Isolation and screening of antibacterial and enzyme producing marine actinobacteria to approach probiotics against some pathogenic vibrios in shrimp *Litopenaeus vannamei*

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Abstract

The application of new probiotics is a good strategy in the biological control of infectious diseases in aquaculture. Approximately 100 marine actinobacteria isolates were obtained from 10 sediment samples of shrimp farms. Heat treatment of sediment samples resulted in a selective reduction of the non actinobacterial heterotrophic microflora. Starch nitrate agar medium exhibited more efficacy than glycerol arginine agar medium for isolation. Twenty seven percent of actinobacterial isolates showed antagonistic activities against pathogenic *Vibrio* spp. All the antagonistic isolates showed the typical morphology of genus *Streptomyces*. Exoenzymatic activity screening showed that 44 %, 26%, 37% of antagonistic isolates represented amylase, lipase and protease activities, respectively. MNM-1400 strain exhibited highest antagonistic and exoenzymatic activity. The pathogenicity experiment revealed that MNM-1400 strain did not cause disease in *Litopenaeus vannamei* larvae. Extraction of produced antibacterial compounds by MNM-1400 strain showed that the active constituent didn't have non polar property. Morphological, physiological and biochemical identification confirmed that MNM-1400 strain belonged to the genus *streptomyces.* Phylogenetic analysis by 16S rRNA gene sequencing showed a high similarity between MNM-1400 strain and *Streptomyces californicus* (similarity: 99%). These results suggest that the MNM-1400 strain can be considered as a potential probiotic in aquaculture.

Keywords: Probiotic, Marine actinobacteria, Biological control, Shrimp, *Litopenaeus vannamei*

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Introduction

Aquaculture is a strategic and rapidly developing industry. Worldwide, it has been growing at an average compounded annual rate of 9.2% since 1970. In the Islamic Republic of Iran shrimp production increased rapidly from 523 tonnes in 1997 to 5128 tonnes in 2009; production levels increasing by approximately 17.29 percent (Iranian Fisheries Organization, 2010). However, the major threat to this industry is disease (Das *et al*., 2009). For instance, shrimp production in the Philippines dropped by 55% in 2 years between 1995 and 1997 due to disease problems (FAO, 2007). Vibriosis is a serious aquatic disease engaging shrimp aquaculture and is responsible for high mortalities and severe economic losses. It is mainly caused by the species *Vibrio alginolyticus, V. anguillarum, V. harveyi*, and *V. parahaemolyticus* (Rattanama *et al.* 2009). Indiscriminating use of antibiotics to control diseases has led to the emergence and spread of antibiotic resistant bacteria resulting in destruction of numerous aquaculture business sectors in many Asian countries (FAO, 2007). Alternatively, probiotics can play a more important role than antibiotics in aquaculture production maintenance by comparably controlling bacterial populations at the optimum level (Maeda *et al.,* 1997). The beneficial effects of microbial probiotics in aquaculture are now extensively approved. (Irianto and Austin, 2002; Vine *et al*., 2006; Wang and Xu, 2006; Wang, 2008). Most bacterial probiotics used for shrimp farming belong to genus *lactobacillus* (Venkat *et al*., 2004) *pseudomonas* (Vijayan *et al*., 2006) and *Bacillus* (Meunpol *et al*., 2003) although other genera have also been reported. Actinobacteria are gram positive and saprophytic bacteria. Marine actinobacteria are extensively found in aquatic habitats and can be potentially used as aquaculture probiotics (Goodfellow and Fiedler, 2010). Structural diversity of secondary metabolites and exceptional biological functions such as antagonistic activity and competitive exclusions against marine pathogenic bacteria have raised great attention to these microorganisms (Liu *et al*., 2010; Lane and Moore, 2011; Zotchev, 2011) The marine actinobacteria can improve water quality through degrading organic matters by producing hydrolytic enzymes (Fuller, 1989). Furthermore, these bacteria produce secretary exoenzymes that promote the digestion of nutrients in the host intestine and improve water quality (Wang *et al*., 2008). Moreover, they can form spores and remain viable to soil desiccation and rough conditions (Doroshenko *et al*., 2005). Selection of antagonistic strains is the initial phase of the integrated study to discover new probiotics (Verschuere *et al*., 2000). The aim of this study was to isolate, screen and identify indigenous marine actinobacteria and evaluate their potential for use as aquatic probiotics against *vibrio* spp.

