

## Screening and Characterization of Halophilic Bacteria with Industrial Enzymes from Salt Lake Razazah, Karbala, Iraq

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A total of 218 halophilic bacterial isolates were obtained from Lake Razazah, west of Karbala, Iraq. Optimum pH and temperature were 7.5 and 37 °C, respectively. According to optimal growth at different salt concentration, the slightly halophilic bacteria were the most abundant isolates with the frequency of 68%. The isolated bacteria were screened for the production of extracellular amylase, alkaline amylase, protease, alkaline protease, lipase, alkaline lipase, pectinase and cellulase. The production of pectinase (55.8%), amylase (52.6%) and lipase (50.0%) were observed in almost half of the halophilic bacteria. Alkaline amylase and alkaline lipase production were reported in less than one third (30%) of isolates. Phylogenetic analysis of 16S rRNA sequences indicated that all isolates were members of eight genera of the domain Bacteria, including *Bacillus*, *Halobacillus*, *Virgibacillus*, *Oceanobacillus*, *Staphylococcus*, *Pseudomonas*, *Idiomarina* and *Halomonas*. The predominant commercial enzymes producers in current study were *Halobacillus* sp. K51 and *Halomonas* sp. K46 with the ability to produce 7 out of 8 exoenzymes. The presented data shows that despite drought, dehydration, increased concentrations of salt and contaminants, Lake Razazah represents an untapped source of halophilic bacteria biodiversity.

**Keywords:** Biodiversity, Halophiles, Hydrolases, Isolation.

One of the widely distributed aquatic ecosystems is saline environments<sup>1</sup>. Despite the high concentration of salt, a great diversity of life forms can be observed in this environment and they found in all the three domains of life, *Bacteria*, *Archaea*, and *Eukarya*<sup>2</sup>. Halophiles include prokaryotic and eukaryotic salt-loving microorganisms of saline environments which are able to balance the high osmotic pressure<sup>3</sup>. To adapt to high level saline, they have developed a number of biochemical strategies to maintain cell structure and function<sup>4-6</sup>. The diversity amongst halophiles

regards to the concentration of salts, temperatures, pH, and redox conditions that the organisms are adapted. Thermophilic, psychrophilic, mesophilic, alkaliphilic, aerobic and anaerobic halophilic bacteria have been described<sup>4-7</sup>. Halophiles play an important role in the carbon and phosphorus cycles in saline environments<sup>8</sup>. Some of them are able to degrade organic compounds, such as crude oil which makes them potential candidates in bioremediation studies<sup>9-10</sup>.

Halophiles are diverse with regard to the concentration of salt which they are adapted. They are able to produce hydrolytic enzymes in hypersaline environment which possess a potential importance in industrial area<sup>11</sup>. Moderately halophilic bacteria represent a group

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of microorganisms that is widely distributed in salinity zones. They show optimum growth at 5-15% NaCl<sup>12-13</sup>. The bacteria grow at salt concentrations above 20% (w/v) to saturation named extremely halophilic<sup>3</sup>. Slightly halophilic microorganisms are capable of growing optimally in media containing 2-5% NaCl<sup>14</sup>.

A variety of industrial applications of microbial hydrolases including textile, food, dairy, pharmaceutical, detergents, cosmetic industries and synthesis of polymeric materials were described in previous studies<sup>15-18</sup>. Microbiological sources of extracellular enzymes are used for a wide range of industrial and biotechnological aims<sup>12, 19</sup>. Many recent studies have focused on extracellular hydrolytic enzymes of the halophilic bacteria in order to use such enzymes in biotechnological processes<sup>20</sup>.

Iraq features various ecosystems including extreme environments such as high salinity soil and water in which microbial diversity has been poorly studied<sup>21-24</sup>. Lake Razazah, also known as Bahr Al-Milh (literally Sea of Salt) is located 10 kilometers west of Karbala province, Iraq (32°45'2 N 43°38'2 E). The covered area ranges from 1050 to 1700 km<sup>2</sup> depends on water evaporation through a year<sup>24</sup>. The water level changes with the seasons but totally, the lake is rather shallow with maximum depth of 17 m. The pH values ranges from 7.1 to 8.5<sup>25</sup>. Due to rapid evaporation of water, especially in summer, the water in this lake is quite saline and in different parts the salinity varies between 7.8 to 11%<sup>26-27</sup>. Lake Razazah fed by the Euphrates River via Sin-Al-Thibban canal<sup>28</sup>. In current study, we identified the microbial diversity of Razazah Lake for the first time in this country. Screening of slightly, moderately and extremely halophilic bacteria were performed using soil, sludge and water samples of the lake. The prevalence of microorganisms producing extracellular hydrolytic enzymes was also described.

