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Discovery of two novel highly tolerant $NaHCO_3$ Trebouxiophytes: Identification and characterization of microalgae from extreme saline–alkali soil

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ABSTRACT

Through identifying and characterizing microalgae from extreme saline–alkaline soils (pH > 10, Songnen Plain, China), which are rich in carbonate ($NaHCO_3$ and Na_2CO_3), we aimed to explore the microalgae carbonate stress mechanism and acquire possible extreme saline–alkali microalgae as genetic resources for crop improvement and cultivation. We identified the microalgae based on optical microscopy (OM), scanning electron microscopy (SEM), and 18S rRNA sequence analysis. We determined strains' salt-tolerance abilities with a range of NaCl and $NaHCO_3$ concentrations. The intracellular ultrastructure was observed with transmission electron microscopy (TEM) after NaCl and $NaHCO_3$ treatments. We observed strain growth response by detecting dry weight and chlorophyll-a (chl-a) content at different $NaHCO_3$ concentrations. We identified 20 novel strains of microalgae as 13 genera based on morphological and sequence analyses, and classified them into six groups by class level, including Chlorophyceae, Trebouxiophyceae, Ulvophyceae, Bacillariophyceae, Xanthophyceae, and Bangiophyceae; Chlorophyta occupied the most dominant algal groups. Most isolates possessed saline tolerance, especially two strains in the class Trebouxiophyceae, which could grow on solid media with high $NaHCO_3$ concentrations. The TEM results showed cell walls of the two Trebouxiophyte strains (JB6 and 17) with more integrity than reference *Chlorella*, and a larger number of starch grains were accumulated under 300 mM NaCl and $NaHCO_3$. Further analysis showed that the dry weights and chl-a contents of two Trebouxiophyte strains were much better than the reference *Chlorella* in liquid media with different concentrations of $NaHCO_3$. The two Trebouxiophyte strains had optimal growth at 100 and 400 mM $NaHCO_3$ concentrations, and both survived at 1000 mM $NaHCO_3$. The results revealed that the two Trebouxiophyte strains showed extreme tolerance to high concentrations of $NaHCO_3$.

1. Introduction

In northeast China, large-scale tree clear-cuts and subsequent intensive grazing have induced soil erosion and increased evapotranspiration, resulting in salt accumulation from groundwater on the soil surface [1,2]. The Songnen Plain, located between N42°30′–51°20′ and E121°40′–128°30′, is an alkalinized–salinized grassland that covers about 23,926 km^2 [3]. Saline–alkali soil has different dominant salts (Na_2SO_4, NaCl, etc.). However, the soil in the Songnen Plain contains mainly salts like sodium bicarbonate and sodium carbonate, also known as “Soda–Saline–Alkali Soils”. Hydrolysis of $NaHCO_3$ and Na_2CO_3 leads to high pH values in soil solution [4,5]. The serious salinization and alkalinization region has many “barren” lands with a pH of about 10.5, and only few plant species are sporadically distributed (Fig. 1). Plants are “carbonate stressed” by $NaHCO_3$ and Na_2CO_3 [4]. The “barren” land is an extreme soil environment for most plant species. Na^+ is the predominant soluble cationic ion. Ca^{2+} and Mg^{2+} were present mainly in soil ash, and the main anion was HCO_3^-. The soil alkalinity exchangeable sodium percentage (ESP) ranged from 70–90% [5]. The soil's structure was very poor and the permeability was very low: saturated hydraulic conductivity was only 0.02–0.22 mm, which is in the low water-permeability range [6], while its electrical conductivity was 3050 μs/cm [5]. Nutritional status of the soil, such as organic matter and total N, P, and K, was low [7]. Although the saline–alkali habitat is harmful to organisms, the environment selects for unique biota (plants, microbes, mosses, etc.).

Aside from well-recognized alkaline- and saline-tolerant plants (*Puccinellia tenuiflora*, *Suaeda glauca*, *Salix linearistipularis*, etc.) [8],

Abbreviations: OM, optical microscopy; SEM, scanning electron microscopy; TEM, transmission electron microscopy; Chl-a, chlorophyll-a; SOD, super oxide dismutase; $NaHCO_3$, sodium bicarbonate; Na_2CO_3, sodium carbonate; BBM, Bold's basal medium.

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Fig. 1. Photograph of the extreme saline–alkali soil of the Songnen Plain (pH > 10) showing the sampling site. The gridline location is the "barren" land (arrow).

microbial life forms are important for biome stability in saline–alkali soil. Recent research on the alkali-salinization soil of northeastern China revealed a few microbial species resources with great development potential, including bacteria, rhizobia, fungi, and microalgae [9–11,5]. The microalgae species are widely distributed opportunistic organisms that are always present in terrestrial systems, and they are important primary producers in various communities. Because algae both possess plant and microbial features, researching algae from extreme saline–alkali soil has great practical significance.

Extreme environments such as Antarctica soil [12,13], Colorado Plateau Desert soil [14], Antarctica lakes [15,16], Arctic freshwater [17], and extreme saline–alkali soil [5], contain microorganisms including Chlorophytes, diatoms, cyanobacteria, and bacteria. Our laboratory previously isolated some microalgae from severe alkali soil, and we performed a rough tolerance analysis of 20 strains using different concentrations of NaCl and $NaHCO_3$ [11]. However, the classification and characterization of these microalgae were not carried out. Here we further characterize microalgae inhabiting extreme saline–alkali soil and acquire potential carbonate-tolerance microalgae as genetic resources for plant breeding. In this study, we (1) identified microalgae from extreme saline–alkali soil based on morphological characteristics and 18S rRNA sequence analysis, (2) discovered and analyzed the highly $NaHCO_3$-tolerant microalgae, and (3) determined dry weights and chlorophyll-a contents of two stress tolerant microalgae at different concentrations of $NaHCO_3$.

