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APPLIED MICROBIAL AND CELL PHYSIOLOGY

# *Bacillus sphaericus* LMG 22257 is physiologically suitable for self-healing concrete

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**Abstract** The suitability of using a spore-forming ureolytic strain, *Bacillus sphaericus*, was evaluated for self-healing of concrete cracks. The main focus was on alkaline tolerance, calcium tolerance, oxygen dependence, and low-temperature adaptability. Experimental results show that *B. sphaericus* had a good tolerance. It can grow and germinate in a broad range of alkaline pH. The optimal pH range is 7 ~ 9. High alkaline conditions (pH 10 ~ 11) slow down but not stop the growth and germination. Oxygen was strictly needed during bacterial growth and germination, but not an essential factor during bacterial urea decomposition. *B. sphaericus* also had a good Ca tolerance, especially at a high bacterial concentration of $10^8$ cells/mL; no significant influence was observed on bacterial ureolytic activity of the presence of 0.9M $Ca^{2+}$. Furthermore, at a low temperature (10 °C), bacterial spores germinated and revived ureolytic activity with some retardation. However, this retardation can be counteracted by using a higher bacterial concentration and by supplementing yeast extract. It can be concluded that *B. sphaericus* is a suitable bacterium for application in bacteria-based self-healing concrete.

**Keywords** Alkaline tolerance · Ureolytic activity · Bio-$CaCO_3$ · Ca tolerance · Oxygen dependence · Temperature adaptability

## Introduction

Microbially induced carbonate precipitation (MICP) has been regarded as an environmentally friendly and economical technique which is and has been under extensive investigation for wide engineering applications (Rodriguez-Navarro et al. 2003; DeJong et al. 2006; Jimenez-Lopez et al. 2007; Jimenez-Lopez et al. 2008; De Muynck et al. 2008a; De Muynck et al. 2008b; Qian et al. 2009; DeJong et al. 2010). Currently, one very promising application is for self-healing of concrete cracks (Jonkers and Schlangen 2007; Jonkers and Schlangen 2009; Jonkers et al. 2010; Wang et al. 2012a; Wang et al. 2012b; Wang et al. 2014a; Wang et al. 2014b; Wang et al. 2014c; Wang et al. 2015). Carbonate-precipitating bacteria together with nutrients are added into the concrete matrix during the process of mixing. Upon cracking, the bacteria present at the crack surface are expected to precipitate $CaCO_3$ to heal the cracks in situ. Compared to conventional manual repair, the bacteria-based self-healing strategy has great potential to reduce the high maintenance and repair cost of concrete structures. In addition, the biogenic $CaCO_3$ has a good compatibility with the inorganic concrete matrix.

Essentially, a proper bacterial strain is of crucial importance in a bacteria-based self-healing system, which makes sure that biogenic precipitation can occur upon cracking. It is noted that the pH inside the concrete matrix can be as high as 12 ~ 13, particularly in freshly cast concrete. However, in the crack zone, the pH can be lower due to carbonation, to values in the range of 8 ~ 11, depending on the exposure time and

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humidity. Therefore, only alkali-tolerant or alkaliphilic strains, which are typically active in latter pH range, can be considered as concrete compatible regarding the alkalinity of concrete, though most bacteria can induce the precipitation of calcium carbonate under suitable conditions (Boquet et al. 1973). Promising results have been achieved using *Bacillus cohnii*, *Bacillus pseudofirmus* and *Bacillus alkalinitrilicus* all alkaliphilic members of the genus *Bacillus* (Jonkers et al. 2010; Wiktor and Jonkers 2011). These strains induce the precipitation of $CaCO_3$ by oxidation of organic compounds into $CO_2$, which transforms to $CO_3^{2-}$ in alkaline environments and subsequently precipitates in form of $CaCO_3$ in the presence of Ca ions available in concrete. Oxygen is an essential factor during oxidation of organic compounds. This indicates that in this kind of bacteria-based self-healing system, the oxygen availability in the crack zone will greatly influence the bacterial carbonatogenesity and hence the crack-healing efficiency. Other frequently used bacteria for concrete application are ureolytic strains, i.e., bacteria that produce urease enzyme in significant quantities (Benini et al. 1996; Bachmeier et al. 2002; Harkes et al. 2010; Okwadha and Li 2010). The strain used in current study is also an ureolytic strain, *Bacillus sphaericus LMG 22257*. Bacterial urease can catalyze urea hydrolysis. The enzymatic reaction rate of urea hydrolysis is approximately $10^{14}$ times faster than the chemical rate (Benini et al. 1996). Urea is decomposed into ammonium and carbonate ions (Eq. (1)) resulting in an increase from neutral pH to a value of about 9 (p*K* value of reaction 1 is 9.2). In the presence of $Ca^{2+}$ in the surroundings, $CaCO_3$ can be formed. The overall reaction can be seen in Eqs. (1) and (2). Typical ureolytic strains are *Sporosarcina pasteurii* (previously named *Bacillus pasteurii*), *B. sphaericus*, and *Bacillus megaterium*. The oxygen dependence of ureolytic strains during the biogenic $CaCO_3$ formation will be investigated in this study.

