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Calcium ion adsorption with extracellular proteins of thermophilic bacteria isolated from geothermal sites—A feasibility study

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ABSTRACT

The scaling of geothermal wells arising from formation of calcium carbonate is one of the major problems associated with the utilization of geothermal energy. A novel eco-friendly biological-based approach for geothermal well descaling was proposed. Thermophilic bacterial strains were isolated from geothermal areas in Taiwan and were evaluated for their calcium adsorption efficiency under the extreme conditions. Among the eight strains isolated, *Tepidimonas fonticaldi* AT-A2 isolated from Antun Hot Spring, Hualien showed the highest calcium adsorption capacity. The calcium adsorbing activity of *T. fonticaldi* AT-A2 was mainly associated with the extracellular proteins and the maximum calcium adsorption capacity (1.94 g Ca/g protein) was obtained at pH 10, 150 °C and 1 atm pressure. This calcium adsorption efficiency is much higher than that of metallothioneins and other bacterial extracellular proteins. The excellent calcium adsorption efficiency of the AT-A2 proteins indicates the potential for their applications in biological geothermal well descaling.

1. Introduction

Geothermal energy is an abundant alternative renewable energy resource. Geothermal energy is the heat energy generated by the radioactive decay of long lived isotopes like potassium, uranium, radium and thorium in the earth's crust. It can be recovered as steam or hot water beneath the earth's surface, such as those in hot springs and used as a source of energy. Geothermal energy provides base-load power just like any other energy sources such as coal, oil or natural gas, and can replace fossil fuels. Geothermal power is cost effective, reliable, sustainable, environmentally friendly and virtually inexhaustible [1]. The geothermal areas or fields can be geographically classified based on their location: Volcanic or high temperature areas that lies close to active volcanoes and non-volcanic or low temperature areas that lies outside the active volcanic belts [2]. A 3 MW power plant was established in Ching-Shui non volcanic geothermal field in 1981 in Taiwan. The plant was decommissioned in 1993 due to continued decline in production. The main cause behind this operational failure was the occurrence of mineral deposits in geothermal wells, pipelines and reservoir fractures [3,4]. The mineral deposits in wells and fractures were identified as calcite ($CaCO_3$).

Calcite is a common formation mineral that often occurs as travertine deposits around neutral Na-HCO_3-SO_4 springs. In general, calcite can be generated by one of the following ways: (i) hydrolysis (involving replacement of calcium alumino-silicates), (ii) boiling of geothermal fluids (from fluids having high dissolved carbon dioxide concentrations and in the absence of mineral pH buffer) and (iii) heating of cooler peripheral geothermal fluids [5,6]. The difficulty of running a geothermal power plant in a non-volcanic area is de-trop calcite growth, when the geothermal fluid boils or degases in response to a pressure drop [7,8]. Most dissolved CO_2 is lost from the liquid phase into gaseous phase at the flash point and this causes a dramatic shift in the following equilibrium to the right [9]:

$$Ca^{2+} + 2\,HCO_3^- \rightarrow CaCO_{3\,(s)}\downarrow + CO_2\uparrow + H_2O$$

Increase in HCO_3^- content in the geothermal fluid also enhances the rate of forward reaction or formation of calcite scales ($CaCO_3$) [7,8].

The major calcite inhibition technologies applied currently include mechanical methods, chemical treatments [10], and

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biological methods [11]. Mechanical cleaning methods (such as using steam shocks and hydro blasts) were used to effectively remove scaling deposits [12]. However, the method is laborious and requires expensive equipment and manpower. Also, much technical skill is required to perform the procedure without causing any inherent damage to the infrastructure [10]. Chemical technologies have also been widely applied both in removal of calcite and inhibition of calcite formation. The chemical removal process generally used is acid wash with the use of mud acid (HCl-HF), sulfuric acid, phosphoric acid, glycine acid and barium nitrate [13]. The chemical-based calcite prevention process uses chemical inhibitors, such as organic phosphate esters, organic phosphonates [14], ethylenediamine tetraacetic acid (EDTA) [35], polyacrylic acid (PAA), polymaleic anhydrides (PMA), sodium polyacrylate (ASAP) [15]. The major disadvantage of the chemical methods is the adverse environmental impacts of the chemicals used and the high operating cost involved [16]. Moreover, both mechanical and chemical methods cannot prevent further scaling and requires periodic repetition, which may affect the productivity of the geothermal site.

On the other hand, the prevention of calcite formation can also be achieved by biological methods, in which calcium ions in aqueous solution can be removed by bacterial biomass or derived products via adsorption. Physical adsorption methods, such as using activated carbon might be feasible for this purpose because of its higher surface area and higher adsorption morphology for adsorption of various pollutants like textile dyes [17] and chromium [18]. However, so far there is no report mentioning the use of such method in the prevention of calcite formation. Biological adsorption of heavy metals has been reported in abundance in literature and heavy metal biosorption is known to be carried out by algae [19], fungi [20], bacteria [21], cyanobacteria [22] and also plant derived products [23,24]. It has been reported that cyanobacterial biomass (of the Genera *Lyngbya, Oscillatoria, Spirulina, Anabaena, Synechocystis* and *Gloeocapsa*) are capable of adsorbing calcium ions in solution [25,26]. This method was originally developed to remove traces of calcium (0.16%) from brine to improve the quality of salt, but the general theme of calcium biosorption can be extrapolated for geothermal well descaling. However, the major difference is the extreme environment involved in geothermal well descaling (such as high temperature and high pressure) compared to rather mild conditions employed in conventional biosorption based calcium removal methods. The advantages of biological method are that it is cost and energy efficient, and does not require heavy labor or expensive equipment. It is also clean and eco-friendly, as it does not generate or release any hazardous chemical at any stage during the treatment.

