

A new medium for the isolation and enrichment of halophilic actinobacteria

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Abstract: This paper is about a new nutritional medium designed for the isolation and enrichment a useful group of bacteria called halophilic actinobacteria. These bacteria can be found in the saline environment, they can be moderate or extremely halophilic. The extremely halophilic require between 15-30% of NaCl for growth, and they can be selectively isolated in different media. The new medium was enriched by the addition of organic and inorganic nutrients appropriate for the growth of these bacteria. It consists of starch, glucose and yeast extract (SGY) supported with artificial sea water for providing a mixture of salts that resemble the composition of concentrated sea water, where halophilic actinobacteria require Na⁺ to grow, In addition to different concentrations of Na⁺, K⁺, and Mg²⁺. The purpose of this medium is for providing the nutritional requirements which can stimulate and support the growth under high salinity conditions during short period of time with high amount of growth compared to other media. Therefore, SGY medium was tested against (Inorganic salt starch agar, Glycerol asparagin agar, Oat meal agar and Yeast extract malt extract agar) supported with 10 % NaCl to enhance the growth of halophoicilic actinobacteria. According to the results, SGY medium achieved the highest bacterial growth during short period of incubation (4-6 days) than other different culture media which extended for (2-3 weeks). Consequently, the (SGY) medium can be considered an alternative to the media traditionally used for the study of halophilic actinobacteria.

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1. Introduction

Actinobacteria (actinomycetes) are the most economically and biotechnologically valuable prokaryotic microorganisms. They are well known as a rich source of antibiotics and bioactive molecules. Actinobacteria are widespread in nature and may occur in extreme environments (Meklat *et al.*, 2011). Microorganisms found in extreme environments have attracted a great deal of attention, due to the production by such microorganisms of various natural compounds and their specialized mechanisms for adaption to extreme environments (Tang *et al.*, 2002).

Among the various extremophiles, halophilic microorganisms have evolved several adaptations to survive and function in extraordinary hypersaline ecosystems, such as solar salterns, the dead sea, solar lake and other hypersaline environments (Jothi Basu *et al.*, 2015).

Halophilic bacteria can be distinguished to different groups on the basis of their physiological responses to salt. Several classifications have been proposed; one that is very well accepted considers the optimum growth at different salt concentrations,

Thus, Kushner & Kamekura (1988) defined several categories of micro-organisms on the basis of their optimal growth: non-halophiles are those that grow best in media containing less than 0.2M NaCl (some of which, the halotolerant, can tolerate high salt concentrations), slight halophiles (marine bacteria) grow best in media with 0.2 to 0.5M NaCl, moderate halophiles grow best with 0.5 to 2.5M NaCl and extreme halophiles are those show optimal growth in media containing 2.5 to 5.2M (saturated) NaCl.

Scientists have known that halophilic bacteria could endure and grow at high salt concentrations; however, they were unaware of the existence of halophilic actinobacteria until the 1970s, Gochner first discovered a strain of halophilic actinobacteria from a contaminated plate and named it *Actinopolyspora halophila*. Thereafter, research efforts focused on finding new halophilic actinobacteria (Tang *et al.*, 2002). Actinobacteria are ubiquitous in saline environment and there are several techniques for their isolation. In the conventional isolation techniques, several factors

must be considered, namely, choice of screening source, selective medium, culture conditions, and recognition of candidate colonies in the primary isolation. The cultivation of actinobacteria from extreme environments including saline habitats is very difficult than common environments, they are grow very slowly. Furthermore, choosing appropriate media and growth conditions is important, and published media are typically associated with a particular microbial genus or species. As with other microbial discovery research, when working with environmental samples harboring communities of novel microbial populations, the media and growth conditions chosen will enrich for certain populations and not others (Schneegurt, 2012).

Many medium formulations have been claimed to be successful for the selective isolation of actinobacteria. However, such media are not generally designed on the basis of nutritional or physiological requirements of the selected actinobacteria group (s), and many fail to be thoroughly evaluated (William and Vickers, 1988).