$$CO(NH_2)_2 + 2H_2O \xrightarrow{\text{Bacterial urease}} 2NH_4^+ + CO_3^{2-} \quad (1)$$

$$Ca^{2+} + CO_3^{2-} \rightarrow CaCO_3 \quad (2)$$

In a bacteria-based self-healing system, biogenic $CaCO_3$ functions as the crack healing/sealing material. The crack healing/sealing efficiency is directly related to the amount and rate of biogenic $CaCO_3$ formed, which relies on the amount and rate of urea decomposed, and the process is, as mentioned above, much faster in the presence of urease. Therefore, bacterial urease activity and urea concentration are the two most important determining factors for the rate and amount of $CaCO_3$ precipitation. Therefore, in a previous study, a spore-forming ureolytic strain, *B. sphaericus* LMG 22257 (Belgian coordinated collection of microorganisms, Ghent) was selected for studying MICP as this strain is characterized by a high urease activity (Dick et al. 2006). Concerning the harsh condition of concrete and the difficulty to predict cracking time, spores instead of vegetative cells have to be used for incorporation in concrete because they have a much longer "life-span" (from several years to hundreds of years) and higher resistance against chemical- and mechanical stress (Setlow 1994). Also to further protect bacterial spores during the concrete mixing and hardening process, different bacterial carriers have been used to encapsulate bacterial spores beforehand (Jonkers et al. 2010; Wang et al. 2012a; Wang et al. 2012b; Wang et al. 2014a; Wang et al. 2014b).

So far, most of the published studies have been focused only on the crack healing effect after incorporation of bacteria. While the bacterial behavior in cracked concrete is still unrevealed, in spite of the fact that it actually directly determines the biogenic precipitation and hence the crack healing efficiency. The main objective of the present study is to disclose the influence of crack zone conditions on bacterial activity, and hence to understand whether the bacterial strain is really suitable for the self-healing application or not. As above-mentioned, one feature of concrete is its alkaline pH, but the actual pH in the crack region is not precisely known. In this study, the alkali tolerance of *B. sphaericus* LMG 22257, i.e., the effect of pH on bacterial growth, spores germination, and bacterial urease activity, was first investigated. Furthermore, the amount of available nutrient in the crack zone is a limiting factor because the dosage of added nutrient has to be restricted due to its negative effect on concrete strength (Wang 2013). Therefore, the effect of nutrient concentration on spore germination and urease activity was examined and the minimum dosage needed for significant MICP was determined. Furthermore, Ca tolerance and oxygen dependence of *B. sphaericus* were explored since concrete functions as a Ca reservoir and oxygen diffusion in the crack zone could be a limiting factor for bacterial $CaCO_3$ precipitation. Regarding practical application, temperature adaptability of this specific bacterial strain was also considered. The activity of *B. sphaericus* with respect to bacterial growth, spore germination, and revival of urease activity at low temperature was therefore studied.

## Materials and methods

### Bacterial strain and cultivation conditions

*B. sphaericus* LMG 22257 (Belgian Coordinated Collection of Microorganisms, Ghent) was used in this study. Cultures were obtained after subsequent culturing (two times and 1% inoculum by volume) from a −80 °C stock culture (a 2-mL sterile vial). The growth medium consisted of yeast extract and urea (UYE medium). The yeast extract solution was first autoclaved, and the filter sterilized urea solution was added to this medium. In this study, the final concentration of yeast

extract and urea in UYE medium were both 20 g/L if not mentioned otherwise. The pH of the medium was 7. The bacteria were incubated aerobically at 28 °C on a shaking table at 130 rpm for 24 h. For some of the following experiments, the cells were harvested by centrifugation (8397 g, 7 min, Sorvall RC 6+, Thermo Scientific™), and the pellets resuspended in a sterile saline solution (NaCl, 8.5 g/L). The concentration of the bacteria in the suspension was around $10^9$ cells/mL.

*B. sphaericus* spores were cultivated in sterile liquid minimal basal salts (MBS) medium (Kalfon et al. 1983), which was composed of $MgSO_4.7H_2O$ (0.3 g/L), $MnSO_4$ (0.02 g/L), $Fe_2(SO_4)_3$ (0.02 g/L), $ZnSO_4.7H_2O$ (0.02 g/L), $CaCl_2$ (0.2 g/L) amended with tryptose (10 g/L), and yeast extract (2 g/L). The pH of this MBS medium was 7.4 (if not, it was adjusted by using 1 M HCl or NaOH). Mature spores were transferred as inoculum (1% by volume) into MBS medium. The cultures were incubated at 28 °C on the shaker at 100 rpm for 14 ~ 28 days until more than 90% of the cells sporulated. For subsequent experiments, the spores were harvested by centrifugation (8397 g, 10 min, Sorvall RC 6+, Thermo Scientific™) and resuspended in sterile saline solution (NaCl, 8.5 g/L). The spores suspension was then subjected to pasteurization (80 °C for 20 min, then 5 min in ice-cold water) to kill present vegetative cells. The concentration of the spores suspension was also established at $10^9$ cells/mL. Both the bacterial (vegetative cell) suspension and spores suspension were stored in a fridge at 4 °C until use.

