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The Interactive Effect of Sodium Chloride and Diatomaceous Earth (DE) on *Bacillus aquimaris*

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The growth of *Bacillus aquimaris* was inhibited from 6 - 20 % of NaCl but it showed some tolerance when Diatomaceous earth (DE) added from 2 - 12% NaCl. Concerning the effect of NaCl on polyol production, we can conclude that, the test bacterium showed some tolerate to NaCl by producing glycerol up to 8 % of NaCl. Then decreased sharply. The addition of DE decrease the amount of polyol and glycerol remarkably and this due to the productive effect of DE to the bacterial cells. The SEM figures represented the presence of electron dense bodies due to the accumulation of small particles of DE as protective molecules.

Key words: *Bacillus Aquimaris*, Diatomaceous earth (DE), Osmoticstress.

In plants and mammalian cells, osmoregulation (adaption to osmotic stress) is mediated through low- molecular weight organic compounds like glycine, proline, glycerin, betanie, proline[26]. These molecules accumulate and transported from cells during osmotic stress and balance the osmotic stress of the cytoplasm with environment, thus reducing damage due to dehydrationas previously explained by[6,36]. Halophilic and halotollerant bacteria accumulate high levels of betanie[16]. And an increase in the survival of *E. coli* in sea water is accompanied by the synthesis of glycine and betanie[28]. Schobert[30] has suggested that the main function of the polyols is specific rather than osmotic, the –OH groups of the polyols may participate in the water structure and preserve the hydration of the biopolymers in the cell. Organisms that thrive under conditions of decreased water availability must be able to adapt to the environmental osmotic pressure to prevent water movement across the membrane[9]. This is achieved through the accumulation of osmotically active compounds inside the cell. The extremely halophilic bacterium *Ectothiorhodospira halochloris* (at 20 % salinity) was isolated from alkaline sodium chloride sulphate lake at Wad Natrun, Egypt[22]. The moderately halophilic bacteria need to maintain an osmotic equilibrium between outside and inside of the cells. They can achieve osmotic balance by the accumulation of salts and/or organic molecules[34]. Among this heterogenous group of bacteria, *T. halophila* can tolerate high salt concentrations, which suggests that it has a high osmotic adjustment capacity in order to maintain positive cell turgor. Osmoregulation has been studied extensively in nonhalophilic bacteria such as *Escherichia coli*, *Sinorhizobium meliloti*, and *Bacillus subtilis*[24, 32]. Both halophilic and nonhalophilic bacteria have evolved mechanisms that enable them to adapt to high salinity. They protect themselves against deleterious

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hyperosmotic injury by the uptake or synthesis of a limited number of compounds, termed compatible solutes[24]. *Bacillus aquimaris* was used in this study, which is a moderately halophilic or halotolerant, Gram-positive or-variable, endospore-forming rods have been commonly isolated from marine environments and related regions or materials[37]. This bacteria were isolated from a tidal flat of the Yellow Sea in Korea, colonies are circular to slightly irregular, slightly raised, pale orange-yellow in colour and 2–4 mm in diameter after 3 days at 30ºC on nutrient agar (NA). Optimal growth temperature is 30–37 ºC. Growth occurs at 10 and 44 ºC, but not at 4 or above 45 ºC. Optimal growth occurs in the presence of 2 – 5 % (w/v) NaCl. Growth is poor in the absence of NaCl, but occurs in the presence of up to 18 % (w/v) NaCl. Growth does not occur under anaerobic conditions on NA.

The term 'Diatomaceous earth' (DE) refers to a sedimentary rock those results from the deposition of silica-rich unicellular life forms known as 'diatoms'. Other names for diatomaceous earth include Diatomite, Tripoli and Industrial earth. Diatoms are aquatic algae, and hence DE forms in water, both salt and fresh[31]. Currently freshwater lake beds are a source of DE. The cell walls of these dead diatoms consist of amorphous silica ($SiO_2.H_2O$). The fossilized skeletal remains (a pair of symmetrical shells - frustules) vary in size but are typically 10 to 200 microns across and have a broad variety of shapes, from needles to discs or balls. DE usually light in colour and is highly porous and has a low density which varies. This gives it capacity to absorb large amounts of moisture from its surroundings. The typical composition of diatomaceous earth is 80 – 90 % silica in the amorphous form, plus various metal oxides. Free crystalline silica content (cristobalite) is an important consideration when sourcing DE products[5].