Based on the hypothesis that anti-scaling mechanism in geothermal wells primarily replies on the chelation of anti-scaling agents with calcium ions so that calcium ions cannot react with bicarbonate to form calcite deposition, we tried to develop eco-friendly and effective biological anti-scaling agents for the use in geothermal site. Thus, in this study, thermophilic proteins with high calcium ion binding ability were examined for their feasibility to be used as anti-scaling agents for prevention of calcite scale formation in geothermal wells. To achieve this, thermophilic bacteria were isolated from geothermal areas in Taiwan and the proteins secreted from these bacterial isolates were collected, characterized, and evaluated for their calcium adsorption capability under the geothermal site conditions. The performance of these protein-based antiscalant was justified based on their ability to bind to calcium ions under the conditions of geothermal sites (e.g., high temperature and high pressure). To the best of our knowledge, this is the first study attempting to utilize biosorption of calcium for the purpose of geothermal site anti-scaling.

2. Materials and methods

2.1. Isolation and identification of bacterial strains from geothermal sites

The bacterial strains used for the study were isolated from geothermal areas in northern and eastern Taiwan. Geothermal water was collected and 300 ml of the sample was filtered using a 0.45 μm pore size membrane. The membrane loaded with filtered bacteria was placed on Tryptic Soy Broth (TSB) agar plates and incubated at 55 °C for 5 days. The composition of the TSB medium is as follows (g/l): pancreatic digest of casein, 17; enzymatic digest of soybean meal, 3; dextrose, 2.5; sodium chloride, 5; dipotassium phosphate, 2.5. Single colonies were picked up and transferred to fresh undiluted TSB agar plates for further growth and analysis. The cycle was repeated at least three times to ensure that pure culture was obtained. Eight isolates thus obtained were used for further studies. The pure strains were cultured in 5-fold diluted TSB medium under aerobic conditions with 200 rpm agitation unless mentioned otherwise. The strains were maintained on undiluted TSB agar plates and incubated at 55 °C for 2–5 days. Cultures were preserved at −80 °C as a 20% *v/v* glycerol suspension. Identification of the strains were done based on 16S rDNA sequencing and analysis as described in our recent work [27].

2.2. Calcium adsorption with proteins produced by the isolated strains

Proteins present in the extracellular and intracellular fraction were assessed for their calcium adsorption efficiency to locate the calcium adsorption activity. The isolated strains were grown in 5 fold dilution TSB broth at 55 °C for 2–5 days, depending on the growth of individual strains. The extracellular proteins were collected by centrifugation of the culture (6000*g*, 10 min) and the supernatant obtained was concentrated by membrane filtration (10 KDa molecular cut, Amicon, Model 8200, Millipore Co., U.S.A). The concentrated protein was stored at 4 °C until further use. The remaining cell pellet was re-suspended in phosphate buffer and disrupted by sonication: 50% amplitude for 5 min, 10 s on, 5 s off in a sonicator (S-4000, MISONIX Co., New York, U.S.A). The clear cell free extract thus obtained was used as the intracellular protein for measurement of calcium adsorption. The concentrations of the intracellular and extracellular proteins were measured via Bradford method using bovine serum albumin (BSA) as the standard. Calcium adsorption assays were performed at 25 °C, pH 7 and normal atmospheric pressure for 30 min.

2.3. Measurement of calcium adsorption ability

A 25 ml of protein solution (at a protein concentration of 50 mg/L) and 25 ml of calcium chloride solution (100 mg/L $CaCl_2{\cdot}2H_2O$) were mixed by vortexing and incubated at 25 °C for 30 min. The reaction time was determined by preliminary experiments and it was performed until the adsorption reached steady state. Then, the reaction mixture was filtered through a 10 KDa membrane (Millipore) to separate the calcium bound protein and residual calcium present in the filtrate was measured by Ion Chromatography (IC 790/792, Metrohm Co., Herisau, Switzerland). The Ca^{2+} adsorption efficiency was measured as mg of Ca^{2+} adsorbed per mg of protein used.

2.4. Effect of pH, temperature and pressure on the calcium adsorption efficiency of the proteins obtained from the isolated strains

Since the calcite may form along the up-flow path and at the flash point, the depth of anti-scalant injection should be below the flashpoint but above the deepest feed zone. The temperature and pressure of flash point in Ching-Shui geothermal well was used as reference for experimental design in this study. In this geothermal well, the flash point pressures ranged from 6.3 to 10 kg/cm^2 (i.e., 6.1 to 9.67 atm), while the temperatures ranged from 164 to 177 °C (note that the geothermal reservoir temperatures in Taiwan are within the range of 100–200 °C). For experimental safety, we conducted the temperature experiment under 150 °C, which could in general represent the temperature of most geothermal wells in Taiwan.

To determine the effect of pH, calcium adsorption assay with the proteins harvested from the eight bacterial isolates was measured at different pH using various buffers (Tris HCl for pH 2 and 4; Phosphate buffer for pH 6, 7 and 8; Tris base for pH 10) at 25 °C and 1 atm pressure. Effect of temperature on calcium adsorption was studied by measuring calcium adsorption capacity at 100 °C, 125 °C and 150 °C at pH 7. The effect of pressure on calcium adsorption efficiency was determined by measuring the adsorption efficiency at 10, 30 and 50 atm pressure at 25 °C and pH 7.

