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Isolation and Characterization of Nisin-Like Peptide Produced by Lactococcuslactis Isolated from Indian Curd

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ABSTRACT

Twenty isolates of lactic acid bacteria (LAB) from traditional dairy Indian curd were tested for sugar fermentation test, strains were preliminarily identified as Lactococcuslactis sp. lactis by physiological and biochemical test. The identification by PCR analysis and partial 16S rRNA confirmed that N5 were 100% identical to Lactococcus. lactissp. lactis. The results revealed that only the bacteriocin produced from strain N5 was shown as being active against mostly gram positive bacterial indicators. The bacteriocin produced also exhibited a strong inhibitory effect on the foodborn pathogen, *Staphylococcus aureus*, which is mostly reported as the dominant foodborn pathogen in foods. This bacteriocin producing LAB The bacteriocin has a broad antibacterial spectrum similar to that of nisin and inhibited several related species of lactic acid bacteria and other Gram-positive bacteria. The inhibitory activity of the bacteriocin was found to be stable over a wide range of pH and temperature. The molecular weight of the peptide was judged to be 3.5 kDa by SDS-polyacrylamide gel electrophoresis. These results indicate that bacteriocin produced by L. lactis sp. lactis N5 is a nisin-like bacteriocin.

Keywords: food pathogens, lactic acid bacteria, bacteriocins, nisi

عزل وصفات البروين الشبيه بالنايسن المنتج من بكتريا Lactococcuslactis المعزولة من الالبان الهندية

الخلاصة

عزلت عشرين عزلة من بكتريا حامض اللبنيك من الالبان الهندية واختبرت قابليتها على تخمير السكريات,تركيز ٦,٥ من ملح الطعام والنمو بدرجات حرارية مختلفة.من خلال اختبار الفسيولوجية والبيوكيميائية وتحليل PCR و١٦SrRNA أن N5 كان تتطابق ١٠٠ ٪ جينيا مع بكتريا Lactococcuslactis عند مقارنتها مع بنك الجينات البكتيرية. تمتاز هذه البكتريا بقابليتها على انتاج

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عدد من البكتريوسينات الفعالة ضد البكتريا المسببة للتلوث الغذائيومنها بكتريوسين النايسين المعروف بطيف الفعالية الواسع ضد بكتيريا إيجابية لصبغة كرام.تم تنقية البكتريوسن الشبيه بالنايسين جزئيا بطريقة الترسيب الملحي باستخدام كبريتات الامونيوم وقد أثبت استقراره على مدى واسع من الرقم الهيدروجينيودرجة الحراة وعند معاملته بالانزيمات كما اثبت فاعليته على تثبيط البكتريا الموجبة لصبغة كرام ومنها المكورات العنقودية الذهبية وعند اجراء تحليل الوزن الجزيئي بطريقة الترحيل الكهربائي في الهلام (SDS PAGE)كان الوزن الجزيئي للبكتريوسن موضوع البحث ٣,٥كيلو دالتونوهذه النتائجتشير إلى أنالبكتريوسين الذي عزل من بكتريا N5Lactococcuslactis هوشبيه النايسين.

الكلمات المرشدة : الممرضات الغذائية , بكتريا حامض اللبنيك, البكتريوسينات, النايسين.