Despite these properties, many bacteria extending survival onto solid surfaces for their ability against predation, detrimental environmental conditions and nutrient availability[14]. For example, *Vibrio cholerae* strains CA401 and RV79 can be attached to celite (diatomaceous earth) under selective condition with NaCl ions. The highest rate of attachment occurred with DE including NaCl from 1 - 1.5 % (20). Other reports shown *Thiobacillus ferrooxidans* growth activity and ferrous ion oxidation were significantly enhanced by its adsorption onto diatomaceous earth. DE was increased the bacterial ferrous ion oxidation rate about 20 % [10-15] h shorter than that of the control (free cells), and growth rate was 1.2 times from 0.077 to 0.099 $h^{-1.}$ (23). Also biotreatment of wastewaters and bioremediation of contaminated soils is well known by microorganisms. The DE used to be a bacterial carrier and supported materials for the immobilization of bacteria. It was found that this carrier is able to be immobilized on *pseudomonas* sp strain M285 to remove 3, 5,6-trichloro-2-pyridinol from industrial wastewater. The ED as well, was used in-situ bioremediation of contaminated soil and ground water for treatment with nonpathogenic bacteria[13].

Accordingly, the aim of the present study was to investigate the diatomaceous earth ability on protection of *Bacillus aquimaris* growth rate in high concentrations of NaCl ions, and determination of osmoregulants like glycerol and polyhydroxy alcohols produced to explore how the bacterium can tolerate NaCl stresses. The isolate was selected based on a halophilic organism.

MATERIALS AND METHODS

The effect of Diatomaceous earth (DE) on *Bacillus aquimaris* (stock department, Biological Science Department, King Abdulaziz University, Jeddah, Saudi Arabia) was tested. The bacterium was inoculated into Erlenmeyer flasks (250 ml) containing 100 ml nutrient broth medium , amended with 1 gm Diatomaceous earth (Sigma 90% SiO2, United Kingdom) sterilized in a dry oven at 1600C for overnight. Salinity tolerance was tested by adding 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20% of NaCl to the medium. All flasks were incubated at 25 ºC under shaking at rmp for 24 and 48 hr , then the growth rate was measured at OD_{600} nm using spectrophotometer.

Oxygen measurement

Bacterial cells were separated by centrifugation, the supernatant was discarded and the bacterial pellet washed twice by the same medium, re-suspended in a small volume of the medium containing the appropriate concentration of NaCl and DE .Oxygen consumption was measured with a Clark-type electrode (Hansatech

Instrument, England) as described by (10). Temperature was maintained at 25 ºC.

Total Polyol and Glycerol determinations

The total polyol content was estimated by methods described by (Lambert and Neish,1950). Enzymatic glycerol determination was analyzed using a commercial enzyme combination[7] based on the method of Eggestein and Kuhlman[11]. Readings were made on Spectronic 2000 at 340 nm. Results were recorded as µmol glycerol per liter culture medium.

Scanning electron microscope (SEM) studies

For SEM studies the cutlers of *Bacillus aquimaris* were suspended in distal Water. A small drop of this suspension placed on the double side carbon tape on Al- Stub and dried in air. All samples were sputtered with a 15 nm thick gold layer (JEOL JFC- 1600 Auto Fine Coater). The specimens were examined with a scanning electron microscope Quanta FEG 450, FEI, Amsterdam, Netherland. The microscope was operated at an accelerating voltage of 20 kV. The specimens were analyzed without coating by using energy dispersive analyzer unit (EDAX, Apollo X) which attached the scanning electron microscope (Quanta FEG 450). Quantitative method ZAF, and characterization method as pure[21]

RESULT AND DISCUSSION

In the present study, the strain of *Bacillus aquimaris* was purified by visual, microscopic and cultivation methods and was maintained on the nutrient agar media, which was used for isolation, at 4ºC.

B. aquimaris was grown on nutrient broth at ten different NaCl concentrations (%), including 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 % for 24h, 48 h respectively. Addition of NaCl led to a decrease in bacterial growth and respiration rate beyond of 4% and above. However the growth rate of the *B. aquimaris* was inhibited from 6 to 20 % NaCl (Fig.

