Extracellular enzymes produced by microbes in hypersaline environments have been reported to possess a wide range of biocatalytic activities capable of functioning under the prevailing extreme conditions. To facilitate the industrial application of microbial enzymes sourced from extreme environments, we investigated the physiochemical properties and extracellular enzyme activities in solar saltern sediments. Our results indicate that high salinity induces an increase in the levels of dissolved organic carbon (DOC), which enhances the activities of cellulase, chitinase, and sulfatase by increasing the energy supplied to microbes. Therefore, enzymes and microbes that can be potentially used in future industrial applications was found in solar saltern.

**Keywords:** dissolved organic carbon, extracellular enzyme activity, hypersaline, solar saltern

A hypersaline environment is an extreme habitat dominated by high salt levels (> 35‰; salinity higher than seawater), and microorganisms living in these environments are called halophiles (Rich and Maier, 2015). Halophiles have unique mechanisms for producing nontoxic solutes inside their cells in order to be isosmotic with their high salt environments, and have pink or orange pigments (carotenoids and bacteriorhodopsins) for protection from high UV levels (Rich and Maier, 2015). Extracellular enzymes produced by halophilic microorganisms in extreme environments demonstrate a wide range of biocatalytic activities capable of functioning under the prevailing extreme conditions (Debashish et al., 2005). This implies that these extracellular enzymes can be used in many harsh industrial processes where high salinity would otherwise inhibit such enzymatic activities (de Souza and de Oliveira Magalhães, 2010).

A solar saltern is a set of pools where seawater is left to evaporate to obtain salt, and is characterized by high temperature, salinity, high levels of UV light, high osmotic pressure, and the presence of toxic compounds. Despite these harsh conditions, there are various halophilic bacteria, archaea, and algae with diverse and unique microbial adaptations that are able to survive these highly saline conditions. In addition, various extracellular enzymes released from these microbes are involved in nutrient cycles of carbon and nitrogen (cellulase, glucosidase, and chitinase), phosphorus (phosphatase), and sulfur (sulfatase), and are able to degrade complex molecules such as lignin (phenol oxidase). Accordingly, investigating the physiochemical properties and extracellular enzyme activities in solar salterns will help to facilitate the industrial application of microbial enzymes in extreme environments (Oren, 2009; Cantrell et al., 2013).

To investigate six extracellular enzyme activities (EEAs)
Chung et al. (Table 1) and physiochemical properties in a solar saltern, samples were collected from the Gomso solar saltern (E35°35'39.38", W126°36'33.15") occupying an area of 0.4 km² near the western coast of South Korea in July 2018 (Fig. 1A). The seawater in this solar saltern is evaporated over a series of ponds to the point where NaCl and other salts precipitate out of the saturated brine, allowing the purified salts to be harvested. This process creates a gradient of salt concentrations over a series of ponds, ranging from typical seawater salinity to saturation. For this study, surface sediments and seawater were collected from five individual ponds (GS1, GS2, GS3, GS4, and GS5), with the GS1 pond located adjacent to a crystallizer pond (hypersaline pond) (Fig. 1B). The samples were moved to laboratory on ice and stored in the 4°C before analyses.

The salinity of sediment (ratio of 1 g of wet sediment to 1 ml of DDI water) and seawater were measured using a salinity refractometer (PAL-106S, ATAGO Inc.). Sediment temperature was measured in situ using a thermometer. Seawater was filtered using a 0.45 µm HDPE syringe filter and DOC was analyzed using a Shimadzu TOC-5050A Analyzer (Shimadzu Corp.). Levels of sediment organic matter (OM) were calculated based on the mass loss ignition.

