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Cloning of *nis* gene and Nisin purification from *Lactococcus lactis* subsp. *lactis* Fc2

D. E. El-hadedy[1]* and Eman W. El-gammal[2]

[1]Department of Radiation Microbiology, National Center for Radiation Research and Technology (NCCRT), Cairo, Egypt.
[2]Chemistry of Natural and Microbial Products Department, National Research Center (NRC), Cairo, Egypt.

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The present study was focused on nisin produced by *Lactococcus lactis* subsp. *lactis* (Fc2 isolate). The isolate was identified by 16S rRNA using specific universal primer. The obtained cell-free supernatant inhibits the growth of *Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus* and *Bacillus subtilis* by using agar diffusion assay. Nisin structural gene was detected by polymerase chain reaction with nisin gene-specific primer followed by cloning in PGEM-T easy vector, transformation in *E. coli* JM109 and direct sequencing of the construct. The Nis gene which encoded nisin Z showed identical sequences to Nis Z in Genbank with accession number AB727286, as indicated by the substitution of asparagine residue instead of histidine at position 27. In this way, it was established that the nis Z gene for nisin Z production is widely distributed. The purified nisin by chloroform extraction was analyzed on 20% SDS-PAGE and gave sharp band at ~ 3.4 kDa. The 3 dimension structure of the purified Nisin was studied by CPHModels as pdb with chimera program.

Key words: *Lactococcus lactis*, *nis* cloning, 16S rRNA, chloroform extraction and SDS-PAGE.

INTRODUCTION

Nisin, antimicrobial peptide (3.4 kDa), is produced by *Lactococcus lactis* ATCC 11454 during its exponential growth phase (Vessoni-Penna and Moraes, 2002). Nisin is a bacteriocin commercially used as natural agent for food biopreservation. It has recently been considered safe by the World Health Organization (WHO) and by the Food and Drug Administration (FDA), with the denomination of generally recognized as safe (GRAS) (de Vuyst and Vandamme, 1994; Arauz et al., 2009). This bacteriocin has large antimicrobial activity spectrum against Gram-positive bacteria and their spores, but shows little or no activity against Gram negative bacteria, yeasts or moulds. However, Gram negative bacteria can be sensitized to nisin by exposing to chelating agents (EDTA), sublethal heat and freezing (Vessoni Penna et al., 2006). As a result of its antimicrobial properties, nisin has been accepted as a safe and natural preservative in different areas of food industry and it has also been used as treatment for some health conditions such as stomach and colon ulcers, cosmetic and veterinary products (Delves-Broughton et al., 1996; Liu et al., 2004; Von Sataszewski and Jagus, 2008). The bacteriocin nisin,

*Corresponding author. E-mail: dodyelhadedy@yahoo.com.

which is produced by *L. lactis* subsp. *lactis*, has been extensively studied and used as a food preservative because of its lethal action and wide spectrum of activity (Hurst, 1981; Delves-Broughton et al., 1996). Two nisin variants, which differ only by one amino acid substitution at position 27, have been studied extensively. Nisin A contains histidine and nisin Z asparagine (Mulders et al., 1991). Usually, nisin producer strains are isolated from dairy products (Rauch et al., 1994).

Brotz and Sahl (2000) showed that nisin is the most popular of the lantibiotics. It was first discovered in the early 20th century and has been used for several decades as a natural, safe food preservative, especially in the dairy industry. Nisin's use as a food preservative has been well-studied and well-documented. It is sometimes used as a comparison when studying the effects of other natural antibacterials, such as certain essential oils. Though it is considered a type A lantibiotic, nisin's antibiotic action appears to occur through two separate mechanisms. Study aim to obtain variant strain to enhanced antimicrobial activity of nisin depending on the target microorganism. This enhancement is apparent against Gram positive and Gram negative targets is particularly novel. Further efforts will focus on determining the mechanistic basis for these enhancements.

MATERIALS AND METHODS

Growth media and organism preparation

The isolate *L. lactis* ssp. *lactis* Fc2, was identified by chemical method previously mentioned. MRS had optimum conditions: pH (7), temperature (37°C), Agitation (120 rpm), yeast extract (0.2 g, %), Tween 80 (0.04%) as described previous (Abdel Karem et al., 2005).

