

Isolation and Characterization of Salt-Tolerant Bacteria for Aquaculture Wastewater Treatment

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ABSTRACT

The water at the coastal fishing port shows high amount of COD due to the seafood exploitation and processing activities. The biological treatments are facing a challenge that is high salinity from 3 to 30 g/L of the water because the high saline concentration could affect the growth of microbes commonly used in the treatment system. Thus, the treatment will become more effective in the presence of salt-tolerant microbes. In this study, total 45 species were isolated from seafood processing effluents in Tien Giang province, Vietnam, and cultured in BMS medium at 30% and pH 7.0. The MALDI-TOF method was used to identify the isolated strains. As a result, the strains were characterized as *Micrococcus luteus*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus subtilis*, *Arthrobacter creatinolyticus* and *Lactobacillus pentosus* with the similarity coefficients from 1.52. They were selected based on the highest tolerance in the range of 1 – 7 % salinity to investigate their COD removal. The highest yield of COD removal was over 90 % at the ratio of 1:5 (v/v) of bacteria to wastewater after 96-hour culture in the aerobic bioreactor. In conclusion, the results highlighted the potential applications of these species in the biological treatment of seafood processing wastewater.

Key words: salt-tolerant bacteria, halophiles, hypersaline aquaculture wastewater, seafood processing effluents.

1. INTRODUCTION

Wastewater discharged from domestic activities and many industries such as food processing, plant protection, pharmaceuticals, petroleum and textile productions contains very high concentrations of organic compounds, total dissolved solids (TDS) with at least 10,000–150,000 mg/L [1], and especially high salinity. The salt concentrations come from reactions in the medium and the origin of water. Thus, the salinity increases by the location of its water source. In

the coastal provinces, the seawater is used for toilet-flushing, and cooling and other activities in residential communities, then discharged directly to the sea even without treatment commonly encountered in Vietnam. In addition, aquafarming has discharged untreated water from shrimp ponds to the nearby brackish seawater, such as in Can Gio province, Vietnam [2]. The amount of organic substances is quantified by the amount of chemical oxygen demand (COD) that is measured by potassium dichromate methods in the presence of silver sulfate catalyst in acidic environment [3]. The salinity and COD vary from 0.1 to 330 g/L NaCl, and 800 to over 200000 mg/L, respectively [4] depending on industrial activities. Amongst them, the wastewater from seafood processing has the range of saline amount of 13 – 65 g/L [5], [6], and 150 – 42,000 mg/L COD [4]. The COD could be removed by chemical reactions; however, biological methods are more preferable because of their friendliness to human and environment.

The hyper-salinity poses several difficulties for determination of organic substances to water treatment system. The chloride (Cl⁻) ions causing the salinity are a significant interference of COD measurement. Therefore, dilution is essential prior to oxidation step in the COD analytical workflow. Nevertheless, the higher dilution factor can lead to too small COD amount below the linear range, inherent experimental errors and decrease in measurement accuracy and precision [7]. In addition, the salt amount that could not be removed completely during the treatment will produce potential adverse effects on the environment. Physio-chemical and biological approaches can be utilized to remove saline effluents. Physical and chemical processes are difficult to run with high set-up and operation costs, consume high amount of chemicals and energy and produce by-products, which results in secondary pollution of possible chemical residues [8]. By contrast, biological processes with simpler operation but high efficient and effective cost are often used. A variety of microbial consortia were employed to degrade the target

contaminants [9]. The treatment again has to face the main problem that the reduction of metabolism activities of microbes will occur in the hypersaline environment because salt concentrations ($> 1\%$) can cause primary contraction, plasmolysis, loss of cell functions and even cell deaths [10]. The presence of high amounts of recalcitrant organics and chlorinated hydrocarbons in wastewater results in low BOD₅/COD ratio, low treatment yield and bulking of activated sludge at the end of the treatment [11], [12].

Salt-tolerant microorganisms are the good candidates to tackle the treatment problem. They live in the medium with the salinity under 1% but can endure the salt-rich environment owing to special metabolic mechanisms [13]. According to salt requirements and the extent of halotolerance, they can be divided into five categories: (1) non-halophiles (below 0.2 M NaCl); (2) halo-tolerant (non-halophiles but can tolerate large salt amount); (3) slight halophiles (0.2–0.5 M NaCl); (4) moderate halophiles (0.5–2.5 M NaCl); and (5) extreme halophiles (above 1–1.5 % equivalent to 2.5–5.5 M NaCl for survival and above 2 % for the optimal growth) [14]. Halophiles maintain an osmotic balance between the cytoplasm and external environment by accumulating soluble inorganic ions such as K^+ , Na^+ and Cl^- in the cytoplasm and producing proteins stable and active in the saline medium [15], while moderate halophiles accumulate osmolytes acting as osmoprotectants to balance the osmotic pressure [16].