Materials and methods

Environmental conditions, sampling, treatment and bacterial isolation

Environmental conditions of farms during sampling were recorded. Dissolved oxygen was measured by polarographic method (Hargreaves and Tucker, 2002). pH value was detected by electrometric method (ASTM Standards, 1981) and salinity was assessed using refractometers. Marine sediments were sampled from 10 shrimp farms located at Tiab Port, Iran (N 27°06′36″ and E 56°49′48″) in October 2011. Sediment samples were transferred in sterile 50 ml tubes and kept refrigerated. Isolation of actinobacteria was carried out upon retrieval of the samples. Heat treatment was performed by holding sediment samples in a water bath at 50 ˚C for 60 min to inhibit other bacterial growth. All samples were diluted with sterilized filtered seawater before inoculation (Takizawa, 1993). 0.1 mL of each dilution was inoculated by spreading with a sterile bent glass rod onto starch nitrate agar (Ghanem *et al*., 2000) and glycerol arginine agar (Mincer *et al*., 2002) prepared with filtered seawater and supplemented with cycloheximide (100 mg/L) and nalidixic acid (20 mg/L). After incubation for 1-3 weeks at 28 ˚C, the colonies were picked out and purified.

Screening for antibacterial activity against Vibrio spp

Preliminary screening for antibacterial activity was performed by the top agar layer method (Fguira *et al*., 2005) against 4 shrimp pathogenic *Vibrio spp* consisting of *V. alginolyticus* ATCC 17749, *V. anguillarum* ATCC 19264, *Vibrio harveyi* KF154983 and *Vibrio parahaemolyticus* KF154990. Hickey Tresner agar medium prepared with filtered seawater was employed for screening. Subsequent screening of potent isolates was carried out under submerged fermentation conditions by the cylinder plate method (Sambamurthy *et al*., 2006).

Extracellular enzymatic activity screening

Amylolytic activity of Antagonistic strains was assayed by modified Bennett agar medium supplemented with starch. Appearance of clear zone around the colony after addition of 1% iodine solution confirmed amylase production. Proteolytic activity was detected by skimmed milk medium. Clear halo around the colony indicated protease activity (Williams *et al*., 1983). Lipolytic activity was determined by Tween 80 agar medium**.** Precipitated fatty acids around the colony revealed lipolytic activity (Harrigan and McCance, 1976)**.**

*Pathogenicity experiment of MNM-*1400 *strain to L. vannamei larvae*

The larvae of *L. vannamei* were maintained for 7 days in 5L plastic tanks containing 2 L seawater. Each tank was stocked with 25 shrimps at PL40 stage**.** Aeration was provided to each tank by Millipore (0.22 μ) filters to prevent contamination. Spore suspension was inoculated into the tank

at the final concentration 10^8 CFU/mL**.** Experiments were conducted in triplicate with control. Larval mortality was surveyed for up to 7 days**.**

Extraction of antibacterial compounds

Fermentation broth of MNM-1400 was filtered through Whatman No.1 filter paper and then through Millipore filters (Millipore Millex- HV Hydrophilic PVDF 0.45 μM). The filtrated broth was aliquoted and each part was extracted three times using chloroform and n-hexan in a ratio of 1:1 (v/v) to ensure the complete extraction. The organic fraction was concentrated by rotary evaporation, and the resulting extract was assayed for its activity against the *Vibrio* species by disk diffusion method (Woods *et al*., 2003).

Identification of antagonistic actinobacteria

Antagonistic strains were preliminarily identified according to traditional morphological criteria, including characteristics of colonies on the plate, morphology of substrate and aerial hyphae, morphology of spores, pigment production and other discriminatory factors (Shirling and Gottlieb, 1966).