## Bacterial urease activity

Bacterial urease activity (also named ureolytic activity) is in addition to urea concentration the most important parameter for ureolytic bacterial induced $CaCO_3$ precipitation. It determines the amount of urea per time unit that can be decomposed and hence the rate of $CaCO_3$ precipitation. In this study, urease activity was quantified by the amount of urea decomposed in time and two methods were used, depending on the specific situation.

### *Conductivity method*

The amount of urea decomposed was determined by measuring the conductivity of the urea solution. One mole urea produces 2 mol $NH_4^+$ and 1 mol $CO_3^{2-}$. The generation of ions due to urea decomposition results in a proportional increase of conductivity of the solution. The more urea is decomposed, the higher the conductivity of the urea solution will be. The relationship between urea decomposed and conductivity can be determined by the method described in Whiffin (2004). The specific formula at 10, 20, and 28 °C is shown in Eqs.(3)–(5). It should be noted that with this method, it is not possible to measure the ureolytic activity in presence of $Ca^{2+}$. This is due to the fact that the increase of $CO_3^{2-}$ from urea decomposition and the decrease of $Ca^{2+}$ because of the formation of $CaCO_3$ would make it difficult to relate conductivity with the amount of urea decomposed. A Consort C3010 sensor was used to measure conductivity in this study.

$$\text{urea decomposed (mM)} = \text{conductivity}\left(\text{ms.cm}^{-1}\right) \times 11.19 \quad (3)$$

$$\text{urea decomposed (mM)} = \text{conductivity}\left(\text{ms.cm}^{-1}\right) \times 11.08 \quad (4)$$

$$\text{urea decomposed (mM)} = \text{conductivity}\left(\text{ms.cm}^{-1}\right) \times 10.54 \quad (5)$$

### *TAN method*

In the presence of $Ca^{2+}$ and/or other cations, the urease activity was tested by the total ammonium nitrogen (TAN) method, which measures the N-$NH_4^+$ in the solution. As mentioned above, 1 mol urea produces 2 mol $NH_4^+$. Thus, the amount of urea decomposed can be calculated based on the measured N-$NH_4^+$. The calculation is shown in Eq. (6). The N-$NH_4^+$ concentrations were measured colorimetrically (spectrometer WPA at 425 nm) by the method of Nessler (Ivanov et al. 2005). Samples (3 ~ 5 mL) were taken and filtered to remove the cells and maybe precipitates before applying the TAN method.

$$\text{urea decomposed (g/L)} = \frac{\text{N-NH}_4^+ \text{ (mg/L)} \times 60 \text{ (g/mol)}}{2 \times 14 \text{ (g/mol)}} \times 10^{-3} \quad (6)$$

## Bacterial growth, spore germination, and urease activity under different pH conditions

### *Influence of pH on bacterial growth and spore germination*

Bacterial suspension was inoculated into different UYE media (1% by volume) with the initial pHs of 7, 8, 9, 10, 11 and 12, adjusted by 5 M NaOH. The bacterial culture was immediately added into a 96-well plate (6 wells for each kind). As controls, the media without inoculation were also added to the wells. The plate was then placed into a plate reader (Tecan Infinite M200 Pro). The optical density (OD) of the culture in the filled wells was measured and recorded every 20 min at a wavelength of 610 nm and temperature of 28 °C for 48 h. Growth curves of *B. sphaericus* at different pH values were therefore obtained. Germination and outgrowth of spores at different pH values were obtained in the same way, only that the monitoring time was 72 h instead of 48 h.

### *Influence of pH on urease activity*

In order to investigate the influence of pH on bacterial urease activity, bacterial suspension was added into a series of urea solutions (1% by volume) with pH of 6.6, 7.85, 8.6, 10.2,

10.96, and 11.6. The final concentration of urea was 20 g/L. To reach different pH values, phosphate buffer was used. Bacterial urease activity was measured by TAN method instead of conductivity method to avoid possible interference of the ions in the phosphate buffer. Three replicates were tested.

### Effect of nutrient on spores germination and revival of urease activity

The main nutrient for *B. sphaericus* was yeast extract that has been proven to be an optimal nutrient for robust bacterial growth in previous research (Wang 2013). In order to examine the effect of yeast extract on spore germination and revival of urease activity, spore suspension was inoculated in media consisting of urea (20 g/L) and different concentrations of yeast extract (20, 5, 2, 0.2, and 0 g/L). The composition of each medium can also be seen in Table S1 (in the supplementary file). The germination and outgrowth of spores were monitored by the Tecan plate reader (in the same way as mentioned in the previous section). Meanwhile, the revival of ureolytic activity of germinated spores (active vegetative cells) under different concentrations of yeast extract was determined by TAN method as mentioned above.

