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Research Paper

Investigation of Effects of NaCl Concentration on the Moderately Halophilic Bacteria (MHB) and its Growth

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Abstract

Moderately halophilic bacteria (MHB) form a heterogeneous group of microorganisms and can grow in a relatively wide range of sodium chloride concentrations. These bacteria accumulate high concentrations of osmotic compatible organic compounds (osmolytes) to create osmotic balance. The MHB were isolated by sampling from Persian Gulf water and various parts of a leather factory. The specimens were enriched in a culture medium specific to halophiles and the bacteria were isolated and purified using streak plate method. The effects of different NaCl concentrations and the presence or absence of various salts on their growth were examined through turbidimetry and ELISA reader. A total of eight bacterial strains were isolated from the factory and eight strains from the Persian Gulf, and subjected to extensive phenotypic and biotypic studies. Results of halophilic status of isolated bacteria showed that 10 isolates were halophiles. Moreover, the morphology and the Gram reaction affected the halophilic status and growth of isolated bacteria at various NaCl concentrations. The presence of halophilic-specific salts in the culture medium could improve their growth of isolated bacteria.

Keywords: Moderately Halophilic Bacteria; NaCl; Salts; Turbidimetry

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1. Introduction

Halophilic bacteria are a group of extremophiles that can grow in media containing NaCl and have adapted to survive in saline environments (6,7). These bacteria are divide into two groups of halophiles and halotolerant species. Microorganisms that grow optimally in salt-free media or at slat concentrations lower that of seawater but tolerate relatively high salt concentrations are called halotolerants (13). Halophilic microorganisms cannot grow in salt-free media, grow at different salinity ranges, and have growth optima in media with relatively high salinities. The main difference between halophiles and halotolerants is that, although the growth range of the former at different salinities can be lower or higher than that of the latter, growth optima occur at concentrations greater that seawater salinity (> 3.5%). The need for salt and tolerability of high salt concentrations are a common trait among all MHB species, which vary widely among different species, and halophility depends on such environmental conditions as temperature and culture media composition. Instead of NaCl, some halophiles can grow at high concentrations of alternative salts including $RbCl_2$, CsCl, KCl, $MgCl_2$, etc. Halophilic and halotolerant organisms apply diverse and multiple adaptive mechanisms to maintain intracellular osmotic balance with the external environment, and are generally divided into KCl group and osmotic compatible organic compounds or osmolytes (4, 5, 20). In this study, MHB were isolated from various parts (wastewater, salted skin, and salted gut) of a leather factory and Persian Gulf water. The effects of different NaCl concentrations and the presence/absence of various salts were examined on their growth.

2. Materials and methods

2.1. Bacterial strains and growth conditions

MHB were isolated from various parts of a leather factory and Persian Gulf water. The samples were cultured in a halophile specific and enriching medium containing nutrient broth with a total salt concentration of 71 g L^{-1}. One L of saline solution contained CaCl (0.36 g), KCl (51 g), $MgSO_4$ (9.6 g), $MgCl_2$ (7 g), and NaBr (0.026 g). The medium pH was adjusted to 7.4 using 1 M KOH, and the media were incubated in a shaker with 130 rpm at 34 °C for 24-48 h (1,16). When necessary, nutrient agar medium with the above salt concentration was used for the solid medium.

2.2. Identification of isolated bacteria

The bacteria were identified through macroscopic (colony color) and microscopic (morphology and Gram reaction) characteristics as well as biochemical tests. The strains were identified morphologically on the halophile-specific media with 10% of NaCl (1,16). The Gram staining outcomes were confirmed by KOH test (9), followed by examination of motility, catalase, oxidase, nitrate reduction, carbon hydrate fermentation and acid production, and hydrolysis of gelatin and starch (1, 3, 10). All the tests were experimented using a medium containing 10% (w/v) of NaCl. Besides phenotypic and biotypic studies, Gram-negative bacilli capable of growing in a wide range of NaCl concentrations were identified with a primer pair (ectoine) specific to *Halomonas* bacteria (21). A fragment of 277 base pair long was amplified by these primers. The sequences of the primers are as follows:

Forward ectoine primer: 5'-GGTAAYTGGGAYAGYACRC-3'

