African Journal of Biotechnology Vol. 11(16), pp. 3849-3857, 23 February, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.3950
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Proteolytic activity of alkaliphilic, salt-tolerant actinomycetes from various regions in Saudi Arabia

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Accepted 1 February, 2012

Actinomycetes were isolated from various desert soil samples of Saudi Arabia using alkaline and normal pH media. A total of 42 akaliphilic actinomycetes isolated from yeast extract-soluble starch (YS) agar media (pH 11.0 ± 1) and 102 actinomycetes were isolated from tap water agar media (pH 7.0 ± 1) for comparison. Aerial mycelium colours of actinomycetes were observed for the detection of strain varieties in the different soil samples from different areas. Colour types were more abundant in isolates grown tap water agar (TWA) than in isolates grown on YS agar medium (pH 11.0). On the TWA medium, there were 18 colour types of actinomycetes and on YS agar medium (pH 11.0) there were 10 colour types. The number of neutrophilic actinomycetes isolated in this study is higher than the alkaliphilic actinomycetes. Among the 42 alkaliphilic actinomycetes isolates, 30 isolates were salt tolerant alkaliphilic. Later about 16 alkaliphilic actinomycetes were screened on skim milk agar for preliminary proteolytic activity. A total of 10 isolates showed proteolytic activity. Of the 10 isolates, three isolates were alkaliphilic and seven isolates were salt-tolerant alkaliphilic. All the isolates need to be further studied for the ability of their potential protease enzyme production.

Key words: Alkaliphilic actinomycetes, salt tolerant actinomycetes, desert soil, isolation, proteolytic activity.

INTRODUCTION

Actinomycetes are large group of Gram-positive eubacteria. They are present in a wide range of environments commonly as saprophytes in soil, water, compost and other habitats. They play an important role in soil structure and composting. Actinomycetes are the main producers of antibiotics and approximately at the rate of 300 antibiotics per year had been identified (Coyne, 1999).

In addition to antibiotics screening, enzymes also have important attention. The microbial enzymes in industrial fields are preferable (Thumar and Singh, 2007). Microbial proteases are among the major hydrolytic enzymes that have been studied extensively. The interest of studying microbial protease mainly is because these enzymes not only play an important role in the cellular metabolic processes but also in the industrial community (Gupta et al., 2002). Protease enzymes from alkaliphilic, salt-tolerant bacteria are needed in industrial process. They are preferred due to ease operation, higher activity, enhanced stability, faster reaction, and likely less contamination (Ningthoujam et al., 2009).

Study of proteases from alkaliphilic and salt-tolerant actinomycetes has been reported elsewhere (Thumar and Singh, 2007; Kuchari 1999, Zaki et al., 1980; Ningthoujam et al., 2009; Gurielidze et al., 2010). Early report on isolation of halophilic actinomycetes from Saudi Arabia was reported by Zaki et al. (1980). Other report about isolation of halotolerant pigmented actinomycetes from Jeddah sea shore was reported by Kuchari (1999). However, the research did not discuss their enzymes production especially protease.

To our knowledge, isolation of alkaliphilic, salt-tolerant actinomycetes from Saudi Arabia is promising to discover new findings of bioactive microbial metabolites, including

