetone, chloroform, ethanol and methanol and surfactant like ethylene diamine tetra acetic acid (EDTA) (10mM), sodium dodecyl sulphate (SDS), Tween 20, Tween 80 for 5hrs at 37^{o}C (Todorov and Dicks, 2005). Using the paper disc method residual activity was monitored.

Purification of bacteriocin

Three levels of purification scheme as described by Burianek and Yousef (2000) was followed for the purification of the bacteriocin produced by TLB06FT. Bacteriocin containing cell free supernatant was obtained by centrifugation at 13000g for 20min. and is followed by precipitation of bacteriocin with 80% ammonium sulfate. These precipitates were dissolved in 0.25M Tris buffer and dialyzed through 12000 kDa cut off dialysis tubing (Sigma) and freeze dried at -50 Torr pressure.

Reverse phase high-pressure liquid chromatography (RP-HPLC)

The Freeze-dried sample of bacteriocin was re-suspended in 1ml of Tris- buffer (0.1mol having pH 6.0) incubated at 4^{o}C for one hour and centrifuged at 120000*g* for 15min. The pellets obtained were allowed to re-suspend in ultra pure water and 30µL of this was run on reverse phase high-pressure liquid chromatography (RP-HPLC)

(Shimadzu, Japan). The sample was injected onto C_{18} semi preparative RP-HPLC column (8 by 100mm; diameter, 15µm; 100 Å) calibrated at a flow rate of 1mL min^{-1} with acetonitrile: water as mobile phases and detection was carried out by UV detector at 332 nm.

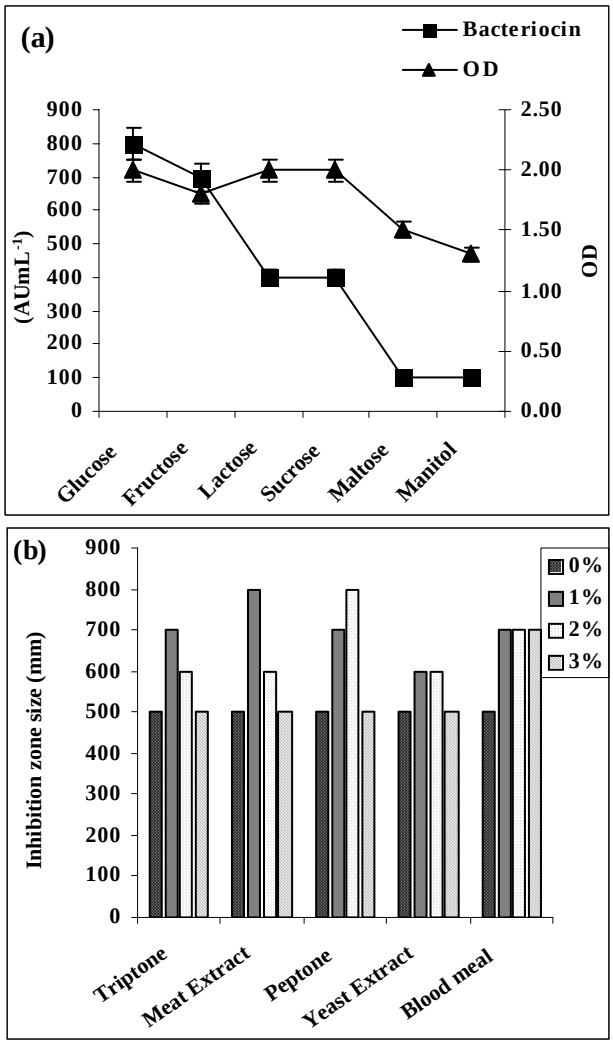

Fig. 1: (a) Effect of various carbon sources in the production of bacteriocin by TLB06FT (b) Effect of various nitrogen sources in the production of bacteriocin by TLB06FT

RESULTS

Screening of L. bulgaricus for antibacterial activities

The isolates of *L. bulgaricus* were screened for antibacterial activities against indicators strains as presented in table 1. Amongst the screened bacteria five isolates showed antibacterial activities, and an isolate TLB06FT had a wide spectrum of this activity against both Gram positive and negative bacteria. Therefore this strain was selected for further characterization for bacteriocin production.