### Influence of urea and $Ca^{2+}$ on bacterial urease activity

Urea is the substrate of urease and the source for production of carbonate. Firstly, the influence of the concentration of urea on urease activity was investigated. Specific amount of bacterial suspension was added to a series of urea solutions of different concentrations. The final concentrations of bacteria were $2 \times 10^7$, $10^7$, $10^5$, and $10^3$ cells/mL and urea were 0.2, 0.4, 0.6, 0.8, 1, 1.5, 2, 2.5, and 3 mol/L in the mixed solutions. The composition can also be seen in Table S2 (in the supplementary file). The conductivity of the solution was tested at certain time intervals to monitor the urea decomposition.

Secondly, specific amounts of bacterial suspension were added to a series of precipitation media which were made of urea (1 mol/L) and different concentrations of $Ca^{2+}$ (0.25, 0.5, 0.75, and 0.9 mol/L). The concentration of bacteria in the precipitation media was 107 or 108 cells/mL. The amount of urea decomposed in different media was measured at certain time intervals by TAN method.

### Influence of oxygen on bacterial growth, spores germination, and urease activity

#### *Oxygen consumption during bacterial growth and spores germination*

Bacterial suspensions and spore suspensions (mentioned in previous section) were inoculated in modified UYE medium which consisted of 5 g/L yeast extract and 20 g/L urea. The medium after inoculation with vegetative cells or spores was added into 15 mL glass bottles. To monitor oxygen consumption during bacterial growth or spores germination, an oxygen meter (Optical Oxygen Meter Fibox 4, Presens) and oxygen sensor (sensor spot PSt3, Presens) were used in this study to measure the concentration of oxygen in the cell suspension. The oxygen sensor was glued to the inner wall of the glass bottle, and oxygen values were read by the optic fiber-based oxygen meter according to the companies specifications. The bottle was completely filled with culture, i.e., no headspace was left, and tightly sealed by a rubber stopper to avoid exchange of oxygen between culture and atmosphere. Therefore, only the dissolved oxygen in the medium was available for bacteria .

#### *Effect of oxygen on bacterial urease catalyzed urea decomposition*

Experiments were performed with cell suspensions in either closed vials or open to the atmosphere. In experiment 1, urea solution (20 g/L) was added to six penicillin bottles (100 mL per bottle), which were then tightly sealed by rubber stoppers. The volume of the penicillin bottle was 125 mL and hence the volume of the headspace was 25 mL. Three of them were then subjected to nitrogen flushing for 50 cycles to remove the oxygen in the headspace as much as possible. The oxygen content in the headspace was around 2% (tested by compact GC). Subsequently, flushed and un-flushed bottles were inoculated with 1 mL bacteria suspension. Therefore, the concentration of bacteria was around $10^7$ cells/mL in the urea solution. At certain time intervals, 4 mL samples from each bottle were taken for conductivity measurement. In experiment 2, urea solution (20 g/L) was added to two glass bottles (100 mL per bottle), which were fitted with oxygen sensor spots. One flask was inoculated with 1 mL bacteria suspension and the other one served as abiotic control. Conductivity and oxygen concentration of the urea solutions (with and without bacteria) were simultaneously and continuously measured every 5 min and recorded automatically (Consort C3020). The bottles were kept open during the whole test period to allow exchange of oxygen between medium and atmosphere. Similarly, vegetative bacteria and spore suspensions were inoculated in the modified UYE media. The evolution of urease activity along with the decrease of oxygen concentration was measured.

### Influence of temperature on bacterial growth, spore germination, and revival of urease activity

#### *Influence of temperature on bacterial growth and spore germination*

Bacteria and spore suspensions were inoculated (1% *v*/*v*) in the UYE medium (pH = 9) and incubated in a conditioned room at a temperature of 4 °C. The bacterial growth and spore

germination and outgrowth were monitored by the Tecan plate reader (in the same way as mentioned in previous section). Only, the temperature in the chamber of the reader was set to be 10 °C during the test to examine the influence of low temperature.

*Influence of temperature on bacterial urease activity*

A series of urea solutions (20 g/L) was made and preconditioned in incubators at 28, 20, and 10 °C for 24 h, respectively. Subsequently, a specific amount of bacterial suspension was added to the urea solutions, and the urease activity under different temperatures was investigated by the conductivity method.

*Influence of temperature on spore germination and urease activity at different concentrations of YE*

UYE media made with urea (20 g/L) and different concentrations of yeast extract (0, 0.2, 2, 5, and 20 g/L) were inoculated with spore suspension (0.5% *v*/*v*) and subsequently incubated at 28, 20, and 10 °C, respectively. The amount of urea decomposed in the media was tested by TAN method after 1, 3, 7, 14, 21, and 28 days.