Cultivation of pathogenic strains

Listeria monocytogenes, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus* and *Bacillus subtilis* were used as indicator strains. They were cultured on plates containing Luria-Bertani (L.B) agar media. The plates were incubated at 37°C for 24 h.

Detection of nisin

LB broth culture of *L. lactis* ssp. *lactis* Fc2 strain was prepared and pH adjusted to approximately 2 to 3 with HCl. This acidified culture was placed into boiling water bath for 5 min to remove cell bound nisin by hot acid extraction. Broth media were centrifuged at 12.000 rpm for 12 min at 4°C. To exclude the inhibiting activity due to acids, the pH of the cell free supernatant (CFS) was adjusted to 6.5 with 1 M NaOH and filter sterilized through (0.45 µm) cellulose membrane.

Quantitative estimation of nisin prepared from the CFS

This was done after cooling the assay medium to about 45°C (Wolf and Gibbons, 1995). Nisin activity was carried out by critical dilution assay according to Pucci et al. (1988). Serial two fold dilutions were made from CFS of the *L. lactis* ssp. *lactis* Fc2 culture. Forty microliter (40 µl) was taken from each dilution and carried out on the surface of filter paper disks on LB agar plates; also, the LB was seeded with 1% (v/v) suspension of log phase cells of every indicator strains culture.

Antibacterial assay comparison of test organisms with nisin

The *Lactococcus* plates were incubated in aerobic condition for 24 h for each of the 6 organisms (*L. monocytogenes*, *P. aeruginosa S. aureus*, *B. subtilis*, *B. cereus* and *E. coli*). A set of standard bio-assay plates (1.5% agar, 1% Tween 20, 7 mm diameter filter paper disks) Lauryl Tryptose Broth (LTB) as assay medium was prepared (stock solution of nisin standard and purified 30 µl was inoculated on sterilized filter paper discs compared with different concentrations of antibiotics discs replicated three times on each set of *Lactococcus* were streaked in the centre of the plates). Plates were incubated for 24 to 48 h at 37°C under anaerobic aerobic conditions, respectively. The zones of inhibition were measured.

Identification of *lactococcus* by 16S rRNA

Full length 16S rRNA (1500 bp) were amplified from isolates (Fc2) by PCR using (universal forward primer P1 and universal reverse primer): Forward: (5'-AGAGTTTGATCCTGGCTCAG-3'), Reverse: (5'-CGGTTACCTTGTTACGACTT-3') under the optimum conditions (denaturation 94°C for 1 min, annealing at 45°C for 30 s and extension at 72°C for 2 min, 35 cycles). Amplified 16S rRNA was purified from 0.8% melting point agarose gel. Bands obtained from polymerase chain reaction (PCR) product were eluted and purified by Qiagen elution kit. PCR instructions, and DNA band desired was excised from ethidium bromide stained agarose gel with a razor blade and transferred to Ependorf tube. DNA was sequenced directly using specific primer with concentration of 20 pmol in Promega company lab. The sequence alignment was prepared with DNA STAR software program. Nucleotides sequences of the products were edited using Bioedit version 5.0.6 (Hall, 1999) and phylogentic tree by mega 4 program.

Gene isolation

Genomic DNA was extracted by a modification of the method of Engelke et al. (1992). The polymerase chain reaction analysis followed procedure described by Horn et al. (1991) with some modifications. The PCR amplification was carried out in a 50 µl mixture in a DNA thermo cycler. The conditions consisted of 35 cycles of 94°C for 1 min, 55°C for 1 min and 72 h for 2 min. The primers were designed from nis A structural gene, which were complementary to regions 17 bp upstream and 2 bp downstream of the coding region (Dodd et al., 1990; Mulders et al., 1991). The restriction sites EcoRI and KpnI were added at 5 end, respectively, for cloning purpose in other studies elsewhere. Forward and reverse primers were as follows: primer F(5'-CCG GAATTC ATA AGG AGG CAC TCA AAA TG-3') primer R(5'-CGG GGT ACC TAC TAT CCT TTG ATT TGG TT-3'). The amplified PCR products were purified from low melting point agarose and the nucleotide sequences in promega lab, Biotechnology Company. *nisZ* expressed nisin Z (PCR product) was purified and ligated to PGEM®-T easy vector; ligated plasmids were transformed into *E. coli* JM109 and transformants were selected using the blue/white screening procedure. After growing on IPTG/X gal agar plates supplemented with 100 µg/ml ampicillin, screening of recombinants was performed according to Sambrook et al. (1989). Purified plasmids were sequenced using a sequencer in promega lab. The sequence

