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PURIFICATION AND CHARACTERIZATION OF NISIN PRODUCED BY LACTOCOCCUS LACTIS ISOLATED FROM INDIAN CURD

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ABSTRACT: *Lactococcus lactis* isolated from traditional dairy Indian curd. Strains were preliminarily identified by PCR analysis and partial 16S rRNA confirmed that N5 were 100% identical to *Lactococcus. lactis sp. lactis*. The results revealed that only the bacteriocin produced from strain N5 was shown as being active against mostly gram positive bacteria The bacteriocin produced purified by precipitation followed by loading with gel chromatography. The partially purified bacteriocin was found to be stable over a wide range of pH, temperature and enzymes. The molecular weight of the peptide was judged to be 3.5 kDa by SDS-polyacrylamide gel electrophoresis.and conform to the result of mass spectrometry by maldi-tof test which calculated the mass of 3354.07 Da for nisin.These results indicate that bacteriocin produced by *L. lactis sp. lactis* N5 is a nisin.

Keywords: Food pathogens, lactic acid bacteria, bacteriocins, nisin.

INTRODUCTION

LAB have for centuries been responsible for the fermentative processing and preservation of many food products including dairy, meat, vegetables and bakery products [1]. *Lactococcus lactis* has been traditionally used as starter in the manufacture of cheese and fermented milk products on account of their function of preservation and contribution to flavor and aroma. Selected strains are used as combined cultures, single or as mixture of single cultures. Preservation of fermented foods is due primarily to the conversion of sugars in organic acids with a concomitant lowering of the pH and removal of large amounts of carbohydrates as nutrient sources. These effects extend the shelf life and safety of the final product [2, 3, 4].

Nisin, which is secreted by *Lactococcus lactis* and [5] although nisin has been used as a food preservative for more than fifty years, no significant bacterial resistance against nisin has been reported [6]. Nisin is one of several ribosomally synthesized small protein antibiotics that contain dehydro residues (dehydroalanine [DHA] and dehydrobutryine [DHB] and thioether cross-linkages (lanthionine and, B-methyllanthionine) that are introduced by posttranslational modifications of ordinary amino acids (serine, threonine, and cysteine). Although these antibiotics are produced by a disparate group of gram-positive bacteria [7, 8]. Nowadays, production of highly purified nisin preparations and enhancement by chelators has led to interest in the use of nisin for human ulcer therapy and mastitis control in the cattle[9].

Nisin from *L. lactis* can be purified directly from the culture medium [10, 11]. It is a cationic peptide and therefore commonly purified using gel permeable chromatography at acidic pH, using high salt concentration for elution, typically a single step elution with 1M NaCl [12, 13, and 14].

Nisin shows promising activity towards clinical isolates of the Methicillin resistant *Staphylococcus aureus* (MRSA) bacterium, *Streptococcus pyogenenes* and several of the most severe human pathogens, including the multi-resistant *Streptococcus pneumoniae* and vancomycin-resistant *Ent. faecium* or *Ent. faecalis*, against which new effective antibiotics are most urgently needed [15,16]. In all these studies purified nisin was used and the bactericidal activity of nisin was measured by we describe the isolation and detection of nisin from *L. lactis strain* Nisin was tested for bactericidal activity using the nisin-sensitive strains through all steps of purification.

MATERIAL AND METHODS

LAB was isolated samples of traditional dairy curd sold in retail markets in Tamil nadu. Samples (25 g) of food were homogenized with 225 ml of 0.85% (w/v) sterile normal saline; 10-fold serially diluted, plated on MRS [16]. All selected strains of LAB were maintained in MRS broth with 20% glycerol at -20oC. Strains were characterized by partial 16S rRNA gene sequencing. A region of the 16S rRNA gene was amplified by 29 cycles of PCR (consisting of 30 s at 94°C, 60 s at 55°C, and 90 s at 72°C, with a final 120-s extension step at 72°C) with purified chromosomal DNA [17] from the strains as template and using universal primers pA (5_ AGA GTT TGA TCC TGG CTC AG 3_) and pE (5_ CCG TCA ATT CCT TTG AGT TT 3_) (3). The amplified 605-bp fragments were harvested from low-meltingpoint gel LM-3 (Genet Bio, Korea), purified with chloroform- propanol [18] , and sequenced with an Autoread sequencing kit with an ALF DNA sequencer (Synergy Scientific Services, India). The sequences obtained were compared against the National Center for Biotechnology Information genome BLAST library (version 2.2.25, accessed 08-AUG-2011; http: //www.ncbi.nlm.nih.gov/BLAST/). The sequence homology (100%) to known sequences of the *L. lactis* subsp. *lactis* 16S rRNA gene confirmed that all strains analyzed represented this species. One *L. lactis* strain was selected for continued characterization.