## MATERIAL AND METHODS

### Sample Preparation

Saline soil and water samples were collected from fifteen different zones, at the surface and various depths of the south and southeast of Razazah Lake (between longitude 43° 85' E and latitude 32° 60' N) on 26<sup>th</sup> February 2014 (Figure 1).

The samples were put into labeled plastic bags or sterile glass jars and transported to the laboratory for further analysis. Even though it was winter, the temperature was between 22 to 25 °C. All samples were cultured in nutrient broth with saline concentration of 2.5%, 5.0%, 7.5%, 10.0%, 12.5%, 15.0%, 17.5%, 20.0% and 22.5%. Sea salt consisted of 175 g NaCl, 20 g MgCl<sub>2</sub>·6H<sub>2</sub>O, 5 g K<sub>2</sub>SO<sub>4</sub>, 0.1 g CaCl<sub>2</sub>·2H<sub>2</sub>O and 2 mL filtered saline lake water (as source of trace elements) in 1 L distilled water. The culture media were adjusted to pH of 7.0, 7.5, 8.0 and 8.5 using NaOH. After autoclaving, all media were incubated at 37°C in orbital shaker, at 120 rpm for 7 days. Cell morphology was examined by using light microscopy (model BH 2; Olympus). Gram staining and KOH lysis tests were carried out according to previous studies<sup>29-30</sup>.

### Hydrolase Activities

Screening of bacteria based on extracellular hydrolase activities including amylase, alkaline amylase, protease, alkaline protease, lipase, alkaline lipase, pectinase and cellulase (CMCase) were performed using agar plates. In order to preparing alkaline media, pH was adjusted using NaOH.

#### Extracellular Protease Activity

A 10% skim milk-agar medium supplemented with a range of sea water concentrations was prepared by dissolving 10 g of skim milk powder in 100 mL of sterile nutrient agar. Ability to create clear zone around the colonies after incubation at 37°C for 7 days was considered as positive proteolytic activity<sup>32</sup>.

#### Extracellular Lipase Activity

Lipase activity was detected using sterilized basal nutrient agar media supplemented a range of sea water concentrations and containing 2.5% olive oil (w/v) at 60°C. Then, 0.001% rhodamine B was added to homogenized mixture at 50°C and poured. Observation of shiny colonies under UV irradiation after incubation of bacteria at 37°C for 7 days indicated lipolytic activity<sup>33</sup>.

#### Extracellular Amylase Activity

Extracellular amylase activity was assayed using 1% starch agar medium (Merck) using various concentration of sea water. To detect the hydrolysis of starch, I<sub>2</sub>-0.6% KI solution or Lugol's iodine was used. After incubation at 37°C for 7 days, presence of dark blue, purple, or black color represented amylolytic activity<sup>33</sup>.

### Extracellular CMCase Activity

Nutrient medium with 1% carboxymethyl cellulose (CMC) containing a range of artificial sea water were incubated at 37°C for 7 days. Clear zone around the colony after flooding in 0.1% congo red solution for 15 minutes showed CMCase activity<sup>34</sup>.

### Extracellular Pectinase Activity

Sterile nutrient medium containing 1% pectin and varying concentration of sea water was used. Cultured media were incubated at 37°C for 7 days and then flooded in 0.3% I<sub>2</sub>–0.6% KI solution. Presence of clear zones around the growth was considered as pectinolytic activity<sup>35</sup>.

### Molecular Analysis

Genomic DNA was extracted by Bioron sample preparation kit (Korea). The 16S rRNA gene was amplified by PCR using the universal prokaryotic primers including 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR mixtures of 50 µL contained 17 µL dH<sub>2</sub>O, 1 µL primers, 1 µL dNTP, 0.75 µLMgCl<sub>2</sub> (50mM), 25 µL PCR amplification buffer (1X), 2 U Taq DNA polymerase, 5 µL template DNA. The amplification was done by initial denaturation 95°C for 5 min, 30 cycles at 95°C 1 min, 58°C 30 s, 72°C 1 min with a final extension of 72°C for 10 min. Techne TC-3000X Thermal cycler (UK) was used for amplification. PCR products were electrophoresed in 1% (w/v) agarose gels and the single ~ 1400 bp of DNA fragments were purified using GeneAll Gel Extraction Kit (Korea). PCR products of the nearly complete 16S rRNA gene were sequenced in both directions using an automated sequencer by Macrogen Biotechnology Company (Korea).