2. Materials and methods

Twenty microalgae (JB1–JB20) previously isolated from severe alkali soil (46°27′N, 125°22′E, Songnen Plain, China, 2011) were maintained

Table 1
Tentative morphological description of 20 microalgae from extreme saline–alkali soil.

Classical designation	Number	Description	Tentative identification
Chlorococcum sp.	JB1	Cells solitary, yellow-green, spherical, perinous or parietal chloroplasts, a pyrenoid, several starch grains, 5–20 μm in diameter.	*Chlorococcum* sp. C1
	JB3	Cells solitary, green, thick cell walls, spherical, perinous or parietal chloroplasts, adult cells divided into 4 or 8 aplanospores (arrow), 15–35 μm in diameter.	*Chlorococcum* sp. C2
	JB8	Cells solitary or in groups, green, spherical, chloroplasts extended over most of the inner cell, a big distinct pyrenoid was embedded in the chloroplast, 10–22 μm in diameter.	*Chlorococcum* sp. C3
	JB19	Cells solitary or in groups, yellow-green, spherical, chloroplasts extended over most of the inner cell, a big distinct pyrenoid was embedded in the chloroplast margin, 18–25 μm in diameter.	*Chlorococcum* sp. C4
Chlamydomonas sp.	JB2	Cells solitary, green, spherical or egg-shaped, with two equal flagella, cup-shaped chloroplast, a jacinth eyepot, a pyrenoid, 8–10 μm in diameter.	*Chlamydomonas* sp. H1
	JB4	Cells solitary, light-green, egg-shaped, with two flagella, chloroplast parietal, a jacinth eyepot, cell fronts had two contractile vacuoles (arrow), a pyrenoid, 11–17 μm length, and 8–15 μm width.	*Chlamydomonas* sp. H2
	JB7	Cells solitary, green, spherical or egg-shaped, with two flagella, chloroplast parietal, a jacinth eyepot, a pyrenoid, 6–10 μm in diameter.	*Chlamydomonas* sp. H3
	JB18	Cells solitary, yellow-green, egg-shaped, with 2 flagella, chloroplast parietal, a jacinth eyepot, a pyrenoid, 7–11 μm length, and 8–15 μm width.	*Chlamydomonas* sp. H4
Scenedesmus sp.	JB5	Cells solitary, 2- or 4-celled coenobia (arrow), green cells alternately arranged in one row, spindle-shaped acute cells, chloroplast perinous or parietal, a pyrenoid, 8–12 μm length and 5–7 μm width, coenobia 12–20 μm width. SEM: wing-like longitudinal ribs on the cell surface (arrow).	*Scenedesmus* sp. S1
	JB11	Cells oval or cylindrical with obtuse, green, 4- or 8-celled coenobia, each cell had a single pyrenoid, chloroplast perinous, 15–18 μm length and 12–15 μm width, 8 celled coenobia (arrow) about 50 μm width. SEM: cell surface was not smooth, and had wrinkles. Cells alternately arranged in one row, blunt ended presence (arrow).	*Scenedesmus* sp. S2
Chlorella sp.	JB6	Cells solitary, green, spherical, chloroplast parietal, cup-, girdle- or saucer-shaped, a distinct pyrenoid, 5–10 μm in diameter.	*Chlorella* sp. X1
Tribonema sp.	JB9	Filamentous, cylindrical cells, unbranched filamentous, comprising serial arrangement of interlocking H-shaped segments (arrow), perinous chloroplast.	*Tribonema* sp. T1
Stigeoclonium sp.	JB10	Plant for a list of cells of branching filamentous, green, the principal branch and lateral branch with no obvious differentiation, erect branches often formed alternate or opposite branches.	*Stigeoclonium* sp. G1
Navicula sp.	JB12	Cell solitary, yellow-brown, ovoid-shaped, a pyrenoid, chloroplast parietal, 15–20 μm length and 3–5 μm width. SEM: raised ribs on front side, girdle view showed lens-shaped (arrow).	*Navicula* sp. N1
	JB16	Cell solitary, yellow-brown, boat-shaped, both symmetrical sides, and two symmetric pyrenoids, chloroplast parietal, 15–20 μm length, and 3–5 μm width. SEM: surface smooth with the frustules showed essentially lens-shaped. Two symmetrical raphes presented on front side.	*Navicula* sp. N2
	JB13	Cells solitary, green, spherical, chloroplast parietal, cup-shaped, 15–20 μm in diameter, covered with several starch grains, and the cell was surrounded with gelatinous vesicles (arrow).	
	JB14	Cells solitary, light-blue, spherical, 10–37 μm in diameter. SEM: each cell showed one spine (arrow).	
	JB15	Filamentous (arrow), yellow-green, thick cell walls, spherical, chloroplast extended over most of the inner cell, and a single cell was 10–22 μm in diameter.	
	JB17	Cells solitary, green, chloroplast parietal, cup-shaped, a distinct pyrenoid, 2–9 μm in diameter.	
	JB20	Most of the vegetative cells formed two sarcinoid aggregations, green, and the chloroplast contained numerous independent starch grains embedded in the matrix.	