Physiological, biochemical and DAP analyses of MNM-1400 strain

Utilization of carbon sources was examined by growth on ISP 9 as the basal medium containing 1% final concentration of carbohydrate sources (Shirling and Gottlieb, 1966). Nitrogen sources utilization and enzyme activity were determined in modified Bennett agar according to available literature (Williams *et al*., 1983). For the determination of diaminopimelic acid (DAP), MNM-1400 strain was inoculated in trypticase soy broth on shaking incubator (200 rpm.) at 28˚C for 7 days. Mycelium was obtained by centrifugation and washed three times with distilled water. Analysis of DAP was done following Staneck and Roberts (1974).

Scanning electron microscopy of MNM-1400 strain

MNM-1400 strain was cultured on ISP 2 agar at 28˚C for 14 days by using the coverslip method (Kawato and Shinobu, 1959). Spore chains and spore surfaces were inspected by scanning electron microscopy (CEM902A; Zeiss).

DNA extraction, PCR amplification and determination of 16S rRNA gene sequences

DNA was extracted and purified utilizing the method of Ezaki (Ezaki *et al*., 1990). 16S rRNA gene sequencing templates were amplified from genomic DNA by PCR using previously described actinomycetes specific primers S-C-Act-235-S-20 and S-C-Act-878-A-19 (Stach *et al*., 2003). 16SrRNA gene PCR products were checked by electrophoresis (70 V, 35 min) and then purified using PCR purification kit (Roche, IN) following the manufacturer's instructions. Purified products were sequenced using a Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems) as

described by Chun and Goodfellow (1995).

Phylogenetic analyses

The 16S rRNA gene sequence was used to search the Megablast software to reveal the closest matches to the 16S rRNA gene sequences for known species. Multiple sequence alignment of 16s rRNA gene was analyzed by CLC main Workbench 6.5. The sequence was aligned with representative actinobacteria 16S rRNA gene sequences and a phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis (MEGA) software version 5.0.

Nucleotide sequence accession number

DNA sequence was deposited to Gene bank under accession number KC012914.

Results

Environmental conditions including temperature, dissolved oxygen (DO), salinity and pH of selected farms during sampling are shown in Table 1. Approximately a total number of 100 actinobacteria isolates were collected. Starch nitrate agar was the one that most frequently gave high percent of isolates (72%) followed by glycerol arginine agar medium (28%) (Fig. 1). All 100 marine actinobacteria were preliminary screened for antibacterial activity against four species of vibrios. In our study about 27% of actinobacteria isolates showed antagonistic activities against *Vibrio* spp. But only 12% of the isolates could inhibit all of 4 *Vibrio* species (Table 2). Preliminary screening revealed that amongst antagonistic isolates three actinobacteria strains consist of MNM-1400, MNM-1402, and MNM-1404 exhibited highest activity against all *Vibrio* species. Extracellular activity screening showed that 50 %, 20%, 70% of antagonistic strains represented amylase, lipase and protease activities, respectively. Antagonistic activity of potent isolates that inhibited all *vibrio* species and their exoenzymatic activity are described in Table 2. Subsequent antibacterial screening of antagonistic isolates showed that among three isolates MNM-1400 exhibited maximum inhibition zone against all *Vibrio* species. Furthermore this isolate showed broad spectrum enzymatic activities. Therefore, MNM-1400 was selected for further studies. The pathogenicity experiment revealed that MNM-1400 strain did not led to any significant mortality after 7 days of challenge with *L. vannamei* larvae. For quantification and validation of antibacterial activity, chloroform and n-hexan extracts of MNM-1400 strain were assayed by disk diffusion method and the inhibition zones were measured against all *Vibrio* species (Table 5). The chloroform extract showed highest activity against the *V. alginolyticus* followed by *V. harveyi*, *V. anguillarum* and *V. parahaemolyticus* while n-hexan extract didn't show any antibacterial activity.

Table 1: Physico-chemical Parameters of farms during sampling*.