The 16S rRNA gene sequence was aligned using BLAST and Ribosomal Database Project (RDP) with the published sequences of closely related bacteria in the GenBank database of the National Center for Biotechnology Information. Phylogenetic trees were constructed with the aid of the MEGA 7 software package<sup>36</sup> using the neighbour-joining<sup>37</sup>, maximum likelihood<sup>38</sup> and maximum-parsimony<sup>39</sup> algorithms. The confidence values for the branches of phylogenetic trees were determined using bootstrap analyses based on 1000 replications<sup>40</sup>. The 16S rRNA gene sequences of the strains have been deposited in the GenBank database.

## RESULTS AND DISCUSSION

The salinity of the Euphrates River has increased gradually to more than double over the last 30 years<sup>27</sup>. Screening isolates from saline Lake Razazah under aerobic conditions led to isolation of 218 isolates from 15 samples. Optimum pH and temperature were 7.5 and 37°C, respectively. The slightly halophilic bacteria were the dominant isolates with the frequency of 68% followed by 32% moderately halophilic bacteria. They consist of 31 gram negative rods, 26 gram negative cocci, 95 gram positive rods and 66 gram positive cocci. No extremely halophilic bacteria were found. About two third (67.4%) of the isolates were halotolerant and only eighteen isolates grew at NaCl concentrations greater than 15.0%. Additionally, 15 protozoa, 3 algae, 5 fungi and yeast were observed (the data not shown).

### Extracellular Hydrolase Production

The priority for further molecular analysis was the number of extracellular enzymes that has produced by the halophilic bacteria. The production of eight hydrolytic exoenzymes by twenty-one isolates was illustrated in table1. *Halobacillus* isolates were the most valuable species in terms of industrial enzyme production in the present study. *Halomonas*, *Staphylococcus* and *Pseudomonas* isolates showed similar strength in the production of the very exoenzymes. *Halobacillus* sp. K51 and *Halomonas* sp. K46 were able to produce 87.5% of abovementioned industrial enzymes. All *Halobacillus* and *Bacillus* isolates showed the ability of extracellular pectinase production. *Halobacillus*, *Bacillus*, *Halomonas* and *Staphylococcus* genera had isolates which were able to produce both extracellular amylase and alkaline amylase. The ability to produce extracellular protease and alkaline protease both were observed in isolates of *Halobacillus*, *Virgibacillus*, *Bacillus*, *Halomonas* and *Staphylococcus*. In addition, *Halobacillus*, *Halomonas* and *Staphylococcus* isolates were found to be potent lipase and alkaline lipase producers.

In this study, among all isolated halophilic bacteria of Lake Razazah (N=218), production of pectinase (55.8%), amylase (52.6%) and lipase (50.0%) were observed in half or more than half of the halophilic bacteria. On the other side, only

less than one third of isolates were able to produce alkaline amylase and alkaline lipase. Near to half of them showed the ability of protease and alkaline protease production (Figure 2). In contrast with the study of Rohban *et al.*, no lipase and amylase activity was reported for *Oceanobacillus* isolates<sup>41</sup>. Kumar, S. *et al.* introduced *Bacillus* isolates as halophilic microorganisms with the maximum ability of lipase and protease production from soil and water samples in India<sup>42</sup>. Similar to our results, they found *Oceanobacillus* strains showed extracellular protease activity. *Halomonas* isolates from the Tunisian Solar Saltern also showed



