Isolation and identification of a calcium-precipitating bacterium and optimization of influential factors

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ABSTRACT
Thirteen morphologically different strains were obtained from mangrove sediment and soda lake sediment. The calcium precipitating activities (CPA) of the strains were evaluated, and strainH4 exhibited the highest CPA value. Strain H4 was identified as *bacillus* species based on the 16S rDNA sequence analysis. Further, effect of variable factors on calcium precipitation of strain H4 was evaluated. The result showed that sodium lactate and sodium nitrate were the best carbon source and nitrogen source for the precipitation of calcium ion, respectively. When the concentration of sodium lactate was above 10g/L, the calcium precipitation reached the top and remained stable. The increase of initial concentration of sodium nitrate from 0 to 1.5 g/L led to a significant increase of calcium precipitation, whereas the concentration of sodium nitrate more than 2g/L inhibited the calcium precipitating process. Under the optimal conditions obtained above, strain H4 achieved its maximal calcium precipitating activity at initial pH 10.6 with the suitable spore concentration being 4.0×10^7 spores/ml. The study will lay a foundation for the further application of bacterial self-healing technology in the repairment of concrete crack.

1. INTRODUCTION
With the development of concrete science and the requirement of environment-friendly concrete maintenance, bacteria-based self-healing technology for concrete has become one of the research hotspots in the field of civil engineering in the last decade. The self-healing of concrete crack by means of bacteria is based on the microbial-induced calcium carbonate ($CaCO_3$) precipitation (MICP), which is a common phenomenon in the natural environment. Among the previous studies, various spore-forming bacteria were employed by different researchers. There are mainly two kinds of carbonate generating metabolic pathway involved in the bacteria-based crack healing of concrete, including oxygen-independent enzymatic hydrolysis of urea and oxygen-dependent (aerobic) respiration. Bang et al.and Wang et al. used urease-producing bacteria *Sporosarcina pasteurii* (*formerly Bacillus pasteurii*) and *Bacillus sphaericus*[1,2], respectively. Urea-hydrolysis has been demonstrated to guarantee the continuous formation of dense calcium carbonate crystals. However, the ammonium ions released from urea lysis might have negative effects on the environment and human health. Recently, a non-ureolytic bacteria based self-healing system with certain nutrients (e.g. calcium lactate, calcium glutamate) instead of urea as CO_2/CO_3^{2-} donor was proposed by Jonkers et al. to avoid such drawbacks[3]. Various facultative aerobes or aerobes such as *Bacillus cohnii*, *Bacillus alkalinitrilicus* and *Bacillus pseudofirmus* have been used to produce $CaCO_3$ in this novel

respiration-based crack healing system[3,4]. In each of these studies, only a part of the cracks could be completely sealed, and the maximum crack width healed by *Bacillus alkalinitrilicus* and *Bacillus sphaericus* were 0.46 mm and 0.5 mm[2,3], respectively.

As shown above, researchers estimated the crack healing effectiveness of various bacteria by roughly measuring the width of repaired crack. However, given the complexity of microstructure inside the concrete, this estimation cannot exactly reflect the capability of bacteria to induce the formation of calcium carbonate. The calcium-precipitating activity (CPA) of bacteria is the key factor for the improvement on self-healing effectiveness of concrete crack. Nevertheless, due to the absence of a suitable detection method, the CPA of various bacteria has not been evaluated in the previous studies. On the other hand, concrete structures in coastal regions are subject to more harmful threats. Until now, however, calcium-precipitating bacteria from marine origin have been less studied. In this study, a high through-put strategy was first established for the determination of CPA of bacteria used for self-healing of concrete crack. Based on the established strategy, marine bacteria with high CPA were screened and identified. Furthermore, the effects of influential factors such as carbon source, nitrogen source, spore inoculums and pH on the performance of bacterial induced calcium carbonate precipitation were investigated. The study will lay a foundation for the further application of bacterial self-healing of concrete crack.