Farm No	Temprature(˚C)	DO[a](mg/L)	Salinity(ppt)	pH
1	33.2	7.2	43.1	8.3
2	32.6	5.7	43.4	8.2
3	32.5	6.3	44.6	8.2
4	32.6	7.4	45.2	8.4
5	31.8	6.2	45.6	8.3
6	33.1	5.5	46.3	8.4
7	31.5	6.7	46.7	8.5
8	32.7	6.3	46.9	8.6
9	32.4	6.7	47.1	8.7
10	32.4	7.3	47.4	8.6

* All parameter were measured in the afternoon
a: Dissolved oxygen

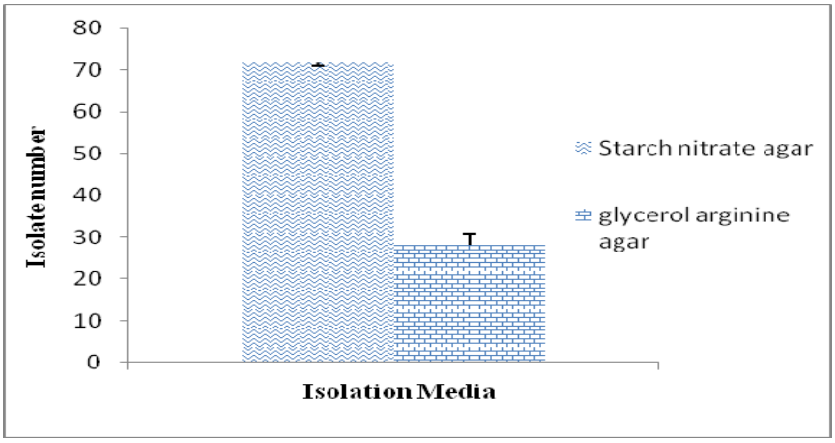

Figure 1: Comparison of isolation of actinobacteria in two isolation media.

Table 2: Antagonistic and enzymatic activity of selected potent isolates.

Strain	Antagonistic activity				enzymatic activity		
	V .alginolyticus	*V. anguillarum*	*V. harveyi*	*V. parahaemolyticus*	Amylolytic	Lipolytic	Proteolytic
MNM-1400	++++	+++	++++	++++	+++	+	++++
MNM-1402	++++	++++	++++	+++	++	+	+
MNM-1413	+++	++	+++	++	-	-	+++
MNM-1424	++++	+++	+++	++++	++	++	+
MNM-1429	+	++	+	++	-	-	-
MNM-1432	+++	+	+++	++	++	+	-
MNM-1445	+++	++	+++	+++	+	+	+++
MNM-1457	+++	++	+++	++	++	-	-
MNM-1472	+++	++	++	++	+++	-	+
MNM-1475	++	++	++	++	+	-	+
MNM-1478	+++	+++	+++	++	-	+	-
MNM-1479	+	++	+	+	-	-	+

Inhibition zone: + <10 mm; ++:10–20 mm; +++: 21–30 mm; ++++ >30 m
Enzymatic activity: ++++: excellent +++: Very good ++: good +: low -: non.

reliminary identification of all antagonistic strains based on traditional morphological criteria revealed that the majority of isolates belonged to *Streptomyces* genus. Cultural characteristics of the strain MNM-1400 are shown in Table 3. The strain exhibited good growth on ISP-2, ISP-3, ISP-4, ISP-5, ISP-6, ISP-7, Czapek-dox agar and Emerson's Agar, while the growth was moderate in nutrient agar medium. MNM-1400 strain showed yellow vegetative and aerial mycelia on all cultural media tested in this experiment and its soluble pigment was found only on ISP-2 medium but it could not produce melanoid pigments. Utilization of carbon resources and production of enzymes are shown in Table 4. D- Glucose, D-fructose, D-xylose, L-arabinose, L-rhamnose, sucrose, raffinose, maltose and mannose were utilized well by the strain MNM-1400 but it did not utilize D-lactose. It could produce enzymes such as protease, amylase, lipase, catalase, nitrate reductase, but could not produce cellulose and hydrogen sulfide. By comparison with the standards using thin layer chromatography, the whole cell wall hydrolysates of strain MNM-1400 showed that it is rich in L, L-diaminopimelic acid (L,L- DAP) which indicates that the cell wall is type I. Scanning electron micrograph of MNM-1400 strain represented typical spore arrangements as expected for most members of the genus *Streptomyces*. Long, straight spore-chains were borne on the aerial hyphae. Spores (0.5 ×1.4 μm) were rod shape and smooth-surfaced (Fig. 3). Morphological, physiological, and biochemical characteristics of the strain MNM-1400 classified it to be *Streptomyces* genus. Consequently 16SrRNA gene of strain MNM-1400 was sequenced about 602 bp (Fig. 4). Comparison of 16S rRNA gene sequence similarity between *Streptomyces sp MNM-1400* and closest strains in NCBI showed a high similarity (99%) between MNM-1400 strain and *S. californicus* (Table 6). Sequence alignment of 16s rRNA gene of *Streptomyces* sp MNM-1400 and two closest strains showed these similarities (Fig. 4). A phylogenetic tree was constructed using the neighbor-joining method with other *Streptomyces* species as representative strains (Fig. 5).