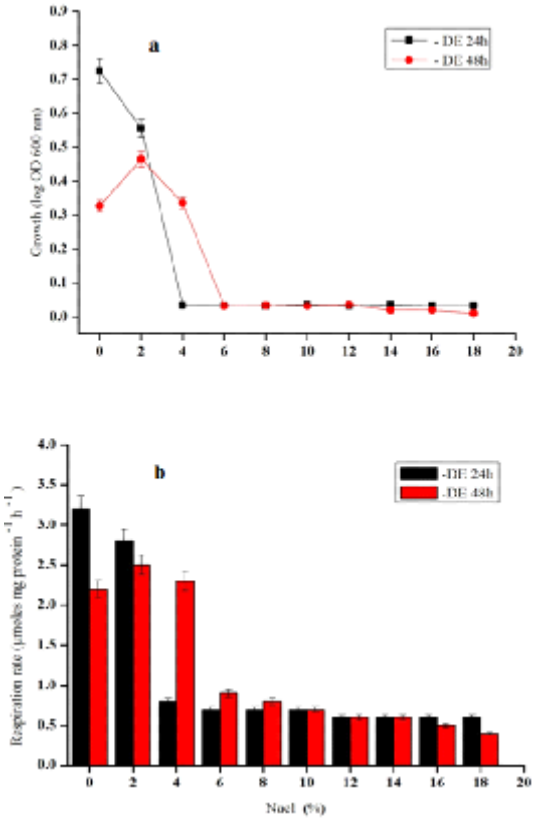

Fig. 1. Effect of different concentration of (%) NaCl on *Bacillus aquimaris* growth rate (a) and respiration rate (b) in the absence of DE.

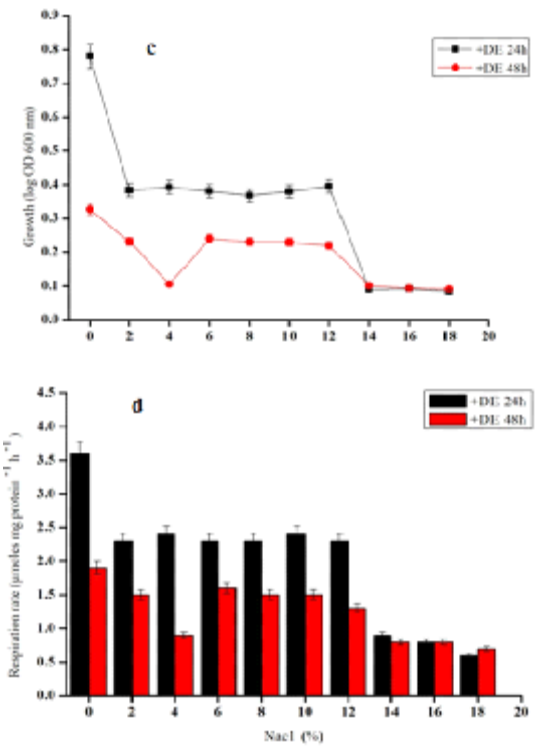

Fig. 2. Effect of different concentration of (%) NaCl on *Bacillus aquimaris* growth rate (c) and respiration rate (d) in the present of DE.

1, a, b). When DE was added under the same conditions, *B. aquimaris* can tolerate the salinity stress from 2 – 12 % then decline sharply thereafter. Maximum growth rates occur at concentration of NaCl ranging from 2 – 12 % with DE (Fig. 2, c, d). The respiration results are also more variable and do not show an increasing inhibitory effect in response to large salt stress except above 12 % of NaCl, these observations were in agreement with that shown by[17, 18].

The additional Diatomaceous earth showed a protective effect on the bacterial dells, particularly in the lower concentration (2 - 12 %) of NaCl (Fig. 2). DE particles have a porous structure, most of the pores are at the nano-scale. That is the reason why DE has such a high specific surface area. Only some particles have hollow inner structures, which can shelter the bacteria that may be absorbed inside. Most of the pores are only 0.1 – 0.5 µm, but the size of the bacteria is about 1 – 2 µm. Therefore, bacterial cells were mainly sorbed on the surface of the particles[35]. Diatomaceous earth was a kind of easily flowable suspension when using low concentrations of DE and the nutrient was distributed homogeneously in the pre-mixture of isolates. The nutrient mixture was incorporated inside the bacterial cells. Therefore, the bacteria could get more nutrients around them from the microenvironment in the flow able suspension[33]. Thus it is obvious from the present findings especially that at low salt concentration where diatomaceous earth play a vital role on the increase of growth and respiration rate for the isolate (Fig. 2, c, d).

Similar results were also showed by[4], who found that the organic and inorganic silicon compounds increased fungal growth in *Alternaria citri* within both in the cell wall and in the hyphae.