Reverse ectoine primer: 5'-GBGGHGTRAAKACRCADCC-3'

y=C or T (pyrimidine) H=A, C or T

K=G or T (keto)

R=A or G (purine) B= C, G or T

D=A, G or T (keto)

2.3. Effects of different NaCl concentrations on the isolated MHB growth and optimum salt concentration for their growth

The isolated strains were cultured at different NaCl concentrations and their mean growth rates wear compared in four replications. This test was done by turbidimetry and ELISA reader (8). Nutrient broth medium was used with different NaCl concentrations (0, 5, 10, 15, 20, and 25%), and, if necessary, the medium pH was adjusted to 7.4 using HCl or 1 M KOH. Microplates were incubated at 34 C for 20-24 h. Then, the optical absorption of the wells was read at 630 nm with an ELISA reader to verify the growth of bacteria (19,20). To prepare bacterial dilutions, a medium with the same salt concentration was used to prevent changes in the experimental salt concentrations.

2.4. Effects of various salts and different NaCl concentrations on the isolated MHB growth and optimum salt concentrations for their growth

This was exactly performed the same as Stage 3, with the difference that instead of nutrient broth, a specific and enriching medium was used with different NaCl concentrations (8,20).

Figure 1. Biochemical characteristics of the bacterial strains isolated in this research

Morphology and Gram reaction	Test / Bacterium	Motility	Pigment	Oxidase	Nitrate	Arabinose	Glucose	Lactose	Trehalose	Manitol	Starch hydrolysis	Gelatin hydrolysis	Salt tolerability
Gram negative bacilli	H30	-	Creamy	+	+	-	-	-	-	-	-	-	0.5-25
	H34	-	Creamy	+	+	-	+	-	+	-	-	-	0.5-25
	H51	+	Creamy	+	+	-	+	-	+	+	-	-	0-25
	H54	+	Creamy	+	+	-	+	-	+	+	-	-	0.5-25
	H59	+	Creamy	+	+	-	+	+	-	+	-	-	0.5-25
	H60	+	Creamy	+	+	+	+	+	+	+	+	-	0.5-25
	H61	-	Creamy	+	-	-	-	-	-	-	-	+	0.5-25
	H62	+	Creamy	+	+	+	+	-	-	-	+	-	0.5-25
	H2d	-	Creamy	+	+	+	+	-	-	-	-	-	0-25
	H8	-	Creamy	+	+	-	+	-	+	-	-	-	0.5-32
Gram positive bacilli	Ar1	+	-	+	+	-	-	-	-	-	-	-	0.5-20
	HB1	-	Creamy	+	+	-	-	-	-	-	+	-	0.5-20
	B1	+	Yellow	+	+	-	-	+	+	-	-	-	0 -20
Gram positive cocci	MA1	+	-	+	+	-	-	-	-	-	+	+	0 -20
	MH1	+	Orange	+	+	-	-	-	-	-	-	-	0.5-30
	NH1	-	-	+	-	+	+	-	-	-	-	+	0.5-32

*NaCl tolerability range is in %.

Gel electrophoresis of PCR products from H29, H30, H34, H46, H51, H54, H59, H60, H61, H62, and H2d strains using ectoin primers. L: ladder, (−): negative control (distilled water), (+): positive control (*Halomonas salina* ATCC 49509)

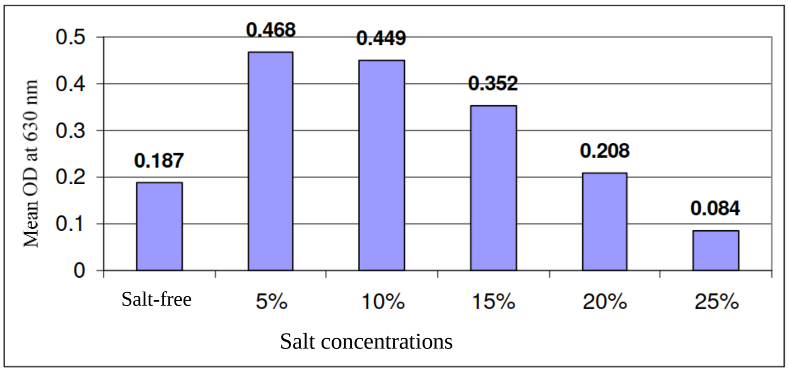

Figure 2. Average bacterial growth at optimal temperature and pH, and different NaCl concentrations