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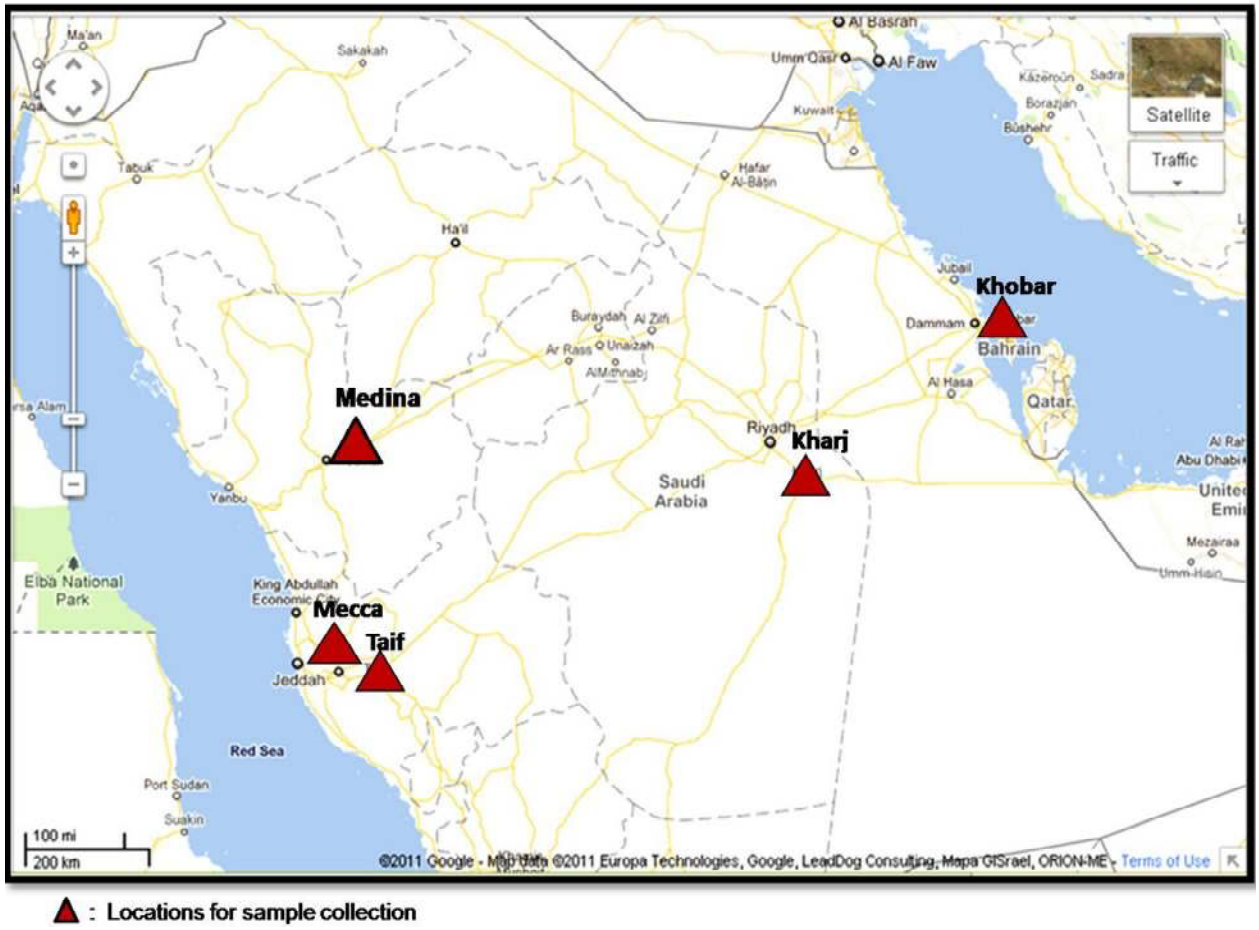

Figure 1. Map showing the different sampling places: Mecca, Medina, Kharj, Khobar, and Taif in the Kingdom of Saudi Arabia**.**

protease enzymes. This study aimed to isolate alkaliphilic, salt-tolerant actinomycetes from Saudi Arabian soils and their preliminary investigation for the proteolytic activity of the potent isolates.

MATERIALS AND METHODS

Collection and processing of the soil samples

Desert soil sample from 9 different places (such as: Mecca, Medina, Kharj, Khobar and Taif) were collected in clean sterile sealed plastic bags (Figure 1). The soil samples were air-dried at room temperature for 7 days and their pH were measured using standard procedure.

Isolation of actinomycetes on alkaline media

Soil suspension were diluted and plated on the modified yeast extract soluble starch agar (YS) (Luedemann, 1971) adjusted at pH 11 (composition: yeast extract, 2.0 g; starch soluble, 10 g; agar, 15 g; distilled water, 1L; pH 7.0 ± 1). The plates were incubated at 30°C for 14 days.

Isolation of actinomycetes on normal pH media

Soil suspension diluted and plated on the TWA medium (composition: agar, 20 g; tap water, 1 L). The plates were incubated at 30°C for 14 days.

Morphological and physiological characteristics

Morphological and physiological studies were carried out with the methods recommended by the International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966). The isolates obtained from yeast extract-soluble starch medium were sub-cultured on the ISP2 medium (composition: yeast extract, 4.0 g; malt extract, 10.0 g; dextrose, 4.0 g; agar, 15.0 g; distilled water, 1 L; pH 7.0 ± 1) with pH 7.0, pH 8.0, and pH 9.0. Similary the isolates also sub-cultured on the ISP2 medium with NaCl at 10% concentration. The plates were incubated at 30°C for 14 days.