2.5. Comparison of calcium adsorption efficiency of proteins from Tepidimonas fonticaldi *AT-A2 with that of other known metal binding proteins*

The calcium adsorption efficiency of the extracellular proteins from *Tepidimonas fonticaldi* AT-A2 isolated from Antun Hot Spring, Hualien County, Taiwan (GPS location, 23° 17′ 37″ N 121° 20′ 13″ E; temperature, 50 °C; pH, 7.3; NaCl, 0%) was compared with other known metal binding proteins. The proteins used were as follows: recombinant fish [28], recombinant mouse metallothionein [28], recombinant human metallothionein [28], Accelerase (DuPont), cellulase isolated from *Pseudomonas* sp. CL3 [29], and cellulases from *Clostridium* sp. TCW1 [30]. The recombinant proteins were prepared as previously reported [26]. The cellulases of *Clostridium* sp. TCW1 and *Pseudomonas* sp. CL3 were the ammonium sulphate fractions of the culture supernatants of the respective cultures. The concentration of the proteins and initial calcium ion concentration used in the biosorption experiments was 25 and 50 mg/L, respectively. The biosorption was conducted under the given conditions: temperature, 25 °C; pH, 7; pressure, 1 atm.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The extracellular proteins from *T. fonticaldi* AT-A2 was analyzed on denaturing polyacrylamide gel electrophoresis. SDS–PAGE was performed using BioRad Miniprotean III system, with 12% separating gel and 5% stacking gel of pH 8.8 and 6.8, respectively. The resolved proteins were visualized by staining with Coomassie Brilliant Blue G250. The molecular mass standard used was GenColor Prestained Protein Marker, (GMbiolab Co. Taichung, Taiwan) [30]. The standard has ten protein bands with the molecular weight range of 10–180 KDa.

2.7. Analytical methods

Cell concentration in the culture was determined by optical density (OD) measurement at 600 nm using a spectrophotometer (model U-2001, Hitachi, Tokyo, Japan). The OD values were then converted to dry cell weight concentration, from the standard calibration curve generated previously. The concentration of soluble protein was measured by the dye binding method of Bradford using the Bio-Rad dye reagent concentrate in micro titer plates. Absorbance was measured at 595 nm (i.e., OD_{595}) after 20 min of incubation at room temperature using Spectrophotometer (U-2001; Hitachi High-Technologies Co., Tokyo, Japan) [31]. Bovine Serum Albumin was used as a standard. Ion chromatography (IC) (IC 790/792; Metrohm Co., Herisau, Switzerland) was also used to detect calcium in the filtered (0.2 μm) supernatant of adsorption sample after adsorption reaction. The column used in IC analysis was Metrosep C4 column (Metrohm Co., Metrohm Co., Herisau, Switzerland). The mobile phase was 1.7 mmol/L HNO_3 and 0.7 mmol/L DPA (dipicolinic acid) with a flow rate of 1.0 ml/min. The column temperature was controlled at 25 °C.

3. Results and discussion

3.1. Isolation and identification of calcium adsorbing bacteria originating from geothermal sites

Eight thermophilic bacterial strains, capable of producing calcium adsorbing extracellular proteins, were isolated from five geothermal areas of Taiwan, including Tatun volcanic area in Taipei City, Chingshui and Renze geothermal areas in Yilan County, Antun geothermal area in Hualien County, and Chinlun geothermal area in Taitung County. The strains were cultured on Tryptic Soy Broth (TSB) medium and re-plated on TSB agar plates to obtain pure cultures. All strains grew well at 55 °C on 5-fold dilution TSB medium under aerobic conditions. The cultures were designated as IC-5, YM, BE-A1, BE-C, LJ-B, GL, AT-A1, AT-A2 for identification purposes. The bacterial strains were identified by 16S rDNA sequencing and the highest similarity for each sequence was determined. As per the similarity values obtained, the strains were named as follows: *Anoxybacillus kamchatkensis* IC-5 (98.7% similarity), *Thermus scotoductus* YM (99.7% similarity), *Mycobacterium hassiacum* BE-A1 (100% similarity), *Brevibacillus thermoruber* BE-C (99.5% similarity), *Anoxybacillus mongoliensis* LJ-B (98.9% similarity), *Bacillus halodurans* GL (99.9% similarity), *Meiothermus ruber* AT-A1 (100% similarity), *Tepidimonas thermarum* AT-A2 (100% similarity). The 16 rDNA phylogenic analysis of the eight strains is depicted in Fig. 1. A further analysis was done to clarify the taxonomic position of *Tepidimonas thermarum* AT-A2 by a polyphasic taxonomic approach and was reassigned as a novel species *Tepidimonas fonticaldi* sp. nov. type strain AT-A2, with a NCBI Genbank accession number of JN713899 [27]. The 16S r DNA sequence of strain AT-A2 is shown in Fig. S1.

3.2. Localization of the calcium adsorbing proteins from the bacterial isolates

Bioremediation of metal contaminated sites or wastewater can be carried out with biosorbents derived from various microorganisms. The active components involved in biosorption can be the actual biomass (alive or dead) or other derived materials like polysaccharides and proteins [32,33,38]. The proteins involved in metal adsorption can be either extracellular (secreted outside to carry out a specific function) or intracellular (to carry out specific metabolic functions). The localization and identification of the active metal-adsorbing proteins is an important step in designing an efficient biological method for geothermal well descaling. In the light of this, the extracellular proteins and the intracellular cell free extract were prepared from all of the eight isolates cultured with 5-fold dilution TSB under aerobic condition at 55 °C and agitation rate of 200 rpm and evaluated for their calcium adsorption activity. The results are summarized in Fig. 2. It can be seen from Fig. 2 that the calcium adsorption activity was predominant in the extracellular