2. MATERIALS AND METHODS

2.1 Establishment of the high-throughput assay of CPA for self-healing bacteria

The assay of CPA was based on the detection of free Ca^{2+} concentration via O-CresolphthaleinComplexone(OCPC) method. The detailed steps were as follows: fresh cultured strains on alkaline LB agar plate were transferred into alkaline LB liquid medium containing (per liter) 5 g yeast extract, 10 g tryptone, 5 g NaCl and 22.13 g 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), and then incubated at 30 °C, 150 rpm. Two μl of the overnight grown culture (OD_{600} is about 1.0) of each strain was used to inoculate 100 μl of the calcium precipitation assay medium (CPM) in the wells of a 96-well microplate I (Corning Inc., US). CPM contained (per liter) 7.5 ml L-sodium lactate (60%), 2 g $NaNO_3$, 0.1 g $MgCl_2$, 0.02 g KH_2PO_4, 22.13 g CAPS,1Lartificial sea water without $MgCl_2$, pH=10.5. For each strain, an additional well inoculated with sterilized overnight grown culture (inactivated strain) was used as the control. Incubation was carried out in a CO_2-free vacuum desiccator at 30°C for 7days. CO_2-free situation of the vacuum desiccator was obtained by vacuumizing the desiccators and maintained by continuous supplying of N_2-O_2 (4:1,v:v) gas mixture. After seven days, cultures were centrifuged and 3.75 μL supernatant of each culture was transferred into the corresponding well of another 96-well microplate II. Then 300μL calcium assay solution containing (per liter) 25 mg OCPC, 0.5 g 8-Hydroxyquinoline and 45 g 2-amino-2-methyl-1-propanol was added into each well. After 10-min reaction, the free Ca^{2+} concentration was measured at 575 nm using a 96-well plate spectrometer (SpectraMax 190, Molecular Devices, US). CPA was calculated as follows: $CPA=(C_C-C_E)/C_C\times100\%$ (Where C_C and C_E are the average residual Ca^{2+} concentrations of the control and the experimental sample respectively).

2.2 Microorganism isolation

Sediment samples were separately collected from Shenzhen Futian mangrove reservation district, China. Each sediment sample (5g) was separately blended with 50 ml 0.1 M Na_2CO_3-$NaHCO_3$ solution (pH is approximately 9.7) in a 250 ml flask and shaken at 150 rpm, 30°C for 30 min. The sediment suspensions were then heated in a water bath at 63°C for 30 min. 5 ml of 25% yeast extract solution was added into each flask.The germinating spores were then harvested by centrifugation

and washed 4 times with denionized water. Subsequently, The suspensions were serially diluted in the Na_2CO_3-$NaHCO_3$ solution and plated onto LNSC (Lactate-Nitrate-Sea water-Carbonate) agar plates containing (per liter) 15 ml L-sodium lactate (60%), 2 g $NaNO_3$, 0.1 g KH_2PO_4, 0.01 g $FeSO_4$, 40 g sea salts (sigma), 10 ml trace element solution SL6, 10.6 g Na_2CO_3, 15 g agar. The initial pH of the suspension was approximately 11.0. The plates were kept in an incubator at 30°C for 2-7 days. The CPA was measured by the high-throughput assay established above.

2.3 Effect of nutritional and environmental factors on calcium precipitation

Influence factors on the bacterial induced calcium precipitation included carbon source (type and concentration), nitrogen source (type and concentration), cell concentration and pH. Glucose, sucrose, soluble starch, sodium formate, sodium acetate and sodium lactate were respectively prepared at 5 g/L in CPM medium without carbon source to optimize the carbon source. For nitrogen source, sodium nitrate, ammonium chloride, ammonium nitrate, urea, beef extract, yeast extract and tryptone were respectively prepared at 2 g/L in CPM medium without nitrogen source. After the best carbon source and nitrogen source were determined, the performance of bacterial induced calcium precipitation was carried out in CPM medium under different pH (7.0, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, 11.0, 12.0), different concentrations of carbon source (0, 2.5, 5, 10, 15, 20 g/L), nitrogen source (0, 0.5, 1.0, 1.5, 2.0, 2.5 g/L) and initial spore concentrations, respectively. The CPA was measured as described above.

3. RESULTS AND DISCUSSION

Fig 1a showed a good correlation between Ca^{2+} concentration and OD_{575}. A linearized standard curve was derived from 0 to 4 mM Ca^{2+} with r^2 being 0.997. The equation of the standard curve was shown as: y=0.2512x+0.2464.Compared with traditional EDTA titration, the high throughput method established in this study can deal with dozens of samples at a time, thus displaying promising potential for screening self-healing bacteria with high calcium precipitating activity.

Thirteen morphologically different strains were obtained during the isolation process. The CPA of resultant 14 strains was detected by the established high-throughput assay and the result was shown in Fig.1b. Among them, strain H4 showed the highest CPA of 94.8%, which was almost 20 times of that of M95.

