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Influence of clay minerals on the *Bacillus halophilus* Y38 activity under anaerobic condition

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ABSTRACT

Studies of interaction between clay minerals and *Bacillus halophilus* Y38 have provided basic information on extremophile growth and physiological characteristics in hypersaline environments, which are of growing concern in microbiology, geobiology, environmental and pharmaceutical researches. In this study, we investigated *B. halophilus* Y38 strain growing in HGM hypersaline medium (sodium ion concentration >55 mg mL^{-1}, chloride ion concentration >80 mg mL^{-1}), with smectite, rectorite and kaolinite of different aggregate sizes (100 μm, 100–50 μm, and <50 μm, respectively). Metabolic heat flow significantly increased in smaller particles, indicating that the stimulation of the *B. halophilus* Y38 growth by the clay minerals increased with decreasing aggregate sizes, and, thus, the close relationship between the bacterial growth and the mineral surface area. The CO_2 flux (FCO_2), total thermal effect (Q_{total}), cell growth rate (K_{cell}) and protein synthesis rate ($k_{protein}$) were the key physiological parameters in metabolic and energy conservation reactions. These metabolic results showed that the stimulation by clay minerals on the *B. halophilus* activity was in the order smectite > rectorite > kaolinite. Our data may provide a strategy to evaluate the influence of clay minerals on the microbial growth and cell activity, and supplied a new experimental procedure to study interactions between minerals and microorganisms.

1. Introduction

The growth of halophilic microorganisms requires the supply of a very great amount of salt (NaCl). These microorganisms are mainly distributed in dry steppes and semidesert areas, such as southwestern Siberia, Northeastern Mongolia, North China, Egypt, India, Pakistan, Hungary and North American Steppes. The main geochemical condition to form such sediments and rocks is the depletion in divalent cations, Ca^{2+} and Mg^{2+}, leading to the accumulation of soluble sodium carbonates. In most cases, they are associated with high-standing saline ground waters and salterns occurring in the depressions where saline alkaline (soda) lakes are formed (hydromorphic solonchaks) (Kondorskaya, 1965; Rodriguez-Valera et al., 1982; Oren and Shilo, 1985). They were even found in Antarctic lakes.

In contrast with relatively well-studied soda lakes, less is known about halophilic microorganism growth and activity in high-saline medium in the presence of some clay minerals. On the basis of the physiological characteristics, some halophilic microorganisms are expected to survive in terrestrial and Martian environmental conditions (Moll and Vestal, 1992). In fact, clay minerals are widely distributed in the high-salt soils, sediments, and marine environments (Gibbs, 1977). These clay minerals not only can be used as an indicator of the original environment, but also influence the environment (Keller, 1970; Warr et al., 2009). Oren (1983) showed that bentonite or kaolinite and starch stimulated the growth of *Halobacterium* strain RD-26 ^{T} by acting as scavengers of toxic metabolities excreted by the bacteria or by adsorbing toxic components from the medium. Thus, environmental microbiology and geobiology should pay more attention to the investigations on the interactions between clay minerals and microorganisms (Dong et al., 2009; Stroes-Gascoyne et al., 2010).

Our previous studies inferred that the substrate composition will change microbial metabolism (Yao et al., 2007; Zhou et al., 2009). The comparison of the measured heat flux with the enthalpy balance may reveal previously undetected and unexpected events, providing a powerful means to extend our physiological knowledge. Admittedly, not every change is energetically important enough to be measurable

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by monitoring the heat flow rate. However, the main distribution of substrate-carbon and substrate-enthalpy may be revealed in this way (Maskow and Babel, 2003).

In this work, the effect of clay minerals on the growth process of *Bacillus halophilus* Y38 is evaluated by using microcosms.

2. Materials and methods

2.1. Clay minerals

Smectite, rectorite and kaolinite were provided by the State Key Laboratory of Geological Processes and Mineral Resources, Chinese University of Geosciences (Wuhan, P. R. China). The mineral particles consisted of 95% clay minerals (in mass) and 5% impurities including mica and traces of halloysites. The aggregate size fraction 100 μm was ground and sieved into 50–100 μm and <50 μm.

2.2. Bacterial strains and media

B. halophilus Y38 (AB021188) was isolated from a hypersaline soil in Yancheng City, Jiangsu Province, East China. It was provided by the State Key Laboratory of Agricultural Microbiology, College of Life Science and Technology in Huazhong Agricultural University (Wuhan, P. R. China) (He et al., 2008).

The nearly full-length 16 S rRNA genes were amplified by PCR using universal primers Uni-27 F and Uni-1492R (Fan et al., 2008). The PCR program consisted of an initial denaturation for 5 min at 94 °C; 30 cycles of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C; and a final extension step at 72 °C for 5 min. The PCR products were purified by using the UltraPureTM PCR Kit (SBS Genetech, Shanghai, P.R. China). DNA sequencing was performed by the Sunbio Company (Beijing, P.R. China). All the 16 S rRNA gene sequences were checked manually and edited for phylogenetic analyses. Sequence alignments were performed using the CLUSTAL W program (Thompson et al., 1994). BLAST N (for 16 S rRNA gene) was used to analyze similarities (www.ncbi.nlm.nih.gov/BLAST). Phylogenetic trees were generated from alignments by the neighbor-joining method and the reliability of inferred trees was tested with bootstrap test using the MEGA3 program (www.megasoftware.net). Some reference sequences from the GenBank were used in generating phylogenetic trees for clarification.