Table 1. Inhibition zones and MICs (Minimum inhibitor concentrations) obtained for purified and Stander nisin Z, using different nisin-sensitive indicator strains.

Indicator strains	Radius of inhibition zone using purified nisin (mm)	MIC Concentration (ug/ml)	Radius of inhibition zone using stander nisin (mm)	MIC concentration (ug/ml)
Listeria monocytogenes	0.9	0.019	1.4	0.017
Pseudomonas aeruginosa	2.1	0.2	2.7	0.018
Staphylococcus aureus	0.5	0.24	0.7	0.2
Bacillus subtilis	2.9	0.014	3.2	0.012
Bacillus cereus	3.4	0.016	3.7	0.015
Escherichia Coli	1.3	0.015	1.6	0.01

alignment was prepared with DNA STAR software program (DNASTAR. INC., Madison, Wis.) and manually edited with GeneDoc (www.NCBI/blast.com), and determined translation encoded regions at www.expasy.org/cgi-bin/dna_aa. Construct contains nisin Z gene fragment diagramed by NTI vector program. Nucleotides sequence of the products was edited using BioEdit version 5.0.6 (Hall, 1999).

Purification of nisin

Chloroform extract (2 L) of a suitable broth medium was inoculated with 1% of an overnight culture of the nisin producing bacterium *Lactococcus* sp. and incubated for approximately 18 h at 35°C. Cells were pelleted at 7100 *g* for 15 min in a refrigerated (12°C) centrifuge; the nisin containing supernatant fluid was collected and purification of nisin was carried out according to Burianek and Yousef (2000).

Gel electrophoresis and detection of nisin molecular weight

One milliliter from overnight culture incubated in shaking incubator at 37°C and 120 rpm of *L. lactis* ssp. *lactis* Fc2 was collected by centrifugation at 10,000 rpm for 15 min. Bacterial pellets were resuspended well in 60 µl distilled water then 20 µl sample buffer and 10 µl protein tracking dye were added. Polyacrylamide gels were prepared with unheated samples as described before (Mulders et al., 1991). Centrifugation for 15 min at 10,000 rpm was done and placed on ice until loading. The components of the separating gel were placed in 50 ml beaker and mixed gently to avoid air bubbles. The solution was pipetted into the assembled vertical slab gel unit in the casting mode leaving 2 ml from the front. A layer of distilled water was pipetted on the top of solution. The gel was allowed to polymerize at room temperature and the water was poured from the upper surface. The stacking gel solution was prepared and added onto the top of separating gel where the comb was inserted. The gel polymerization was allowed. The assembled vertical slab containing the polymerized gels were put into the electrophoresis chamber filled with the tank buffer. The protein samples were loaded into the wells after removing the comb by using Hamilton syringe in loading; a protein marker was also loaded (200 volt and tank buffer 1X). For electrophoresis, the gel was stained in 50 ml of staining solution for 45 min with shaking at room temperature and then distained in distaining solution and finally, gel image was taken.