Optimization of bacteriocin production

Optimization of growth media for bacteriocin production by TLB06FT

For the selection of best-suited growth media for maximum bacteriocin production, TLB06FT was allowed to grow in different preparation and the results obtained were presented in the figure 1a. It was observed that MRS media supplemented with 2% glucose, 1% each of tryptone, yeast extract and NaCl enhance the bacteriocin production. The addition of nitrogen source like tryptone, yeast extract and carbon source like glucose enhanced the level of available nutrients and ultimately increased the bacteriocin production. Similarly, addition of Tween 80 and NaCl increase release of bacteriocin from the cells of producer microorganisms.

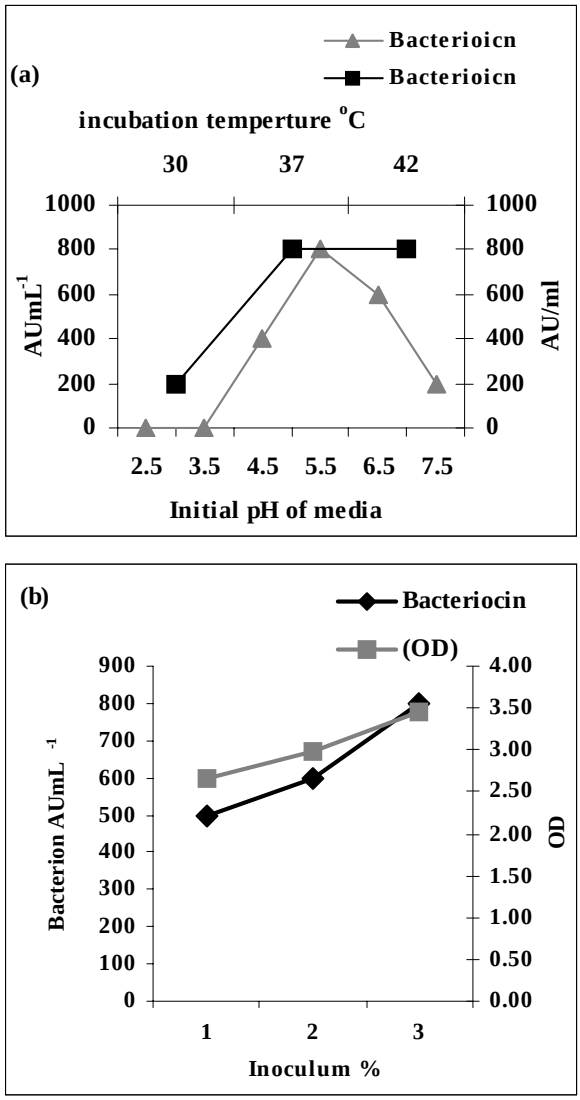

Fig. 2: (a) Role of Incubation temperature and initial pH of medium for the production of bacteriocin by TLB06FT (b) Role of inoculums concentration on the production of bacteriocin by TLB06FT