The total polyol and glycerol destinations tests were conducted basically to determine the capability of bacteria to still survive at salt stress. Results from these experiments indicate that polyol and glycerol levels rose in the culture medium with adding DE. (Fig. 3, a ,b) illustrates that *B. aquimaris* synthesis polyol and glycerol when exposed to salinity stress of NaCl and that increasing still to the maximum amount (217 and 112 µmol/l) respectively at 8 % of NaCl

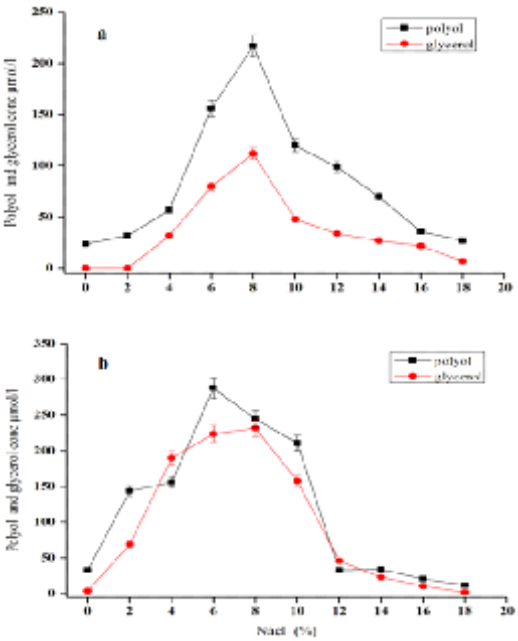

Fig. 3. Effect of different concentration of (%) NaCl on polyol and glycerol (µmol/l) production by *Bacillus aquimaris* (a) 24 h, (b) 48 h in nutrient broth medium in the absence of DE

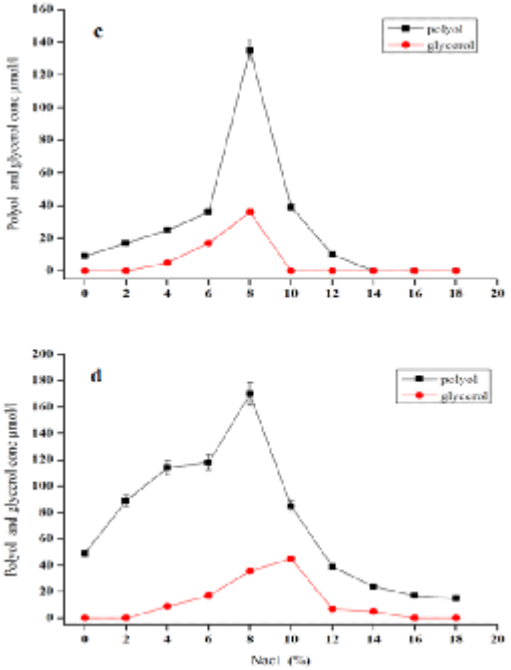

Fig. 4. Effect of different concentration of (%) NaCl on polyol and glycerol (µmol/l) production by *Bacillus aquimaris* (c) 24 h, (d) 48 h in nutrient broth medium amended by 1g DE

after 24 h of incubation. The amount of polyol and glycerol are decreased regularly at the second day of incubation and still at maximum production from 6 - 8 % then decrease sharply. These data confirm that polyol and glycerol were constitutes the most ismoregulant product. The data showed that growth rate of isolates of *B. aquimaris* gradually still survive with the supplementary of 1g DE reaching maxima up to 8 % NaCl, when the DE added to the medium, synthesized polyol and glycerol also take place in response to salt stress after 24 and 48 h of incubation (Fig. 4 c, d), but the amount of the productive compounds were less than that shown in Fig. 3 a, b in the case of NaCl stress only. These findings may attributed to the addition of DE, and confirm that DE utilized by *B. aquimaris* to protect bacterial cells from the salt effect.

Since high concentrations of salt are generally inhibitory to metabolic functions and various kinds of compensatory mechanisms are found among salt-tolerant organisms[15, 29]. This phenomenon had been previously reported in other microorganisms. Similar results demonstrated that *saccharomyces rouxii* and other yeasts accumulate glycerol when grown under salt stress[1,9,19]. It is