In this paper we describe the formulation of a new medium, Starch glucose yeast extract (SGY) which is supported with artificial seawater for the selective isolation and enrichment of halophilic actinobacteria from saline environments based on the nutritional requirements of these organisms, and determine the effectiveness of this medium comparing to other media used for cultivation halophilic actinobacteria.

2. Materials and Methods

2.1 (SGY) medium compositions

(SGY) is a new selective medium for isolation and enrichment of halophilic actinobacteria from saline environments. The ingredients in this medium which are support the growth of halophilic actinobacteria were (Starch 15 g, Glucose 5 g, Yeast extract 5 g, Agar 20 g and artificial seawater 1000 ml pH 6.8-7.2). Media were composed of inorganic salts corresponding in proportions to the artificial seawater solution of (Smibert and Krieg, 1994) which is contain (NaCl 100 g, MgCl₂ 5.0 g, MgSO₄ 2.0 g, CaCl₂ 0.5 g, KCl 1.0 g, FeSO₄ 0.01 g, distilled water 1000 ml). Keeping these proportions, the concentrations of all salts were maintained at seawater concentrations (except NaCl which can increase to give the total concentration required of salinity).

2.2 Preparation of (SGY) medium

Starch, glucose, yeast extract and agar powder are hydrated with water and homogenized in a glass flask. The artificial sea water salts (NaCl, MgCl, MgSO₄, CaCl₂, KCl and FeSO₄) are dissolved in another glass flask with water. The contents of both flasks are mixed to obtain the base agar. The pH of the medium was adjusted to 6.8 - 7.2 with 1 N HCl and NaOH, and they were autoclaved at 121°C for 15 min and tested for the isolation of halophilic actinobacteria from environmental samples.

2.3 Other comparison media

Other media used for comparison were: Inorganic salt-starch agar (ISP, 1964), Yeast extract malt extract agar (ISP, 1964), Oatmeal agar (ISP,1964) and Glycerol-Asparagine agar (ISP,1964). All media were supplemented with sodium chloride at (10%). All inoculated media were incubated at 40°C for 14 days.

2.4 Isolation of halophilic actinobacteria

Halophilic actinobacteria strains were isolated from the coastal salt-marsh sediments samples collected from Jeddah in the west of Saudi Arabia. Serial dilution plate method was used for selective isolation of actinobacteria from the samples suspension to the media according to (Holt and Krieg, 1994). After incubation at (40°C) for 2 weeks a visible colony was transferred and sub cultured until pure culture was obtained. Stock culture were maintained on slant agar and kept at (4°C).

2.5 Identification of actinobacteria

Actinobacteria isolates were identified by using molecular sequence analysis of the gene 16S rDNA through rDNA microseq 500 16S rDNA bacterial sequencing kit (PE Applied Biosystems), as the microseq identification system is an accurate and rapid method for the identification of actinobacteria, where it was rely directly on sequence database (Patel et al., 2000). The identification of the isolates was determined by comparing the 16S rDNA sequence to EMPL database that contain a large number of sequences including 16S rDNA sequences (Patel et al., 2004). The methods for genomic DNA extraction, amplification and the sequencing of DNA followed by sequence data analysis were used as described by (Patel et al., 2000).

2.6 Physiological characteristics

All the tests were carried out at 40°C, unless otherwise specified. Media and procedure used for physiological features and carbon sources utilization were as described by (Shirling and Gottlieb, 1966; Locci, 1989).

2.6.1 Salt concentration range for growth

The NaCl tolerance and requirement for growth was determined by using starch nitrate agar medium supplemented with different concentration of NaCl (10, 15, 20, 25, 30 %).

2.6.2 Temperatures range for growth

The ability of the strain to grow at different temperatures (30, 35, 40, 45, 50, 55 °C) was determined after 7-21 days using starch-nitrate agar supplemented with 10 % NaCl.

2.6.3 pH range for growth

The ability of the strain to grow at different pH (5, 6, 7, 8, 9, and 10) was determined after 7-21 days using starch-nitrate agar supplemented with 10 % NaCl.