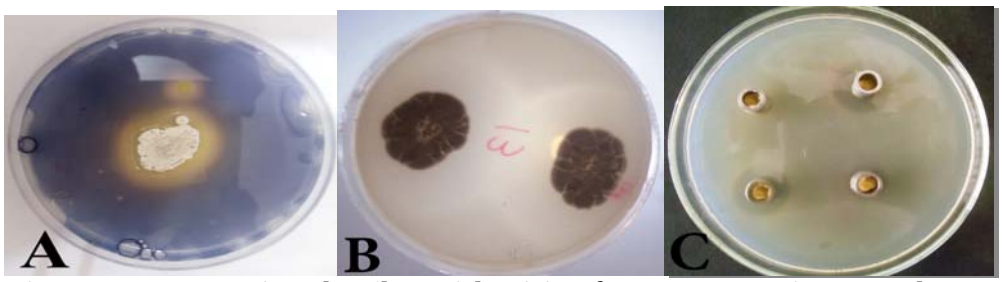

Figure 2: Exoenzymatic and antibacterial activity of MNM-1400 strain: A: Amylase activity b: protease activity c: antibacterial activity.

Table 3: Cultural characteristics of strain MNM-1400.

Medium	Growth	Vegetative mycelia	Aerial mycelia	Spore	Soluble pigment
ISP2	Good	yellow	yellow	Abundant/yellow	+
ISP3	Good	yellow	yellow	Abundant/yellow	-
ISP4	Good	yellow	yellow	Abundant/yellow	-
ISP5	Good	yellow	yellow	Abundant/yellow	-
ISP6	Good	yellow	yellow	Abundant/yellow	-
ISP7	Good	yellow	yellow	Abundant/yellow	-
Czapek-dox agar	Good	yellow	yellow	Abundant/yellow	-
Nutrient Agar	Moderate	yellow	yellow	Abundant/yellow	-
Emerson's Agar	Good	yellow	yellow	Abundant/yellow	-

Table 4:Physiological and biochemical characteristics of strain MNM-1400.

Utilization of	Response	Production of	Response
glucose	+	Protease	+
Fructose	+	Nitrate reductase	+
Xylose	+	Amylase	+
Arabinose	+	Catalase	+
Lactose	-	Cellulase	-
Rhamnose	+	Lipase	+
Sucrose	+	Melanoid pigments	-
Raffinose	+	H2S	-
Mannose	+		
Maltose	+		

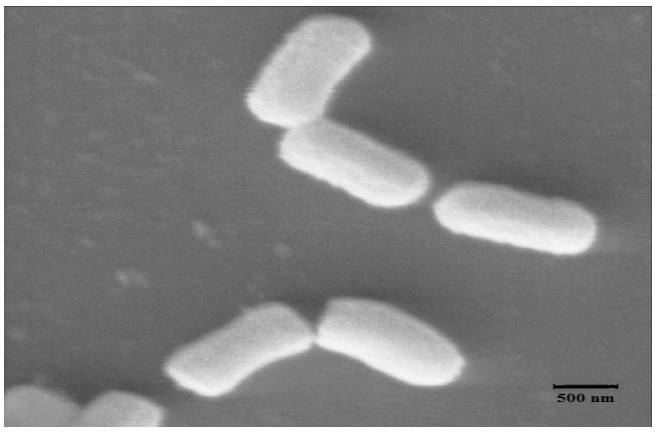

Figure 3:Scanning electron micrograph of strain MNM-1400 after growth on ISP2 medium for 14 days at 28 ˚C. Bar, 500 nm.