3. Results

A total of eight bacterial strains were isolated from the leather factory and eight strains from the Persian Gulf, and subjected to extensive phenotypic and biotypic studies (Table 1). In addition to phenotypic and biotypic studies, Gram-negative bacilli capable of growing in a wide range of NaCl concentrations were identified with a primer pair (ectoine) specific to *Halomonas* bacteria (Figure 1). A total of four (50%) and six (75%) samples of halophilic bacteria were isolated from the Persion Gulf water and the leather factory, respectively. In the presence of different NaCl concentrations, the bacterial growth rates were 0.187 ± 0.024 and 0.468 ± 0.034 in the salt-free and 5% saline media, respectively. Results of bacterial growth at the other salt concentrations are shown in Figure 2.

The results showed that NaCl could be replaced by the salts used in the halophilic-specific media and affect the growth of isolated bacteria. Some of the halophiles (H34) that could not grow in NaCl-free nutrient agar medium were able to grow in the specific, enriching, and NaCl-free medium. Some other strains, however, required the presence of NaCl, which could not be replaced with the other salts in the medium. Some of the halophiles (H30), which did not grow in the NaCl-free nutrient agar medium, faild to grow in the specific, enriching, and NaCl-free medium as well. Average growth rates of the examined bacteria were almost similar at different salt concentrations with/without the other salts (Figure 3).

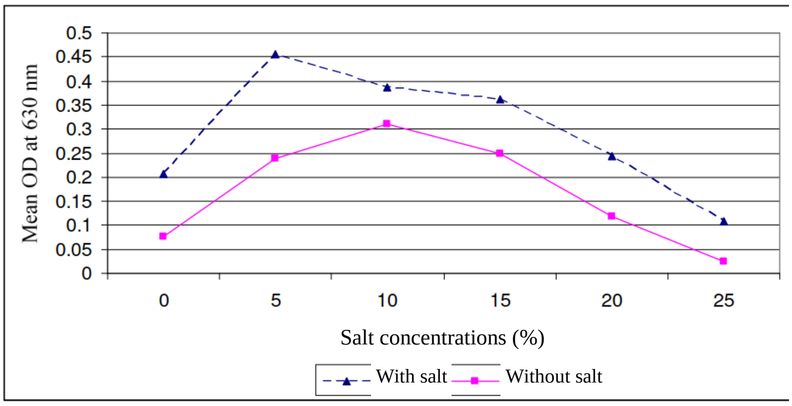

Figure 3. Changes in bacterial growth at optimal temperature and pH, and different NaCl concentrations with or without the other salts

This research also investigated the effects of morphology and Gram reaction on the isolated halophilic bacteria, of 13 bacilli, 10 and 3 strains were halophiles and halotolerants, respectively. All the three cocci were halotolerants. Of 11 Gram negative strains, 9 and 2 strains were halophiles and halotolerants, respectively. And of 5 Gram positive strains, 1 and 4 strains were halophiles and halotolerants, respectively.

The present results also demonstrate that the morphology (Figure 4) and Gram reaction (Figure 5) affect the growth of isolated bacteria at different NaCl concentrations.

As shown in Figure 4, the two bacterial groups have different mean growth rates at various salt concentrations, but grew similarly at a concentration of 25%.

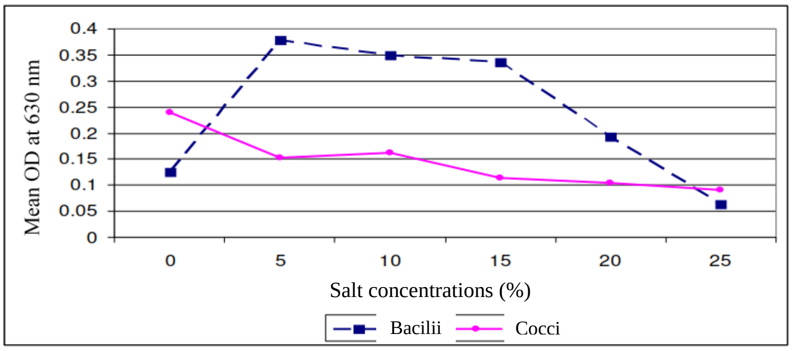

Figure 4. Mean bacilli and cocci growth rates at optimal temperature and pH, and different NaCl concentrations.