RESULTS AND DISCUSSION

The antimicrobial substance produced by *L. lactis* subsp. *lactis* WNC 20 strain had similar enzymatic, pH sensitivity and heat insensitivity pattern to nisin. The identical antimicrobial spectrum and larger inhibition zones in agar diffusion assay of bacteriocin obtained from WNC 20 as compared with those from DL 11, a nisin A producing strain, suggested that the bacteriocin produced by WNC 20 was nisin Z. De Vos et al. (1993) mentioned that the undiluted culture supernatant of NIZO 22186 resulted in much larger inhibition zones than that of NIZO R5 in a standard agar diffusion bioassay. Also, Tramer and Fowler (1964) found the same result with *M. flavus* as an indicator microorganism. Therefore, the antimicrobial activities of the purified and standardized nisin variants were compared. To study possible differences in specific antimicrobial activity, the MICs of lantibiotic nisin were determined in liquid cultures of *M. flavus*. The MICs appeared to be identical (0.015 µg/ml). In this study *L. monocytogenes*, *P. aeruginosa*, *S. aureus*, *B. subtilis*, *B. cereus* and *E. coli* were used as indicator microorganisms against nisin activity of *L. lactis* by using standard agar diffusion bioassay. The minimum inhibitor concentration (MIC) for inhibition zone was 50 µg/ml of nisin solution. Data shown in Table 1 indicate that the sizes of the inhibition zones varied considerably between the different indicator bacteria and were inversely related to the MICs. The most sensitive Nisin was *B. cereus* (inhibition zone was 3.4 and 3.7 mm) with purified and standard nisin, respectively.

The resistance microorganism was *S. aureus* (inhibition zone was 0.5 and 0.7 mm with purified and standard nisin, respectively). De Vos et al. (1993) and Dosler and Gerceker (2012) reported that the physicochemical properties of nisin Z are contributing to the formation of the large inhibition zones. On the basis of the nature of the His27Asn substitution in nisin Z, it is expected that nisin Z solubility is higher than nisin A solubility at pH values above 6 (the pK value of His) since the Asn side chain is more polar than the uncharged His side chain.