## Results

### Influence of pH

The growth of cells and germination of spores, indicated by the optical density in function of time at different pH values, are shown in Fig. 1. One graph, which shows the average of the six replicates, is presented at each pH value. It can be seen that high pH prolonged the lag phase during bacterial growth. As shown in Fig. 1a, the lag phase lasted around 3 h when the pH of the growth medium was 7 to 9. With the pH increased to 10, 11, and 12, the lag phase increased to 4, 8, and 20 h, respectively. The propagation rate (slope of the tangent line of the graph) during log phase was almost the same when the pH was 7 ~ 9 but greatly decreased when the pH increased from 9 to 12. The concentration of the cells after 48 h, indicated by the $Abs_{610}$, followed the same sequence. Similarly, high pH extended the germination of spores. During the germination and outgrowth of the spores, there was also a lag phase period, indicated by the horizontal part of the graphs (Fig. 1b). The germination of spores occurred in this period, followed by outgrowth (van Melis et al. 2011; Pandey et al. 2013). The germination time varied from 6 to 12 h when the pH was in the range of 7 to 9. Much longer time (24 ~ 42 h) was needed for germination when the pH increased from 9 to 12. Spores germinated fastest at pH 8 (6 h) and slowest at pH 12 (around 42 h). The outgrowth rate was quite similar

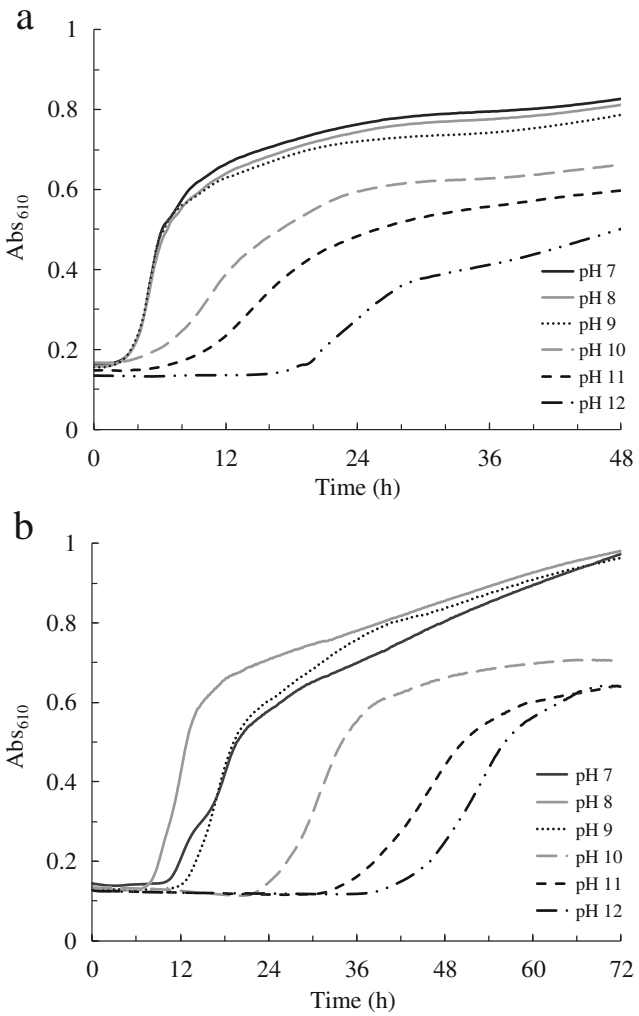


**Fig. 1** Optical density ($Abs_{610}$) evolution during bacterial growth (**a**) and spore germination (**b**) under different pH conditions

at the pH of 7 ~ 9, but significantly decreased at a pH higher than 9. It can be concluded that the optimal pH for *B. sphaericus* growth and germination was 7 ~ 9. At a highly alkaline pH (10 ~ 12), bacteria can still grow or germinate but at a much slower rate.

The urea decomposition under different pH conditions by bacteria is shown in Fig. 2a. The amount of urea decomposed at times 0, 1, 2, 4, 6, and 24 h was measured. The highest urease activity was seen at the pH of 8.5, which was 0.72 $mM.min^{-1}$. The urease activity at the pH of 6.6 and 7.85 was slightly lower, around 0.64 and 0.58 $mM.min^{-1}$, respectively. The urease activity further decreased to 0.5 and 0.44 $mM.min^{-1}$ with pH increase to 10.2 and 10.96. Nevertheless, it can be seen that all urea was decomposed within 24 h when the pH was lower than 11. However, urease activity was sharply decreased at the pH of 11.6, at which a very small amount (around 5%) of urea was decomposed after 24 h. Due to the decomposition of urea, the pH in the solution, depending on the initial pH, either rapidly increased or