The growth media and incubation temperature of the bacterial strains used as indicator bacteria for detection of antibacterial activity of *L. lactis* subsp. *lactis* are shown in Table 1

Table 1: Antibacterial spectrum of bacteriocin produced by L. lactis subsp. lactis N5

Indicator strain	**Inhibition zone of *Lactococcus lactis subsp. lactis* N5**
Staphylococcus aureus	++
Micrococcus luteus	++++
Enterococcus faecalis	++
Bacillus cereus	++
Escherichia coli	-
Pseudomonas aerogenosa	-
S. enterica serotype Typhimurium	-
Salomella typhi	-
Klebsella pneumoniae	-

no zone of inhibition; +: zone of inhibition between 1 and 5 mm in diameter; ++: zone of inhibition between 6 and 10 mm in diameter; +++: zone of inhibition between 11 and 15 mm in diameter; ++++: zone of inhibition 16 mm and over

Bacteriocin production

A 100 ml MRS broth was inoculated with an 18h old culture (1%, v v-1) of strain N5. Incubation was at 30ºC, without agitation. Samples were taken at 1 h intervals to determine the optical density (at 600 nm) of the culture and the antibacterial activity of the bacteriocin produced. Bacteriocin activity was defined as the reciprocal of the highest dilution showing definite inhibition of the indicator strain and was expressed as activity units per milliliter (AU ml-1).

Bacteriocin purification procedure

Bacteriocin was isolated from a 500 ml culture of the *L. lactis* subsp. *Lactis* N5, grown in M17G broth at 30 ºC for 18h. The cells were removed by centrifugation at 15500x g, 4ºC, 20 min (Sigma 3K 30, rotor 12155) and the pH of the cell-free culture supernatant was adjusted to 6.5 by addition of 10 N NaOH. This cell-free culture supernatant was brought to a final ammonium sulphate concentration of 30-70% saturation by slow addition of the salt, and was stirred at 4°C over a night. Then, the mixture was centrifuged at 15500 x g, 4ºC, for 30 min (Sigma 3K 30, rotor 12155), The pellet was resuspended in 2 ml of sterile ultra pure water (Milli Q). This partially purified bacteriocin was stored at –20ºC [15].

Effect of heat, temperature and enzyme treatment

Aliquots 5 ml of sterile partially bacteriocin were heated at 70, 80, 90 and 100C° for 10, 15 and 20 min in a thermostatically controlled water bath (GP-400, Neslab instruments, Newington, USA) and at 121C° for 20 min in an autoclave (Sanyo Vertical Labo Autoclave, NB Scientific, Edison, NJ) .

Aliquots 50 ml of sterile CBS were adjusted to pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 with 0.1 M HCl and 0.1 M NaOH, incubated for 4 h at room temperature.

Effect of enzymes on the bacteriocin activity was determined by treating sterile CBS pH 6.5 with enzyme solutions. Each of catalase (Sigma, St. Louis, USA) and proteinase (Sigma, St. Louis, USA) was added to the pH 7.0 bacteriocin solution to the final enzyme concentration of 1 mg/ml, and then incubated for 2 h at 37C° [19].

The treated samples were tested for antibacterial activity against the indicator bacteria *M. luteus* ATCC *4698*. Untreated culture concentrated served as a positive control.