The number of studies on application of halophilic bacteria in saline wastewater treatment is increasing recently. Kargi and Dincer (2000) obtained good treatment performance of wastewater with the salinity of over 2 % when using salt-tolerant bacteria in activated sludge [17]. Sivaprakasam *et al.* (2008) isolated halophilic microorganisms from seawater, marine soil, salt lake water (15% and 20% NaCl w/v) and salt lake sediment clay in India and applied successfully these halophiles in tannery wastewater treatment [18].

This study aims to isolate several salt-tolerant bacteria from aquafarming wastewater collected in Tien Giang, Vietnam. Microbial strains were cultured in BMS medium at 30°C, pH 7.0 and identified by MALDI-TOF. An experimental model was assembled to investigate the COD removal ability of the characterized bacteria at different ratios of bacteria and aquafarming wastewater in the laboratory.

2. METHODS

2.1. Sampling and designing experimental model

The wastewater was collected in two places – Vam Lang fishing port in Tien Giang province and Minh Thang seafood processing company in Vam Lang town, Go Cong Dong, Tien Giang province. The parameters (pH, DO, COD, total P, N and Cl^-) were tested to give the first insight about water quality that was the cornerstone to design the following experiments.

The experimental model illustrated in Figure 1 includes 10 reaction compartments with the continuous aeration by using air distribution bar at the bottom of the compartments and aeration pump system during the experiments. Each compartment has the dimensions 7 cm (length) x 12.5 cm (width) x 10.5 cm (height) and contained 500 mL of water samples.



Figure 1. Experimental model of wastewater treatment

2.2. Isolation of salt-tolerant microorganisms

The saline wastewater was diluted with physiologic sodium chloride solution (0.9 %). A suitable aliquot of the mixture was dropped in the BMS medium containing 3 g/L $NaNO_3$, 1 g/L KH_2PO_4 , 0.5 g/L $MgSO_4$, 0.5 g/L KCl, 5g/L NaCl, 1 g/L yeast extract and 20% agar in sterile Petri dishes. The culture was incubated at 37°C in 24 hours. Separated clear colonies were selected and sub-cultured until the pure colonies were obtained.

2.3. Selection and identification of salt-tolerant bacteria

The isolated species were cultured in BMS medium supplied with NaCl at different rates: 1, 3, 5 and 7 %. The development of colonies was observed and colonies with the highest salt tolerance were selected for identification. The bacterial characterization was carried out by protein mass spectroscopy method with MALDI-TOF Matrix Assisted Laser Desorption Ionization-Time of Flight.

2.4. Investigation of COD removal performances in saline wastewater

The experimental model (Figure 1) was used to investigate the effects of input amount of bacteria as the treatment agent, input COD amount of the wastewater to find out the best solutions for treating seafood processing wastewater collected in Tien Giang province, particularly:

- Determine the best ratio of species to wastewater by testing at four ratios: 1:5, 1:10, 1:15 và 1:20 in volume during 96 hours.
- Determine the maximum COD threshold that can be adapted by halophiles, and the removal ability of each individual species during the period from 24 to 96 hours.
- Determine the effectiveness of combining species depending on their functions. The treatment yield were calculated in the period from 24 to 96 hours.

3. RESULTS AND DISCUSSION

3.1. Isolation of salt-tolerant microorganisms

Table 1. Input quality of wastewater

Parameters	Vam Lang fishing port	Minh Thang factory
COD (mg/L)	3195	4254
Total P (mg/L)	54,2	84
Total N (mg/L)	87,4	675
pH	8,1	8,4
DO (mg/L)	3,03	0,2
Cl ⁻ (mg/L)	6523	9851

Table 2 is the list of 45 microbial species in total was isolated from 10 wastewater samples with the quality described in Table 1. In all parameters, COD and chloride concentrations were significant high.