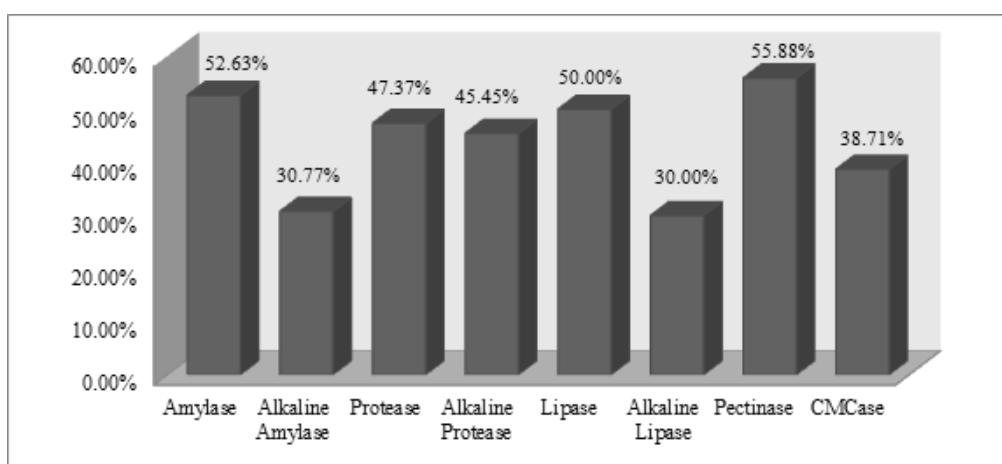
**Fig. 1.** Map of sampling sites at the south and southeast of Lake Razazah, Karbala, Iraq. The map was derived from Satellite World Map in 2014 [31]. Red arrows show the sampling sites

hydrolase producing ability<sup>43</sup>. The environmental isolates of *Salinivibrio*, *Halomonas*, *Bacillus* and *Salibacillus* genera were predominant commercial enzymes producers in hypersaline environments of Spain<sup>12,44</sup>.

Amylase and alkaline amylase derived from the genus *Bacillus* is the main source of these enzymes with commercial applications<sup>45</sup>. Similarly, in the present study, alkaline amylase activity was mainly observed in *Bacillus*, *Halobacillus* and *Oceanobacillus* isolates. In the study of the hypersaline environments in South Spain, only two isolates of *Halomonas* and *Nesterenkonia* genera were detected amylase positive<sup>44</sup>. However, extracellular amylase able to be active in pH more than 8.0 was purified from other microorganisms.

Among our isolates, *Halobacillus*, *Virgibacillus*, *Oceanobacillus*, *Staphylococcus*, *Idiomarina* and *Halomonas* showed the protease activity in pH 8.5. An extracellular alkaline protease of an extreme halotolerant *Bacillus* was introduced from West Sea in Inchon, Korea<sup>46</sup>. Same protease activity was observed in a haloalkaliphilic *Bacillus* *sp.* isolated from a seawater sample in Gujarat, India<sup>47</sup>. An extracellular protease was produced under conditions of high salinity and pH 8.5 by a moderately halophilic *Salinivibrio* *sp.* isolated from Bakhtegan Lake, South of Iran<sup>48</sup>.

The *Staphylococcus* *sp.* K33 showed alkaline lipase activity similar to a newly soil-isolated *Staphylococcus* *sp.* strain with the ability of alkaline lipase secretion introduced by Slim *et al.*<sup>49</sup>. Additionally, alkaline lipase activity was



**Fig. 2.** Hydrolytic activities of halophilic bacteria isolated from Lake Razazah, Karbala, Iraq

**Table 1.** Hydrolytic activities of the representatives of eight genera including *Bacillus*, *Halobacillus*, *Virgibacillus*, *Oceanobacillus*, *Staphylococcus*, *Pseudomonas*, *Idiomarina* and *Halomonas* isolated from Lake Razazah, Karbala, Iraq.