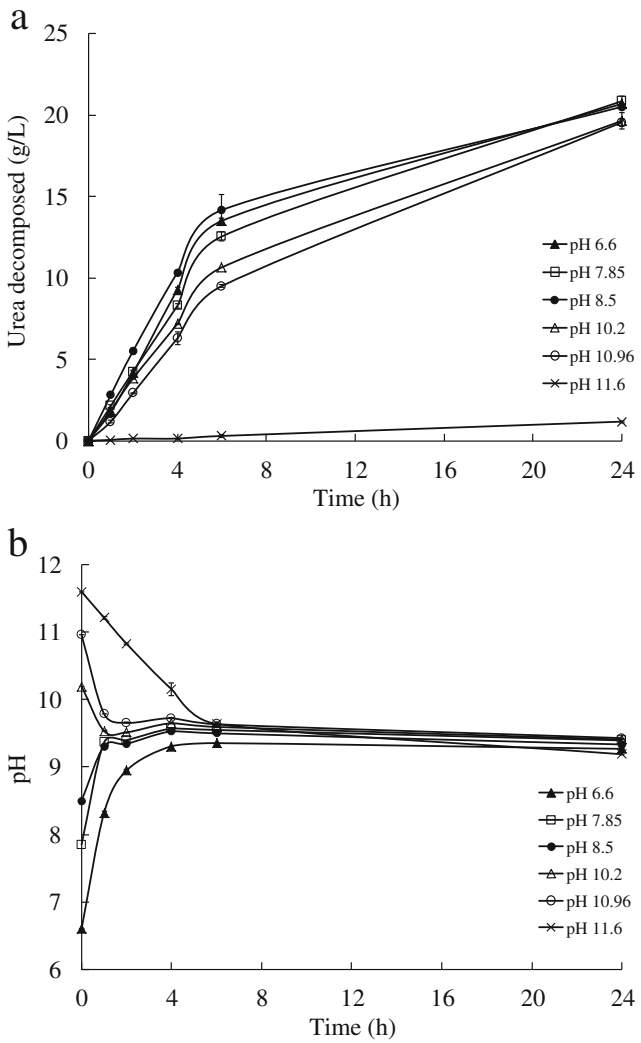


**Fig. 2** Urea decomposition (**a**) and pH evolution (**b**) under different initial pH conditions

decreased to 9.2 in the first 4 h except for the one at pH of 11.6, as shown in Fig. 2b. With the ongoing of urea decomposition, the pH values kept stable at a pH of 9.2. The pH of the urea solution which had an initial pH of 11.6 decreased to around 9.2 after the addition of bacterial cells (6 h after the addition).

## Effect of yeast extract

The germination and outgrowth of spores were greatly influenced by the concentration of the nutrient (yeast extract) in the medium. As shown in Fig. 3a, the higher the concentration of yeast extract, the higher the $Abs_{610}$ value. The germination was much faster, and the outgrowth of spores was much more remarkable when the concentration of yeast extract was 5 and 20 g/L than that in other series. The germination took more than 20 h at a low concentration of yeast extract (2 and 0.2 g/L), while no obvious outgrowth was seen with no presence of yeast extract. The revival of ureolytic activity of germinated spores was also affected by the amount of yeast extract. As shown in Fig. 3b, spores in the media with yeast extract concentrations of 20 and 5 g/L had a fast revival of ureolytic activity. About 70 ~ 95% of the urea in the media was decomposed in the first day. Spores in the media with 2 and 0.2 g/L yeast extract showed a greatly increased ureolytic activity after 3 days. Within 1 week, all the urea in the media of yeast extract was completely decomposed. For the spores in the media without yeast extract, the revival of ureolytic activity was much slower but still gradually increased. About 50% (10 g/L) and 85% (17 g/L) of the urea was decomposed after 7 and 28 days, respectively. This implied that yeast extract greatly facilitated the revival of ureolytic activity due to spore germination and outgrowth of cells. Without yeast extract, the spores can still revive the ureolytic activity but at a much slower rate.

## Effect of urea, $Ca^{2+}$, and bacterial concentration

At each concentration of urea, the higher the bacterial concentration was, the more urea was decomposed, as shown in Fig. 4a, c. The amount of decomposed urea was significantly decreased about two orders of magnitude when the concentration of the bacteria was decreased from $10^7$ to $10^5$ and $10^3$ cells/mL. At higher urea concentrations, higher bacterial concentration showed a beneficial effect: more urea was hydrolyzed in the series with bacterial concentration of $2 \times 10^7$ cells/mL, about 14 ~ 50% higher than that of $10^7$ cells/mL. The highest amounts of urea decomposed were obtained at urea concentrations of 2 and 1.5 M for bacterial concentration of $2 \times 10^7$ cells/mL (850 mM) and $10^7$ cells/mL (800 mM), respectively. When the bacteria concentration was $2 \times 10^7$ cells/mL, the total amount of urea decomposed increased with the increasing concentrations of urea until 2 M but then started to decrease at urea concentrations of 2.5 and 3 M. Similarly, for the series with a bacteria concentration of $10^7$ cells/mL, the total amount of urea degraded increased with the increasing urea concentrations until 1.5 M and then decreased from 1.5 M onwards. When the urea concentration was less than 1 M, the total amount of urea decomposed was similar in both series (Fig. 4a). The amounts of urea decomposed at 2.5 and 3 M urea were less than those obtained at urea concentration of 1 ~ 2 M, which indicated that for a certain amount of bacteria ($10^7$ ~ $2 \times 10^7$ cells/mL), the urea concentration, higher than 2 M, had a negative effect on bacterial ureolytic activity.

