

Corrosive Lesions at Concrete Infrastructures as Promising Source for Isolating Bioactive Actinobacteria

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Abstract: The aim of this paper is isolating rare actinobacteria from new ecological source as corrosive lesion at concrete infrastructure and screening their ability to produce biological products. Ten pure actinobacteria isolates were isolated from corrosive lesions at concrete infrastructures of irrigation channel using cement extract media at different pHs (9- 12) and incubated at 30°C for 7d. All of the isolates produced variable levels of cellulase and lipase, and nine of them displayed variable levels of alkaline protease and amylase products. Only Four isolates produced extracellular alkaline phosphatase in liquid media. The antagonistic activities of these isolates were screened against four pathogenic microorganisms including Gram positive and negative bacterial species and two species of fungi. Only the isolate ROR40 exhibited antagonism activity against *Staphylococcus aureus* and *Escherichia coli*, whereas nine isolates showed different degrees of antagonism activities against *Microsporium canis* and *Trichophyton mentagrophyte*. The potential isolate (ROR40) to produce extracellular alkaline phosphatase was selected and identified depending on phenotypical, physiological and molecular according to partial sequences of 16S r RNA gene. It had 98% similarity with *Pseudonocardia alni* 20049 and *P. alni* 44104. Finally the isolate is named *Pseudonocardia sp.* ROR40 (Genbank accession no KJ 725072). The isolate produced extracellular alkaline phosphatase in liquid medium at optimum conditions were pH 8.5 of production medium, 37 °C for 4d. in stand incubator. Conclusion: Our study would be the first instance in comprehensive characterization of concert deteriorating actinobacteria for producing commercially valuable primary and secondary metabolites and it may facilitate us to isolate and characterize more bioactive species.

Keywords: Concrete Corrosive Lesions, Actinobacteria, Secondary Metabolites, Pseudonocardia, Alkaline Phosphatase

1. Introduction

Actinobacteria are Gram positive bacteria exhibiting wide variety of morphologies including coccoid, coccobacilli, filamentous and highly differentiated branched mycelium (Goodfellow, 2012). Actinobacteria are exploited in many trends of economical and biotechnological applications, therefore they are became highly valuable and hold a prominent position as targets in screening programs due to their diversity and their proven ability to produce extracellular enzymes and the important secondary metabolites like antibiotics and other therapeutic compounds of pharmaceutical importance (Berdy 2005). These organisms are existent in various ecological habitats such as soil, fresh water, lakes, compost and sewage environments and they also are dispersed in extreme habitats like marine sediments and high alkalinity and salinity environments, and

therefore they have developed a complex stress management for their survival (Sigrid *et al.*, 2008). An extreme environment is the major reservoir of biological diversity and their microorganisms are recognized to be rich sources of novel compounds.

One of the bioactive compounds is enzymes as lipases, proteases, acid and alkaline phosphatases and so on. Actinobacteria and thermophilic bacteria produce alkaline phosphatases that show higher velocity and thermo-stability. The thermo- stabile alkaline phosphatase is preferred in many applications such as molecular biology, genetic engineering, immunology and industrial applications (Baranov *et al.*, 2008)., Whereas a thermo- labile alkaline phosphatase having higher specific activity, it can be used further in electrochemical biosensors to detect of hazardous chemicals in water bodies at low temperature (Dhaked *et al.*, 2005).

The finding from previous study on microbial communities fouling concrete infrastructure that have been

performed in our Lab., numerous alkaline microbial species were isolated including the unique archaea, bacteria and fungi which having ability to produce various primary and secondary metabolites (Khazal, 2013). Therefore, the corrosive lesions or stains at concrete infrastructures can be used as new sources for isolating numerous divers genetic lineage of archaea, alkalotolerant bacteria and fungi. Because of the unique characteristics of these organisms that acquired during adaptation to harsh conditions of environment and they are carrying novel genes and biochemical pathways, they became powerful sources for metabolites (enzymes, antibiotics, organic acids etc.) production. The aim of the present study is isolating rare actinobacteria from corrosive lesions at concrete infrastructure and screening on their ability to produce several secondary metabolites as extracellular enzymes, alkaline phosphatase particularly, as well as antibacterial and antifungal substances.

2. Materials and methods

2.1. Bacterial Isolation from Deteriorating Concrete Infrastructures

The samples were collected from corrosive lesions at concrete infrastructures of irrigation channel passing through date palm grove in Babylon province / Iraq. The collection was occur by chipping or scraping off the concrete surface to a 5-10mm depth and transferred into plastic bags at ice container. In Biotechnology Lab., the samples were grinded, one gram of powdered from each one was weighted and suspended in 100 ml of physiological solution, mixed well and 1ml of the supernatant was cultured on modified alkaline medium, cement extract- nutrient agar, and incubated at 30°C for 7d. After that the single colonies of actinobacteria were selected and re-cultured on the same medium. The pure bacterial isolates were maintained in nutrient agar slants and maintained at 4°C.