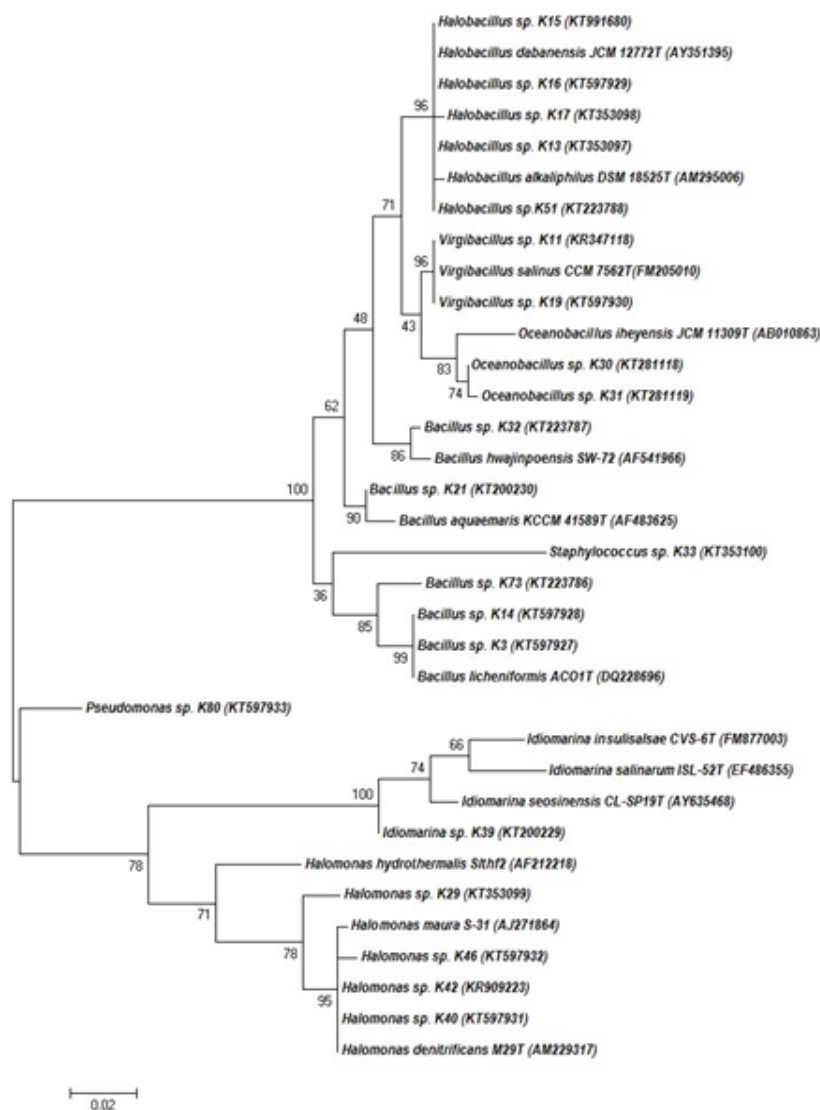
Isolates	GenBank Accession Number	Amylase	Alkaline Amylase	Protease	Alkaline Protease	Lipase	Alkaline Lipase	Pectinase	Cellulase	Enzyme production (%)
<i>Halobacillus</i> sp. K13	KT353097	-	-	-	+	+	+	+	-	50.0
<i>Halobacillus</i> sp. K15	KT991680	+	-	-	+	+	-	+	-	37.5
<i>Halobacillus</i> sp. K16	KT597929	+	+	-	+	-	+	+	+	75.0
<i>Halobacillus</i> sp. K17	KT353098	-	+	+	+	+	-	+	-	62.5
<i>Halobacillus</i> sp. K51	KT223788	+	+	+	-	+	+	+	+	87.5
<i>Virgibacillus</i> sp. K11	KR347118	-	-	+	+	+	-	+	+	62.5
<i>Virgibacillus</i> sp. K19	KT597930	+	-	-	-	+	-	-	-	25.0
<i>Oceanobacillus</i> sp. K30	KT281118	-	+	-	+	-	-	-	-	25.0
<i>Oceanobacillus</i> sp. K31	KT281119	-	+	+	-	-	-	+	+	50.0
<i>Bacillus</i> sp. K3	KT597927	+	-	+	-	-	-	+	-	37.5
<i>Bacillus</i> sp. K14	KT597928	+	+	-	-	-	-	+	-	37.5
<i>Bacillus</i> sp. K21	KT200230	+	-	+	+	-	-	+	-	62.5
<i>Bacillus</i> sp. K32	KT223787	+	+	+	+	+	-	+	-	75.0
<i>Bacillus</i> sp. K73	KT223786	-	+	+	-	-	+	+	-	50.0
<i>Halomonas</i> sp. K29	KT353099	+	-	+	-	-	+	-	+	50.0
<i>Halomonas</i> sp. K40	KT597931	+	+	-	-	+	-	-	-	37.5
<i>Halomonas</i> sp. K42	KR909223	-	+	+	+	+	+	-	+	75.0
<i>Halomonas</i> sp. K46	KT597932	-	-	+	+	+	+	+	+	87.5
<i>Staphylococcus</i> sp. K33	KT353100	+	+	+	+	-	+	-	+	62.5
<i>Idiomarina</i> sp. K39	KT200229	+	-	-	+	-	-	-	+	37.5
<i>Pseudomonas</i> sp. K80	KT597933	+	-	+	-	+	-	+	+	62.5



observed in a *Pseudomonas* sp. strain, isolated from the peninsular coast of India<sup>50</sup>. However, same ability was not detected for our *Pseudomonas* sp. K80. Also, extracellular alkaline lipase was purified from a new *Bacillus* sp. strain capable of withstanding pH 8.0 to 9.0<sup>51</sup>. In the present study, same ability at pH 8.5 was observed mostly in the isolates of *Halobacillus* and *Halomonas* genera.