EEAs of cellulase, glucosidase, chitinase, phosphatase, and sulfatase were assayed using fluorogenic 4-methylumbelliferone (MUF) substrate analogues (Table 1). One gram of sediment sample was added to 1 ml DDI water and vortexed for 10 min. Two hundreds microliter of sediment sample diluted with DDI water was added into each well of microplates. Since all substrate had minimal solubility in pure water, they were pre-dissolved in methylcellosolve (final concentration of 1.5%) and then diluted in ultrapure water as a final concentration of 500 μM. The solvent, methylcellosolve, was reported not to interfere with enzyme assay (Hoppe, 1983). All substrate solutions were freshly prepared for each assay. One hundred microliter of 500 μM...
µM substrate solution (resulting in 50 nM substrate per each well in the microplate) were added to microplate wells containing 200 µl of mixture with sediment and DDI water. The plates with substrates and sediments were incubated for 9 h at 20°C. The fluorescence intensity was measured immediately upon substrate addition and after 9 h incubation using an automated fluorometric plate reader (Spectra Max i3X, Molecular Devices). The excitation wavelength was 360 nm for fluorescent substrates, and emissions were measured at 460 nm for MUF substrate treatments. Enzymatic activities were calculated as the amount of MUF released during the 9 h incubation based on appropriate standard curves. Standard curves were prepared for each sediment sample to account for different levels of fluorescence quenching due to the presence of phenolic compounds. The activity was calculated based on the sediment organic matter (OM) and expressed as µM MUF OM/kg/day (Kang and Freeman, 1999; Kim et al., 2016).

The phenolic compound L-dihydroxy phenylalanine (L-DOPA) was used as a model substrate for analyzing phenol oxidase activity. The simple and sensitive L-DOPA method measures the formation rate of red colored compounds of 2-carboxy-2,3-dehydroindole-5,6-quinone from the enzyme oxidation of L-DOPA (Pind et al., 1994). One gram of sediment sample was added to 5 ml solution of 10 mM L-DOPA, and vortexed throughout for 3 min at 20°C due to poor solubility at higher concentrations. The samples were incubated for 1 h at 20°C, and the reaction was terminated by immediate centrifugation at 12,000 rpm for 10 min at 20°C. Two mililiter of supernatant sample was added to a cuvette cell and absorbance was measured at a wavelength of 460 nm using a spectrophotometer (UH5300, Hitachi High-Tech). The absorbance of sediment samples in ultrapure water served as negative controls (Kim et al., 2016). Activity was expressed as µM of 2,3-dihydroindole-5,6-quinone-2-carboxylate (diqc), a compound obtained upon enzymatic oxidation of L-DOPA produced in OM kg⁻¹ day⁻¹ using Beer’s Law and a molar absorbance coefficient for diqc of 3.7 × 10⁴ (Mason, 1948).

Statistical analysis was conducted using JMP version 13.0 (SAS 2016). One-way ANOVA was used to evaluate differences in EEs between test sites, with p < 0.05 indicating significant differences. All comparisons of means were accomplished using a Tukey-Kramer HSD test that protects the overall error rates.

Salinity levels ranged from 44‰ (GS1) to 10‰ (GS5) for surface sediments and from 64‰ (GS1) to 32‰ (GS5) for seawater (p < 0.05, Table 2 and Fig. 2). The DOC levels ranged from 15 mg/L (GS1) to 6 mg/L (GS5) and the highest level was

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**Table 2. Physical and chemical properties of surface sediments and water collected from the Gomso solar saltern**

<table>
<thead>
<tr>
<th>Site</th>
<th>Sediment salinity (%)</th>
<th>Sediment temperature (°C)</th>
<th>Water content (%)</th>
<th>Organic matter (g/kg)</th>
<th>Total nitrogen (g/kg)</th>
<th>C:N ratio</th>
<th>Sediment δ¹³C (%)</th>
<th>Sediment δ¹⁵N (%)</th>
<th>Water salinity (%)</th>
<th>Water pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS1</td>
<td>44</td>
<td>28</td>
<td>30</td>
<td>41</td>
<td>0.35</td>
<td>16.5</td>
<td>-17.7</td>
<td>0.6</td>
<td>64</td>
<td>7.98</td>
</tr>
<tr>
<td>GS3</td>
<td>18</td>
<td>25</td>
<td>27</td>
<td>37</td>
<td>0.49</td>
<td>13.6</td>
<td>-19.4</td>
<td>1.8</td>
<td>41</td>
<td>7.64</td>
</tr>
<tr>
<td>GS5</td>
<td>10</td>
<td>25</td>
<td>27</td>
<td>28</td>
<td>0.48</td>
<td>13.4</td>
<td>-19.6</td>
<td>3.0</td>
<td>32</td>
<td>7.66</td>
</tr>
</tbody>
</table>