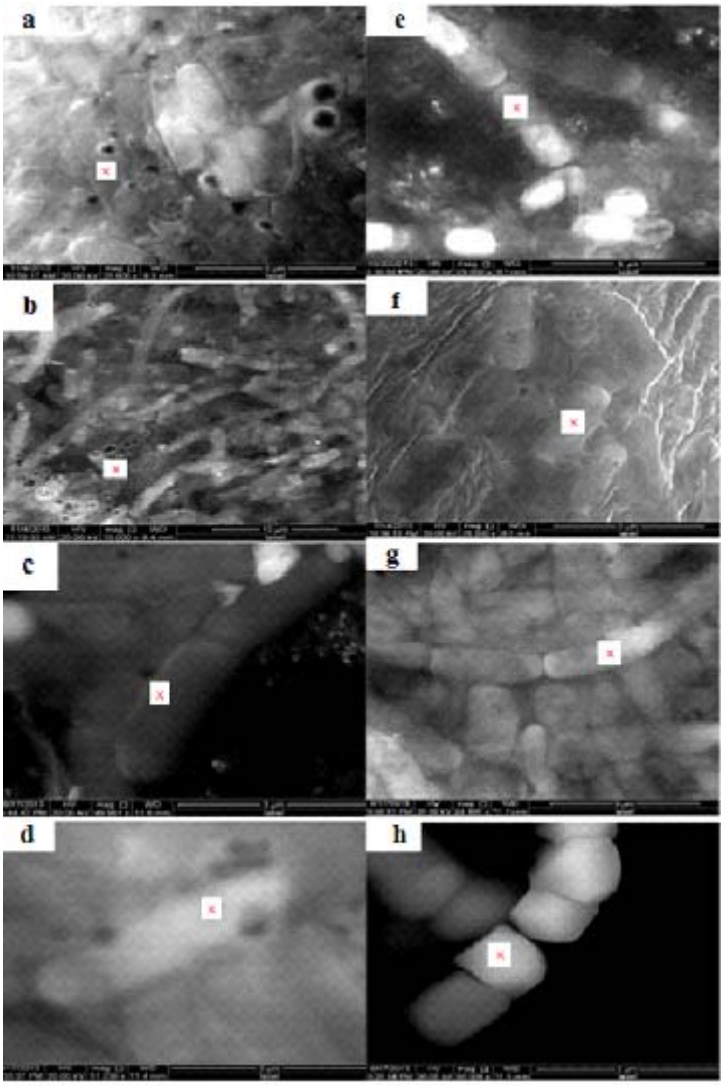

Fig. 5. SEM of *Bacillus aquimaris* growing in liquid medium with 2, 4, 12 and 18 % NaCl (a-d) in the presence of DE arrowed showed electron dense materials of DE, and (e-h) in the absence of DE (control).

also reported that the filamentous fungi *Eurotium amstelodami* strain 110-13 and *Asprgillus wentii* IMI 023010 proved to be a high glycerol producer and increased with the increase of NaCl concentration[12]. There are, however, indications that with increasing salt stress polyols accumulation by *Aspergillus niger* and *penicillium chrysogenum* were shifted towards the synthesis of lower polyols which may serve the purpose of minimizing enzyme inhibition in growing cells. The accumulation of mannitol by mycelia in low salt media may, on the other hand, serve to protect stress (2). A compatible role for glycerol has also been demonstrated for the salt –thriving algae of the genus *Dunaliella*[38]. Accumulation of intracellular glycerol enabled the algae to recover in part or whole process of photosynthesis, results from Gilmour *et al*,[17] indicate that the green algae *Dunaliella Tertiolecta* induced glycerol synthesis in response to increase concentrations of NaCl of the external medium, thus inhibited the overall process of photosynthesis in *Dunaliella*. Related outcomes were previously reported that Glycerol and other polyols are poor inhibitors of enzyme function even at very high concentrations[3,8]. Brown[8] also found that enzyme inhibition by polyol decreased with decreasing number of hydroxyl groups of the polyol.

It is clear that from Fig.5 (a - d) *B. aquimaris* was found to accumulate electron dense bodies located inside the cell (only in the presence of DE). This change in morphology and growing cell culture were found better and associated with DE uptake by *B. aquimaris*. EDAX analysis revealed that these bodies contained large amounts of element silicon including other elements (data not shown), compared with the control (in the absence of DE) Fig.5 (e - h). The SEM and EDAX data on the chemical composition of the electron dense bodies from *B. aquimaris* were similar to those reported by (4).

It could be argued however, that the observed growth of *B. aquimaris* in medium containing a high concentration of NaCl with Diatomaceous earth was due to the ability of this bacterium to adsorb particles to their surface. As mentioned before in the introduction, Diatomaceous earth as natural compounds being used as attachment and adsorption substrate for some bacteria[20,23], as well as a good support materials for the immobilization of bacteria[27]. Finally we can conclude that the growth and respiration activity of *Bacillus aquimaris* was still tolerate the low (%) concentrations of NaCl up to 12 % with Diatomaceous earth, as well as growth curves for *B. aquimaris* growing with DE indicate that there was no evidence that the DE prevented the bacterial growth and suggested this may protect bacteria from the high concentrations of NaCl. This finding is valuable for some industrial and biotechnological process, which may have potential in commercial applications. Further researches are needed to find out the mechanism involved in the bacteria attached to diatomaceous earth and its effect on the growth and metabolic activities of such organisms grown in halophilic environments.

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