Table 5: Antibacterial activities of organic extract of MNM-1400 against selected *Vibrio* species.

Strain	Inhibition zone diameter (mm)			
	V. alginolyticus	***V. anguillarum***	***V. harveyi***	***V. parahaemolyticus***
Chloroform extract	28	20	25	25
n-Hexan extract	0	0	0	0

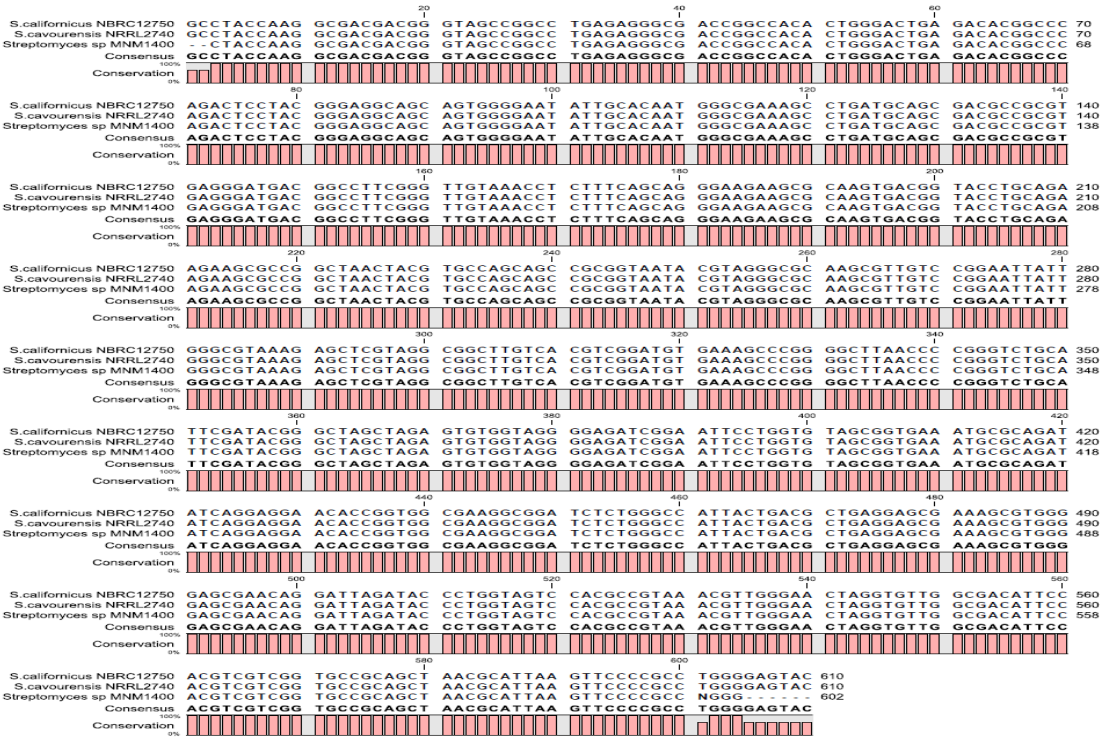

Figure: 4 Sequence alignment of 16s rRNA gene of *Streptomyces sp MNM-1400* and two closest strains

Table 6: Comparision 16S rRNA gene sequence similarity between *Streptomyces sp MNM-1400* and closest strains in NCBI.