Table 2. The list of microbial strains isolated from 10 wastewater samples

Wastewater	Number of strains	Symbols
1A	3	1A1 → 1A3
1B	5	1B1 → 1B5
1C	4	1C1 → 1C4
2A	5	2A1 → 2A5
2B	4	2B1 → 2B4
2C	3	2C1 → 2C3
3A	5	3A1 → 3A5
3B	3	3B1 → 3B3
4A	7	4A1 → 4A7
4B	6	4B1 → 4B6

The color, homogeneity and surface of colonies were described in Figure 2 and was compared with the standard species in the literature. The cell strains appeared in flat or convex round with slightly marginal edge. The colonies looked like spreading-out root with smooth or slightly wrinkled surface, and had transparent white, milky white, light yellow or orange colors. These colors were fairly similar to those mentioned in the studies of Oren *et al.* (1986, 1992) [19], [20], which are transparent white, white, opalescent white, light yellow, yellow and orange colors depending on the carotenoid in their cells.

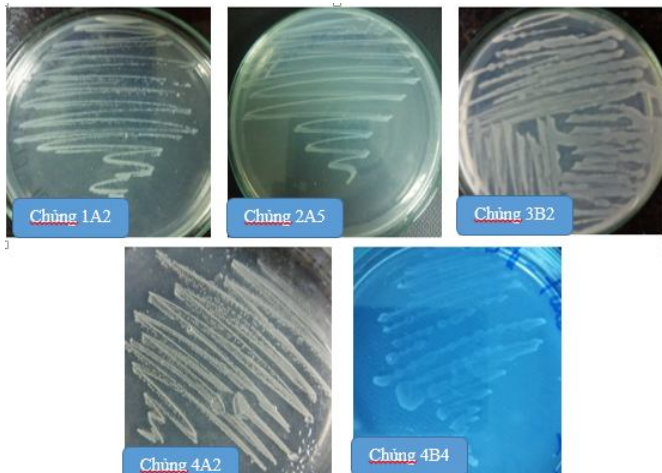


Figure 2. The description of microbial colonies isolated from 10 wastewater samples

According to Larsen *et al.*, 1962, the change of colors was also affected by the salinity of the medium, in which they appear in few colors, such as cream or yellow [15]. In addition, mucosa were found in some colonies with the protrusive or expanded leading edges. The size of cells was smaller or more sensitive in the saltier environment, particularly in the micro-size, which made the cell staining more difficult. After 24 – 36 hours, mature colonies were found in the attachment to the surface of agar medium.

3.2. Identification of isolated salt-tolerant bacteria

As shown in in Figure 3, all 45 strains were developed well in the medium containing 1 % NaCl. However, only 33 strains could develop in higher medium with 3 % NaCl after 24-hour culture. In higher saline concentration, their growth rate reduced, and after 45 hours, only 14 strains and 12 strains could live in the environment with the salinity of 5 and 7 %, respectively. The high salinities caused direct impacts on transferring ions in and out of the cell membrane, affecting on the osmotic pressure of the membrane. Only three strains 1B1, 2A2, 3A5 grew up most strongly in these hypersaline media. The lowest proliferation in the 7 % salinity was observed at 1B5 and 3A3 of 12 strains.

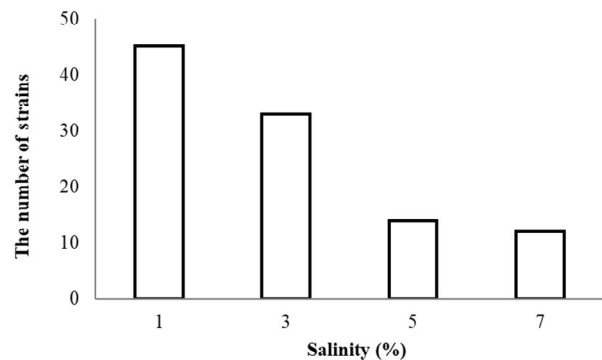


Figure 3. The salt tolerance of strains isolated from saline wastewater

Strains isolated from different samples belonged to halophilic species with various colors and cellular morphologies. The appearance of colonies was not enough to distinguish microbial species and to determine the exact species' names because morphological characteristics depend on growth conditions and change by the change of saline concentrations, temperature, pH, and other components of the medium [21]. Therefore, the strains were determined based on the mass spectroscopic measurement of proteins produced by the colonies in the typical medium by MALDI-TOF. This technique is less time-consuming and cost-effective compared with PCR and gene sequencing methods that are based on the gen bank to conclude the microbial species. Thus, ten strains 1B1, 1C2, 2A2, 2B1, 2B3, 3A5, 3B3, 4A1, 4A7, 4B6 which could survive in the medium of 7 % salinity were selected for identification with MALDI-TOF.