Cement extract –nutrient agar was prepared by dissolving half amount of nutrient agar medium as instructed manufacture company supplemented with 1% agar-agar, sterilized by autoclave at 121 °C (15 lbs pressure) for 20 min. When the medium was cooled to 80°C, mixed with sterile cement extract at a ratio 1:1 (vol.: vol.). After that antifungal, 100 µg.ml⁻¹ as a final concentration of nystatin was added to the medium to prevent fungal growth. Cement extract was prepared by suspending 250 gm of regular Portland cement in one liter of tape water and continuously stirred for two hours at 30°C; the supernatant was filtrated through Whatman No.1 filter after cement particles setting. Subsequently, the extract was sterilized by filtering through 0.22µm pore size membrane filters and stored at room temperature. pH of the extract is maintained between 10-12.

2.2. Primary Screening for some Extracellular Enzymes and Antibiotics

After primary identification of these isolate depending on morphological characteristics including microbial and

cultural features as well as oxidase and catalase tests, they were subjected to detect their ability to produce extracellular enzymes and antibiotics using selective media.

Degradation of cellulose: 1% of soluble Carboxymethyl Cellulose (CMC) was added to the ISP2 media. The plates were inoculated and incubated at 30°C for 7-15 d. Control plate was used as standard to check the growth of actinobacteria after 7- 15 d. for cellulose degradation activity which may be visually observed (Leon *et al.*, 2007; Meena *et al.*, 2013).

Lipolytic activity: The formation of lipase is demonstrated by adding water-soluble Tweens to a nutrient medium. Around the colonies with lipolytic activity there appears a well visible halo which is due to crystal of the calcium salt of the fatty acid liberated by lipolysis. The results are directly visible. This test was done by taking 1% Tween 40, 60 & 80 (Hi media) separately with ISP2 media, inoculated and incubated at the temperature of 30°C for 7 to 10 d.

Proteolytic activity: This test was studied with skimmed milk (Hi media) medium, ISP2 medium supplemented with 2% skimmed milk. The plates were inoculated and incubated at 30°C. The hydrolysis halo appeared around actinobacteria colonies were recorded after the 7th and 10th days of incubation (Leon *et al.*, 2007).

Starch hydrolysis: For this test, cultures should be grown at 30°C for 5-7 d. on starch medium at pH7.5 consists of 0.3% beef extract, 2% agar-agar and 1% starch. The development of clear zone around the culture streaks, when the plates were flooded with Lugol's iodine solution should be recorded as the hydrolysis of starch. The medium is composed of ISP2 Media supplemented with 1% starch, which serves as the polysaccharide substrate (Leon *et al.*, 2007; Meena *et al.*, 2013).

2.3. Secondary Screening for Extracellular Alkaline Phosphatase Production

Extracellular alkaline phosphatase production was screened using liquid production medium according to Pandey and Banik method (2010). The flasks containing 10ml of liquid media were inoculated with 5% of culture suspension (OD 0.5 at 600nm) for each isolate separately, and incubated at 30°C for 5-7 d. After that enzyme activity and protein concentration were estimated.

2.4. Screening of Concrete Actinobacteria for Antibacterial Potential

Preliminary screening for antibacterial activity was done by the cross-streak method (Madigan *et al.*, 2012), on potato dextrose agar (PDA) medium using Gram positive and negative bacteria pathogenic as test bacteria including *Staphylococcus aureus* and *Escherichia coli*. Whereas primary screening of antifungal activity was performed according Crawford *et al.* (1993) method using PDA medium and *Microsporium canis* and *Trichophyton mentagrophyte* as test pathogenic fungi. All pathogenic isolates were obtained from Biotechnology lab. at Babylon University.

2.5. Phenotypic and Molecular Identification of Potential Strain

Strain was identified on the basis of their phenotypic characterizations and physiological and biochemical characteristics and molecular identification using the partial sequence of 16S rRNA of the selected isolate.

2.6. Phenotypic Characterizations

The classification of actinobacteria was originally based largely upon the morphological observations. These observations are best made by the variety of standard cultivation media. Several of the media suggested for the International Streptomyces Project (Shirling and Gottlieb, 1966). The Tryptone yeast extract agar (ISP 1), yeast extract-malt extract agar (ISP-2), oatmeal agar (ISP-3), inorganic salt-starch agar (ISP-4), glycerol-asparagine agar (ISP-5), peptone-yeast extract-iron agar (ISP-6), tyrosine agar (ISP-7) were inoculated with the isolate and incubated at 30° for 10 d. Aerial mass color, Reverse side pigment, Melanoid pigments, Spore chain morphology were examined. For the grouping and identification of actinobacteria the Chromogenicity of the aerial mycelium is considered to be an important character. Spore chain morphology; spore bearing hyphae and spore chains are determined by the direct microscopic examination of the culture area. Adequate magnification used to establish the presence or absence of spore chains and to observe the nature of spore chains is 40X. By the standard protocol of cover slip culture technique and direct culture plate, the plates were prepared and after the incubation of 7 to 10 d. it is observed.

2.7. Physiological and Biochemical Characterization

Assimilation of carbon sources: The ability of the actinobacteria isolate in utilizing various carbon compounds as sole source of energy was studied by following the method recommended in International Streptomyces Project. Stock solution of 10 sugars like D-glucose, L-arabinose, sucrose, D-fructose, D – xylose, raffinose, D-mannitol, cellulose, rhamnose, inositol having concentration of 10x was prepared in autoclaved water and sterilized by filtering through 0.22µm pore size membrane filters and stored at 4°C. Growth of actinobacteria isolate was checked by addition 1% carbon source into ISP2 media separately (Biehle *et al.*, 1996). Plates were streaked by inoculation loop by flame sterilization technique and incubated at 30°C for 7 to 10 d. Growths were observed by comparing them with positive and negative control.