Screening for proteolytic activity of the selected actinomycetes

Preliminary screening for extracellular protease production and activity was done using modified agar plate assay technique (Chu,

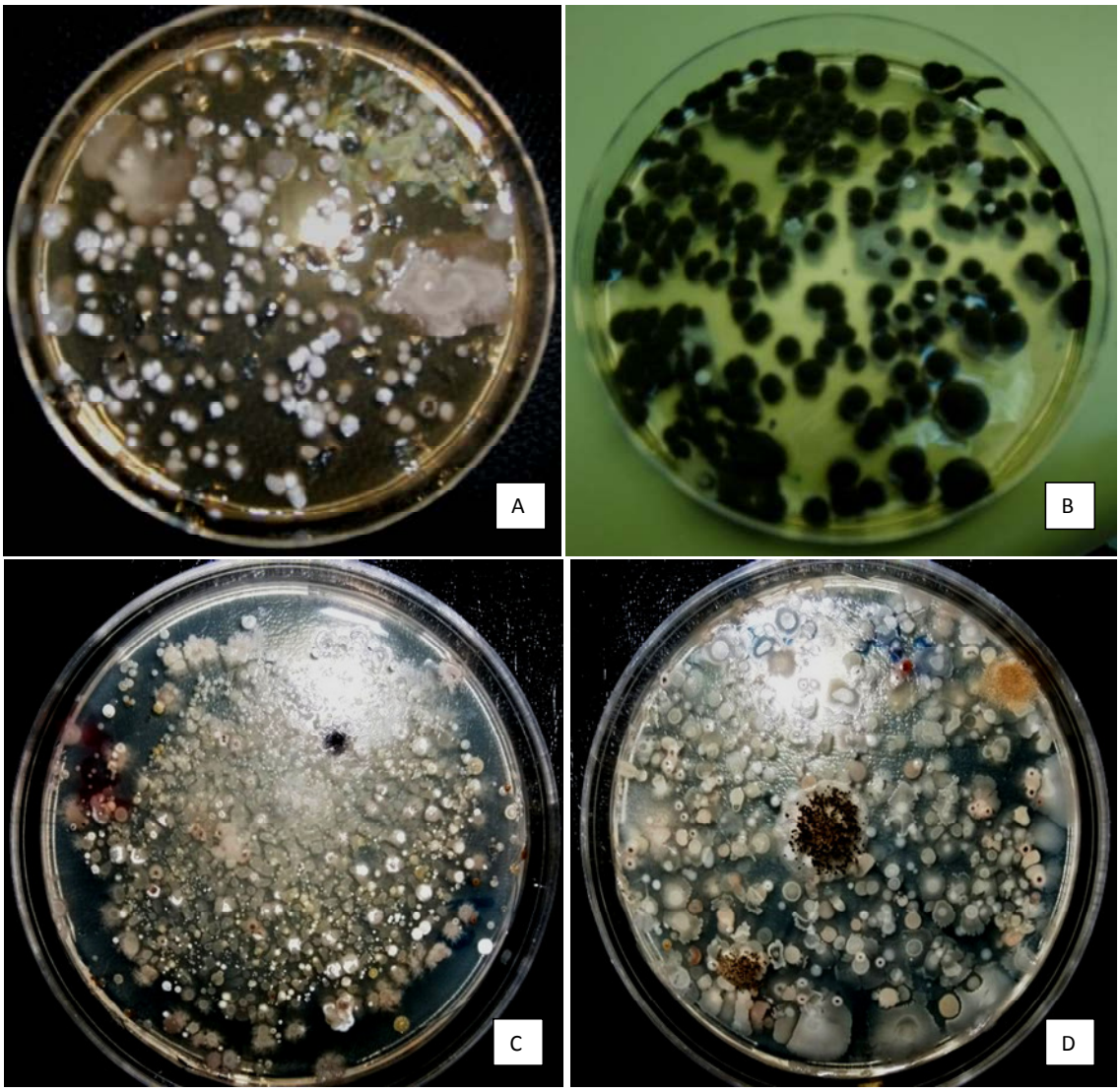

Figure 2. Actinomycetes colonies on yeast-starch agar medium with pH 11.0 (A and B); Actinomycetes colonies on tap water agar medium (C and D).

2007) with 10% skim milk agar medium (w/v) (composition: skim milk, 100 g; agar, 15.0 g; distilled water, 1L; pH 7.0 ± 1). The plates were incubated at 30°C for 7 days. Proteolytic enzyme production and their activity was observed based on the clearance of opaque milk protein formation surrounding the isolates (Chu, 2007; Bajaj and Sharma, 2011).

RESULTS AND DISCUSSION

Isolation of actinomycetes: morphological and physiological characteristics

A total of 42 isolates were isolated from the soil samples on the YS medium and 102 isolates on the TWA medium (Figure 2). The number of isolates grew on the YS agar (pH 11.0) was less compared to those grew on the TWA medium (Figure 3).

Aerial mycelium colours were observed for the detection of strain varieties in the different soil samples from different areas. Colour types were more abundant in isolates grown TWA medium than in isolates grown on YS agar medium (pH 11.0). On the TWA medium there were 18 colour types and there were 10 colour types on YS medium (pH 11.0) (Table 1).

Yeast starch medium (with or without agar) has been used widely for actinomycetes growth, purification or physiological, morphological test. This medium used for the observation of spores and sporophores development in actinomycetes (Luedemann, 1971; Shimizu et al., 2000). It was also noted as medium for characterizing actinomycetes colony development and the colour change after the development of sporulation(Luedemann, 1971).