The bacterial names were called in Table 3 based on the mean of score values of similarity coefficients produced from the instrument software. If the coefficients fall in the range of 2.3 – 3.0, marked as (+++), the sample species are highly

probable with the standard species. The range of 2.0 – 2.299 marked (++) means secure genus identification and probable species identification. The decreased coefficients show the less possibility of identification. The coefficients in the range of 1.7 – 1.999 indicate the probable genus identification (+). Finally, when the coefficients are less than 1.699, marked (–), there are no reliable identifications, and strains having score values in this range were negligible.

Table 3. Identification of ten salt-tolerant bacteria isolated from wastewater

Strains	Proposed species' name	Similarity coefficients	Degree
1B1	<i>Staphylococcus epidermidis</i>	2.17	++
1C2	<i>Micrococcus luteus</i>	2.22	++
2A2	<i>Bacillus lichiniiformis</i>	1.93	+
2B1	<i>Bacillus lichiniiformis</i>	2.21	++
2B3	<i>Bacillus subtilis</i>	1.73	+
4A1	<i>Bacillus subtilis</i>	2.11	++
4A7	<i>Bacillus subtilis</i>	1.57	–
4B6	<i>Bacillus cereus</i>	2.23	++
3A5	<i>Arthrobacter creatinolyticus</i>	1.96	+
3B3	<i>Lactobacillus pentosus</i>	1.52	–

Bacteria cannot perform phagocytosis because of hard cell walls. Instead, they secrete enzymes such as proteolytic protease enzymes, amylases to hydrolyze large macromolecules into smaller molecules that will then be transported into the cell for the cellular metabolism. Most species of salt-loving bacteria are aerobic or aerotolerant anaerobes. Gram-negative bacteria are believed to be members of different genera *Halomonas*, *Deleya*, *Volcaniella*, *Flavobacterium*, *Paracoccus*, *Pseudomonas*, *Halovibrio*, or *Chromobacterium*. Based on their phenotypic and phylogenetic data, they have closely relations and are grouped in *Halomonadaceae* family consisting of two genera: *Halomonas* and *Chromohalobacter*. Gram-positive bacteria include *Bacillus*, *Halobacillus*, *Marinococcus*, *Salinicoccus*, *Nesterenkonia*, *Tetragenococcus*, *Actinopolyspora* and *Nocardiopsis*.

Among species, *Staphylococcus sp.* has commonly been isolated from soil and wastewater with the halophilic characteristics and thereby applied in hypersaline wastewater treatment in several studies. For example, Kubo *et al.* (2001) isolated *Staphylococcus sp.* and *Bacillus cereus* from soil that could grow well in the environment having up to 15 % salinity and successfully applied in treatment of grape-production wastewater in Japan [22]. Ganzert *et al.* (2011) isolated *Arthrobacter* from salt-contaminated soil; however, they did not found *Arthrobacter creatinolyticus* as our study [23]. Most recently, Pham Kim Lien and Nguyen Bang Phi (2017) from Thu Dau Mot University (Vietnam) has successfully

used *Bacillus subtilis* to treat domestic wastewater in Thu Dau Mot city [24]. The genus *Bacillus* is known for diverse strains of bacteria with high adaptability and can use various sources of C, N nutrients. On the other hand, this genus has the ability to produce spores under the harsh conditions including high-salt-content environment. Indeed, among 10 named strains, 6 strains of *Bacillus* genus were found to be able to grow relatively well in 7 % salinity, with the optimal 3 -5% NaCl.

Halophiles have the ability to change their properties with living conditions. An osmotic balance must be established between the intracellular and external environment of the microbial cells in order to resist high salinity and frequent fluctuations of salt concentrations. By this way, they can be resistant to the high osmotic pressure in the environment. This balance is established by the accumulation of salts and dissolved organic matter [16] and itself controlling the movement of water in and out of the cells to keep the osmotic balance in the intracellular space. The natural water in Tien Giang belongs to Mekong River. However, the current salt intrusion has caused the unstable salt concentrations in the water here, and salt-tolerant bacteria could adapt to the salt fluctuations thanks to these special mechanisms.

3.3. Determination of the best seed rate of bacterial species for saline wastewater

The change of concentration ratio of bacteria and wastewater affected the COD removal yields in the same treatment time of 96 hours, which was illustrated in Figure 4. The highest treatment yield was observed in strain 1C2 (reduced by 100% COD) at the ratio of 1: 5. The color of treated wastewater was much clearer than that of other samples. At the seed rate of 1:10, treatment efficiencies varied from 78.77% to 90.15%, except that strain 3A5 gave low yield of only 50.77%. At the seed rates 1:15 and 1:20, COD treatment efficiencies were generally low, compared with the higher ratios. This could be because the amount of treatment agent (bacteria) was lower to some extent than the amount of organic substances in the wastewater. The lowest yield was 33.92% at 1:20 ratio of strain 3B3.