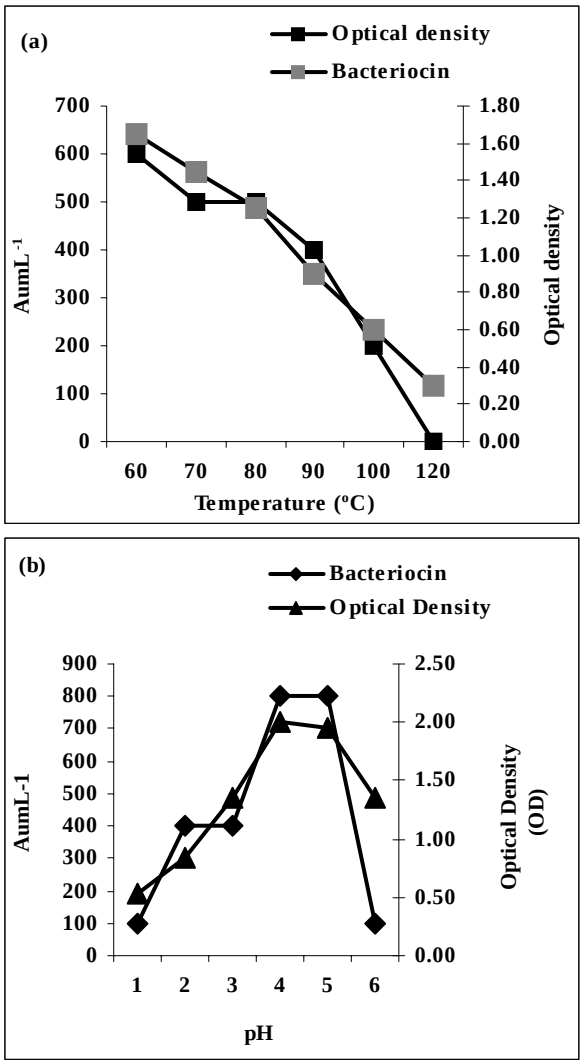

Fig. 3: (a) Effect of various temperature range on the bacteriocin produced by TLB06FT (b) Effect of different pH on the bacteriocin produced by TLB06FT

Optimization of growth conditions for bacteriocin production by TLB06FT

Results obtained for initial pH of culture media and the incubation temperature on the production of bacteriocin was given in the figure 2a. It was observed that both of these parameters had significant (P>0.005) effect on the bacteriocin production and maximum bacteriocin production was obtained at pH 5.5 and temperature 37°C. This might be due to stress exerted on TLB06FT which implicated the immunological changes and resulted in the increased production.

Characterization of bacteriocin production

A. Acid and heat tolerance of bacteriocin produced by TLB06FT

The bacteriocin showed heat stability to some extent, however its activity ended at temperature above 100°C as presented in figure 2a. Data revealed that bacteriocin production is inversely proportional to temperature. It was noted that with the increase of temperature from 60 to 100°C activity reduced from 600AUmL^{-1} to 200AUmL^{-1} and above this temperature it retarded completely. It was further established that bacteriocin showed maximum activity between pH of 5.5 to 6.5 as presented in the figure 3b.

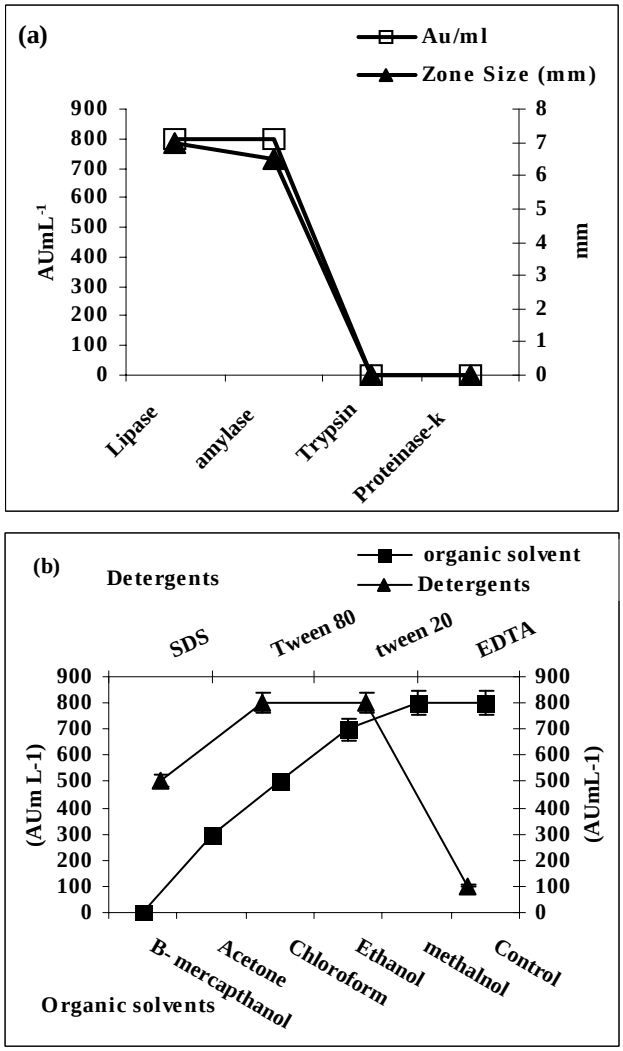

Fig. 4: (a) Effect of different enzymes on bacteriocin produced by TLB06FT (b) Effect of different detergents and organic solvents on bacteriocin produced by TLB06FT.