Sodium chloride tolerance: Different concentrations of sodium chloride (0, 3, 5, 7, 10, 15, 20 and 25 %) solution were added to the starch casein agar medium to check the sodium chloride tolerance test. The isolate was streaked on the agar medium, incubated at 30°C for 7-15 d. and the presence or absence of growth was recorded on 7th day onwards (Biehle *et al.*, 1996).

Hydrogen-sulfide production: The inoculated Tryptone-Yeast extract agar slants (ISP1 at pH7.5 supplemented with

0.06% of L-cysteine, 0.03% of $C_6H_5FeO_7$ and 0.03% of $Na_2S_2O_3$) were incubated at 30°C for 7 d. for this test. Observations on the presence of the characteristic greenish-brown, brown, bluish-black or black color of the substrate, indicative of H_2S production were recorded on 7th, 10th and 15th days. The incubated tubes were compared with uninoculated controls.

Ability to grow in different pH: This test was carried out on ISP2 media. pH was adjusted to different ranges of 4- 12. Duplicate slants were prepared for strain of each range and inoculated. After the incubation of 10-12 d., the results were recorded (Biehle *et al.*, 1996).

Gelatin liquefaction test: The medium at pH 7.5 consists of nutrient (0.3% beef extract and 0.5% peptone) supplemented with 12% gelatin this high gelatin concentration results in a stiff medium and also serves as the substrate for the activity of gelatinase. Gelatin liquefaction is studied by sub-culturing the strain on gelatin agar medium and incubated it at 30°C. Observation should be made after 7 d. The extent of liquefaction should be recorded after keeping the tubes in cold conditions (5-10°C) for 2h. Cultures that remain liquefied were indicative of slow gelatin hydrolysis.

Finally, after all these results have been matched with the keys given in Bergey's Manual of Systematic Bacteriology (2012), and the isolate identification was done.

2.8. DNA Extraction, PCR Amplification and Sequencing

Genomic DNA was extracted using Gene Jet™ Genomic DNA purification Kit (Fermentas Scientific, Inc.) according manufacturer's instructions. PCR reactions were conducted using iCycler Thermal Cycler (Bio-Rad, USA Laboratories, Inc.) and PCR (recipe and cycle) conditions were optimized, based on the mentioned references (Reysenbach *et al.*, 1994; Rudi *et al.*, 1997) in order to get the best possible yield utilizing about 40ng of the extracted DNA.

The 16S rDNA genes were amplified from genomic DNA using bacterial primers E8F: 5'AGAGTTTGATCCTGGCTCAG 3' and Ab939R: 5'CTTGTGCGGGCCCCCGTCAATTC 3'. The oligonucleotide primers were synthesized by Sentromer DNA Technologies LTD., Istanbul, Turkey.

The PCR program used was as follows: Pre-denaturation at 95°C for 10 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 52.3 °C for 1 min, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min followed by holding at 4°C. The PCR product of actinobacteria was run on 0.8% agarose gel in 1XTAE electrophoresis buffer (0.04µM Tris-acetate and 1mM Na_2EDTA) with DNA ladder (1Kb gene ruler, Ferment as) as a molecular size reference. The gel was run at 90V for 50 min, then it was checked and photographed over UV transillumination using gel documentation system (Gel Doc., Bio-Rad Laboratories, Inc.). The bands of interest were cut by a sterile blade over a UV transilluminator with as minimum as possible of UV exposure time, then transferred to a sterile eppendorf tube. The PCR products were purified with QIAquick PCR Purification Kit (QIAGEN, USA) according

manufacturer's instructions. The quality (A260/280 ratio) of each sample was checked using the NonoDrop ND-1000 Spectrophotometer (NonoDrop Technologies, Inc., Wilmington, DE, USA) in order to fulfill the sequencing experiment requirements where the ratio of template DNA should be ≥ 1.8 which recommended by the manufacture. The purified DNA was kept at -20°C . The sequencing experiments were done at Izmir Institute of Technology Biotechnology Bioengineering Research and Application Center (BIYOMER), Izmir, Turkey. The purified PCR product of actinobacterial isolate was subjected to the sequencing reactions following the manufacture's recommendation using the BigDye® Terminator v3.1 sequencing reaction Kit (Applied Biosystems, Foster City, CA, USA). The reaction mixture was set up in 10 μl final reaction volume in thin-wall tube and all reagents were kept on ice during experiment. The primer E939R was used for partial 16S rRNA gene sequencing of actinobacteria. The reaction tubes were transferred to the BioRad C1000 Thermal Cycler (BioRad, USA Laboratories, Inc.). The sequencing reaction and cycle conditions were optimized in order to get long good quality sequence and as following: Pre-denaturation at 98°C for 5 min; 30 cycles of (denaturation at 96°C for 20 sec., annealing at 56°C for 10 sec., extension at 60°C for 4 min); final extension at 60°C for 5 min then hold at 4°C . The BigDye Terminator ready reaction mix was added after the pre-denaturation step. The annealing temperature was increased in order to proven non-specific noise in the late sequences (Sultana and Khan 2007). The sequencing product was purified using Sephadex G-50 (Sigma-Aldrich) through spin column. The tubes were spin at 5400 rpm for 2 min and DNA was collected. The collected DNA was transferred to a special multi-well plate and put into the ABI Prism 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) for sequence recording.