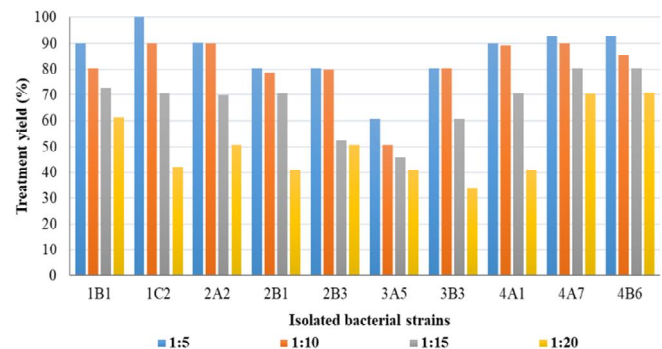


Figure 4. Effects of the seed rates on COD removal yields. As can be seen from Figure 4, at the seed rate of 1:5, the treatment yields of *Micrococcus luteus* (1C2) were highest, which reached 100 %, followed by *Bacillus subtilis* (4A7),

Bacillus cereus (4B6) with 92.77 % and *Staphylococcus epidermidis* (1B1) with 90.15 %. In a general overview, *Bacillus sp.* strains (2B3, 4A1, 4A7, 2A2 và 2B1) gave high treatment abilities (over 70 %) at seed rates of more than 1:15. Only three strains of *Staphylococcus epidermidis* and *Bacillus subtilis* were able to remove COD by over 60 % even in the low input cell concentrations (1:20 ratio). The good treatment performance of *Staphylococcus sp.* and *Bacillus sp.* has also discovered in previous studies. Motoki Kubo *et al.* (2001) also reported *Staphylococcus sp.* and *Bacillus cereus* isolated from the grape processing plant could reduce 70% COD of salty wastewater after 72 hours and up to 90 % when applied at a pilot scale with the 1m³ water volume for 7 days in the factory [22]. Sohair I. Abou-Elea *et al.* (2009) used *Staphylococcus xylosus* isolated from agricultural processing wastewater with activated sludge containing 3% salinity to get the yield of 90 % [25]. Moreover, *Staphylococcus sp. BH4*; yeast *Candida sp. YH*; *Desulfovibrio sp. BH* and salt-tolerant Anammox bacteria were able to adapt to the saline concentration of 5 – 10 g/L in wastewater and remove by 70 – 80 % COD in average [26]. On the whole, *Micrococcus sp.*, *Staphylococcus sp.*, and *Bacillus sp.* were great candidates for treatment of seafood processing wastewater at the ratio of 1:5.

3.4. Determination of the COD tolerance and removal of each bacterial strain

In addition to the seed rates, the COD of input wastewater should be taken into account. Although microorganisms can absorb certain kinds of organic pollutants, they will be overwhelmed if the organic amounts are too high. Thus, a limit COD input of 500 mg/L is recommended for biological treatment methods. Besides, chloride concentration play a vital of role in inhibiting the microbial proliferation. Results of the preliminary tests show considerable differences in organic treatment of 10 bacterial strains at two COD levels – 200 and 400 mg/L during the exposure time of 24, 48, 72 and 96 hours.

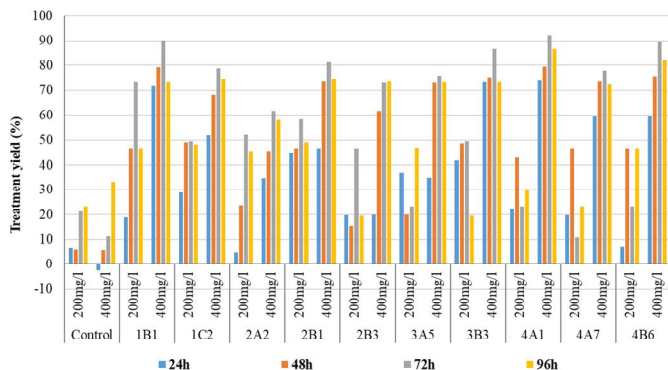


Figure 5. COD removal of individual bacterial species by the time

At the low COD content of 200 mg/L, the highest treatment efficiencies were 73.33% for strain 1B1 (*Staphylococcus epidermidis*) in 72 hours. However, the yield increased by the increase of COD content in the same treatment period, particularly, 90 %. At this level, strain 4A1 (*Bacillus subtilis*)

gave the highest yields after 48 hours compared with other strains (Figure 5).