B. Effect of enzymes, organic solvents and surfactants on bacteriocin produced by TLB06FT

To confirm the protein nature of bacteriocin produced by TLB06FT and its behavior toward various organic solvents and detergent, it was treated with these compounds and results obtained were presented in figure 4a and 4b. It was observed that bacteriocin is only inactive by proteolytic enzymes (Proteinas-K and Tripsin) while glucocidic enzymes (Lipases and Kinases) had no effect. It was revealed from the results that bacteriocin of

Table 1: Screening of *L. bulgaricus* isolates for antibacterial activities against food pathogens

Name of Strains	Indicator strains			
	E. coli ATCC25922	*S. aureus* ATCC 6538	*P. eruginosa* ATCC 25923	*L. monocytogenes* ATCC 19115
L. bulgaricus TLB01FT	-	-	-	-
L. bulgaricus TLB02FT	-	-	-	-
L. bulgaricus TLB03FT	+	+	++	+
L. bulgaricus TLB04FT	-	-	-	-
L. bulgaricus TLB05FT	+++	+++	+++	+++
L. bulgaricus TLB06FT	++++	+++	+++	++
L. bulgaricus TLB07FT	-	-	-	-
L. bulgaricus TLB08FT	++	+++	+	+++
L. bulgaricus TLB09FT	-	-	-	-
L. bulgaricus TLB10FT	++	++	++	+++
L. bulgaricus TLB11FT	-	-	-	-
L. bulgaricus TLB12FT	-	-	-	-
L. bulgaricus TLB13FT	-	-	-	-
L. bulgaricus TLB14FT	-	-	-	-

Inhibition pattern in term of zone size: (-) 0 mm, (+) ≤ 3 mm, (+++) 4≤ 8 mm, (++++) 8≤ 12 mm

TLB06FT is protein in the nature as it was only denature by proteolytic enzymes. Similarly, organic solvents like beta macrcapthanol, acetone and chloroform reduced its activity.

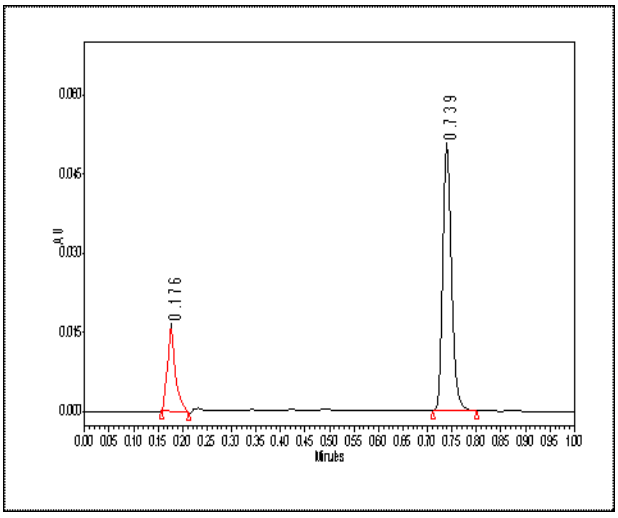

Fig. 5: Elution of different fractions of purified bacteriocin produced by TLB06FT on RP-HPLC.

Purification of bacteriocin

The detail scheme for the purification of bacteriocin was summarized in the table 2. In the first level of purification, bacteriocin was recovered by centrifugation and filtration pertaining 100% recovery (5369.13AUmg^{1}). At second level, ammonium sulfate precipitation was carried out with 80 % recovery (16494.85AUmg^{-1}). At the end partially purified bacteriocin was run on reverse phase column chromatography (RP-HPLC) and a single peak was obtained having 6400AU mL^{-1} activity.