Inhibitor activity of partially purified nisin

The antibacterial effect of the partially purified nisin from *L. lactis sp. Lactis* N5 was investigated by adding partially purified nisin to a log-phase culture (OD600=0.6) of *M. luteus* ATCC *4698* in BHI at 35 °C. Ten milliliter of the culture was distributed aseptically in different tubes. Each tube contained 0·5 ml of the partially purified nisin, except for the control that only contained sterile distilled water. The OD600 and cell counts (cfu/ml) were measured after 10 min, 1 h, 2 h, and 4 h of incubation. Cell counts were determined on BHI agar plate

Purification of bacteriocin by chromatography

Partially purified nisin was purified by gel-chromatography Superdex 75prep was selected as the matrix as it was suitable for low molecular weight proteins like nisin. The wet Superdex 75 and 50 mM phosphate buffer (pH 6.5). Five grams of Superdex 75was soaked in 200 ml of 50 mM phosphate buffer (pH 6.5) containing 0.1 gm of sodium azide and incubated for 72 hours at room temperature. After soaking the gel was deaerated and poured in a 0.9 x 60 cm column. Void volume was determined by passing blue dextran (2000 kDa) through the column. The sample was loaded to the column 2.0 ml at a time. The above mentioned buffer was used to elute the sample fractions each of 3.0 ml were collected at a flow rate of 0.2 ml/min Protein elution was monitored by measuring the absorbance at 215 nm. Unfortunately, it is not possible detect nisin at 280 nm because it does not contain any aromatic amino acids, therefore the 215nm was chosen as wavelength.

Tricine-SDS-PAGE and detection of antibacterial activity

Tricine-SDS-PAGE was essentially carried out as described in[20]. For analysis, 16 µl sample was supplemented with 4 µl 5x SDS Sample Buffer (0.2 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 40% (v/v) glycerol, 0.02% (w/v) bromophenol blue and 10 mM DTT) and loaded on a tricine gel consisting of a stacking gel containing 5% acrylamide and a separation gel containing 16% acrylamide. The gel was run at 100 V for 2 hours and proteins were detected via silver staining. For all purification fractions, 3.2 µg of total protein was analyzed.

MALDI-TOF Mass spectrometry

Samples obtained from gel chromatography were lyophilized For mass spectrometric analysis the samples were dissolved in water with 1% (v/v) formic acid Mass spectrometric data will be collected using an ABI 4800 MALDI TOF/TOF (Applied Biosystems, Foster City; CA). Depending on the samples mass data acquisition and processing will be done. In most of the cases the data will be acquired in reflector mode from a mass range of 600 – 4000 Daltons

Five most intense ions from the MS analysis, which are not on the exclusion list, will be subjected to MS/MS with appropriate precursor mass range. In most the cases for MS/MS analysis, the mass range is between 60 to precursor ion.

GPS Explorer software (Applied Biosystems, Foster City; CA) is used to generate peak list from the raw data generated from the ABI 4800. The peak list generated based on signal to noise filtering, exclusion list and de-isotoping parameters. The resulting peak list file is then searched and compared against existing data bases like Swissport and NCBI using Mascot (Matrix Science, Boston; MA).

Unless and specified default search parameters* will be used analysis. Additional or more detailed search can be done on request at additional cost, as this may need lot of man hours to do the analysis.

RESULTS AND DISCUSSION

Fermented dairy products namely imported processed cheese, local processed cheese, fresh milk, yoghurt, butter, spoiled dairy products and chicken sausages were used for isolation of lactic acid bacteria. Predominant colony types were selected and purified by continuous streaking on MRS media**.**

The isolate was tentatively identified as *L. lactis* having close similarity with *L. lactis subsp. lactis*. In order to study the phylogenetic position of *L. lactis* N5, 605 nucleotides of the 16S rRNA of the bacterium was amplified by PCR, sequenced, and subjected to 16S rRNA sequence analysis. The BLAST result shows 100% nucleotide homology with the 16S rRNA sequences of *L. lactis* in the database. The analyses indicate that the isolate is a strain of *L. lactis* having close similarity to subspecies lactis (Figure 1).