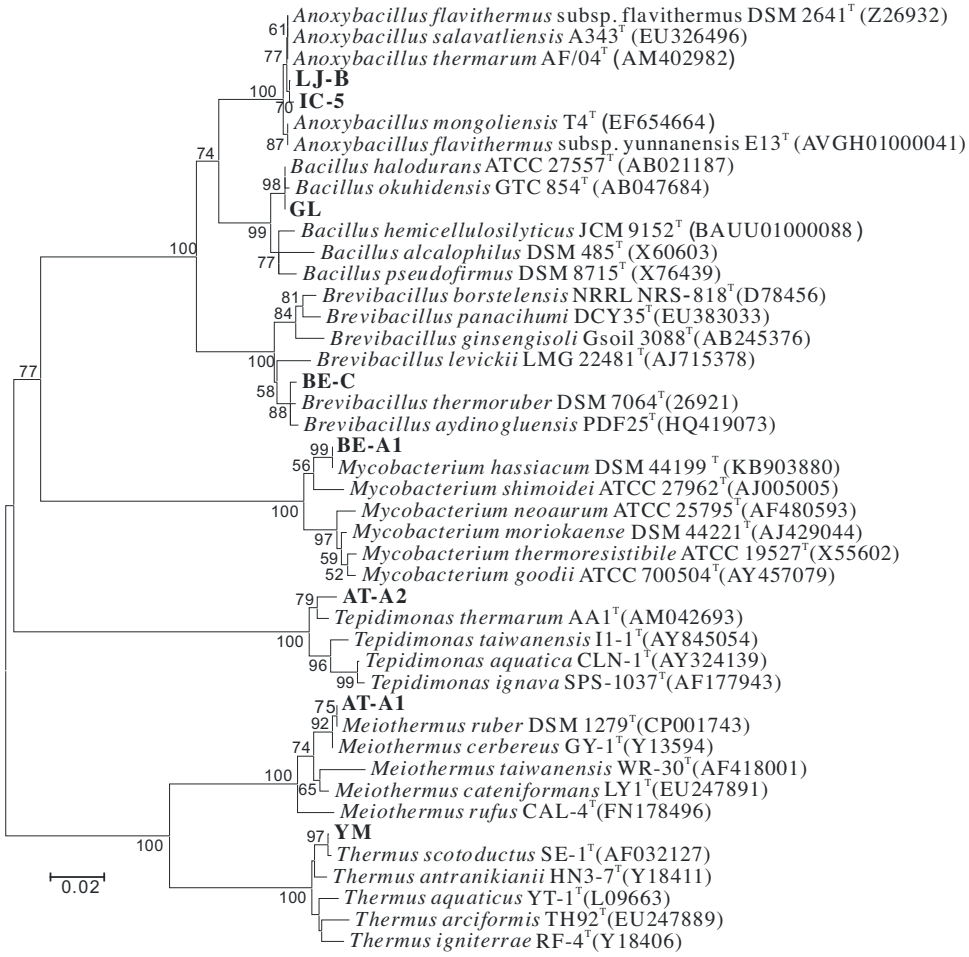

Fig. 1. A 16S rDNA phylogentic tree of eight isolated bacterial strains producing calcium-binding proteins.

fraction. The strains GL and BE-C shows calcium adsorption activity in both intracellular and extracellular fractions, but the activity in the intracellular fraction was less compared to the extracellular fraction. The presence of residual calcium adsorption activity in the intracellular fraction could be due to the various calcium binding proteins of the cytoplasm involved in cellular activities. The maximum calcium adsorption capacity (0.327 mg Ca^{2+}/mg protein) was seen in the extracellular fraction of AT-A2. For further investigation, only the extracellular fraction from the bacterial isolates were used.

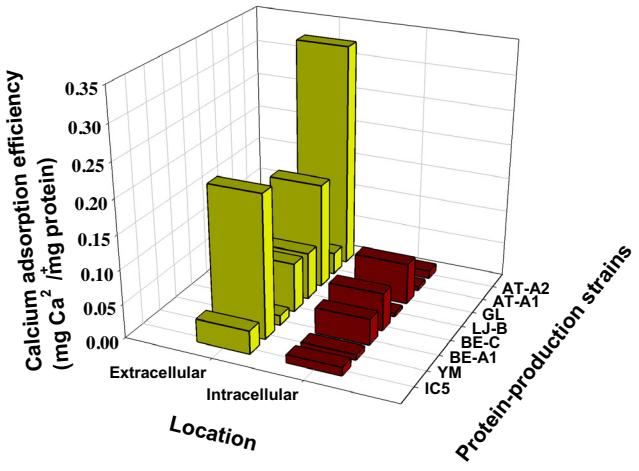

Fig. 2. Location of protein production based on calcium adsorption efficiency. Intracellular and extracellular proteins were collected from eight isolated strains grown on tryptic soy broth (TSB) medium at a temperature of 55 °C and an agitation rate of 200 rpm.