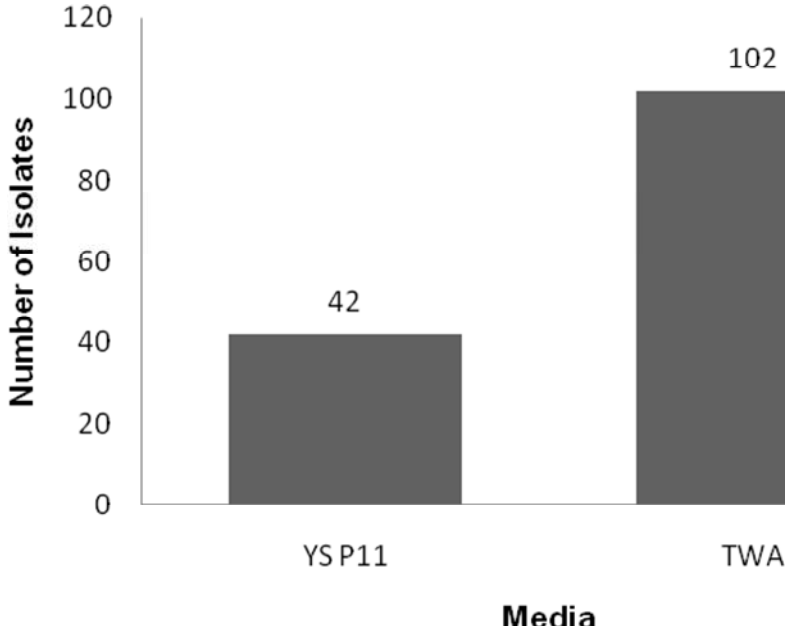

Figure 3. Total isolates grew on yeast-starch agar (pH 11.0) (YS P11) and tap water agar (TWA) medium.

Table 1. Aerial mycelium colour of actinomycetes on yeast starch and tap water agar medium.

S/N	Colour	YS P11*	TWA*
1	Black	-	+
2	Brown	-	+
3	Black grey	-	+
4	Colour less	+	+
5	Cream	+	+
6	Pale cream	+	+
7	Grey	+	-
8	Grey white	+	+
9	Pale grey	+	-
10	Pale greenish	+	-
11	Orange yellow	-	+
12	Orange	-	+
13	Pink	-	+
14	Pink red	-	+
15	Purple	-	+
16	White	+	+
17	White brown	+	-
18	White grey	-	+
19	White black	-	+
20	Off white	+	-
21	Pale white	-	+
22	Yellow	-	+
23	Yellowish black	-	+

*YS P11: Yeast-starch agar (pH 11); TWA: tap water agar; +: Present; -: Absent.