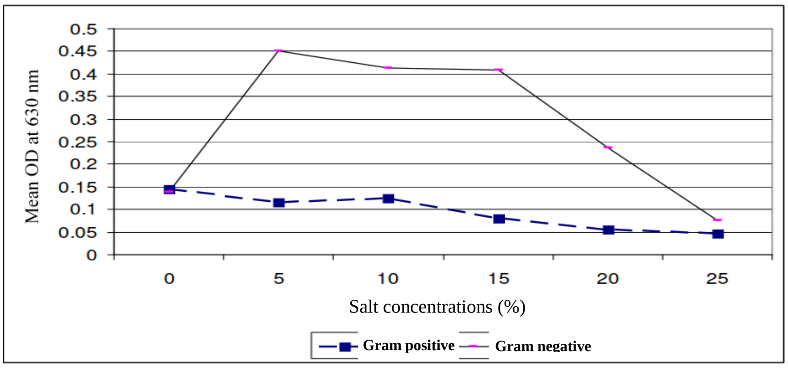

Figure 5. Mean growth rates of Gram negative and Gram positive bacteria at optimal temperature and pH, and different NaCl concentrations.

Figure 5 represents different growth rates of Gram negative and Gram positive bacteria at various NaCl concentrations. Both types of bacteria grew differently at salt concentrations of 5-20%, but had almost similar growth rates in the salt-free and 25% salt media.

4. Discussion and conclusion

Moderately halophile bacteria are of a high potential for biotechnology and nanotechnology. Many of these bacteria not only produce industrial compounds such as enzymes, polymers, pigments, and so on, but also have specific physiological traits facilitating their exploitation (18,19). A variety of environmental factors, in particular NaCl concentration and the presence of different cations and anions, influence considerably on the growth of halophiles. Therefore, the effects of above factors were examined in here to optimize their growth conditions in terms of culture media composition (11, 15, 19, 20).

The majority of our isolated bacteria were Gram negative and mostly belonged to *Halomonas*. There were only two Gram positive bacilli and three Gram positive cocci among the isolated bacteria. As mentioned above, 10 and 6 strains of the isolates were halophiles and halotolerants. It is noteworthy that NaCl could be replaced by the other salts in some of the isolated halophiles, but NaCl was necessary in some other strains and NaCl could not be replaced by the other salts.

According to Ventosa (1998), some halophiles can grow at high concentrations of replaced salts (e.g., MgCl2, KCl, CsCl, etc.) in the absence of NaCl. However, NaCl can partially be replaced by other soluble salts (e.g., KCl) in some halophilic bacteria (19,20). In addition, since most of laboratory salts contain little amounts of NaCl, it will be very difficult to prove that microorganisms do not need NaCl and that it can be replaced by other salts (20).

NaCl concentrations of 5% or 10% were found as optimal levels for the bacterial growth (Figure 2). ANOVA results revealed significant differences between bacterial growth rates at various NaCl concentrations ($P < 0.001$). The isolated bacteria had different mean growth rates in the presence or absence of NaCl. Halophiles presented the highest growth rates at optimal NaCl concentrations, and increasing concentrations higher than the optima lead to reduced substrate decomposition, increased reproduction time, and slow growth. Accordingly, the bacterial growth decreased markedly at 25% salt concentration.

Mean growth rates of the isolated bacteria (Figure 3) at various NaCl concentrations with or without the other salts indicated that bacterial growth rates were different at optimal NaCl concentrations with the presence (5%) and absence (10%) of the salts. Predictive ANOVA demonstrated significantly different bacteria growth at various NaCl concentrations with the presence of the salts ($P = 0.02$). Similarly, Ventosa (1898) reported that the presence of salts in media specific to halophiles resulted in improved bacterial growth rates (9).