2.6.4 Carbon substrate utilization

The ability of the isolates to grow with a single organic compound as sole source of carbon and energy was determined in the basal minimal medium (ISP-9) which is containing 9 different carbohydrates, by replacing glucose with the test substrate at a final concentration of 1% (w/v).

3. Results and Discussion

3.1 Salt concentration range for growth

The results presented in table (1) show that the tolerance of the halophilic actinobacteria to NaCl differs with soil source and actinobacteria species. They were found to be resistant to the inhibitory effects of wide range of NaCl concentration with the optimal growth ranging between 10-30 %.

The results showed increase in the growth rate with increasing salt concentration of sodium chloride. This result agree with the finding of (Mudryk and Donderski, 1991) who found that an increase in the amount of sodium chloride in the medium used to culture halophiles results in an increase in their metabolic activity.

This enhancement of metabolic activity when cells are cultured in increased amount of salts may be due to an increase activity of enzymes as suggested by (Forsyth and Kushner, 1970) who found that Na⁺ ions facilitate the transport of amino acids and sugars across the cytoplasmic membrane.

In the other hand, the isolate (R2) reported decrease in the growth rate with an increase of the salt content. Similar results were described by (Mudryk and Donderski, 1991 ; Buckmire and Macleod, 1965) whose reported that an increase of the salt content results in a decrease in the metabolic activity of the moderately halophilic bacteria. Since the salt concentration inside halophilic bacteria is normally lower than that of habitat, the rate of normal metabolism may be lower in the presence of high extreme salt concentrations, because a great deal of energy must be used to maintain an appropriate salt balance between the interior and external environment. However, high salt concentration can cause disturbance in the metabolism of halophilic bacteria under some circumstances due to inhibition of particular enzymes.

Table (1): The effect of different concentrations of sodium chloride on the growth of actinobacteria isolates by estimating the dry weight of the isolates grown on starch-nitrate agar at 40°C for 14 days.

Isolate	The average amount of growth Dry weight (gm/100 ml) ± Standard error				
	10%	15%	20%	25%	30%
G1	0.395 ± 1.3	0.645 ± 1.1	0.648 ± 0.6	0.748 ± 1.5	-
X3	0.444 ± 1.1	0.547 ± 2.6	0.692 ± 1.2	0.743 ± 1.4	-
K2	0.505 ± 0.2	0.649 ± 0.3	0.652 ± 2.6	-	-
R2	0.571 ± 1.2	0.525 ± 0.1	0.350 ± 1.2	-	-
T4	0.452 ± 2.3	0.614 ± 0.2	0.647 ± 2.8	0.726 ± 2.9	0.812 ± 1.6
U1	0.549 ± 2.4	0.674 ± 1.9	0.725 ± 3.5	0.844 ± 2.4	0.928 ± 3.3

The results also revealed the ability of the isolates to grow at different concentration of sodium chloride considering that the isolate (T4-U1) showed their ability to grow until (30%), whereas the isolates (G1-X3) can grow until (25%), and the isolates (K2-R2) until (20%), Which indicates that there are extents appropriate for salt tolerance among the isolates. It is evident from this study according to the definition of halophiles that proposed by (Kushner, 1978) that the isolates (G1-X3-T4-U1) were an extremely halophilic actinobacteria, whereas the isolates (K2-R2) were moderate halophilic.

3.2 Temperatures range for growth

The study was considered to test the ability of the isolates to grow at different temperatures and the effect of temperature on the growth of actinobacteria isolates, as the needs of salinity and the ability to tolerate it based on some important conditions for development such as temperature and food components of the media. The results in table (2) revealed the ability of the isolates (G1-K2-R2-U1) to grow at (50°C) with limitation the highest growth rate of all isolates at (40°C) with the exception for the isolate (K2) which recorded the highest growth rate at (45°C). The optimum growth temperature of the isolates was 40°C. No growth occurred at 55°C and the upper limit of growth temperature was 50°C, which indicates that the isolates in this study are a moderate thermophilic. This finding is in agreement with that in Hochstein report (1988) who suggested that most halophiles isolates come from warm climates and many of them can be considered (moderate thermophiles). Though halophilic bacteria are usually grown at 37°C, their optimal temperature for growth is probably higher; with values of up to 50°C for this have been reported.