Identification Fc2 by 16S rRNA

Molecular techniques, such as PCR have been used

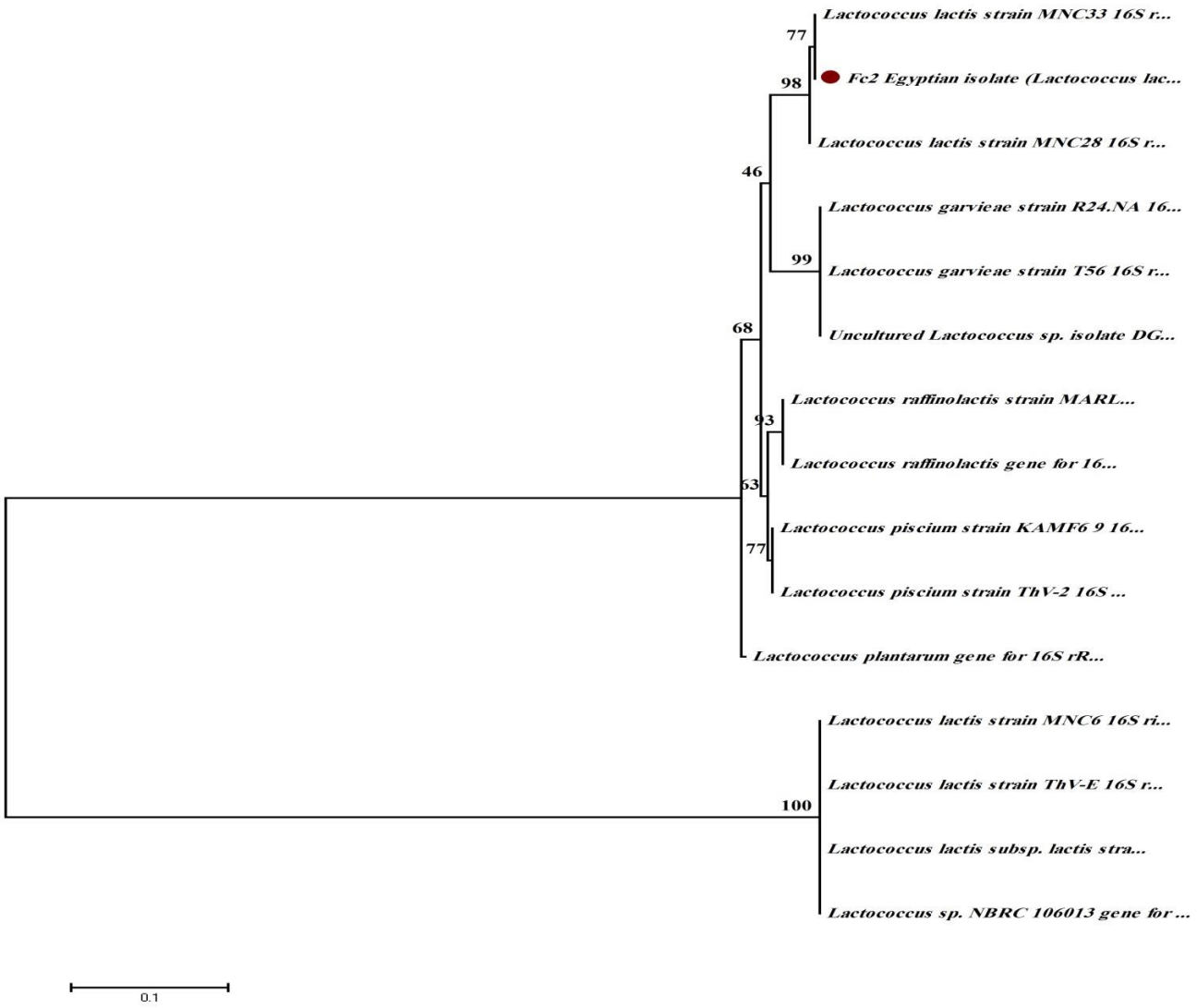

Figure 1. Phylogentic tree average distance tree using neighbor-joining (NJ) including those of *Lactococcus lactis* spp.

extensively for several years for identification and characterization of food borne pathogens in food samples (Hill, 1996; Wang et al., 1997). Here, we tried to use PCR method based on 16S rRNA gene accurate method for identification_and confirmation of Fc2 strain. 16S rRNA gene bands_which were detect by specific primer at 1500 bp. The ribosome consists of two unequal subunits, which associate via numerous intersubunit contacts. Medium-resolution structural studies have led to grouping of the intersubunit contacts into 12 directly visualizable intersubunit bridges. Most of the intersubunit interactions involve RNA. The relevant sequences were downloaded and phylogenetic analysis has been carried out as shown in Figure 1. Small rRNA gene sequencing, particularly 16S rRNA sequencing in bacteria, has led to advances on multiple fronts in microbiology. First, the construction of a universal phylogenetic tree classifies organisms into three domains of life: bacteria, Archaea, and Eucarya (Olsen et al., 1992; Olsen and Woese, 1993; Thompson et al., 1994; Arto pulk et al., 2010). Second, it revolutionists the classification of microorganisms, and makes the classification of non-cultivable microorganisms possible (Relman et al., 1990; Relman et al., 1992). Third, it helps to elucidate the relation of unknown bacterial species to known ones.

Gene isolation

Total DNA was isolated from these strains, and the structural nisin gene was amplified by PCR, resulting in a 350 bp DNA fragment. The presence of nis A or nis Z genes was determined by direct sequencing of the PCR product. Sequence ladder was obtained, indicating that a