B. halophilus Y38 was cultivated at 30 °C on HGM agar slants and stored at 4 °C. During microcalorimetric measurements, *B. halophilus* grew in HGM medium containing (g L^{-1}) $MgCl_2.6H_2O$ (19.5), $MgSO_4.7H_2O$ (29.0), $CaCl_2.2H_2O$ (1.1), KCl (6.0), NaBr (0.5), $(NH_4)_2SO_4$ (10.0) and NaCl (58.0), yeast extract (2.0). Tris–HCl buffer (10 mM) was added to maintain the pH at 7.5. In addition, 2% agarose was added to HGM solid medium for pure culture in Petri dish. It was then sterilized at high-pressure at 121 °C for 30 min.

2.3. Culture experiments

B. halophilus Y38 cultures were carried out in our sterile bioreactor with a working volume of 1.0 L. The growth process of *B. halophilus* Y38 was continually cultivated and measured, and simulated the ampoule environment in microcalorimetre. The initial pH and cell density of bioreactor cultures were kept at 7.5 and OD_{700} of 0.2, respectively. The culture experiments were carried out in triplicate. Anaerobic atmosphere was maintained in a glovebox by sparging some filtersterilized nitrogen. No antifoaming agents were used.

The culture process is described as follows. The *B. halophilus* Y38 was continually cultivated in HGM medium without any clay minerals. This process was repeated three times as an initial step in every experiment. To measure the influence of clay minerals on bacterial metabolism, strain Y38 was inoculated in HGM medium containing 1 g L^{-1} smectite, rectorite and kaolinite.

2.4. Determination of mixed substrate interference

B. halophilus Y38 was pre-cultured in HGM medium at 30 °C overnight before microcalorimetric measurements. The bacterial suspensions were mixed by inoculating *B. halophilus* Y38 into the same medium of an optical density of 0.2 at 700 nm.

A TAM III multi-channel thermal activity microcalorimeter (Thermometric AB, Sweden) was used to measure the metabolic heat flux. The heat flux measurement was based on the isothermal ampoule method. The 4.0 mL stainless steel ampoules were sterilized to hold the strains. All determinations were performed in triplicate in ampoules containing 3.0 mL bacterial suspensions plus different substrates. An ampoule containing only 3.0 mL bacterial suspensions was used as the control. The temperatures of the calorimeter system and the isothermal box were maintained at 30 °C. A computer continuously recorded the power–time curves of the growth of isolated strains.

The thermokinetic parameters, such as the growth rate constant (k) and the total heat evolution (Q_{total}), which reflects the biochemical reactions, were determined by analyzing the power–time curves. The growth power–time curves of strains obey the thermokinetic equation at the exponential phase of growth (Yao et al., 2007):

$$\ln P_t = \ln P_0 + kt \quad (1)$$

where t is the time, P_t is the power output at time t, and P_0 is the power at the beginning of the exponential growth phase. Q_{total} was calculated by integration of the curves at the end of the exponential increase of the signal (Barros et al., 2000). At the same time, the interference of mixed substrates on the bacterial metabolic process was determined by combination modes to monitor some continually parameters in the bioreactor. In all experiments, the samples were incubated at 30 °C on a shaker at 200 rpm. Cell growth was monitored by measuring OD_{700} of 5 d culture. Non-inoculated treatments were used as control. Each ampoule was filled in a tent with a nitrogen atmosphere and an oxygen content of less than 0.2 % (v/v).

2.5. Analysis

The biomass concentration was determined gravimetrically in quadruplicate after oven-drying at 105 °C. Cell growth was measured over time by optical density at 700 nm (OD_{700}).

The CO_2 flux was monitored by a Pasco PS-2110 carbon dioxide (CO_2) gas sensor (Pasco Scientific, Roseville, CA, USA). Usually, this sensor measures the carbon dioxide concentration in the atmosphere or an enclosed space, such as a terrarium or bioreactor by the NDIR (non-dispersive infrared) technology. The sensor consisted of an infrared (IR) source and an IR thermopile element. The thermopile element was covered with a narrow band filter that only passed IR energy at 4.27 μm. As CO_2 concentration increased, the IR energy 'seen' by the thermopile decreased. The relationship between the CO_2 gas concentration and the absorption of IR energy followed Beer's Law. This sensor had to be calibrated before any use. The signals of FCO_2 were captured and processed by the Xplorer GLX Interface PS-2002, serial cables, a power supply, and control by DataStudio software (version 1.9.8, Pasco Scientific, Roseville, CA, USA). By using this sensor, the physicochemical property was monitored on-line during the microbial growth in the mixed substrates.

The protein concentration of the supernatant was measured by the method of Bradford (1976), using bovine serum albumin as the standard.

3. Results and discussion

3.1. Identification of the strain B. halophilus Y38

After growth on HGM solid medium, the strain *B. halophilus* Y38 formed some round and convex colonies, 1–2 mm in diameter, slightly