Sequencing results were analyzed for chimeras using DECIPHER version 1.4.0 program (Wright *et al.*, 2012) for 16S rRNA gene. (We introduce DECIPHER (<http://DECIPHER.cee.wisc.edu>), a publicly available web-based tool specific for detection of chimeric 16S rRNA sequences by the use of the novel search-based approach. For standalone implementations, the DECIPHER R package, source code, and associated documentation are available for download under the terms of the GNU General Public License.)

Sequences similarity was accomplished through sequence alignment to the existing relevant sequences available in database at National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) program : BLASTN 2.2.30+ (Zhang *et al.*, 2000) Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) online gene database to determine taxonomic classification through finding the most similar sequence in the database to the isolated species and its significant polygenetic relationship represented by similarity percentage.

Phylogenetic neighbor-joining tree was constructed in MEGA version 6 software (Tamura *et al.*, 2013) using

method of Saitou and Nei (Saitou and Nei, 1987).

2.9. Extracellular Alkaline Phosphatase Production from Potential Strain

Extracellular alkaline phosphatase production from selected isolate and optimization of environmental conditions were studied using the same production liquid medium. The production medium composed of (g.l^{-1}) glucose 0.2%; peptone 0.5%, $(\text{NH}_4)_2\text{SO}_4$ 3.0 g/L, CaCl_2 0.2 mmol, NaCl 0.08 mol, KCl 0.02 mol, NH_4Cl 0.02 mol, MgSO_4 1 mmol, ZnCl_2 0.004 mmol, Na_3PO_4 200 μmol , $\text{Ca}(\text{NO}_3)_2$ 50 mmol (Dhaked *et al.*, 2005). 10 ml of culture medium was taken in 100 ml Erlenmeyer flask with an initial pH maintained at 8, and Flasks were autoclaved at 121°C for 20 min. The sterilized media was cooled at room temperature. 5% of actinobacteria suspension culture (OD 0.5 at 600nm) was inoculated in each flask and the flasks were incubated at 30°C for 10d. Subsequently the culture broths were centrifuged at 5000 $\times g$ for 15 min at 4°C ; the biomass was separated while the supernatant that containing extracellular ALPase was stored at -18°C for further analysis.

2.10. Assay of Alkaline Phosphatase

ALPase activity was measured by spectrophotometrically by monitoring the release of *p*-nitrophenol from *p*-nitrophenyl phosphate (pNPP) at 405nm according to method (Dhaked *et al.*, 2005) with some modification. A typical reaction mixture contained 1.9 ml of 20 mM *p*-nitrophenyl phosphate (pNPP) diluted in 1 M diethanolamine buffer (pH 9.8) and 0.1ml of enzyme filtrate. The reaction was performed at 37°C for 5 min, and then was stopped by adding 50 μl of 4 M sodium hydroxide solution. The color intensity was measured spectrophotometrically at 405 nm against blank. Blank was prepared by replacing the enzyme with 0.1 ml distilled water. One unit of enzyme defined as the amount of enzyme required to liberate 1 μmol of *p*-nitro phenol per minute under assay conditions.

The protein concentration was estimated using Bradford method (1976) with bovine serum albumin as the standard protein.

2.11. Effect of some Environmental Conditions on Extracellular Alkaline Phosphatase Productions

The effect of initial pH on alkaline phosphatase production was observed by adjusting initial pH of fermentation medium in the range 6-12 by using available buffers and incubation at temperature 30°C for 4d.

The effect of temperature on production of alkaline phosphatase was checked by incubation fermentation media of actinobacteria in temperature ranging from 20 to 50°C with interval 5°C for 4d.

The optimal incubation period of alkaline phosphatase was determined by incubation of fermentation media of actinobacteria for different periods ranging 1-8d. under optimal conditions.

The effect of shaking rate on production of alkaline

phosphatase was checked by incubation of fermentation media of actinobacteria in shaker incubator at different shaking speed 40, 80, 120, 160, 200 rpm, also it was incubated in stand incubator under optimal conditions.

3. Results

In current study, ten pure actinobacteria isolates were isolated from three out of five samples collected from corrosive lesions at concrete infrastructures of irrigation channel using cement extract media at pHs ranged from 9 to 12 and incubated at 30°C for 7d. The cement extract medium proved it had a powerful influence to enhance bacterial isolation particularly the actinobacteria which implicated in destruction of concrete infrastructure due to the ability of these microorganisms to grow in low level of nutrition and alkaline environment.