INTRODUCTION

Lactic Acid Bacteria have for centuries been responsible for the fermentative processing and preservation of many food products including dairy, meat, vegetables and bakery products [1].*L.*lactishas 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, 3and 4]. In recent decades, it has become clear that the overall inhibitory action is due to more complex antagonistic systems produced by the starter cultures. Those systems included the production of the hydrogen peroxide, diacetyl, secondary reaction products, and bacteriocins [2]. Competition for essential nutrients, the accumulation of D-aminoacids, a lowering of oxidation-reduction potential orcoaggregation may also be involved in antagonism [3]. Bacteriocins produced by LAB can be defined as biologically active proteins or protein complexes displaying a bactericidal mode of action exclusively towards Gram-positive bacteria and particularly closely related species. They form a heterogeneous group with respect to producing bacterial species, molecular size, antibacterial spectrum, mode of action, stability and physical and chemical properties [5, 2]. Bacteriocin productions were detected in all genera of lactic acid bacteria and have been well described for the subspecies of *L. lactis*. Kozak*et al*. [6] observed 88% of bacteriocin-producing among 67 nisin-non-producing *L. lactis* subsp. *lactis*strains. The frequency was of 1% in *L. lactis* subsp. *lactis* var. *diacetylactis* and 9% and 7.5% in *L. lactis* subsp. *lactis*and *L. lactis* subsp. *cremoris*, respectively. Out of 600 strains isolates from raw buffalo milk by Gupta and Batish[7], 34 (5.7%) inhibited at least one or more indicator strains.The frequency of production ranged from 3.8% in *L. lactis* subsp. *cremoris* to 6.8% and 7.6% in *L. lactis* subsp. *lactis* var. *diacetylactis* and *L. lactis* subsp. *lactis*, respectively. Piard*et al.* [8] found 18% of *L. lactis*bacteriocin-producing of all 50 strains examined. Vanderberg*et al*. [9] reported the production of nisin-like for 8

(14.5%) of all 55 strains examined. Villani*et al*. [10] founded activity bacteriocin-like in 12 (16.7%) of *L. lactis* subsp. *lactis* in a total of 72 strains.

With the emergence of psychrotrophic food-borne microorganisms, the development of new food technologies and the search of consumers for natural food products, the bacteriocins and/or producing microorganisms have been recognized as a potential source of biopreservatives for foods. sincenisin was accepted by the U.S. Food and Drug Administration in 1987 as a generally recognized safe food additive in dairy products. Today nisin is a permitted preservative in at least 48 countries, in which it is used in a variety of products, including cheese, canned food and cured meat [3]. Although information on nisin in the literature is extensive, little information has emerged regarding potential use in traditional fermented products. Hence this study is to report on the detection and characterization of nisin producing *L.lactis*N12 isolated from a traditional dairy Indian curd.

MATERIALS AND METHODS

Isolation of lactic acid bacteria (LAB)

LAB were isolated from 10 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 [11], + 0.5 % Calcium carbonate (CaCO3) agar plates and followed culture condition as described by Swetwiwathana(Ivanova 2000). Colonies were either selected randomly or all colonies were sampled if the plate contained less than 10, according to Leisneret al. [12]. The purity of the isolates was checked by repeated streaking on fresh (MRS, CaCO3) agar plates, followed by microscopic, physiological and biochemical determination. All selected strains of LAB were maintained in MRS broth with 20% glycerol at -20oC.

PCR analysis and DNA sequencing

The 20 isolated inhibitory 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 [13] 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 [14],and sequenced with an Autoread sequencing kit with an ALF DNAsequencer (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.

Detection of antimicrobial activity

The growth media and incubation temperature of the bacterial strains used as indicator bacteria for detection of antibacterial activity of *L. lactis*subsp. *Lactis.*

Bacteriocin production

A 100 ml M17 broth was inoculated with an 18h old culture (1%, v/v) of *L. lactis*. Incubation was at 30ºC, without agitation. Samples were taken at 1 h intervals to determine the optical density (at 600nm) 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*N5, grown in M17 broth at 30 ºC for 18h. The cells were removed by centrifugation at 15500x g, 4ºC for 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].

Tricine-SDS-PAGE and detection of antibacterial activity

Tricine-SDS-PAGE was essentially carried out as described in [16]. 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.

Results and Discussion

From the preliminary results of catalase test, cell morphology and carbohydrate fermentation pattern of N12 (Table 1) indicated that N12 was gram positive, coccoid shape and catalase negative, VP-positive, and is able to hydrolyze arginine. The isolate could grow at 10ºC but not at 45ºC nor in 6.5% NaCl. The isolate did not produce gas from glucose. The data indicate that the strain belongs to the genus Lactococcus. It not utilized L- arabinose,lactose and starch similar to many species of *Lactococcus*[17, 7]. The isolate fermented glucose, fructose, maltose, sucrose, ribose and mannitol, but not xylose which shown in table 3. However, the isolate also utilized esculin, in contrast to the strain MTCC 440, the type strain of *L. lactis* subsp. lactis[7]. On the basis of acid production from 49 carbohydrates, 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 sequence 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 and Table 1).