Description	Max score	Total score	Query coverage	E value	Ident	Accession
Streptomyces californicus strain NBRC 12750 16S ribosomal RNA gene, partial sequence	1109	1109	100%	0.0	99%	NR_112257.1
Streptomyces cavourensis strain NRRL 2740 16S ribosomal RNA gene, partial sequence	1109	1109	100%	0.0	99%	NR_043851.1
Streptomyces griseobrunneus strain NBRC 12775 16S ribosomal RNA gene, partial sequence	1109	1109	100%	0.0	99%	NR_112577.1
Streptomyces californicus strain NBRC 3386 16S ribosomal RNA gene, partial sequence	1109	1109	100%	0.0	99%	NR_112486.1
Streptomyces floridae strain NBRC 15405 16S ribosomal RNA gene, partial sequence	1109	1109	100%	0.0	99%	NR_112456.1
Streptomyces bacillaris strain NBRC 13487 16S ribosomal RNA gene, partial sequence	1109	1109	100%	0.0	99%	NR_041146.1

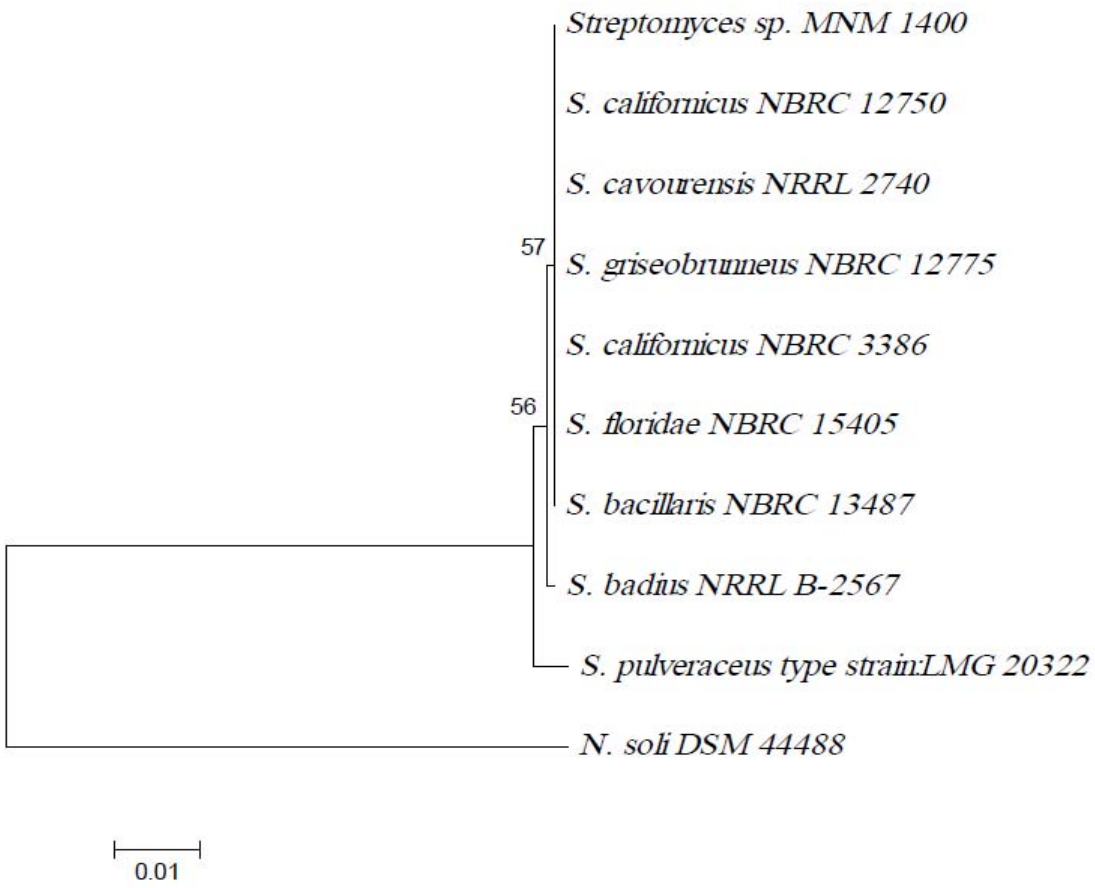

Figure 5: Phylogenetic dendrogram based on 16S rRNA gene sequence analysis, reconstructed from evolutionary distances by using the neighbour-joining method, showing the phylogenetic position of strain MNM-1400 and the most closely related species. Bootstrap values are indicated at the relevant branching points. Only values >50% are shown Bar, 0.01 substitutions per nucleotide position.