In terms of treatment time, at the COD level of 200mg / L, the yields started increasing only more than 48 hours. Strains 1B1, 1C2, 2A2, 2B1, 2B3, 3A5, 3B3 achieved the highest efficiencies after 72 hours, then the yields decreased by the time. Nevertheless, the yields of three strains 4A1, 4A7, 4B6 were highest in after 48 hours, then decreased if the treatment was continued lasting. The COD efficiencies were highest at 72 hours in all strains especially *Staphylococcus epidermidis* and *Bacillus subtilis*, maximum of 92.14 % for 4A1 and minimum of 73.08 % for 2B3. Again, these two bacterial species were mentioned with their great COD removal performances. *Staphylococcus sp.* and *Bacillus cereus* were applied in grape processing wastewater treatment by Kubo *et al.*, 2001. The COD levels were reduced by 70 % after 72 hours in a treatment pilot and by up to 90 % after 7 days in the practical scale of 1m³ in the plant [22].

3.5. Effectiveness of combination of strains in COD removal from saline wastewater

Based on the COD removal ability of each individual species, strains were selected to combine and classified into three groups:

- Group 1 includes 5 strains with the highest treatment yields: 1B1, 2B1, 3B3, 4A1 and 4B6.
- Group 2 consists of 5 strains with the best salt tolerance: 1B1, 1C, 2A2, 3A5 and 4A7.
- Group 3 is a simple combination of 10 strains.

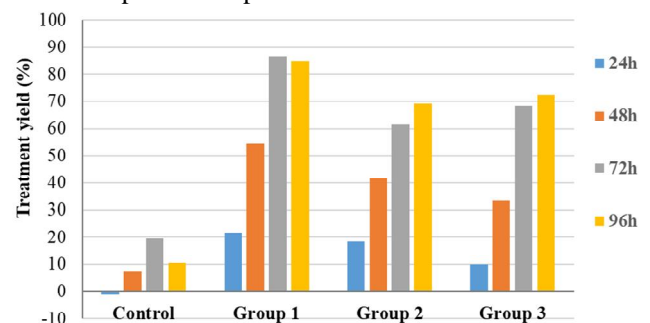


Figure 6. COD treatment of combined bacterial species

It is obviously seen that combining bacteria with great treatment abilities could give better than the mixture of salt-tolerant species and the random combination. Particularly, the yield of group 1 was higher four times than the control samples. Furthermore, there was no noticeable difference about the exposure time from 72 hours in all groups. The efficiencies of both group 2 and 3 were similar and increased significantly compared with the control if the incubation time was expanded (from 24 hours). This phenomenon highlighted the key role of salt-tolerant species in the removal of organic compounds in saline aquafarming wastewater taken in Tien Giang province of Vietnam.

4. CONCLUSION

A total of 45 microbial strains were isolated from 10 saline wastewater samples. The investigation showed all 45 strains

proliferated very well in the presence of 1 % NaCl. The number of survival rate decreased by the increase of the salinity. 33 strains could grow at 3 % salinity, 14 strains develop fairly well at the salinity of 5 %, and only 12 strains could multiple at the saline concentration of 7 %. From the salt tolerance outcomes, 10 strains with the greatest salt tolerance was chosen - 1B1, 1C2, 2A2, 2B1, 2B3, 3A5, 3B3, 4A1, 4A7, 4B6 strains that could endure the medium containing 1 – 7 % salinity and develop after 24 hours at 30°C and pH 7. The MALDI-TOF results showed the selected strains belonged to genus *Arthrobacter*, *Micrococcus*, *Staphylococcus*, *Bacillus* and *Lactobacillus*.

The first evaluation showed all 10 strains were able to reduce COD level in the 3 % saline mixture. To obtain the best treatment efficiency, bacterial strains should be mixed with the waste water at the ratio of 1:5 and incubated for at least 96 hours in the continuous aeration. As a result, *Micrococcus luteus* (1C2 strain) gave the most effective treatment while the COD removal ability of 3A5 strain (*Arthrobacter creatinolyticus*) was lowest. Other strains produced relatively high treatment efficiencies in general at the seed rate of 1:5.

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