3.3. *Effect of pH on calcium adsorption efficiency of proteins from pure bacterial isolates*

Like other heavy metal biosorption activities, calcium adsorption can also be affected by changes in pH [34,37]. The changes in the pH of the medium or reaction conditions can alter the net charge of the protein and affect the ionic interactions that forms the basis for most adsorption methods. The solubility of the protein is also subject to change based on the pH values. Hence, the calcium adsorption activities of the extracellular fractions of the eight strains were studied at different pH values (2, 4, 6, 7, 8 and 10) under constant temperature and pressure (25 °C and 1 atm, respectively). The results are summarized in Fig. 3. The calcium adsorption efficiency of *Brevibaillus thermoruber* BE-C and *Mycobacterium hassiacum* BE-A1 was the highest at pH 2. *B. thermoruber* BE-C showed the highest calcium adsorption capacity (0.82 mg Ca^{2+}/mg protein) of all the pure strains in the acidic pH range (pH 2). All other strains showed the highest calcium adsorption efficiency at pH 10. Higher calcium adsorption efficiencies at pH 10 were obtained with *Tepidimonas fonticaldi* AT-A2, *Thermus scotoductus* YM, and *Meiothermus ruber* AT-A1, exhibiting a biosorption capacity

of 1.56–1.66 mg Ca^{2+}/mg protein. For the other strains, the highest adsorption capacity was in the range of 0.6 to 0.8 mg Ca^{2+}/mg protein (Fig. 3). Anburaj et al. showed that calcium adsorption was higher at the alkaline range of pH 8.5, but in their study whole cells of *Gleocapsa* sp. were used as the biosorbent [26]. Based on the overall assessment of the protein-based calcium adsorption efficiency over the pH range of 2–10, the extracellular proteins obtained from *M. ruber* AT-A1, *T. fonticaldi* AT-A2 and *T. scotoductus* YM seem to be better biosorbents for the removal of calcium (Fig. 3).

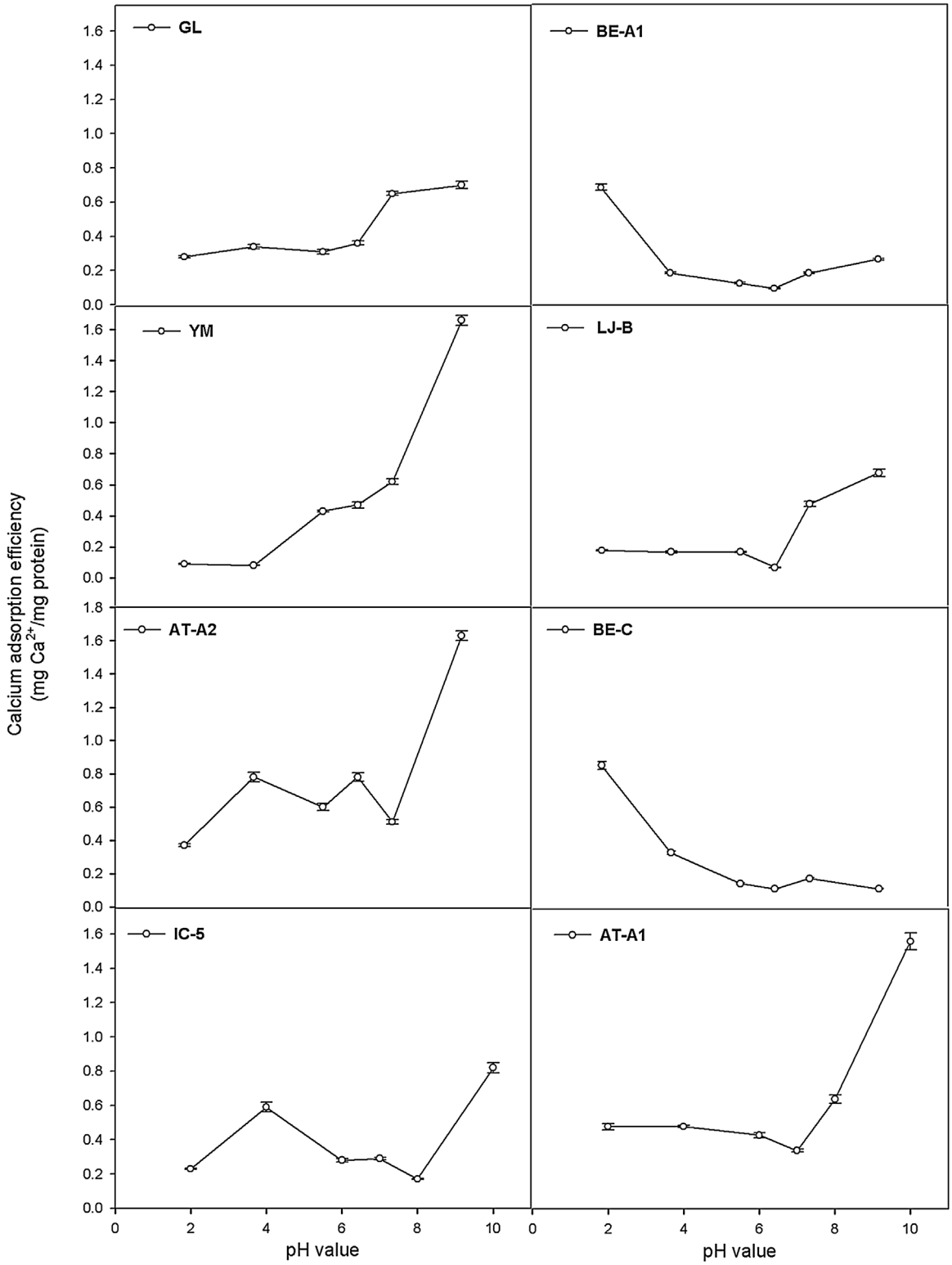

Fig. 3. Effect of pH on calcium adsorption efficiency of proteins obtained from eight isolated strains. The operating temperature and pressure was 25 °C and 1 atm, respectively. IC-5: *Anoxybacillus kamchatkensis* IC-5, YM: *Thermus scotoductus* YM, BE-A1: *Mycobacterium hassiacum* BE-A1, BE-C: *Brevibacillus thermoruber* BE-C, LJ-B: *Anoxybacillus mongoliensis* LJ-B, GL: *Bacillus halodurans* GL, AT-A1: *Meiothermus ruber* AT-A1, AT-A2: *Tepidimonas thermarum* AT-A2.