The presence of different salts (e.g., $MgCl_2$, $MgSO_4$, $NaHCO_3$, KCl, $CaCl_2$, and NaBr) in saline environments and in media specific to halophiles implies the impacts of these cations and anions on the growth of such microorganisms. In addition to partial involvement in adjusting osmotic pressure, potassium ions are needed for the stability and activity of various enzymes including 2,4-diaminobutirate aminotransferase. The enzyme involves in the biosynthesis of ectoine, which is one the most important osmolites for maintaining osmotic pressure and adapting salinity stress (2, 13). In addition to storing osmolites to maintain osmotic pressure, some MHB accumulate relatively high concentrations of Cl^-, K^+, and Na^+. Of these, K^+ is preferable due to partial inhibitory effects on enzyme activities or even activation of enzymes (e.g., α-amylase) the ribosomal enzymatic system of halophiles. Ca^{2+} and Mg^{2+} stimulates the activity of a halophile nuclease isolated from *Bacillus halophylus*. Mg^{2+} concentration is vital for the growth of extreme halophiles, the growth of which is not supported at low Mg^{2+} concentrations. The presence of $MgCl_2$ stabilizes the membrane and even influences the maintenance of respiratory chain. Bi- and polyvalent cations form bridges between adjacent negative charges and are, therefore, more effective than monovalent ions in neutralizing the surface charges of polymers. Bivalent cations contribute to the formation of complexes. Magnesium included in halophiles can be replaced by a high NaCl concentration, hence optimum growth in a salt-free medium occurs at a higher salt concentration (10%) than a salt-containing (5%) medium (6, 12, 17, 19).

The results of frequency distribution of halophilic bacteria based on the morphology and Gram reaction indicated significant differences in terms of morphology ($P = 0.036$) and Gram staining ($P = 0.036$) according to Fishers' test. The morphology and Gram reaction of the bacteria affect their halophile status.

As reported by Ventosa (1898), most MHB were Gram-negative bacilli, most halotolerants were Gram-positive, and Gram-positive cocci were mostly partial or moderate halotolerants (19), which corresponds to our observations.

Based on the obtained results, mean growth rate of bacilli was uppermost at 5% salt concentration. The highest growth rate of cocci occurred in the salt-free medium (Figure 4). According to the figure, mean bacterial growth rates were different in the two groups at various salt concentrations, but both groups had almost similar growth rates at a concentration of 25%. ANOVA results revealed that mean bacterial growth rates were significantly different in terms of morphology at various salt concentrations ($P = 0.001$) suggesting a significant interaction between salt concentrations and bacterial morphology.

The highest growth rates of Gram positive bacterial were recorded in the salt-free medium and the lowest rates belonged to those grown at 25% salt concentration. A salt concentration of 5% was optimal for the growth of Gram negative bacteria (Figure 5). As shown in the figure, mean bacterial growth rates were different in the two groups at salt concentrations of5-20%, but both groups had almost similar growth rates at 25% salt concentration and in salt-free media. According to ANOVA, the status of bacteria concerning Gram staining was an affecting factor in the growth of bacteria at different salt concentrations. In other words, there is a significant interaction between salt concentrations and the Gram status of the bacteria ($P < 0.001$). Ventosa (1898) reported that most MHB bacilli were Gram negative and most halotolerants were Gram-positive (19), as was also detected in our samples.

Changes in NaCl concentrations of culture media lead to altered lipid content of cell membrane. Increasing salt concentrations elevates the amounts of negative relative to neutral phospholipids. For example, levels of neutral phosphatidil ethanolamine decrease at higher salinity and, instead, negative phosphatidil ethanolamine or diphosphatidil glycerol (kardiolipin) shows an elevation. In fact, increasing salt concentration results in elevated negative charge on the membrane. Cell membrane in Gram negative facultative anaerobic MHB consists of phosphatidil ethanolamine and phosphatidil glycerol that together comprise 90% of lipids with little amounts of kardiolipin, glycolipid, and other lipids. The ratio of phosphatidil ethanolamine to phosphatidil glycerol drops with rising salinity. In Gram positive species, an increase in anionic lipids is mostly related to the rise of kardiolipin, which reduces the growth of cells and growth rates decline at a high salt concentration. Glyco(phospho)lipids are rarely found in Gram negatives but are often detected in Gram positives (14, 19, 20). In other words, increasing kardiolipin levels reduce the growth rates of Gram positives.

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