The antibacterial activity of L. lactissubsp. *lactis*N5 was tested against Gram-positive and Gram-negative strains Table (2) as described by van Belkumetal. 1989. *L. lactis*subsp. *lactis*N5 strain displayed broad spectrum of antibacterial activity, as it inhibited 5 indicator strainsTable (2). However Gram-negative bacteria (Escherichia coli, and Salmonella entericaserotype Typhimurium were not inhibited while Pseudomonas aeroginosashowed less inhibitory effect compared to gram positive bacteria.

The effect of pH, heat and enzymes on bacteriocin activity compared to that of nisinproducing strain *L. lactis*subsp. *lactis*MTCC440 used as an experimental control is shown inTable (4).

The inhibitory activity produced by *L.* lactisN5 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]. The antibacterial activity of *L. lactis*subsp. LactisN5 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 Table (4). These results indicate that the antibacterial compound produced by *L.lactis*subsp. LactisN5 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].

Inhibitory activity of neutralized culture supernatant of N5 strain did not decreaseby 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. Table(4) showed that nisin was remained stable after autoclaving at 115.6ºC 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 [24]. 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 (6200 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 varied dependent on the pH of the media[25, 26].which show good activity in pH (2, 3 and 4) while this activity inhibited with decrease the acidity of media [29, 30, 1726 and 23] as confirmed in this study. Bacteriocin of L. lactissubsp. LactisN5 strain gave identical results to those obtained with nisin produced by L. lactissubsp. lactisMTCC 440 strain used as an experimental control under different pH, heat and enzyme treatments. On the basis of these observations, it appeared that L. lactissubsp. lactisN5 produced a nisin-like bacteriocin.

The antibacterial effect of bacteriocin produced by strain N5 was observed by recording the cell density (at 600 nm) of Micrococcus luteusover 9 h. Addition of the filtersterilized cell free supernatant containing bacteriocin (6200 AU ml-1) to early exponentialphase cells of Micrococcus luteus(3h old, OD600nm= 0.4) resulted in growth inhibition after1h (OD600nm= 0.2), followed by complete growth inhibition for the following 5 h Table (2).

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 Alantibiotics such as subtilin, epidermin, gallidermin. Nisin affect a rapid killing of sensitive bacteria [27]. For instance, Streptococcusagalactiaewas killed within 10 min after addition of nisin [28], whereas Ogden and Waites[9] noted that the majority of cells of sensitive *Lactobacillus* strains were killed within 1 min of nisin treatment.

Nisin-like bacteriocin N5 production occured throughout exponential phase, with the highest activity recorded at the end of this phase. 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 (6200AU ml-1) was recorded after 8h of growth in M17G broth at 30ºC (Table 3). De Vuyst and Vandamme[10] reported that nisin production with *L.* lactissubsp. lactisNIZO 22186 clearly parallels that of biomass, and thus shows primary metabolite kinetics. Moreover, the highest nisintitre is reached at the end of the exponential phase, as confirmed this study.

Tricine-SDS-PAGE analysis showed that the molecular weight of active peptide bandwas 3.5 kDa Figur (3). Previous studies showed that the molecular weight of nisin extractedfrom growth media was about 3.5 kDa[29, 30, 7 and.23] The molecular weight of bacteriocins partially purified by ammonium sulphate precipitation of *L.* lactissubsp. *N5* cell free supernatant showed similarity with nisin.

CONCLUSIONS

Consequently, the strain identification of N12 by both phyological, carbohydrate fermentation test and about 605 base pairs of 16S rRNA sequences, inhibitory spectrum of the bacteriocin produced from N12, all lead to the conclusion that LAB strain N12 isolated from traditional Indian curd is *Lc. lactis*subsp. lactis and can produce bacteriocin which have been identified as nisin by SDS-page. As a result, this potent nisin producer strain has been selected for further study aimed at its potential use as a starter culture in traditional production.

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Table (1) Phenotypic characterization and PCR results of isolates.