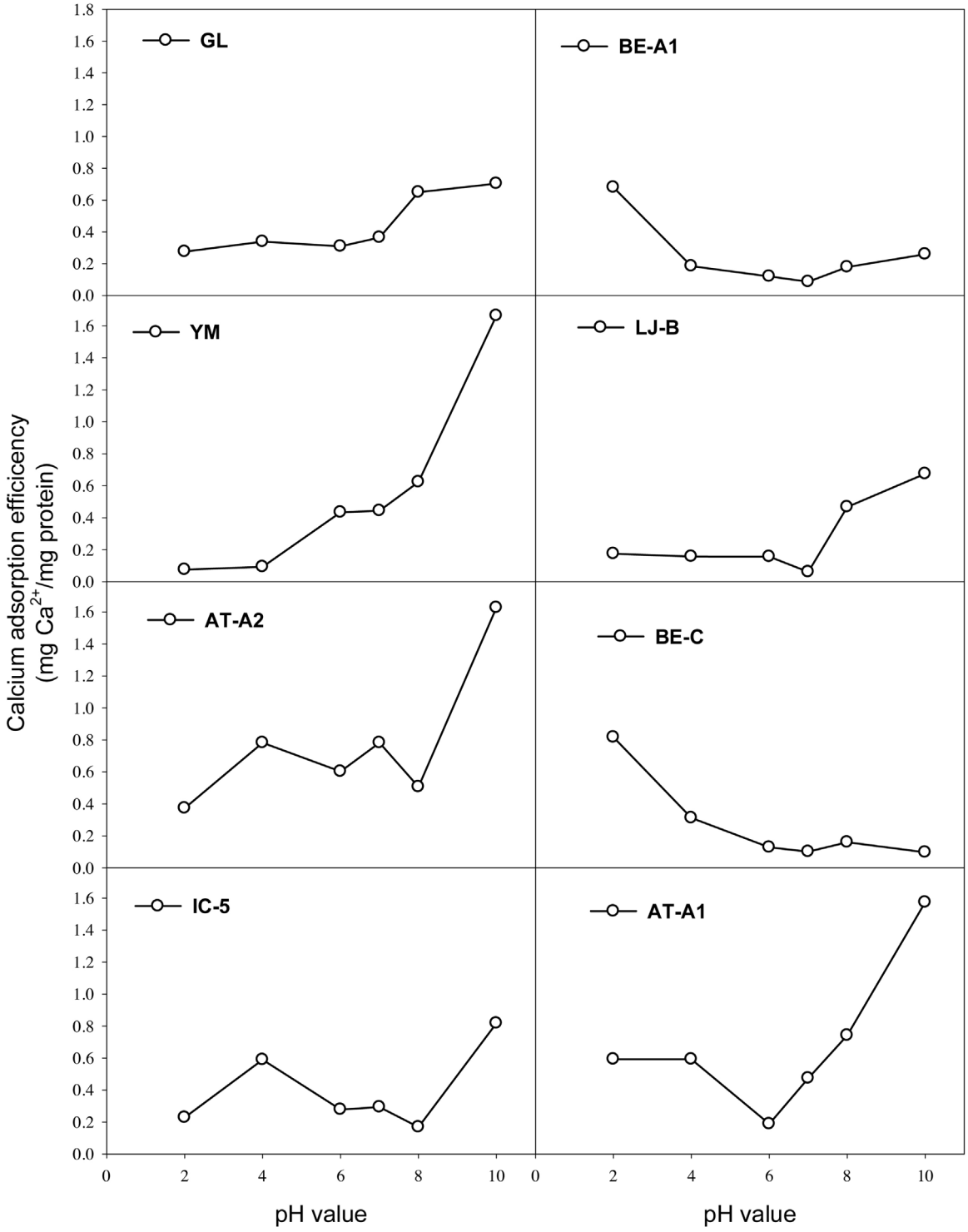

Fig. 3. (*Continued*)

3.4. Effect of pressure on calcium adsorption efficiency of protein from pure bacterial isolates

The pure strains used in this study were isolated from different geothermal areas in Taiwan. Pressure is apparently an important factor influencing the biodiversity of the species isolated from hot springs as these waters rise from deeper regions through cracks in the earth's crust [1]. Since geothermal site descaling needs to be operated at high pressure, the effect of pressure on calcium adsorption efficiency with the protein-based biosorbent should be investigated. The calcium adsorption efficiencies under different pressures (i.e., 1, 10, 30 and 50 atm) were thus examined at constant temperature 25 °C and pH 7. The results are shown in Table 1. It was observed that increasing the pressure from 10 to 50 atm did not significantly influence the calcium adsorption capacity for the proteins from *B. thermoruber* BE-C, *M. hassiacum* BE-A1, *A. kamchatkensis* IC-5 and *A. mongoliensis* LJ-B. However, for proteins from *B. halodurans* GL, *M. ruber* AT-A1 and *T. scotoductus* YM, the calcium adsorption capacity increased with increase in pressure from 1 atm to 30 atm, while it decreased when the pressure was further

Table 1
Effect of pressure on calcium adsorption efficiency of proteins obtained from eight pure strains. The operating temperature and pH was 25 °C and pH 7, respectively.