DISCUSSION

Dahi locally made fermented milk product contain diversifying microflora. The predominated group in lactic acid bacteria (Dewan and Tamang, 2007). In the literature, it was reported that *L. bulgaricus* exhibited antibacterial activities against the different food pathogen (Balasubramanyam and Varadaraj, 1998). Simova *et al.* (2008) applied *L. bulgaricus* in combination with *S. thermophilus* to confirm its pathogenicity against closely related useful and other harmful bacteria. Recently, Tufail *et al*. (2011) also investigated its growth related phenomena with reference to bacteriocin production. It was revealed from the findings that *dahi* contained high bacteriocin producing strains of *L. bulgaricus.* However, we selected single strains with unique characteristics and wide array of antibacterial activity against food born pathogens.

Bacteriocin production is a growth linked phenomena (Simova *et al*., 2008) that need optimization for various condition that directly effect on the production of bacteriocin (Drider *et al*., 2006). Supplementation of the growth media by various constituents like Tween 80 and NaCl increase release of bacteriocin from the cells of producer microorganisms (Todorov and Dicks 2005). This important role of media constituents was also established previously by Ogunbanwo *et al.* (2003) who reported that bacteriocin production increased many fold in a medium that was supplemented with growth limiting factors (C, N, P) and major and minor minerals.

Physical growth conditions also have major effect on the production of bacteriocin (Sullivan *et al.,* 2002) that ultimately effect on the rate on bacteriocin production.

Table 2: Purification Scheme of bacteriocin produced by TLB06FT

Purification Stages	Volume (mL)	Activity ($AumL^{-1}$)	Total Activity (AU)[a]	Protein ($mgmL^{-1}$)[b]	Total Protein (mg)	Specific Activity (AU mg^{-1})[c]	Purification Factor[d]	Recovery (%)[e]
Centrifugation and Filtration	250	800	200000	0.149	37.25	5369.13	1.00	100.00
Ammonium Sulphate ppt	100	1600	160000	0.097	9.70	16494.85	3.07	80.00
HPLC (Reverse Phase-C_{18})	0.03	6400	192	0.006	0.00018	1066666.67	198.67	0.10

Various studies related to pH and temperature phenomena elaborated that maximum activity was obtained in acidic pH (3.5-5.5) against pathogenic and spoilage bacteria Balasubramanyam and Varadaraj (1998). Similarly Todorov and Dicks (2005) and Simova *et al.* (2008) also reported that maximum bacteriocin production was obtained at an initial pH of 5.5-6.5 and at temperature 37^{o}C.

Bacteriocins are categories in the bases of various characteristics like acid and heat tolerance (Erdogrul and Erbilir, 2006). Our results were different from the finding of Ghrairi *et al.* (2008) who found bacteriocin activity over 100^{o}C. The reason for this difference might be strain specificity or chemical nature of the bacteriocin. However, our findings regarding the effect of enzymes coincided with the previous work of De Kwaadsteniet *et al.* (2005) and Simova *et al*. (2008) who reported that bacteriocin was completed destroyed by proteases. In the same way results were in line with the findings that stated except Beta mercapthanol and EDTA all surfactant and organic solvent did not affect the activity (Khalil, 2009).

Purification of bacteriocin is very important fro the commercialization of the product. The purification scheme that was applied in the present investigation were previously reported by many researchers for the purification of bacteriocin like Chumchalova *et al*. (2004) applied this and recovery all active fractions in the same way Batdorj *et al*. (2006) use this technique for the purification of bacteriocin.

CONCLUSION

Lactobacillus bulgaricus has been found in many traditional fermented milk products. The rapid growth and compatible behavior toward primary starter species ensure its survival in yogurt like products. Therefore, it is important to study technological and therapeutic properties of this bacterium. Present study provides an insight regarding its bacteriocin potential and Purification Scheme. In the same way, bacteriocin producing *L. bulgaricus* TLB06FT sharing same homology with other reported bacteriocins. Although bacteriocin production is a inherited trail but supplementing the growth medium with growth limiting factors could enhance bacteriocin production. In this consequence, purification of these compounds would be further helpful to meet the thirst of new and more effective bacteriocin.

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