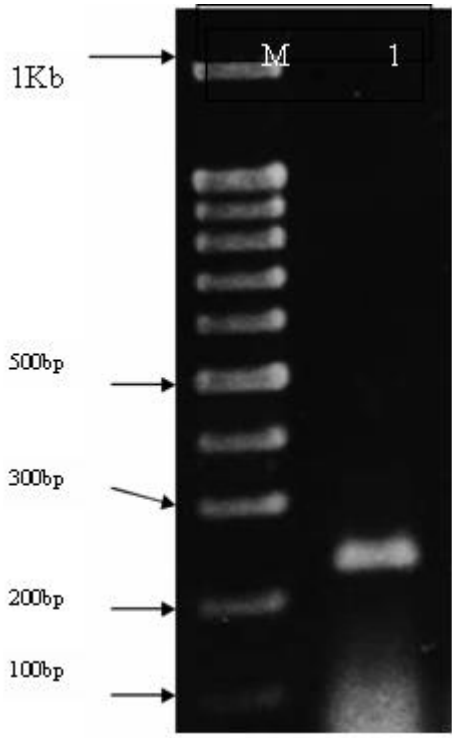

Figure 2. Agarose gel electrophoresis of PCR products with nisin gene-specific primer. Lane 1, shows nisin gene from *Lactobacillus plantarum*; lane M, was loaded with 100 bp ladder DNA markers.

```
atgagtacaaaagattttaacttgcagttggtatctgtttcgaagaaagattcaggtgca
 M  S  T  K  D  F  N  L  Q  L  V  S  V  S  K  K  D  S  G  A
tcaccacccattacaagtatttcgctatgtacacccggttgtaaaacaggagctttgatg
 S  P  P  I  T  S  I  S  L  C  T  P  G  C  K  T  G  A  L  M
ggagctaacatgaaaacagcaacttgtcattgtagtattcacgtaagcaaataataagca
 G  A  N  M  K  T  A  T  C  H  C  S  I  H  V  S  K  -  -  A
```

Figure 3. Gene sequence.

single nis gene species was present in the strains. In strain NCDO 2118, a small insertion of 16 bp was detected between positions 180 and 181 (Mulders et al., 1991), which was located just downstream of the nis Z structural gene. This insertion consists of a tandem duplication of the octanucleotide AACCAAAT present at positions 173 to 180. Sequences were as reported for the nis A or nis Z genes, differing only in the nucleotide at position 148 (Buchman et al., 1988 and Mulders et al., 1991). Data appear in Figure 2 illustrated nis gene expressed nisin Z product detect at ~ 227 bp. PCR product was eluted and cloning in PGEM-T easy vector (Figure 5) follow sequenced using alignment via genebank sequence identical nis Z gene expressed nisin Z with similarity 96% with new nucleotide sequence 4%. Figure 3 showed the gene sequence with 180 bp and 60 amino acids.

Results in this study are in agreement with Noonpakdee et al. (2003) who found that the bacteriocin produced by *L. Lactis* WNC 20 was nisin, PCR analysis using the published sequences of the nisin structural gene (Dodd et al., 1990) was performed. Two primers complementary to sequences occurring proximal to the 3 and 5 ends of the nisin A structural gene were used to amplify nisin gene from the genomic DNA of *L. lactis* DL 11 and *L. lactis* WNC 20. A 227 bp fragment was amplified from the genomic DNA of *L. lactis* WNC 20, which was identical to that amplified from a nisin-producing strain of *L. lactis* DL 11. Results indicated that sequences were 100% identical to that of nisin A except

A B

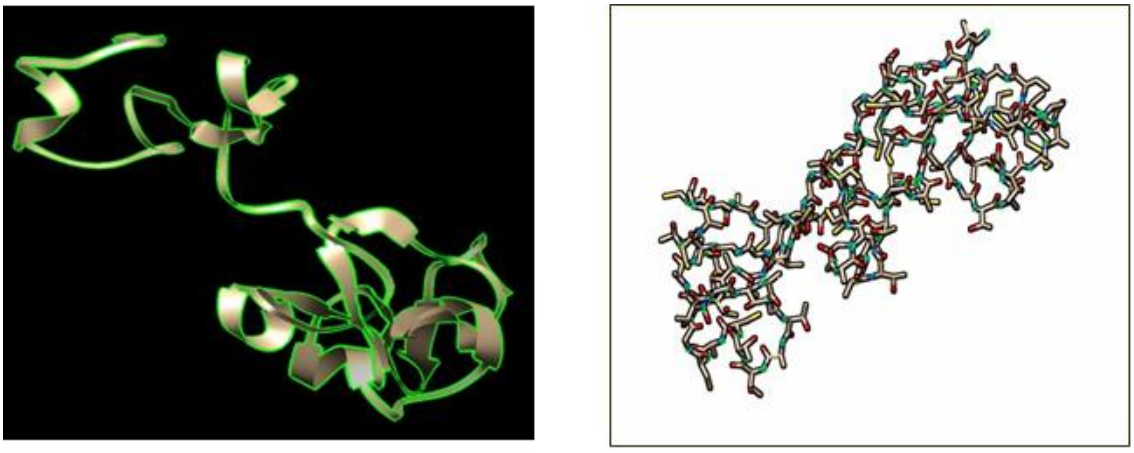

Figure 4 (a). Nucleotide sequence and deduced amino acid sequence of the nisZ gene isolated from *Lacctococcus . lactis subsp. lactis* (Fc2). The amino acid sequence is shown below the coding sequence. **(b).** 3 D structure of purified nisin Z from *lactococcus*.

for a C-to-A transversion at position 148. This resulted in an asparagine (AAT) residue at position 27 of the nisin peptide, instead of histidine (CAT). This indicates that the bacteriocin produced by *L. lactis* is nisin Z. The gene sequence (180 bp) had a similarity of 96% with *L. lactis* nisin (nis Z) gene. Hence, the purified Nisin from Egyptian strain which was identified by 16S rRNA in this study. The 3 Dimension structure of it obtained by CPHModels as pdb with Chimera program 1.7rc is as indicated in Figure 4b.