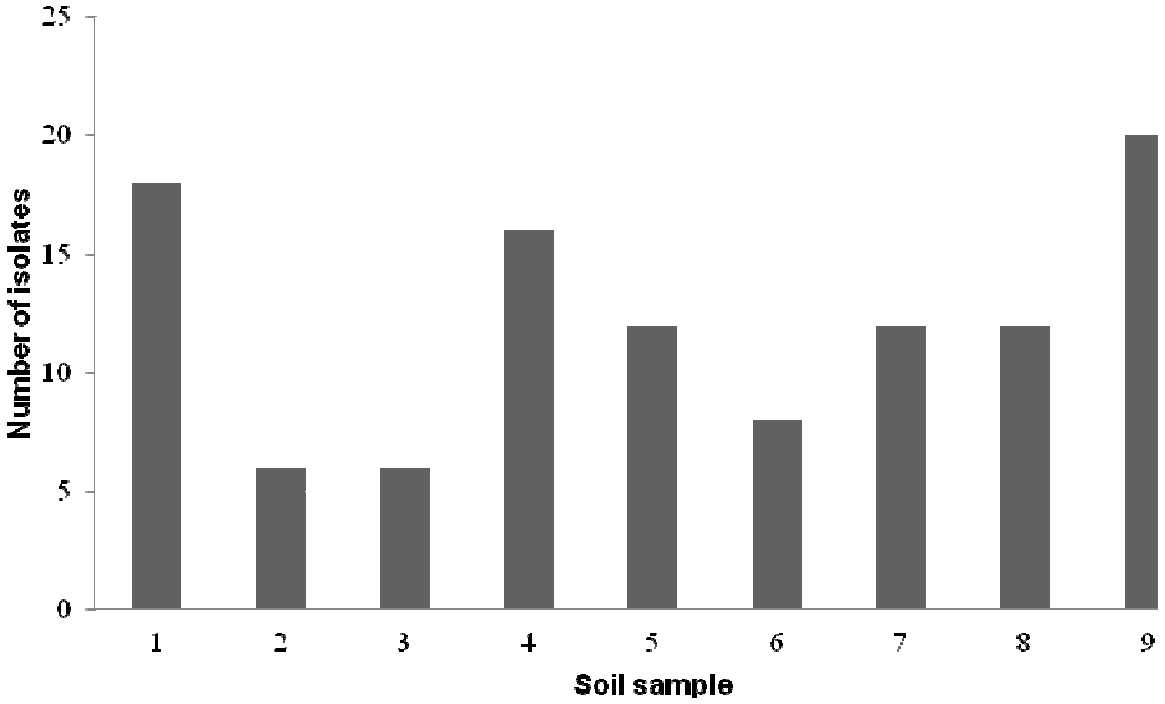

Figure 4. Number of actinomycetes from each soil samples isolated on tap water agar.

Regardless the media differences, all the isolates from yeast-starch (pH 11) could grow at pH 7, pH 9, and pH 10 on ISP-2 medium. These data proved that all the isolates are alkaliphilic organisms. Alkaliphilic organism is known to be able to grow optimally at pH above 9, usually between 10 and 12. Nonetheless, it cannot grow or grow slowly at the near neutral pH value of 6.5 (Horikoshi, 1999). In addition, Park et al. (1991) reviewed that neutrophiles *Streptomyces* species are able to grow between pH 5.0 and 9.0 with optimum growth close to neutrality.

The soil samples collected in this study were sandy and alkaline (range of pH 7.76 and 8.80). Isolation of actinomycetes from sandy soil had been done with various media. The media were humic-acid-vitamin agar (HV), soil extract agar, glucose-yeast-extract agar and the newest media was the minimum media (Ara et al., 2007; Hozzein et al., 2008, 2011). Actinomycetes can survive and grow in poor nutrient media as already reported, water agar can be used as selective medium for the isolation of actinomycetes (El-Nakeeb and Lechevalier, 1962). Berd (1973) has noted that tap water agar was better to study actinomycetes isolates for distinct sporulation. Tap water agar was used in this research as a control media to compare the isolation of alkaliphilic actinomycetes and neturophilic actinomycetes result from Saudi Arabian desert soils. The result showed 102 isolates grew on tap water agar. The 102 isolates displayed 18 colour types of aerial mycelium. Our result showed that the tap water agar is appropriate to screen actinomycetes from sandy desert soil. It displayed that the neutrophilic actinomycetes were present in the sandy soils. In addition, Neutrophilic species *Streptomyces synnematoformanns* sp. nov. and *Nonomuraea aegyptia* sp.nov. from sand dune soil in Egypt had been reported (Hozzein and Goodfellow, 2007a b).

Actinomycetes isolates from different media and different regions

The highest number of isolate on tap water agar medium was sample number 9 from Al-Khobar and the lowest was sample number 2 and 3 from Mecca (Figure 4). The highest number of isolate on YS agar medium (pH 11.0) was sample number 8 from Al-Kharj and the lowest was sample number 4 from Mecca (Figure 5). These results show neutrophilic actinomycetes were more abundant than the alkaliphilic actinomycetes in our soil samples. Moreover, more media should be included in actinomycetes isolation to obtain the optimal screening of actinomycetes.

Morphological and physiological characteristics

Almost all the isolates grew on ISP2 medium with pH 7.0, pH 9.0 and pH 10. The highest growth percentage on