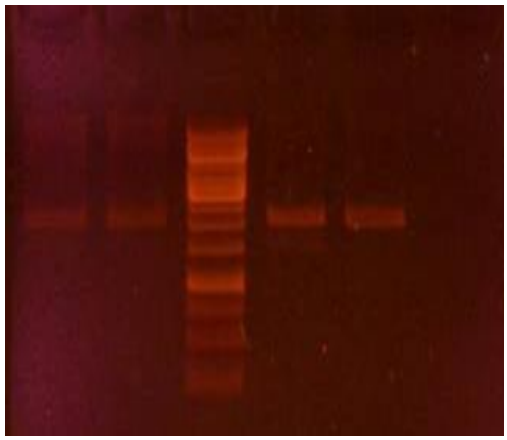

Figure (1). PCR-amplified 16S rRNA from isolated *Lactococcus* strains on 0.9% agarose gel (lane 1) and (lane 2) reference strains Lactococcus lactis MTCC440: lane 3, marker(gene); lane 4,Lactococcus lactis N4 ; lane 5,Lactococcus lactis N5.

The antibacterial activity of *L. lactis* subsp. *lactis* N5 was tested against Gram-positive and Gram-negative strains as described by L. De Vuyst [21]. *L. lactis* subsp. *lactis* N5 strain displayed broad spectrum of antibacterial activity, as it inhibited 9 indicator strains (Table 1). However Gram-negative bacteria (*Escherichia coli*, *Salmonella enterica* serotype Typhimurium and *Pseudomonas aeroginosa* were not inhibited.

The effect of pH, heat and enzymes on bacteriocin activity compared to that of nisin producing strain *L. lactis* subsp. *Lactis* N5 used as an experimental control is shown in (Figure2, 3 and 4). Inhibitory activity of neutralized culture supernatant of N5 strain did not decrease by heating at 100ºC for 5, 10, 15 and 20 min. Compared with unheated neutralize supernatant, inhibitory activity was reduced by 50%, 75% and 94% for treatments at 100ºC for 30 and 60 min and 121ºC for 15 min, respectively. Tramer [18] showed that nisin was remained stable after autoclaving at 115 ºC at pH 2.0, but loses 40% of its activity at pH 5.0 and more than 90% at neutral pH (6.8). Heat stability is a very useful characteristic in case of using bacteriocin as a food preservative because many food-processing procedures involve a heating step [23] .The supernatant from strain N5 was active in a wide range of pH from 2.0 up to 11.0. The antibacterial activity of bacteriocin N5 was highest (51200 AU ml-1) when the pH of the supernatant was between 2.0 and 4.0. Bacteriocin produced by N5 strain was stable at pH values between 5.0 and 8.0, but lost 50%, 75% and 87.5% of its activity at pH 9.0, 10.0 and 11.0, respectively. The biological activity of nisin is highly dependent on the pH of the solution [24, 25] .In acidic conditions nisin shows greatest activity [26, 27 and 17] but its activity decreases in alkaline conditions On the basis of these observations, it appeared that *L. lactis* subsp. *lactis* N5 produced a nisin-like bacteriocin.

The inhibitory activity produced by *L. lactis* N5 is lost upon treatment with proteolytic enzymes such as trypsin, papain or proteinase K. This is in contrast to nisin, which is resistant to trypsin[18, 19 and 20], Non proteolytic enzymes did not affect the activity. The antibacterial activity of *L. lactis* subsp. *lactis* N5 supernatant was not affected by treatment with lipase, catalase, pepsin and trypsin; however treatments with proteinase K, α-amylase resulted in substantial decreases in antibacterial activity.

These results indicate that the antibacterial compound produced by *L. lactis* subsp. *Lactis* N5 have a proteinaceous nature and could be classified as bacteriocin. Nisin is inactivated by α-chymotrypsin [21, 8] but not pepsin [8] and trypsin [21] as bacteriocin produced by N5 strain in our study. The bacteriocin produced by N5 was lost activity upon treatment with α-amylase. This indicated that bacteriocin produced by N5 is active only when they form aggregates with carbohydrate residues. Although by definition all bacteriocins are made of proteins, some have been reported to consist of combinations of different proteins or are composites of proteins together with lipid or carbohydrate moieties [22, 23].