Nisin Z purification

Purification of nisin by chloroform was an easy method that gave high yield of nisin. Data shown in Table 2 indicated that nisin activity and specific activity were increased after chloroform extraction. Nisin yield was 0.5 g/l in MRS medium. Purification fold was also higher after using chloroform extraction than the culture supernatant. Solubility of chloroform in water is only 0.815%, therefore, chloroform is a solvent with intermediate polarity and is immiscible with water. These properties make chloroform most suitable for bacteriocin concentration from culture media. Nisin is amphiphilic peptide with high affinity towards lipid membrane (Breukink and Kruijff, 1999; Burianek and Yousef, 2000). Similar results were obtained by (Yang et al., 1992; Meghrous et al., 1997).

Gel electrophoresis and detection of nisin molecular weight

Nisin had a molecular weight of 3.4 kDa. Kuipers et al. (1992) reported that nisin separation SDS-PAGE use certain amount of purified nisin and use coomassie blue have molecular weight of 3 kDa. Monomer could arise as reaction products from hydroxyl addition to dehydro groups or from intramolecular additions involving nucleophilic groups within the peptide. Electrophoresis of purified nisin after storage for two weeks gave the same result but nisin appeared in dimmer form means that it has molecular weight of 7 kDa. Nisin on SDS-PAGE with coomassie blue stain give one band, after storage for two weeks or more, produced several bands, some of which were diffused, nisin as monomer very closely to 3.5 or 7 kDa as other references on the other hand Hansen et al. (1990) and Yildirim and Johnson (1997) nisin have 2.5 kDa. Figure 6 showed that the chloroform extraction gave a purified nisin from *L. lactis* (Fc2) had ~ 3.4 kDa.

Conclusion

The purified Nisin by cheap method (chloroform extraction) from Egyptian strain was identified by 16S rRNA (producer nisin) with high activity compared with other data published previously 14000 u/ml, so we can consider this strain as variant to enhanced antimicrobial activity depending on the target microorganism. This enhancement is apparent against Gram positive and Gram negative targets is particularly novel. Further efforts will focus on determining the mechanistic basis for these enhancements. Also, nisin was studied in 3 Dimension structure of it by CPHmodels as PDB with Chimera program 1.7rc, *nis* gene which encoded nisin Z in; Egyptian strain was isolated, sequenced and submitted in GenBank with accession number AB727286, indicated by the substitution of asparagine residue instead of histidine at position 27. In this way, it was established that the nis Z gene for nisin Z production is widely distributed.