* The n.d. in the column means the sample data that were not measured.

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**Fig. 2. Sediment and water salinity, and dissolved organic carbon (DOC) concentrations collected from the Gomso solar saltern in summer season.**

The alphabets with different characters on the bars mean statistically significant difference.
observed in GS1 (Fig. 2). Similar to salinity levels, the DOC levels increased from seawater pond (GS5) to hypersaline pond (GS1), suggesting a positive relationship between salinity and DOC concentrations in the water column \( (p < 0.01, \text{Fig. 2}) \). The results of EEAs analyses revealed that activities of cellulase, chitinase, and sulfatase in GS1 (hypersaline pond) were higher than those of other ponds \( (p < 0.01, \text{Fig. 3}) \), showing positive associations between salinity, DOC and EEAs. In contrast, glucosidase, phosphatase, and phenol oxidase activities did not significantly differ among the ponds tested (Fig. 3).

The positive relationship between salinity and DOC observed in seawater can be explained by the monovalent cation effect. Monovalent cations such as Na\(^+\) can disrupt divalent cation bridges and disperse more soil particles instead of bonding or stabilizing them, while divalent cations like Ca\(^{2+}\), Fe\(^{3+}\), and Mg\(^{2+}\) bind organic matter with soil particles through the divalent cation bridge effect. Thus, high levels of Na\(^+\) can promote the desorption of labile organic carbon from soil particles, resulting in the release of more DOC from soil particles to the water column (Greenland, 1971; Reemtsma et al., 1999). In addition, anions (Cl\(^-\) and SO\(_4^{2-}\)) in salts compete with DOC for absorption to the soil particle surface, releasing more DOC into the seawater. Through these two processes, more energy sources (labile organic carbon; DOC) are supplied to microbes, which enhances their activities such as EEAs. Several studies have also shown a positive relationship

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**Fig. 3.** Extracellular enzyme activities in the surface sediments collected from the Gomso solar saltern in summer season. The alphabets with different characters on the bars mean statistically significant difference.
between the DOC and EEAs, likely because DOC is a potential carbon source for microbial metabolism or because DOC can be produced as a byproduct from microbes upon degradations of polymers to monomers through EEAs (Li et al., 2012; Chambers et al., 2016; Kim et al., 2016). In this study, the high DOC observed in GS1, the hypersaline pond, was caused by high salinity, which enhanced EEAs by supplying a carbon source to the microbes. Thus, high salinity, DOC, and EEAs observed in hypersaline conditions (GS1) were correlated, implying that a solar saltern is an optimal location to detect useful EEAs.

The incomplete microbial degradation of organic matter due to inhibition by hypersaline conditions is a possible alternative explanation for the results observed. Hypersaline conditions are known to negatively influence enzymatic activity because osmotic stress can interfere with enzyme synthesis (Rietz and Haynes, 2003; van Ruykgem and Verbeke, 2005). In addition, hyper-salinity can change the quantity and quality of substrates and mineralization rates, resulting in the inhibition of enzymatic activity (Jackson and Vallaire, 2009). However, majority of the studies demonstrating negative effects of hyper-salinity on EEAs have been performed in freshwater soils and sediments with relatively low salt concentrations ranging from 3‰ to 1 5‰ in water, in which microbes are already well adapted to freshwater salinity (Bouvier and del Giorgio, 2002). These conclusions cannot be applied to extremely hypersaline conditions. Thus, the responses of EEAs during hypersaline conditions should be investigated using microbial populations that are well adapted to long term survival in a particular condition.