Isolates	Morphology	6.5% NaCl	Growth in 10ºC	Growth in 45Cº	Catalase activity	PCR identification
N1	Cocci	+	+	-	-	NI
N2	Cocci	+	+	-	-	NI
N3	Rods	-	+	-	+	NI
N4	Cocci	-	+	-	-	L. lactissubsp. Lactis
N5	Cocci	-	+	-	-	L. lactissubsp. Lactis
N6	Rods	+	+	-	+	NI
N7	Cocci	+	+	+	-	NI
N8	Cocci	+	+	-	-	NI
N9	Cocci	-	+	-	-	NI
N10	Rods	-	+	-	+	NI

N11	Cocci	-	+	-	-	L. lactissubsp. Lactis
N12	Cocci	+	+	-	-	NI
N13	Cocci	+	+	-	-	NI
N14	Cocci	-	+	-	-	NI
N15	Cocci	-	+	-	-	NI
N16	Cocci	-	+	-	-	L. lactissubsp. Lactis
N17	Rod	+	+	-	+	NI
N18	Cocci	-	+	-	-	NI
N19	Cocci	-	+	-	-	NI
N20	Cocci	-	+	-	+	NI

NI: not identified**.**

Table (2) Antibacterial spectrum of bacteriocin produced by *L. lactis*subsp. *lactis*N5and MTCC440.

Indicator strain	Media*	Temperature c°	Inhibition zone**	
			N5	MTCC440
Staphylococcus aureus	TSB	37	++	++
Micrococcus luteus	LB	30	++++	++++
Enterococcus faecalis	M17G	30	++	+
Bacillus cereus	TSB	37	++	+
Lactobacillus plantarum	MRS	37	++++	++++
Escherichia coli	LB	37	-	-
Pseudomonasaerogenosa	LB	37	-	-
S. entericaserotype Typhimurium	LB	37	-	-
Salomellatyphi	LB	37	-	-
Klebsellapeumoniae	LB	37	-	-

* M17: M17 broth
MRS: De Man Rogosa Sharpe Broth;
LB: Luria Bertani Broth; TSB: Tryptic Soy Broth
** -: 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

Table (3) carbohydrate fermentation test.

Test	**Bacterial isolation**	
	Lactococcuslactis sp. Lactis N5	***Lactococcuslactis sp. Lactis MTCC 440***
Carbohydrate fermentation pattern		
Control	+	+
L-Arabinose	-	-
Ribose	+	+
D-Xylose	-	-
L-Xylose	-	-
Mannitol	+	+
Sorbitol	-	-
Salicin	+	+
Cellibiose	+	+
Maltose	+	+
Lactose	-	-
Starch	-	-
Milibiose	+	-
Sucrose	+	+
Galactose	±	-
Inulin	-	-
D-Raffinose	+	+
D-Glucose	+	+
D-Mannose	+	+
D-Fructose	+	+
Gluconate	-	-
Esculin	+	+
produce gas from glucose	+	+
Arginine hydrolysis	+	+

+ = positive; - = negative; ± weak fermentation.

Table (4) Sensitivity of culture supernatants from L. lactissubsp. lactisN5 and MTCC440 towards differentenzyme, pH and heat treatments.

Treatment	**Bacteriocin activity (AU ml-1)**	
	***L. lactis*subsp. *Lactis* N5**	***L. lactis*subsp. *Lactis* MTCC440**
Control	3100	3500

Trepsin	3100	3500
Pepsin	3100	3500
Proteinase K	210	0
Catalase	3100	3300
Lipase	3100	3500
α-Amylase	590	600
pH 2.0	6200	7000
pH3.0	6200	7000
pH4.0	6200	7000
pH5.0	3100	3500
pH6.0	3100	3500
pH7.0	3100	3500
pH8.0	3100	3500
pH9.0	1600	1750
pH10.0	800	830
pH 11.0	400	420
100°C 5 min	3100	3500
100°C 10min	3100	3500
100°C 15 min	3100	3500
100°C 20 min	3100	3500
100°C 30 min	1600	1750
100°C 60 min	620	830
121C° 15 min	325	410