All isolates were preliminary characterized depending on microbiological, cultural characteristics and their reactions with catalase and oxidase tests. Subsequently five extracellular enzymes production were screened and the

results revealed that all of the isolates appeared their ability to produce variable levels of cellulase and lipase on CMC-agar and Tweens-nutrient agar media respectively. Also extracellular protease and amylase were screened on skimmed milk agar and starch agar respectively, nine of ten isolates displayed variable levels of alkaline protease and amylase products but not the isolate ROR40. Four out of ten isolates have ability to produce extracellular alkaline phosphatase (Table 1). The antagonistic activities of these isolates were screened against four pathogenic microorganisms including Gram positive and negative bacterial species and two species of fungi. Only the isolate ROR40 exhibited antagonism activity against both *S. aureus* and *E. coli*, whereas most isolates showed different degrees of antagonism activities against *M. canis* and *T. mentagrophyte*, but not the isolate 12R (Table 1). Secondary screening of alkaline phosphatase in liquid media exhibit that the best isolate was ROR40 depending on ALP specific activity (10.66 U/mg proteins) and it was selected to produce alkaline phosphatase (Table 2).

Table (1). Primary screening on biological products from actinobacterial isolates.

No	Actinobacterium isolates	Enzymes ^a					Antagonism activity ^b	
		Cellulase	Lipase	Alkaline protease	Amylase	Alkaline phosphatase	Antibacterial	Antifungal
1	7R	++	+++	+	+	+	-	+
2	ROR40	+	++	-	-	+++	++	+++
3	11R	+++	++	+	+	+	-	++
4	12R	+	+	+	++	++	-	-
5	13R	++	+++	++	+	-	-	+
6	14R	+	+++	+	++	-	-	+
7	15R	+++	+	+	+	-	-	++
8	16R	+++	+	++	+	-	-	+
9	17R	+	++	++	++	-	-	+
10	20R	+	+	+	++	-	-	++

a. Ratio diameter of clear zone / colony diameter : + (0.5-1); ++ (1.1-2.5); +++ (2.6- 3.5); - (no product)

b. Antagonism activity against both *S. aureus* and *E. coli*,

antagonism activities against *M. canis* and *T. mentagrophyte* ; + (weak); ++ (reasonable) ; +++ (very strong); - (no inhibition)

Table (2). Secondary screening on extracellular alkaline phosphatase from actinobacterial isolates.

No.	Actinobacterium isolates	ALP activity (U/ml)	Protein Conc. (mg/ml)	ALP specific activity (U/mg proteins)
1	7R	1.38	1.9	0.73
2	ROR40	14.5	1.36	10.66
3	11R	2.19	1.6	1.37
4	12R	7.8	1.5	5.2

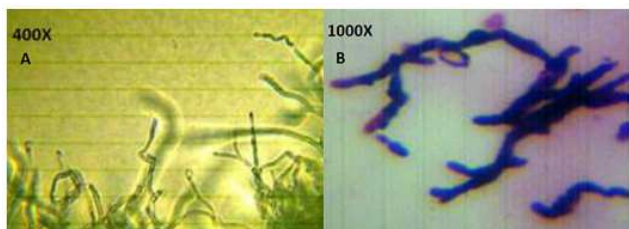


Fig. 1. Microbiological characteristics of *Pseudonocardia* sp. ROR40 areal and substrate mycellium.

A: Substrate mycelium on ISP1 medium after incubation at 30°C for 10 d. (magnified power: 400X)

B: Gram stain of fragmented areal mycelium (magnified power: 1000X)

The potential isolate (ROR40) was Gram positive actinobacteria, the cultural characteristics: Substrate and aerial mycelia fragment into rod-shaped and oval elements. Substrate hyphae were characterized by the presence of swellings, polygonal shaped cells, and their conglomerates. Swollen hyphal segments were present and become divided by transverse septa (Fig.1). The aerial mycelium was white to gray colored and the substrate mycelium was brown on PDA medium. The actinobacterial colonies had wrinkled appearance on ISP media (International Streptomyces Project), aerial and substrate mycelium of the isolate had different pigmentation on ISPs media [ISP1: brown(areal), pale brown(substrate); ISP2: brown, creamy brown; ISP3:

gray, gray; ISP4: gray, yellow; ISP5: gray, gray; ISP6: gray, gray and ISP7: gray, creamy] and produced diffused brown melanoid pigment on most especially ISP7. Most ISP media had shown very luxuriant growth but ISP4 was less growth.

The isolate had ability to grow at 20–50°C (optimum at 30–35°C) and pH 6–12 (optimum at pH8–9). Moderate growth occurs on water agar and good growth on media containing malt extract, tryptone, peptone and yeast extract. It degraded gelatin, cellulose and Tweens 40, 60 and 80, but not starch or casein. Another major milestone in the identification of actinobacteria was the assimilation of carbon by actinobacteria. Utilization of carbon sources d-glucose, arabinose, xylose, inositol, mannitol, fructose, rhamnose, sucrose and raffinose were analyzed for classification. Test includes ten carbon sources which are sterilized by membrane filtration method. These carbon sources were separately supplemented (1%) in each ISP 1 medium. The isolate had ability to assimilate d-glucose, d-xylose, maltose, lactose, inositol, fructose, sucrose and raffinose as carbon sources and had shown very luxuriant growth but not arabinose, mannitol and rhamnose. Acids were produced from fructose, d-glucose, d-xylose, maltose, inositol, and sucrose, but not from raffinose or lactose. The isolate utilized Arginine, asparagine, glutamine, histidine, lysine, alanine, phenylalanine, tyrosine, proline, potassium nitrate, ammonium chloride and ammonium phosphate as nitrogen sources, but not cysteine or methionine. The isolate had

ability to grow in the presence of sodium chloride (1–7% w/v) lysozyme (10–100 µg/ml) and potassium tolerate (0.001%, w/v). Also it was susceptible to phenol (0.001%, w/v), crystal violet (0.0001 w/v) and sodium azide (0.01% w/v). It degraded L-Tyrosine (0.5 %w/v) and xanthane (0.4% w/v). H₂S production and Nitrate reduction were negative.