Among the carbon sources and nitrogen sources applied in the experiment, lactate and nitrate conferred the highest CPA on H4 (Fig. 1c and 1d). The advantageous effect of these carbon and nitrogen sources on bacterial induced calcium precipitation has been confirmed by previous studies.The effect of concentrations of sodium lactate and sodium nitrate on calcium precipitation was also shown in Fig. 5a and b.It was clear that the presence of carbon source is very important to calcium precipitating ability of the bacterial stains.The result in Fig.1e showed that H4 maintained more than 80% of CPA in the range of pH 9.5 to 11.0. The pH lower than 9.5 resulted in a sharp decrease of CPA to 20%. The decrease was attributed to the alkaliphilic characteristic of the strain.Our experimental result in Fig 1f showed that 4.0×10^7 spores/ml was an economically suitable spore concentration.

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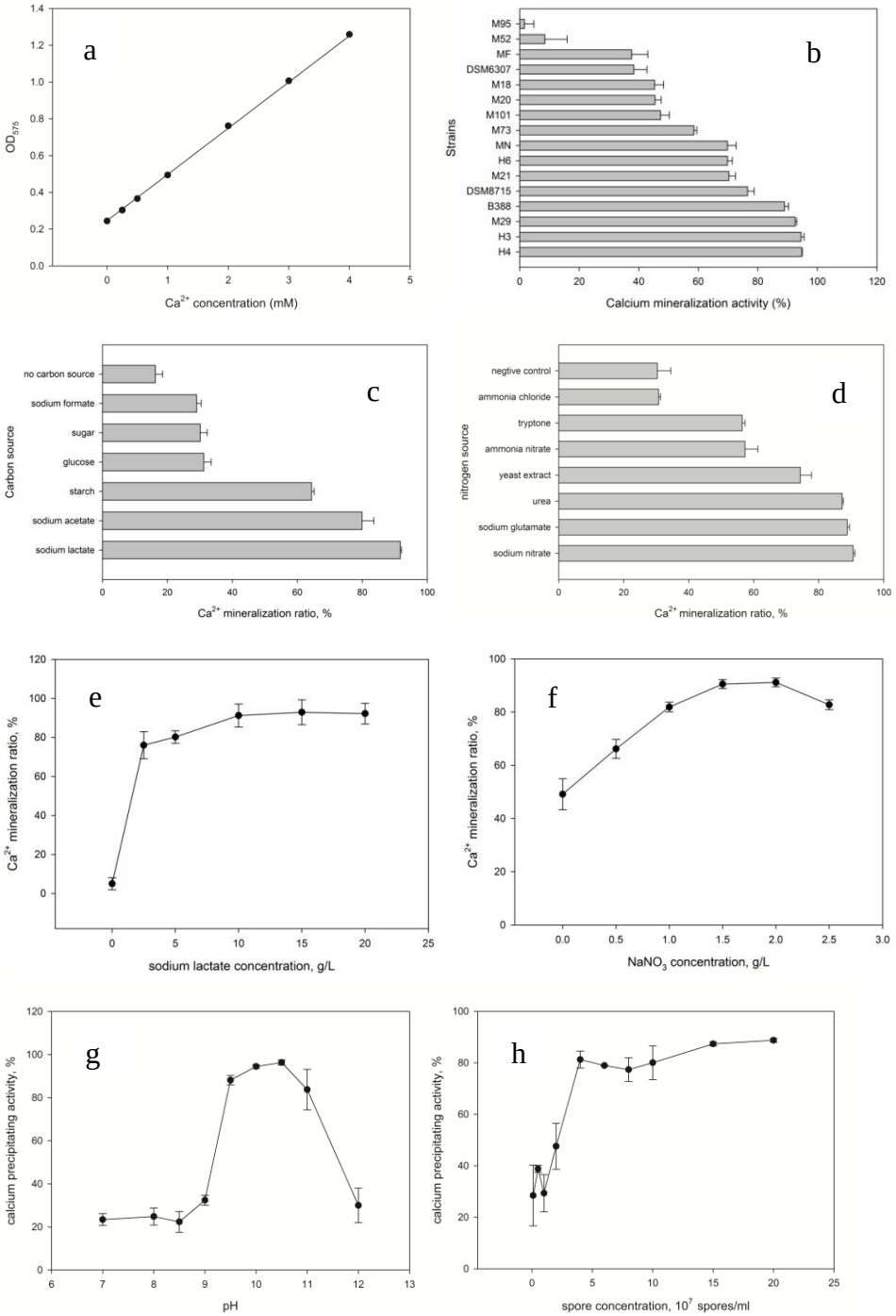

Figure 1. The linearized standard curve (a) ,the calcium precipitating activity of the isolates (b) and effect of carbon source type (c), nitrogen source type (d), carbon source concentration (e), nitrogen concentration (f), pH (g) and initial spore concentration (h) on the calcium precipitating activity of strain H4.