Microorganism	Calcium adsorption efficiency (mg Ca^{2+}/mg protein)			
	1 atm	10 atm	30 atm	50 atm
Anoxybacillus kamchatkensis IC-5	0.29 ± 0.02	0.42 ± 0.03	0.40 ± 0.04	0.44 ± 0.01
Tepidimonas fonticaldi AT-A2	0.78 ± 0.02	0.91 ± 0.10	0.85 ± 0.05	0.86 ± 0.02
Thermus scotoductus YM	0.08 ± 0.01	0.90 ± 0.01	0.91 ± 0.03	0.82 ± 0.04
Bacillus halodurans GL	0.36 ± 0.01	0.52 ± 0.03	0.73 ± 0.02	0.66 ± 0.05
Meiothermus ruber AT-A1	0.34 ± 0.02	0.92 ± 0.04	1.24 ± 0.06	1.05 ± 0.05
Brevibacillus thermoruber BE-C	0.10 ± 0.01	0.07 ± 0.01	0.13 ± 0.01	0.02 ± 0.01
Anoxybacillus mongoliensis LJ-B	0.06 ± 0.01	0.16 ± 0.02	0.17 ± 0.01	0.15 ± 0.03
Mycobacterium hassiacum BE-A1	0.09 ± 0.01	0.30 ± 0.01	0.20 ± 0.02	0.10 ± 0.04

increased from 30 atm to 50 atm. Among all the strains examined, proteins from strain AT-A1 exhibited the highest calcium adsorption capacity of 1.24 mg Ca^{2+}/mg protein at a pressure of 30 atm. Overall, an increase in pressure led to increase in calcium adsorption capacity and a pressure of 30 atm seems to be optimum for most of the strains (Table 1). To our best knowledge, there is no study reporting the effect of pressure on biosorption performance in metal-containing aqueous solutions. Thus, this study seems to be the first attempt in the literature to reveal the influence of operating pressure on the biosorption of calcium with protein-based biosorbents.

3.5. Effect of temperature on calcium adsorption efficiency of proteins from pure bacterial isolates

The temperatures of the hot springs across Taiwan vary between 40 and 100 °C in the surface and can be even higher inside the geothermal wells. Temperature is an important factor in determining the nature of the proteins and the activity decreases outside of their optimal temperature range. The influence of operating temperature on metal adsorption efficiency also depends on the nature of biosorption reaction (i.e., exothermic or endothermic). In this study, the calcium adsorption capacity of the proteins from eight pure strains at different temperatures (namely, 25, 100, 125 and 150 °C) were determined. The results are indicated in Table 2. For all the strains except *M. hassiacum* BE-A1, the calcium adsorption capacity increased when the temperature was raised from 100 °C to 125 °C, and a slight increase when the temperature was further increased to 150 °C. For *M. hassiacum* BE-A1, the calcium adsorption capacity increased when the temperature was increased from 25 to 125 °C, and the adsorption capacity decreased with further increase in temperature to 150 °C. (Table 2). The maximum calcium adsorption capacity of 1.9 mg Ca^{2+}/mg protein was achieved for *T. fonticaldi* AT-A2, *T. scotoductus* TM, *B. halodurans* GL and *M. ruber* AT-A1 (Table 2). In general, increase in temperature increases the calcium adsorption efficiency for most of the isolated strains. This suggests that the calcium biosorption with the proteins is endothermic. It was previously reported that calcium adsorption with ion exchange resins (Zeolite A) are endothermic and adsorption efficiency increases with an increase in temperature [35]. Ion exchange has been proposed as the mechanism of heavy metal biosorption by other macrophytic biomass [36].

3.6. Comparison of calcium adsorption efficiency of extracellular proteins of Tepidimonas fonticaldi AT-A2 with other metal binding proteins

The calcium binding efficiency of the extracellular proteins from the eight isolated strains were studied in detail with respect to the effect of temperature, pH and pressure. Of all the strains, *M. ruber* AT-A1, *T. fonticaldi* AT-A2 and *T. scotoductus* YM showed higher calcium adsorption capacities. Among these three strains, cell growth rate of *T. fonticaldi* AT-A2 was much higher than that of *M. ruber* AT-A1 and *T. scotoductus* YM (data not shown). Furthermore, *T. fonticaldi* AT-A2's extracellular proteins also showed higher calcium adsorption activities under all conditions tested. Thus, for further studies extracellular proteins from *T. fonticaldi* AT-A2 was selected as the biosorbent.

The calcium adsorption capacity of AT-A2 protein biosorbent was compared with known metal binding proteins, such as metallothioneins from various sources (fish, mouse and mammalian) [28], Accellerase (commercial cellulase from DuPont), cellulases from *Pseudomonas* sp. CL3 [29] and cellulases from *Clostridium* sp. TCW1 [30]. Metallotioneins are small molecular weight, intracellular proteins with high affinity and specificity for metals, implying their role in metal ion homeostasis in mammals and resistance to heavy metal toxicity in other organisms [28]. Cellulase enzymes are used as similar extracellular enzymes from bacteria to study the calcium adsorption efficiency. As shown in Fig. 4, the extracellular proteins from *T. fonticaldi* AT-A2 showed the highest calcium adsorption capacity of 0.78 ± 0.02 g Ca^{2+}/g protein when compared to the other proteins. Hence, it is clear that the efficiency of calcium adsorption by AT-A2 proteins are much higher than known metal binding proteins. This demonstrates that the AT-A2 protein is a suitable candidate as the biosorbent for calcium removal via biosorption.