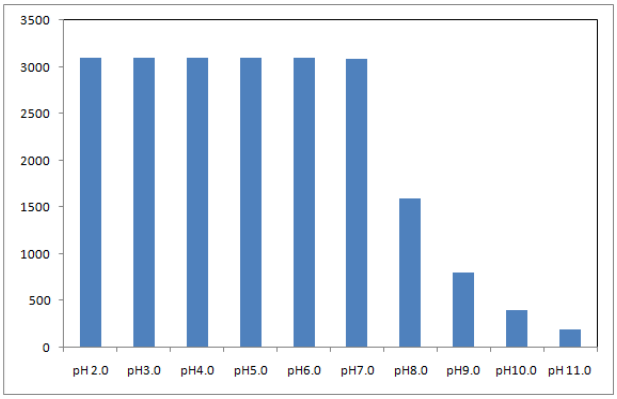

Figure (2) Effect of pH on partially purified nisin

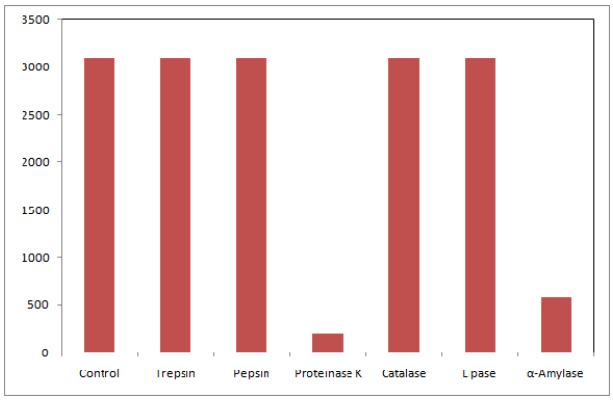

Figure (3) Effect of enzyme on partially purified nisin

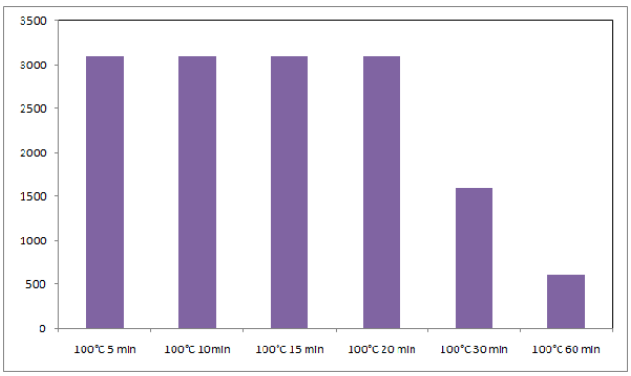

Figure (4) Effect of temperature on partially purified nisin

The antibacterial effect of bacteriocin produced by strain N5 was observed by recording the cell density (at 600 nm) of *Micrococcus luteus* over 9 h. Addition of the filter sterilized cell free supernatant containing bacteriocin (51200 AU ml-1) to early exponential phase cells of *Micrococcus luteus* (3h old, OD600nm= 0.4) resulted in growth inhibition after 1h (OD600nm= 0.2), followed by complete growth inhibition for the following 5 h (Table2).

Table (2): Antibacterial activity of selected bacteriocin-producing strains using *M luteus* as indicator strains

Cell free culture			Ammonium sulfate precipitation			Gel filtration chromatography		
AU/ml	mg/ml	AU/mg	AU/ml	mg/ml	AU/mg	AU/ml	mg/ml	AU/mg
3100	14.6	212.32	51200	8.3	6168.67	6500	0.57	11403.50

Antibacterial activity (AU/ml) = Reciprocal of the highest dilution x 1000 / volume of the sample used.
Protein conc. (mg/ml) = measured by Bradford assay taking BSA as a standard.
Specific activity (AU/mg) = Total activity of the sample / Total protein of the sample.