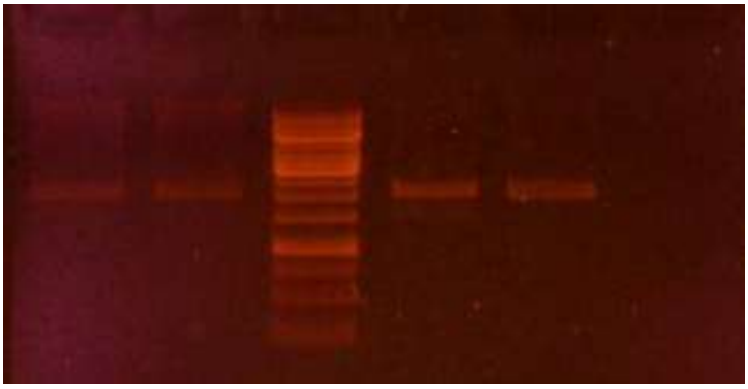

Figure (1) PCR-amplified 16S rDNA segments derived from total DNA.
Lane (1and 2) reference strains Lactococcuslactis MTCC440.
Lane 3, PCR marker.
Lane 4, Lactococcuslactis N4.
Lane 5, Lactococcuslactis N5.

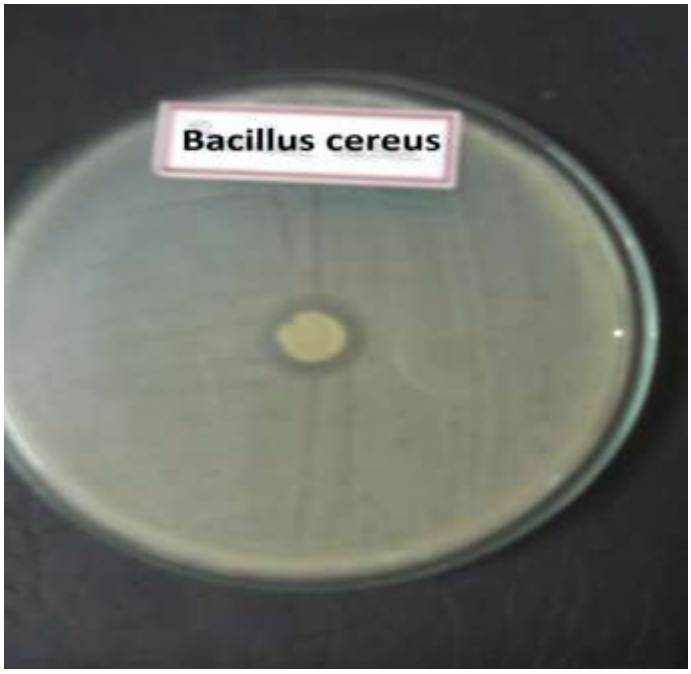

Figure (2) Effect ofLactococcuslactissubsp. LactisonBacillus cereus.

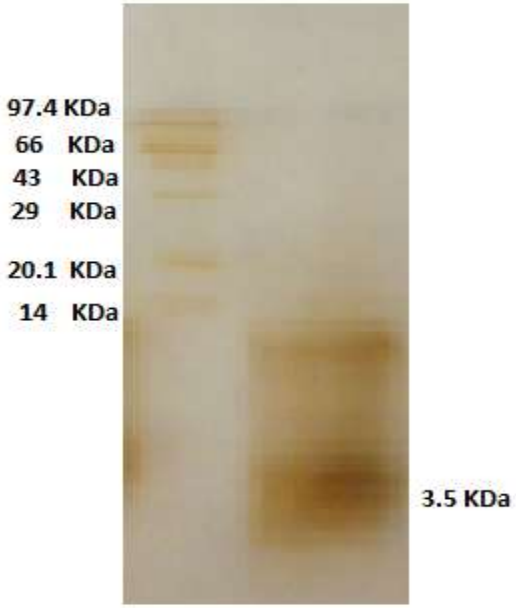

Figure (3) Tricine-SDS-PAGE of partially purified nisin.
Line 1 protein marker.
Line 2 isolatedprotein.