The urea decomposition ratio in all series decreased with increasing concentrations of urea, from about 95% at 200 mM urea to 15% at 3 M in the high bacterial concentration series (Fig. 4b) and from about 0.65% at 200 mM urea to 0.1% at 3 M in the low bacterial concentration series (Fig. 4d). Compared with the series of $10^7$ cells/mL bacteria, the series

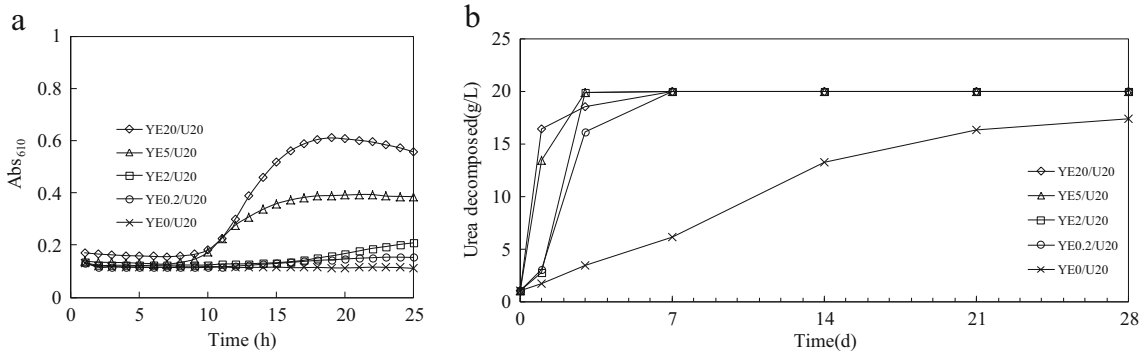


**Fig. 3** The germination and outgrowth indicated by optical density (**a**) and revival of ureolytic activity indicated by urea decomposition (**b**) of spores under various concentrations of yeast extract

of $2 \times 10^7$ cells/mL bacteria had similar urea decomposition ratios at lower urea concentrations (less than 1 M) and higher values at higher urea concentration range (1.5 ~ 3 M). For the series with $10^3$ ~ $10^5$ cells/mL bacteria, the ureolytic activity was much lower. The total amount of urea decomposed was in the range of 1 ~ 3 mM and the decomposition ratio was 0.1 ~ 0.6%. The results indicated that there was an optimal concentration of urea for each specific amount of bacteria.

The effect of $Ca^{2+}$ on bacterial ureolytic activity varied at different bacterial concentrations. As shown in Fig. 5a, the amount of urea decomposed was increased with increasing $Ca^{2+}$ concentration when the bacteria concentration was $10^8$ cells/mL. In the first 24 h, urea was completely decomposed in the media with a $Ca^{2+}$ concentration of 0.75 and 0.9 M, while 90 and 75% of the urea were degraded in the media with the $Ca^{2+}$ concentration of 0.5 and 0.25 M, respectively. Overall, within 3 days, urea was completely decomposed in all media with different concentrations of $Ca^{2+}$ at a bacteria concentration of $10^8$ cells/mL. Differently, when the concentration of bacteria was decreased to $10^7$ cells/mL, $Ca^{2+}$ had a negative effect on ureolytic activity. The amount of urea degraded was decreased with increasing $Ca^{2+}$ concentration (Fig. 5b). In the first day, about 4 and 20 g/L urea were decomposed in the media with 0.9 and 0.25 M, respectively. After 3 and 5 days, no significant increase of hydrolyzed urea was observed in the series with 0.9 M $Ca^{2+}$. For the ones with 0.75 M $Ca^{2+}$, the amount of urea decomposed after 5 days was slightly increased, around