nisZ gene expressed Nisin Z product

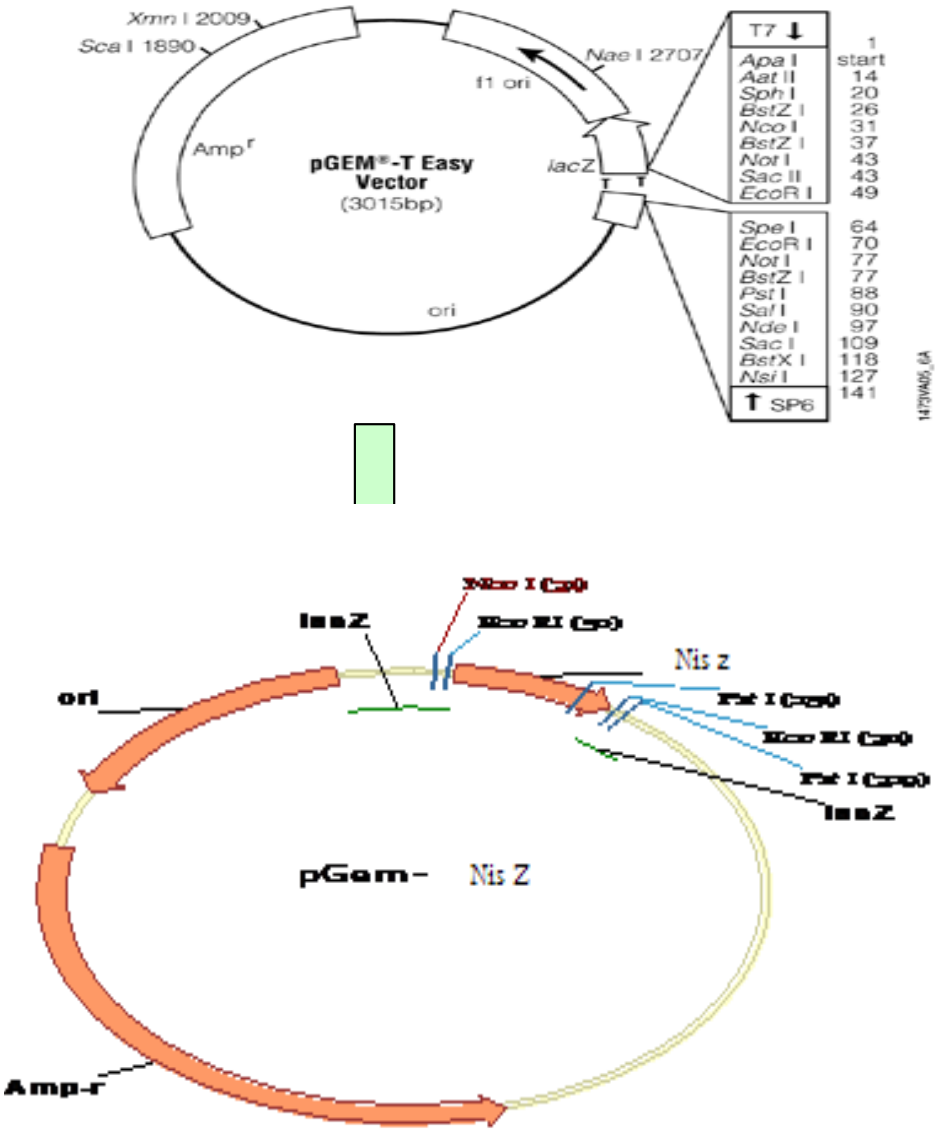

Figure 5. Diagram indicating the ligation process for nisZ gene fragment into PGEM –T-easy vector (construct). Nisin Z purification.

Table 2. Nisin Z purification at different stages of purification.

Purification steps	Nisin activity (Au/ml)	Specific activity (Au/mg)	Purification Fold (%)	Purified nisin (g/l)
Culture supernatant	10000	20	1.00	--
Chloroform extraction	14000	28	1.4	0.5

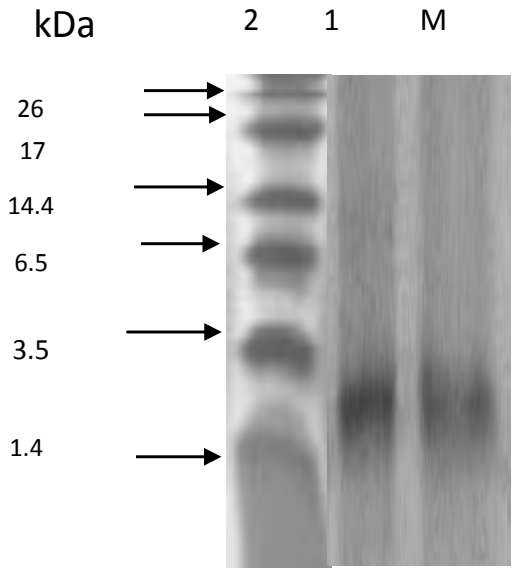

Figure 6. SDSPAGE purified nisin from *L. Lactis* sp lactis. Lane M, Polypetide SDSPAGE Standards (1.4 to 26.6 kDa); lane 1 Standand nisin; lane 2, purified nisin Z by chloroform extraction.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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