3.3 pH range of Growth

The results illustrated in table (3) showed that all tested strains, were able to grow in the pH range of 5.0-10.0, with an optimal pH at 6.0-7.0. This result agree with other studies reporting that most halophiles and halotolerant microorganisms isolated to date are neutrophiles, growing best in media with pH from 6.8 to 7.5 (Caton et al., 2004 ; Jothi Basu et al., 2015).

Table (2): The effect of different temperature on the growth of actinobacteria isolates by estimating the dry weight of the isolates grown on starch-nitrate agar for 14 days.

Isolate	The average amount of growth Dry weight (gm/100 ml) ± standard error				
	30°C	35°C	40°C	45°C	50°C
G1	0.316 ± 4.0	0.322 ± 2.1	0.395 ± 3.1	0.337 ± 0.9	0.321 ± 0.5
X3	0.302 ± 0.8	0.320 ± 1.4	0.465 ± 1.5	0.444 ± 1.6	-
K2	0.260 ± 0.1	0.277 ± 0.9	0.505 ± 2.3	0.611 ± 1.2	0.495 ± 0.8
R2	0.276 ± 1.3	0.278 ± 2.5	0.571 ± 0.8	0.545 ± 0.5	0.440 ± 1.2
T4	0.322 ± 0.9	0.282 ± 2.2	0.460 ± 0.2.2	0.442 ± 1.4	-
U1	0.303 ± 1.1	0.315 ± 0.3.1	0.549 ± 1.9	0.514 ± 1.3	0.504 ± 1.2

Table (3): The effect of different pH on the growth of actinobacteria isolates by estimating the dry weight of the isolates grown on starch-nitrate agar at 40° C for 21 days.

Isolate	The average diameter of the colony (mm) at graded concentrations of hydrogen ion					
	5	6	7	8	9	10
G1	22	31	30	30	30	29
X3	23	31	30	30	30	29
K2	18	19	22	18	18	17
R2	16	16	17	15	14	14
T4	-	12	14	13	12	11
U1	19	20	18	18	17	16

3.4 Carbon substrate utilization

The ability of the isolates to assimilate (9) different carbon sources is presented in table (4). The isolates assimilated well most of the tested carbohydrate as sole carbon source for growth, suggests a wide range pattern of carbon sources assimilation. This finding agree with what was found by (Litzner et al., 2006 ; Ventosa et al., 1982) that halophilic actinobacteria have wide metabolic abilities than actinobacteria, thus, many carbon sources are suitable. All the isolates assimilated the glucose, fructose, and starch efficiently. Similar results were described by (Burgess and Raw, 1967). As for the other carbon sources it has been recorded significant variation between the isolates in their ability for consuming.

3.5 Identification of actinobacteria isolates

A total of 36 strain of halophilic actinobacteria were isolated from (28) soil samples collected from different coastal salt-marsh sediments of Jeddah province. Representative (6) strains were selected for further extending study based on the ability of the isolates to tolerate high concentration of salts.

Molecular sequence analysis (500 pb 16S rDNA gene sequence analysis) were used for identification the isolates which are selected for the study. Preliminary comparison of the sequences against the EMPL data base indicated that the isolate (G1-X3-K2-R2) were closely related to the member of the genus *Nocardiopsis*, were the isolates (T4-U1) were most closely related to the genus *Actinopolyspora*. The isolates (G1-X3-K2-R2-T4) had been identified to the species level, were the isolate (U1) were identified only to the genus level. The isolate (G1-X3) belong to the species *Nocardiopsis halophila*, were the isolate (K2-R2) belong to the species *N. rosea*. For the isolate (T4) it is belong to the species *Actinopolyspora salina*. While the isolate (U1) considering as a new species of the genus *Actinopolyspora*. It showed from the results of identification that most actinobacteria isolates from Jeddah saline soil were extremely halophilic.