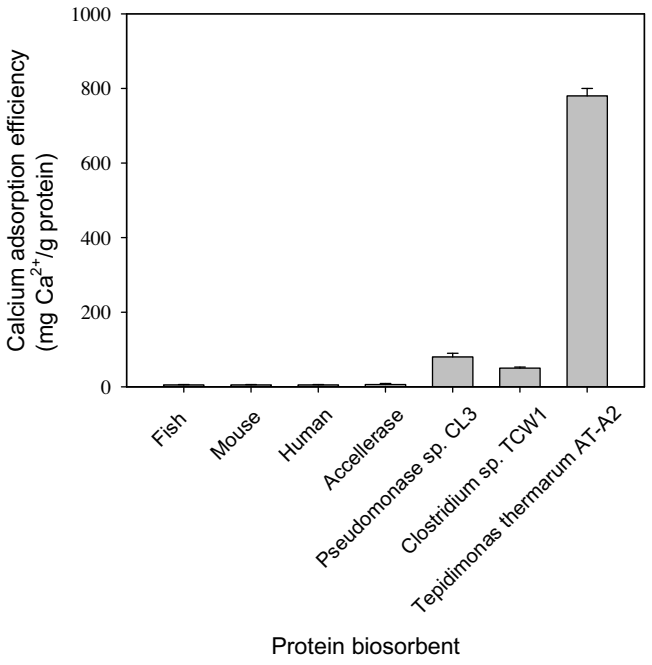

Fig. 4. Comparison of calcium adsorption efficiency of metal-binding proteins obtained from different biological sources. The biosorption was conducted under the conditions of temperature, 25 °C; pH, 7; pressure, 1 atm. The concentration of the proteins and initial calcium ion concentration used in the biosorption experiments was 25 and 50 mg/L, respectively.

Table 2
The effect of temperature on calcium biosorption efficiency of proteins obtained from eight pure strains. The operating pressure and pH was 1 atm and pH 7, respectively.

Microorganism	Calcium adsorption efficiency (mg Ca^{2+}/mg protein)			
	25 °C	100 °C	120 °C	150 °C
Anoxybacillus kamchatkensis IC-5	0.29 ± 0.02	0.22 ± 0.01	0.54 ± 0.02	0.74 ± 0.03
Tepidimonas fonticaldi AT-A2	0.78 ± 0.02	1.08 ± 0.05	1.84 ± 0.07	1.94 ± 0.05
Thermus scotoductus YM	0.08 ± 0.01	1.26 ± 0.03	1.94 ± 0.06	1.98 ± 0.04
Bacillus halodurans GL	0.36 ± 0.01	1.18 ± 0.08	1.82 ± 0.05	1.84 ± 0.06
Meiothermus ruber AT-A1	0.34 ± 0.02	1.30 ± 0.04	1.88 ± 0.02	1.94 ± 0.06
Brevibacillus thermoruber BE-C	0.10 ± 0.01	0.08 ± 0.01	0.20 ± 0.01	0.28 ± 0.01
Anoxybacillus mongoliensis LJ-B	0.06 ± 0.01	0.42 ± 0.02	0.72 ± 0.03	1.16 ± 0.06
Mycobacterium hassiacum BE-A1	0.09 ± 0.01	0.20 ± 0.01	0.48 ± 0.02	0.36 ± 0.02

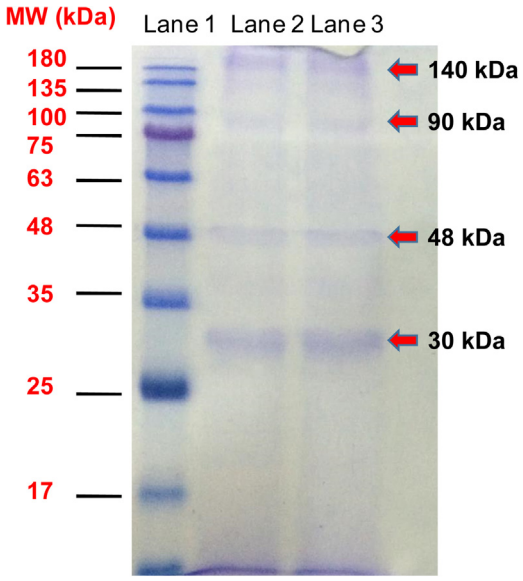

Fig. 5. SDS-PAGE gel of extracellular proteins produced by *Tepidimonas fonticaldi* AT-A2. Lane 1: molecular marker; Lane 2 and Lane 3: AT-A2 protein samples.

3.7. *SDS-PAGE analysis of the extracellular proteins of* Tepidimonas fonticaldi *AT-A2*

The extracellular proteins were isolated from *T. fonticaldi* AT-A2 and concentrated before analysis with SDS PAGE. The protein samples (20 μg) were resolved on 12% polyacrylamide gel. After coomassie brilliant blue staining, the protein bands were visualized. The SDS-PAGE results are indicated in Fig. 5. Lane 1 shows the molecular weight marker and lanes 2 and 3 shows the resolved AT-A2 extracellular proteins. There are a significant number of proteins present and the major protein bands present were estimated to be of a molecular weight of 30, 48, 90 and 140 KDa, respectively. Further studies needs to be done to identify the main proteins responsible for the calcium adsorption activity of the extracellular fraction. This will be taken up as the objective for future studies and using pure protein as the biosorption agent than a mixture of extracellular proteins.

4. Conclusions

This study demonstrated a novel and eco-friendly method to remove calcium from geothermal fluid for control of scaling. Eight thermophilic strains were isolated from Taiwan's geothermal sites. Among them, *Tepidimonas fonticaldi* AT-A2 had better growth rate and higher calcium adsorption capacity. The extracellular proteins from AT-A2 showed the maximum adsorption capacity of 1.94 mg calcium/mg protein, which is much higher than that of other known metal-binding proteins (e.g., metallothioneins). Thus, the AT-A2 proteins seem to have the potential to become an effective biosorbent to remove calcium in the geothermal wells to reduce scaling.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bej.2016.09.010.

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