3.1. Molecular Identification and Phylogenies of Potential Strain

The analysis of the 16S rRNA gene is a more vital tool for correct identification of microbial species; the 16S rRNA gene of the isolate was sequenced partially. The phylogenetic tree, which was constructed by neighbor joining method for comparison of the 16S rRNA gene sequences, indicated that the strain belonged to the genus *Pseudonocardia*. The levels of similarity between the 16S rRNA gene of *Pseudonocardia* species are shown in Figure 2. The National Centre for Biotechnological Information (NCBI) sequences data shows that the isolate had 98% similarity with *P. alni* 20049 & *P. alni* 44104 and 97% with *P. antracta* DVS 5a1, *P. carboxydivoran* Y8, *P. autotrophica* NRRL B11275 and *P. tropica*. Whereas it had 96% similarity with *P. compacta* NRRL B16170 and *P. antitumoralis*. Finally the isolate was named *Pseudonocardia* sp. ROR40 (Genbank accession no KJ 725072).

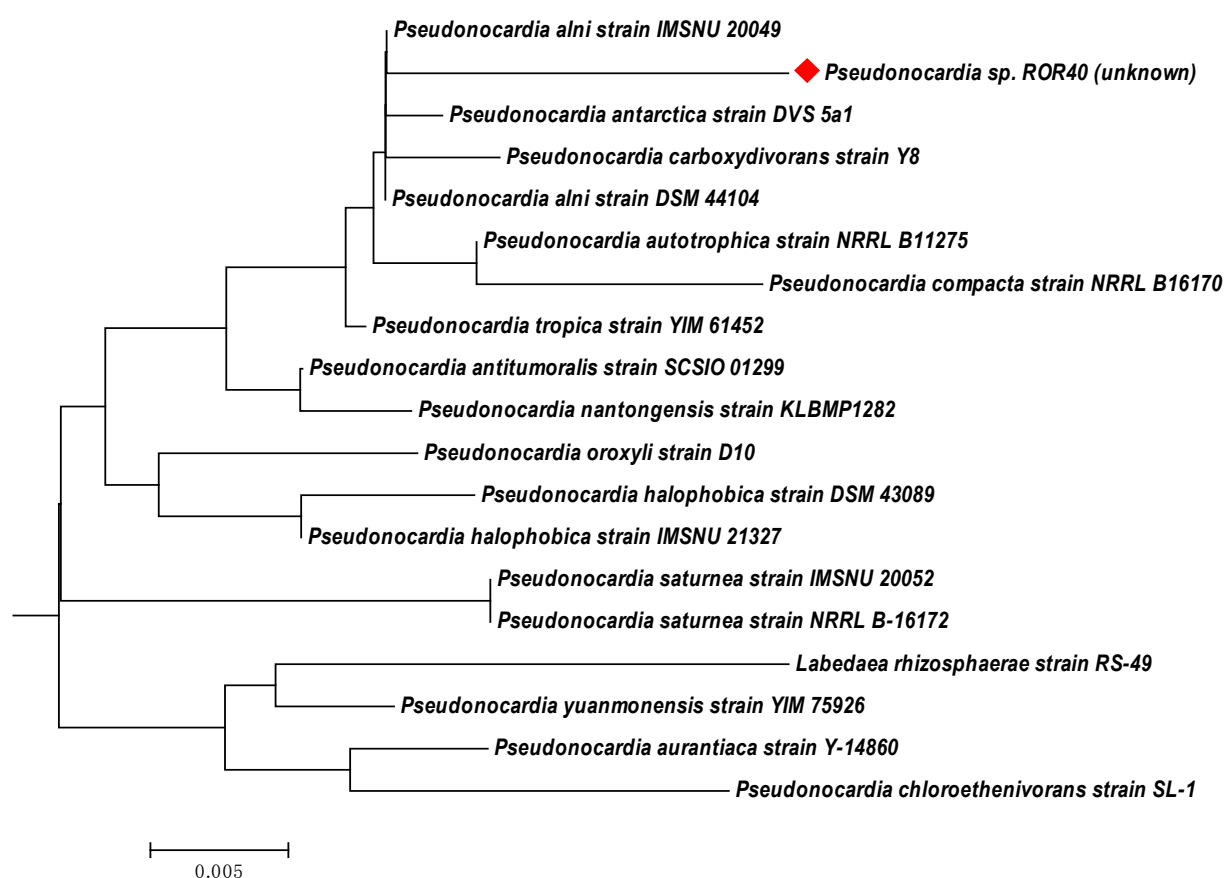


Fig. 2. Phylogenetic tree based on 16S rRNA sequences using neighbor-joining method. Branch distances represent nucleotide substitution rate and scale bar represent the number of change per nucleotide position.