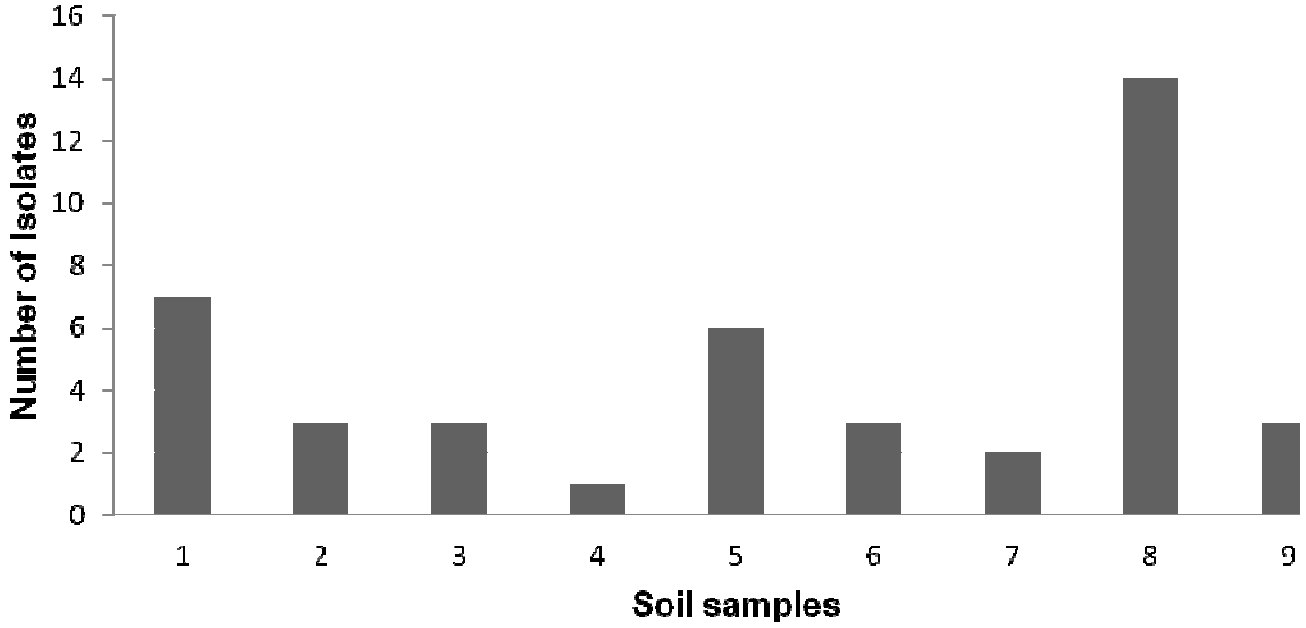

Figure 5. Number of actinomycetes isolated from each soil samples on yeast-starch agar medium.

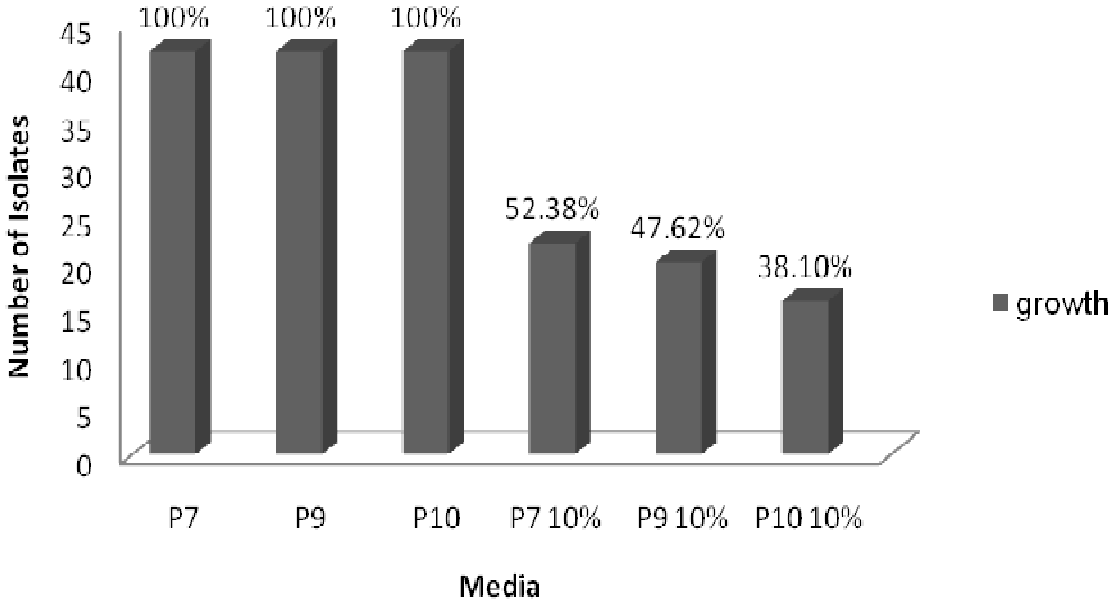

Figure 6. Growth percentage on ISP2 medium with various pH and salt concentration. Medium: P7: ISP2 pH 7; P9: ISP2 pH 9; P10: ISP2 pH 10; P7 10%: ISP2 pH7 and 10% NaCl; P9 10%: ISP2 pH9 and 10% NaCl; P10 10%: ISP2 pH10 and 10% NaCl.

ISP2 medium with NaCl 10% was at pH 7 followed by pH 9 and pH 10 (Figure 6). Numbers of isolates were declining at higher pH. A total of 42 isolates grew on the ISP2 medium and all isolates were alkaliphilic, 30 isolates were salt-tolerant alkaliphilic (Table 2). The decreasing numbers of bacterial isolates with 10% NaCl concentration in the medium showed that 10% NaCl concentration inhibited the alkaliphilic bacterial growth. Combination of 10% NaCl and higher alkaline pH (pH 9.0 and pH 10.0) also inhibited the alkaliphilic bacterial growth as seen in Figure 6.