Discussion

Tiab region as a major center of shrimp culture in Iran was investigated as a source for probiotics. Analysis of physico-chemical parameters of selected farms revealed that water quality of all shrimp farms was within acceptable ranges for growth of *L. vannamei* although salinity rate was higher than optimal level due to high evaporation rate in this region (FAO, 1986). Selection of sampling sites is an important phase in probiotic discovery. There are very suggestions in this context that emphasize on isolation of probiotics from native environments (Maeda *et al*., 1997; Defoirdt *et al*., 2007). Resident candidate probionts can easily grow and be accepted by the host. Therefore they can exhibit controlling roles in the culture ponds (Wang *et al*., 2008). Isolation of actinobacteria using two different isolation media caused more diversity that could be detected with different morphological characters and

pigmentation. Preference of starch nitrate medium to glycerol arginine agar is likely due to presence of nitrate and starch in its composition. Nitrate is used as a common nitrogen source by *Streptomyces* (Ghanem *et al*., 2000) and starch is a common carbon source that is applied as part of feed in shrimp farming and abundant in aquaculture ponds (Guillaume *et al.*, 2001) Many studies on the antibacterial activity of marine actinobacteria have been carried out, but the results are different. Zheng reported that 43.6% of marine derived actinobacteria showed antibacterial activities, but only 12.8% of the actinobacteria produced antibacterial metabolites against *Vibrio* spp (Zheng *et al*., 2000). In the other study You *et al*. (2005) found that about 51.1% of actinobacteria isolated from marine aquaculture showed antagonistic activities against *Vibrio* spp. In the current study about 27% of actinobacteria isolates showed antagonistic activities against *Vibrio* spp. It seems that these differences are mainly due to various isolation and screening techniques and different diversity, distribution and frequency of actinobacteria in marine ecosystems. Exoenzymatic activities of potent isolates give them the ability to decompose residual feed and feces and improve water quality of ponds (Zhou *et al.*, 2009). Moreover colonization of these potent isolates in the digestive tract of shrimp could promote feed utilization and digestion. It is worth pointing out that due to the employment of seawater in the composition of all screening media, antagonistic strains were adapted to marine conditions and physiologically active in the marine environment (Jensen *et al*., 1991). Results of pathogenicity experiment are in agreement with this statement that *Streptomyces* doesn't infect target animals in aquaculture due to deficiency of virulence factors and pathogenicity islands on *Streptomyces* genome (Das *et al*., 2009). Bioassay guided extraction of antibacterial ingredients by two solvents (chloroform and n-hexan) with different polarity indicated that n-hexan fraction didn't show any antibacterial activity thus active constituents didn't have non polar properties (Rostagno *et al*., 2013). Preliminary identification experiments revealed that *streptomyces* genus was prevalent among antagonistic strains. Frequency and dominance of *Streptomyces* among actinobacteria in various near shore marine sediments were reported by several studies (Bredholt *et al*., 2008; Ramesh *et al.*, 2009; Suthindhiran *et al*., 2010). 16S rRNA gene studies provide a reliable method for more accurate identification of microorganisms. The results of 16s rRNA gene analysis suggest that MNM-1400 strain **is** closely related to but not identical with other *streptomyces* spp submitted in NCBI. Phylogenetic analysis confirmed that MNM-1400 strain located in the same cluster with the closest strains that were submitted to NCBI and they have a common ancestor. It should be noted that MNM-1400 strain could produce spores abundantly that were resistant to severe

conditions such as desiccation. This trait extends shelf life and gives advantage to commercialization in preservation and storage of products. To conclude, these findings further support the idea of employing actinobacteria in marine aquaculture as potential probiotics. However more extensive experimental studies should be done to investigate mode of action of MNM-1400 strain *in vivo* conditions, evaluation of its effects on other animals in aquaculture and determination of water quality improvements. Furthermore commercial cost–benefit analysis must be done. Further studies on MNM-1400 strain are in progress in the experimental phase.

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