Of the high EEAs observed in the hypersaline ponds, cellulase is particularly interesting for industrial utilization because it can break down strongly bound cellulose strands into shorter polysaccharides and oligosaccharides. The industrial applications of cellulase include the production of pulp, food products like beer, wine, and animal feed, textiles, laundry detergents, biofuels, and combating plant pathogens (Bhat, 2000; Farrell et al., 2006; Bamforth, 2009; Adav and Sze, 2014). One bacterium that releases cellulase is the halophile *Halocella cellulolytica*, which was first isolated from hypersaline lagoons by Simankova et al. (1993) and whose complete genome was sequenced using bacterium isolated from a Salt Evaporation Pond in Thailand (Heng et al., 2019). The reported optimal growth conditions for *Halocella cellulolytica* were 15% NaCl, 39°C, pH 7.0, and it is an obligate anaerobe (Simankova et al., 1993). These conditions are very similar to those of solar saltern environments, indicating that a solar saltern is an optimal location to find and culture cellulase-releasing microbes.

Chitinase is that hydrolytic enzyme that breaks down glycosidic bonds in chitin. Industrial applications of chitinase include treating agricultural fields to control pathogens, in health care for asthma treatment, contact lenses, artificial skin and sutures, and in the treatment of chitinous waste (Hamid et al., 2013). The ChiNi and HschiA1 genes encoding chitinase were cloned from the *Halobacterium salinarum* NRC1 and *Halobacterium salinarum* CECT395 and displayed optimal activity at the 58‰ to 87‰ salinity, pH 7.3, and 40°C, similar to solar saltern environments. Shrimp and crab, the main industrial sources of chitin, are still hydrolyzed by acid treatment; thus the isolation of chitinase from solar salterns could lead to industrial applications for crustacean degradation (Stoykov et al., 2016).

High sulfatase activities observed in the hypersaline ponds indicate that sulfatase degrades and utilizes sulfated glycopolymers as carbon sources. This was also observed in the marine strains of *Planctomycetes* (Glöckner et al., 2003; Wagner and Horn, 2006) and *Verrucomicrobia* isolated from a hypersaline microbial mat (Spring et al., 2016). In addition, sulfate reducing bacteria (SRB) are abundant in hypersaline environments due to a positive relationship between salt and sulfate concentrations and are largely responsible for the mineralization of organic matter. As the SRB mineralize organic matter, labile organic carbon (DOC) and HCO$_3$ are produced and introduced into the sedimentary pore fluids (Berry et al., 1981). In a previous study, high acetate concentrations in hypersaline conditions were attributed to enhanced fermentation processes related to SRB (Foti et al., 2007).

In summary, solar salterns showed high levels of DOC because high salinity increases the solubility of nutrients and organic carbon, supplying more energy to microbes. Consequently, dynamic EEAs including those of cellulase, chitinase, and sulfatase, were observed in the surface sediments of
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 hypersaline environments. These results imply that the solar saltern environment can be potentially used to screen and isolate enzymes and microbes for industrial applications in the future.

## 적 요

고염분 환경에 서식하는 미생물들은 극한 환경에서도 유기물을 분해하기 위하여 다양한 형태의 체외효소를 만들어낸다. 특히 극한 환경에서 만들어진 효소들은 산업적으로 이용 가능성이 높기 때문에, 고염분 환경인 염전의 퇴적물에서 발생되는 체외효소 활성도를 측정하였다. 그 결과, 유기용존탄소의 양과 염분농도간의 강한 상관관계가 나타났으며, 미생물에 대한 용존용존탄소의 공급 증가는 cellulase, chitinase, sulfatase의 활성도를 증가시켰다. 따라서 향후 산업적 가치를 고려하였을 때, 고염분의 환경인 염전에서의 체외효소활성도에 대한 연구가 필요할 것으로 판단된다.

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