Actinomycetes isolated from sandy soil were reported

Table 2. Growth of the isolates from 9 soil samples on ISP2 medium with various pH and saline concentration.

S/N	IC*	P7*	P9*	P10*	P7 10%*	P9 10% *	P10 10% *
1	SA 1	+	+	+	+	+	+
2	SA 2	+	+	+	+	+	+
3	SA 3	+	+	+	+	+	+
4	SA 4	+	+	+	-	+	-
5	SA 5	+	+	+	-	+	-
6	SA 6	+	+	+	-	+	-
7	SA 7	+	+	+	+	+	-
8	SA 8	+	+	+	-	+	+
9	SA 9	+	+	+	+	+	+
10	SA 10	+	+	+	-	+	+
11	SA 11	+	+	+	-	-	-
12	SA 12	+	+	+	-	+	-
13	SA 13	+	+	+	-	-	-
14	SA 14	+	+	+	+	-	-
15	SA 15	+	+	+	-	-	-
16	SA 16	+	+	+	-	-	-
17	SA 17	+	+	+	+	-	-
18	SA 18	+	+	+	+	-	+
19	SA 19	+	+	+	-	-	-
20	SA 20	+	+	+	-	-	-
21	SA 21	+	+	+	+	+	+
22	SA 22	+	+	+	+	+	+
23	SA 23	+	+	+	+	-	-
24	SA 24	+	+	+	-	-	-
25	SA 25	+	+	+	-	-	-
26	SA 26	+	+	+	-	+	+
27	SA 27	+	+	+	+	+	+
28	SA 28	+	+	+	+	+	+
29	SA 29	+	+	+	+	-	-
30	SA 30	+	+	+	-	-	-
31	SA 31	+	+	+	+	-	-
32	SA 32	+	+	+	-	-	-
33	SA 33	+	+	+	+	-	-
34	SA 34	+	+	+	+	-	-
35	SA 35	+	+	+	+	-	-
36	SA 36	+	+	+	-	-	-
37	SA 37	+	+	+	+	+	+
38	SA 38	+	+	+	+	+	+
39	SA 39	+	+	+	+	+	-
40	SA 40	+	+	+	-	-	-
41	SA 41	+	+	+	-	-	+
42	SA 42	+	+	+	+	+	+

*IC: Isolates code; P7: ISP2 pH 7; P9: ISP2 pH 9; P10: ISP2 pH 010; P7 10%: ISP2 pH7 and 10% NaCl; P9 10%: ISP2 pH9 and 10% NaCl; P10 10%: ISP2 pH10 and 10% NaCl; +: growth; - : no growth.

as alkaliphilic or alkalitolerant, halophilic or halotolerant species. Earlier report on halophilic actinomycetes from Saudi Arabia was reported by Zaki et al. (1980). They isolated halophilic actinomycetes from rhizosphere and soil apart of six littoral salt marsh plants at Shuaiba Lagoon. The soils have pH 8.0-8.5 and total soluble salt from 24.42 to 54.83 in 1:5 soil water extract. Moreover, halotolerant actinomycetes from Jeddah sea shore, Saudi