3.2. Production of Extracellular Alkaline Phosphatase

The isolate showed its ability to produce extracellular alkaline phosphatase in liquid medium after 4d at 30 °C. The effect of some parameter on production enzyme were studied

and the results revealed that the optimum conditions were pH 8.5 of production medium , 37 °C for 4d. in stand incubator (Fig.3)

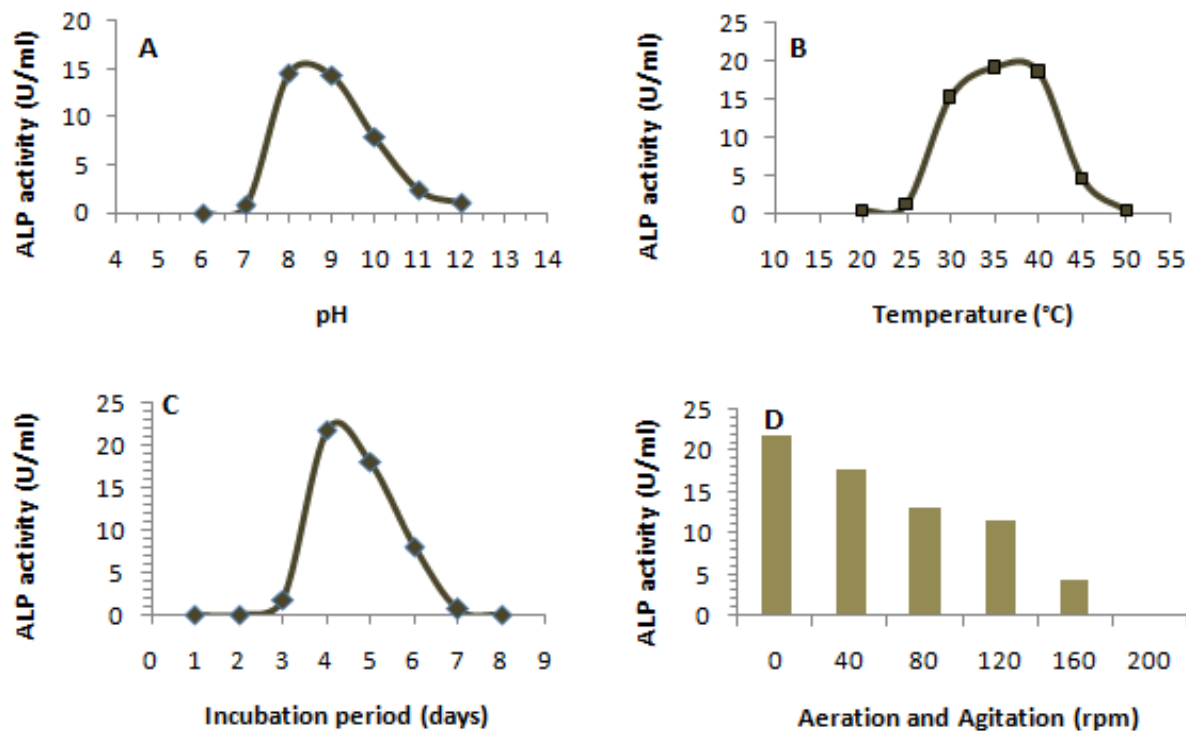


Fig. 3. Optimization environmental production conditions of extracellular alkaline phosphatase produced from *Pseudonocardia* sp. ROR40.

A: effect of different pHs of media on enzyme production at 30°C for 4d. in stand incubator.

B: effect of the temperature on ALPase production from the strain that cultured in liquid media at pH 8.5 and incubated at different temperature for 4d. in stand incubator.

C: effect of different incubation periods on ALPase production from the strain that cultured in liquid media at pH 8.5 and incubated at 37°C in stand incubator.

D: effect of aeration & agitation on ALPase production from the strain that cultured in liquid media at pH 8.5 and incubated at 37°C for 4d.

4. Discussion

Research on the actinobacteria isolated from deteriorating concrete structure is very scanty and till date these resources have not been properly explored to identify novel microorganisms with important biological properties. With this outlook, the present research has been initiated to identify novel actinobacterial isolates from corrosive lesions at irrigation concrete channel infrastructures. Rare actinobacterial strains were isolated using modified growth medium as cement extract medium. Many researchers already described the usage of aged seawater enriched modified media or selective media for the isolation and enumeration of marine actinobacteria (Ramesh and Mathivanan, 2009).

Majority of the isolates in this study possessed zigzag shape mycelia and the same morphology has been reported by Ara *et al.* (2011) and outlined in Bergey's Manual of Systematic Bacteriology (Goodfellow, 2012). Based on isolation and growth studies, it was made known that

majority of the isolates can be isolated using modified cement extract medium and grew well in ISP media. Varied pigment production pattern was also observed among our isolates. Shirling and Gottlieb (1966) stated that the pigmentation prototype can be used as markers for identification. Majority of our isolates exhibited considerable antibacterial activity against tested clinical pathogens including bacteria and fungi.