We found *Bacillus* and *Halobacillus* genera as potent extracellular pectinase producer

in this study. CMCase activity was detected for the genus *Halobacillus*, *Virgibacillus*, *Oceanobacillus*, *Staphylococcus*, *Pseudomonas*, *Idiomarina* and *Halomonas*. In a survey of halophilic microorganism in Orissa and West Bengal, India, all halophilic bacteria were negative for pectinase and cellulose activities<sup>52</sup>. In a screening of bacterial strains from Brazilian soil samples, 60.7% of isolated bacteria presented pectinolytic activity<sup>35</sup>. The main microorganisms of Howz Soltan playa



**Fig. 3.** Phylogenetic tree showing the position of the halophilic isolates, based on the partial 16S rRNA sequence comparison, obtained by the maximum likelihood method. The accession numbers for the reference strains are included in brackets. Bootstrap values are indicated on the branches

which were able to produce extracellular pectinase and cellulase belonged to the genus *Gracilibacillus* and *Virgibacillus*<sup>41</sup>.

#### Taxonomic Characterization of the Isolates

According to morphological (macro and microscopic) and molecular (sequences of genes encoding for 16S rRNA) analysis, only 21 isolates were selected and examined in greater details. Phylogenetic analysis of 16S rRNA sequences of the isolates revealed eight different taxonomic groups. Isolates clustered with *Bacillus*, *Halobacillus*, *Virgibacillus*, *Oceanobacillus*, *Staphylococcus*, *Pseudomonas*, *Idiomarina* and *Halomonas* with bootstrap values (36-100%). Figure 3 shows the phylogenetic tree of isolates. In contrast with the study of Rohban *et al.* the dominant genus of our study was *Halobacillus* and *Halomonas* while they presented *Salicola* as the most frequent genus in Howz Soltan Lake<sup>41</sup>. We found two members of genera *Staphylococcus* and *Pseudomonas* which previous studies have not reported. In the study of microbial diversity of Qinghai Lake, China, *Actinobacteria* and *Acidobacteria/Holophaga* was reported as the dominant gram-positive bacteria while we have not found them among our isolates<sup>53</sup>. The most common bacteria from different saline environments in South Spain were assigned to genera *Salinivibrio* while we had not found them in our study<sup>44</sup>. Although species of the genus *Salinivibrio* can be found in low salt concentration, in this study we did not identify any member of this genus<sup>41</sup>. The present study as well as study of an alkaliphilic soda lake showed that the most frequent isolates belonged to genera *Halomonas* and *Bacillus*<sup>54</sup>.

#### CONCLUSIONS

To our knowledge, this is the first microbiological study on biodiversity of halophilic bacteria in Iraq. Lake Razazah is subjected to drastic physico-chemical conditions including changes in salinity, temperatures and dryness which make it a study target for microbiologists. In the course of a program of screening for halophilic and bacteria in Razazah Lake, 218 bacteria were isolated from soil, water and sludge samples. We found halophilic bacteria belong to eight genera including *Bacillus*, *Halobacillus*, *Virgibacillus*, *Oceanobacillus*, *Staphylococcus*, *Pseudomonas*, *Idiomarina* and

*Halomonas*. Additionally, the potential of various industrial hydrolase productions by halophilic bacteria demonstrated in this study. The genus *Halobacillus*, *Halomonas* and *Bacillus* had members with the most ability to produce industrial extracellular enzymes. Further studies focusing on molecular and biochemical characteristics recommend in order to estimating the best hydrolytic exoenzyme producers. A large part of the oil resources in Iraq located in high temperature and hypersaline sites which are contaminated by crude oil. Apart from all industrial applications, discovery of new microorganisms which are able to treat these contaminations would be a perfect solution in environmental cleanup.

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