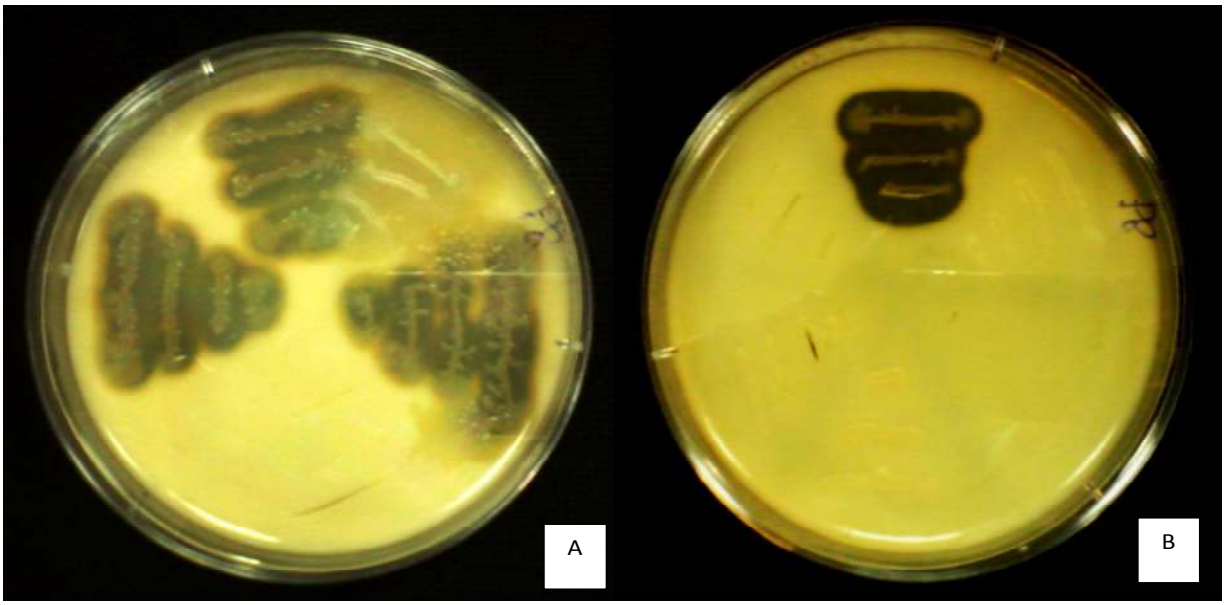

Figure 7. Akaliphilic actinomycetes showed proteolytic activity on skim milk agar plates (A and B).

Arabia were able to grow at a concentration of 9.0% NaCl (Kuchari 1999). Hozzein et al. (2004) reported *Nocardiopsis alkaliphila* sp. nov. a novel alkaliphilic actinomycete isolated from desert soil in Egypt. It grew optimally at pH 9.5 to 10.0 and limited or no growth at pH 7.0. Other species, *Nocardiopsis Arabia* sp. nov. was isolated from Egyptian sand dune soil (Hozzein and Goodfellow, 2008). The species showed growth with 15.0% NaCl concentration and at pH 7.8. In addition, haloalkaliphilic actinomycetes were reported by Norovsuren et al. (2006). The isolates grew successfully at pH 8.0 to 9.0 with 5.0% salt concentration.

Screening for proteolytic activity of the actinomycetes

Out of 16 different isolates with different colour of aerial mycelium (5 alkaliphilic and 11 salt-tolerant alkaliphilic), 10 isolates showed proteolytic activity (Figure 7) on skim milk agar medium. All of the 10 isolates showed proteolytic enzyme activity grew on skim milk agar plate in the range of 1 to 2 days (Table 3). Of the 10 isolates, three isolates were alkaliphilic and seven isolates were salt-tolerant alkaliphilic.

The skim milk agar medium had been used to detect bacterial proteolytic enzyme activity (Ningthoujam et al., 2009; Bajaj and Sharma, 2011). Frazier and Rupp, (1928) noted that according to Freudenreich in 1895, an organism is considered to be proteolytic if there is a clear zone around the colony.

Protease production from alkaliphilic, and salt-tolerant alkaliphilic had been reported before. Previous study by Gurielidze et al. (2010) reported that alkaliphilic isolates isolated from soils of Georgia showed protease activity. Three of the protease producers strains were *Streptomyces globisporolactis* 203A, *Streptomyces streptomycini* 204A, *Nocardia polaris* 206A. Thumar and Singh (2007) reported an alkaline protease produced by a salt-tolerant alkaliphilic *Streptomyces clavuligerus* strain MIT-1 isolated from Mithapur, western coast of India. In addition, moderately halophilic alkali thermo-tolerant indigenous actinomycetes isolate HA4, has been reported to have protease activity (Ninghoujam et al., 2009).

Conclusion

The study shows that the soil samples from Saudi Arabia are good sources of diverse alkaliphilic, salt-tolerant alkaliphilic and neutrophilic actinomycetes. Further, this preliminary study displays that the large numbers of diverse isolates have proteolytic activity. Therefore, further study will help biotechnological industry for the detection and purification of protease enzymes from locally isolated actinomycetes in the Kingdom of Saudi Arabia.

ACKNOWLEDGEMENT

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding the work through the research group project No. RGP-VPP-072.

Table 3. Proteolytic activity of actinomycetes isolates on skim milk agar medium.

S/N	Isolate code	Incubation day							
		1	2	3	4	5	6	7	8
1	SA 1	-	+	+	+	+	+	+	+
2	SA 6	-	-	-	-	-	-	-	-
3	SA 7	-	-	-	+	+	+	+	+
4	SA 16	-	+	+	+	+	+	+	+
5	SA 17	+	+	+	+	+	+	+	+
6	SA 20	-	+	+	+	+	+	+	+
7	SA 23	-	-	-	-	-	-	-	-
8	SA 24	-	+	+	+	+	+	+	+
9	SA 25	-	-	-	-	-	-	-	-
10	SA 26	-	+	+	+	+	+	+	+
11	SA 27	-	+	+	+	+	+	+	+
12	SA 28	-	+	+	+	+	+	+	+
13	SA 29	-	+	+	+	+	+	+	+
14	SA 30	-	-	-	-	-	-	-	-
15	SA 34	-	-	-	-	-	-	-	-
16	SA 35	-	-	-	-	-	-	-	-

+: Halo (clear zone around the isolate); -: no halo present (no clear zone around the isolate).

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