Based on the cell lysis treatments, nisin-like bacteriocin produced by N5 was determined as acting bactericidal activity against *Micrococcus luteus*. Nisin is characterized by a strong bactericidal mode of action as other subtype A lantibiotics such as subtilin, epidermin, gallidermin. Nisin affect a rapid killing of sensitive bacteria [24]. For instance, *Streptococcus agalactiae* was killed within 10 min after addition of nisin [25]. Detectable level of bacteriocin produced by strain N5 was recorded from 1 h after inoculation, indicating that peptide is primary metabolite. The highest level of bacteriocin activity (6168.67AU ml-1) was recorded after 8h of growth in M17G broth at 30ºC (Table 2). De Vuyst and Vandamme [21] reported that nisin production with *L. lactis* subsp. *lactis* NIZO 22186 clearly parallels that of biomass, and thus shows primary metabolite kinetics. Moreover, the highest nisin titre is reached at the end of the exponential phase, as confirmed this study.
Total 15 fractions each of 3.0 ml were collected at a flow rate of 0.2 ml/min and monitored at 215nm with the spectrophotometer. Antimicrobial assay was performed using Micrococcus luteus ATCC4698 as indicator strain. The separation profile resulted in one well-separated peaks. The active fractions were collected and subjected to lyophilization. The specific activity of lyophilized sample was increased to 11403.50 AU/mg resulting in 54 fold purification (Table 3). All constitutively synthesized peptides, regardless of sub-classification, share a net positive charge which causes them to fold into anamphiphilic conformation upon interaction with bacterial membranes Gujarathi14 reported that Sephadex G-75 was used for purification of nisin from the culture filtrate of Lactococcus lactis MTCC440 They suggested that GPC was generally employed in the final stages of purification, or when the proteins to be fractionated had considerable differences in molecular weights. Purification of nisin from Lactococcus lactis N5 was found simple and generally required one or two chromatographic steps. The GPC system used in the study was standardized to achieve the best possible fractionation of the nisin from Lactococcus lactis N5 In the present study nisin isolated from Lactococcus lactis N5 was successfully purified through salting out with ammonium sulphate and gel permeable chromatography on Superdex 75, and they got a fold purification of 54.

The purified nisin (5 µg) was analyzed on SDS-PAGE (16 % w/v) and stained with silver nitrate. On sodium dodecyl sulfate poly acrylamide gel electrophoresis the purified nisin showed a single band indicated that it was electrophorotically homogeneous. The molecular mass of the purified nisin was determined as ca. 3.5 kDa by comparing with relative mobility of the molecular mass of the protein markers (Figure 7).

Table (3): Summary of purification of bacteriocin from crude culture filtrate of Lactococcus lactis N5

Treatment	Total activity (AU/ml)	Protein (mg/ml)	Specific activity (AU/mg)	Fold Purification
Cell free crude filtrate	3100	14.6	212.32	1
Ammonium sulfate precipitation	51200	8.3	6168.67	29
Gel filtration chromatography ((Superdex 75	6500	0.57	11403.50	54

1) Activity (AU/ml) = Expressed in terms of arbitrary units defined as reciprocal of the highest dilution x 1000 / volume of the sample used.
2) Protein (mg/ml) = measured by Bradford's method taking BSA as a standard.
3) Specific activity (AU/mg) = Total activity of the sample / Total protein of the sample.
4) Fold purification = Specific activity of the subsequent step / Specific activity of crude sample.