The selected potential isolate was further identified on using cultural characteristics and utilization of carbon by the isolate in different media (ISP-2 to ISP-7) also play a major role in identification of actinobacteria to generic level. It is also verified that different physiological characteristics will undoubtedly influence the growth rate of actinobacteria (Takizawa *et al.*, 1993). Growth survival studies of our selected isolate also able to tolerate in varied NaCl and pH levels, this isolate resemble to actinobacteria that isolated from marine sediments were moderate alkaliphilic and moderate halophilic in nature (Ramesh and Mathivanan, 2009). Also our isolate appeared variable ability to produce

different organic and inorganic acid that lowering extreme alkaline pHs (10-12) to moderate alkaliphilic (8 -9.3). To cope with the external stress, these organisms have developed adaptive metabolic characteristics to persist under extreme conditions (Rosenberg and Ron, 2001). *Nesterenkonia alba* sp. nov., an alkaliphilic actinobacterium was testified to grow optimally at pH 9–10, also a halophilic marine actinomycete, *Nocardiopsis litoralis* sp. nov., isolated from a sea anemone (Gandhimathi *et al.*, 2009; Singh *et al.*, 2010).

According to all these experiments results have been matched with the keys given for actinomycetes included in ISP (International Streptomyces Project) and Bergey's Manual of Systematic Bacteriology (Goodfellow, 2012) the species identification was done and it was found that the isolate had been grouped under *Pseudonocardia* genus. The isolate was nearest to type strain *P. alni* DSM 44104, with the presences of differences in some characteristics such as acid production from carbohydrate as L-arabinose, rhamnose, inositol or sucrose, as well as starch hydrolysis, grows in the presence phenol (0.001%, w/v) and susceptibility to lysozyme (Goodfellow, 2012).

Phylogenetic analyses also made known that *Pseudonocardia* sp. ROR40 form the same cluster with *P. alni*, *P. antracta*, *P. carboxydivoran*, *P. autotrophica* and *P. tropica*. *P. compacta* and *P. antitumoralis*, respectively. To the best of our knowledge, this is the first report on detailed characterization on enzymes with industrial and pharmaceutical importance from the rare actinobacteria isolated from deteriorating concrete structure.

To know the overall activity of the potential isolate (ROR40) various enzymatic screening has been done including oxidase, β -galactosidase, cellulase, caseinolytic activity (protease), amylase, gelatinase, alkaline phosphatase and lipolytic activities. Oxidase, β -galactosidase, cellulose, gelatin hydrolysis, alkaline phosphatase and lipolytic activities were shown by the strain which some of them were used in its identification. But the amylase or protease activities are not. Actinobacteria are physiologically diverse group in synthesizing various enzymes and metabolic products of industrial interest and are well recognized to produce most valuable pharmaceuticals and agrochemicals (Luo *et al.*, 2009). These results on enzymatic production authenticated the capability of our isolate to over synthesize the valuable enzymes of industrial importance.

Our isolates may play a role in mineralizing stingily phosphate sources at irrigation channel through extracellular alkaline phosphatase production. Franco-Correa *et al.* (2010) estimated phosphate mineralization ability of thirty actinomycete strains were isolated from the rhizosphere of field-grown plants (*Trifolium repens* L.) by assayed intracellular acid and alkaline phosphatases activities and found the activities of enzymes ranged from 15-25U/ml for acid phosphatase and 10- 15 U/ml for alkaline phosphatase. These isolates play a role in plant growth promotion and mycorrhiza helping activities through their capabilities of solubilizing/mineralizing sparingly phosphate sources, N₂-fixation and/or siderophore production.

The optimization of alkaline phosphatase production parameters were studied and the results appeared the optimum conditions were pH 8.5 of production medium, 37°C for 4d. in stand incubator. The optimum conditions for alkaline phosphatase production or other enzymes depend on category of the microorganisms such as actinobacteria or *Bacillus* sp. (Omran and Qaddoorior, 2014), genetic contents and the conditions of their environments.

Many studies had been reported that actinobacteria species such as *Streptomyces* and *Streptomyces griseus* had ability to produce acid and alkaline phosphatases (Moura *et al.*, 2001; Franco-Correa *et al.*, 2010).

Most of the actinomycetes strains were isolated from soil and aquatic environment appeared to have the ability of solubilizing sparingly available inorganic P sources or mineralizing some P from the organic P sources in soil. Actinomycetes developed several potential mechanisms for phosphate solubilization those involving the production of chelating compounds, like organic acids or by means of a modification of pH of the medium by the secretion of organic acids or protons (Richardson *et al.*, 2009; Vasconcellos *et al.*, 2010). Also they produced acid or alkaline phosphatases would be able to mineralize organic P sources (Richardson *et al.*, 2009; Ghorbani-Nasrabadi *et al.*, 2012). Actinomycetes are widely distributed in different habitats and involved in important processes. They are not only able to survive under extreme soil condition such as low level of moisture or high salinity, but actinomycetes are also reported to promote plant growth (Hamdali *et al.*, 2008). Therefore, evaluation of their distribution is important in understanding their ecological role.

5. Conclusion

In the currents scenario over the world, both academic and industrial research mainly focuses on isolation alkaliphilic and halophilic microorganisms from marine sediments and halophilic environments due to its novel characteristics. These authorizations urge to initiate the recent research to isolate salt and alkali tolerant novel actinobacteria from unexplored environment as corrosive lesions at old concrete bridges, dams and irrigation channels. Our study would be the first instance in comprehensive characterization of concert deteriorating actinobacteria for producing commercially valuable primary and secondary metabolites. It is concluded that a very frequent and systematic screening of concert deteriorating actinobacteria from different sources in Iraq may facilitate us to isolate and characterize more bioactive species.

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