3.6 (SGY) medium compositions

The results of carbon substrates utilization in this study showed that the best carbon sources for the isolates were starch and glucose. These results agree with other studies reporting that **Glucose** is the most common simple carbohydrate added to

complex hyper saline media which is used as energy and carbon source for most organisms and most bacteria do best when glucose is provided as the primary energy source, because they many not able to digest other carbohydrate (Schneegurt, 2012). Accordingly, glucose was induced at (5g/l).

Starch was also found to be essential for the growth of halophilic actinobacteria (Kushner, 1993) and was included at high concentration (10g/l) in the medium, as secondary carbon sources. **Yeast extract** was also included at (5g /l) in the medium, considering that it is the most popular organic ingredients used in hyper saline medium, for providing vitamins and other growth factors to stimulate growth of most halophilic bacteria (Kauri et al., 1990 ; Schneegurt, 2012).

It is noteworthy that the predominant salt in hyper saline media is nearly always NaCl, but, in fact, NaCl is not the only salt found in high salt environments such as saline soils and seawater. For example, most saline soils also have high concentrations of Na^+ , K^+ , Mg^{2+} and Ca^{2+} . Therefore, studies of halophilic actinobacteria focusing only on NaCl concentrations are inadequate for gaining an overall understanding of these microorganisms (Tang et al., 2002). For some media, NaCl is supplemented with other salts in an effort to mimic the composition of concentrated sea water. Therefore, we supported SGY medium with **artificial sea water** which consists of MgCl_2 , MgSO_4 , CaCl_2 , KCl , FeSO_4 , According to (Smibert and Krieg, 1994). The fact that adding mixture of salt including Na^+ , K^+ , Mg^{2+} , Ca^{2+} , SO_4^- at 15 to 25 % total concentration are necessary for selective isolation of halophilic actinobacteria was pointed out by (Jiang et al., 2013).

3.7 Evaluation of (SGY) medium

The results in this study revealed that the components in (SGY) medium were enhanced and stimulate the growth of halophilic actinobacteria under high salinity conditions at short period of time (4-6 days) with high amount of growth Fig. (1), Compared to other media (Inorganic salt-starch agar, Glycerol- asparagin agar, Oat meal agar and Yeast extract -malt extract agar) which extended for (2-3 weeks) Figures (2-3-4-5). These results agree with (Kushner, 1993) who reported that for practical purpose, the growth medium for halophilic bacteria should be defined as rich or more accurately, the medium that supports the most rapid growth under optimal salt concentration.

Table (4): The ability of halophilic actinobacteria isolates to consume different carbon sources

Carbon Source	Growth rate of halophilic actinobacteria					
	G1	X3	K2	R2	T4	U1
Cellulose	++	++	±	±	++	++
Fructose	++	++	+	+	++	++
Glucose	++	++	++	++	++	++
Galactose	±	±	±	±	++	±
Lactose	-	-	±	±	+	-
Maltose	±	±	+	+	+	+
Starch	+	+	++	++	++	++
Sucrose	-	-	±	±	±	++
Xylose	+	+	±	±	+	++

(++): Heavy growth, (+): Moderate growth, (±): Weak growth, (-): No growth

**Figure 1.** Cultural characteristics of the isolate (K2) grown on (SGY) medium for 5 days at 40°C.**Figure 2.** Cultural characteristics of the isolate (K2) grown on (Inorganic salt starch agar) medium supplemented with 10 % NaCl for 15 days at 40° C.**Figure 3.** Cultural characteristics of the isolate (K2) grown on (Glycerol-asparagin agar) medium supplemented with 10 % NaCl for 15 days at 40° C.**Figure 4.** Cultural characteristics of the isolate (K2) grown on (Oatmeal agar) medium supplemented with 10 % NaCl for 15 days at 40° C.**Figure 5.** Cultural characteristics of the isolate (K2) grown on (Yeast extract- malt extract agar) medium supplemented with 10 % NaCl for 15 days at 40° C.

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