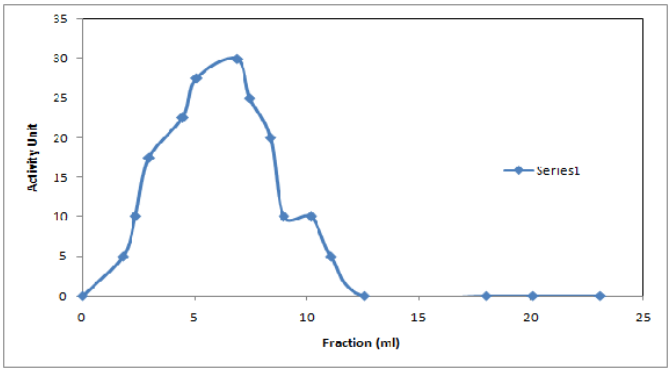

Figure (5) Activity unit of fractions after filtration by gel chromatography

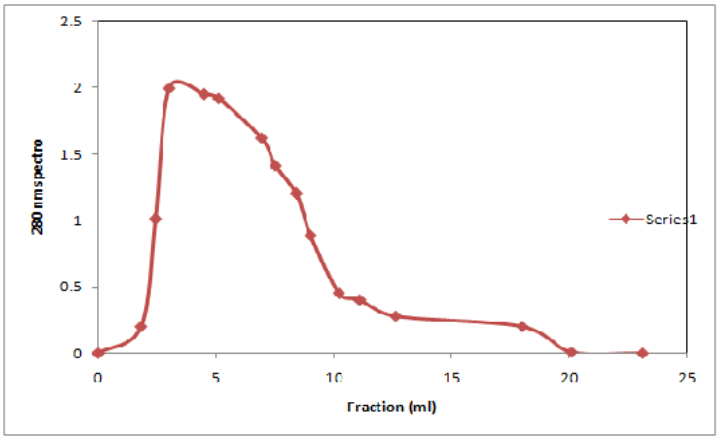

Figure (6) OD of pure nisin fractions after filtration by gel chromatography

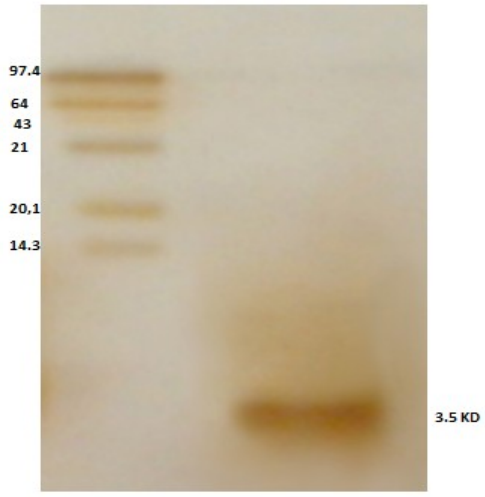

Figure (7). Tricine-SDS-Page : A-Protean marker, B-Pure protean

To assess and confirm the presence of nisin in the lyophilized pure sample after purification with Gel permeable chromatography we applied MALDI-TOF mass spectrometry. The lyophilized sample contained only one peptide with a molecular mass of 3353.09 Da (Figure 8), which is in agreement with the calculated mass of 3354.07 Da for nisin. Peak integration of the total mass spectrum revealed that the 400mM elution fraction contains >98% of nisin, indicating that this fraction is essentially devoid of contaminants. These results are in agreement with tricine-SDS-PAGE analysis. These molecular masses correspond to the molecular mass of nisin A.

The difference in molecular weights obtained from the SDS-PAGE and MALDI-TOF analysis [26] could be explained by high hydrophobicity of the sample[27] or formation of intramolecular rings [28], which physically shortens the peptide. Similar findings were reported by Piard [27].

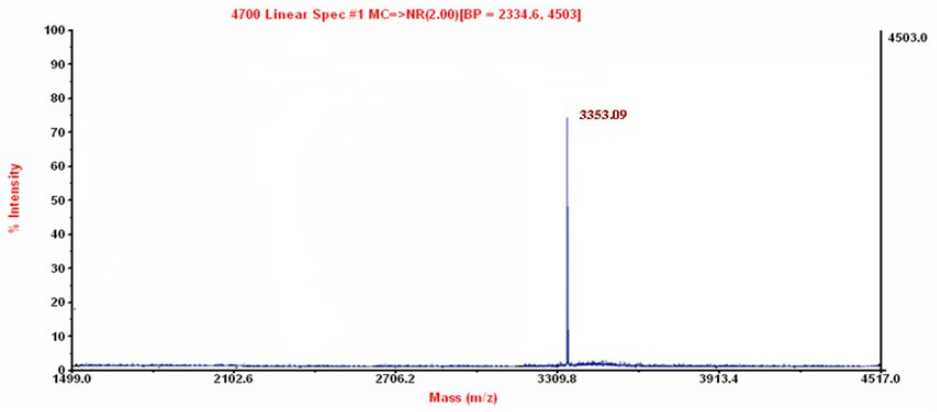

Figure (8). Mass spectrometry of Lyophilized nisin determined by Maldi-tof test

CONCLUSION

Consequently, the MRS medium is excellent for *Lactococcus lactis* isolation and nisin production. Strain identification with 16S rRNA sequences, purification of nisin with ammonium sulfate followed by purification by gel chromatography method was 54 fold The activity of partially purified bacteriocin stable in broad range of pH, temperatures and verity of enzymes inhibitory affects against bacterial pathogenic, beside the result of SDS-page and Maldi-tof test could be concluded that the bacteriocin antibiotic produced by *Lactococcus lactis.*

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