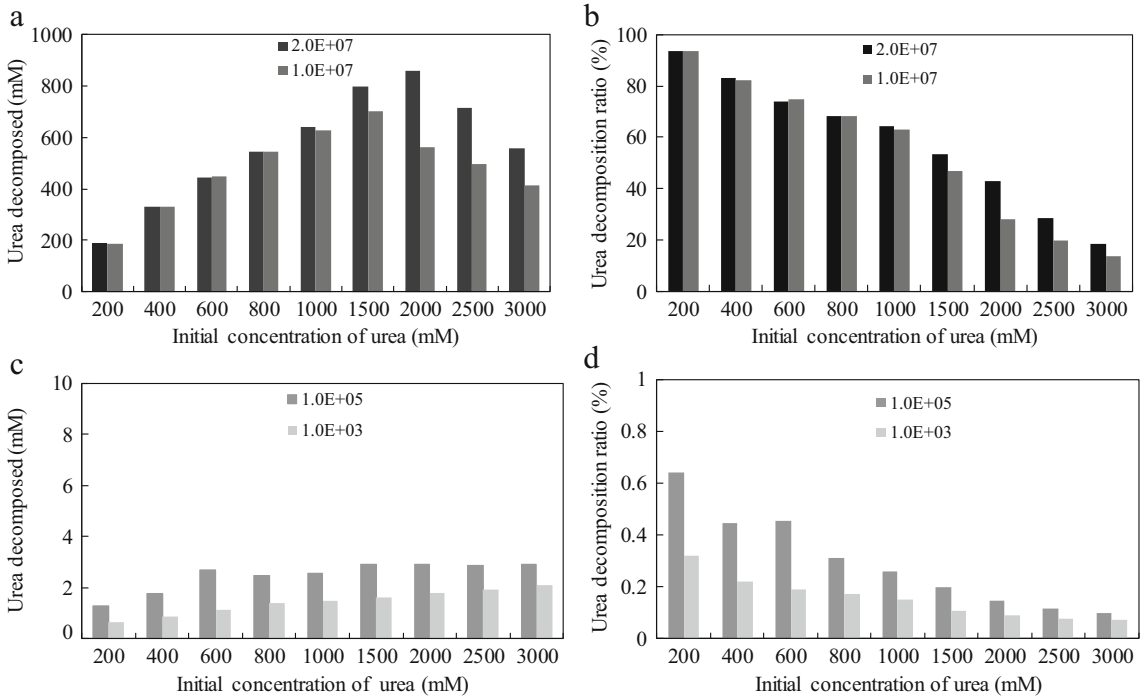


**Fig. 4** Different amounts of urea decomposed by different concentrations of bacteria (total amount of urea decomposed by high (**a**) and low (**c**) concentrations of bacteria; decomposition ratio at high (**b**) and low (**d**) concentrations of bacteria)

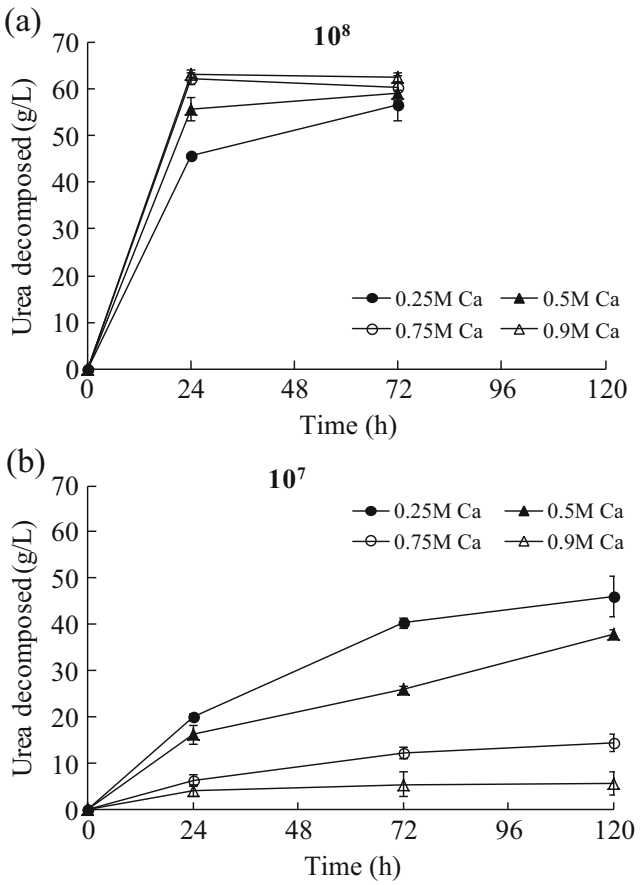


**Fig. 5** Urea decomposition under different concentrations of $Ca^{2+}$ by different amounts of bacteria (**a** $10^8$ cells/mL; **b** $10^7$ cells/mL; initial urea concentration 60 g/L)

12 g/L (20%). The ureolytic activity was much higher in the series with 0.25 and 0.5 M, in which 75% (45 g/L) and 58% (35 g/L) of the urea was degraded after 5 days. However, none of the series had all urea decomposed in the presence of a bacterial concentration of $10^7$ cells/mL. This indicated that $Ca^{2+}$ had more pronounced influence on ureolytic activity at a relatively lower bacterial concentration, for instance, $10^7$ cells/mL.

## Oxygen dependence

Oxygen was needed during bacterial growth and spore germination. As shown in Fig. 6a, the concentration of dissolved oxygen in water almost did not decrease as time went on. After the addition of bacterial cells, a slight decrease of oxygen concentration was noticed, which was due to the respiration of the cells. A more remarkable decrease of dissolved oxygen occurred in the UYE media with bacterial cells added. Oxygen was almost completely consumed by the bacteria within 20 h. Similarly, oxygen consumption was also observed during spore germination. As shown in Fig. 6b, the concentration of dissolved oxygen in the UYE media amended with bacterial spores gradually decreased from about 250 to 180 μM in the first 20 h, caused by the germination of spores. Subsequently, a more drastic decrease from 180 to 0 μM occurred in the next 8 h, which was due to the outgrowth of spores. It was also noted that the media itself also consumed small amount of oxygen, especially in the beginning (in the first 2 h), which was due to chemical oxidation of the nutrients (particularly yeast extract).

No significant difference in the evolution of conductivity (indication of urea decomposition) was observed between the flushed and non-flushed series in which the bacterial cells suspensions were added after flushing with nitrogen gas. Detailed data were not shown here but can be seen in Fig. S1 in the supplementary file. This implied that oxygen had no influence on bacterial ureolytic activity. This was further demonstrated in the tests performed in the open system.

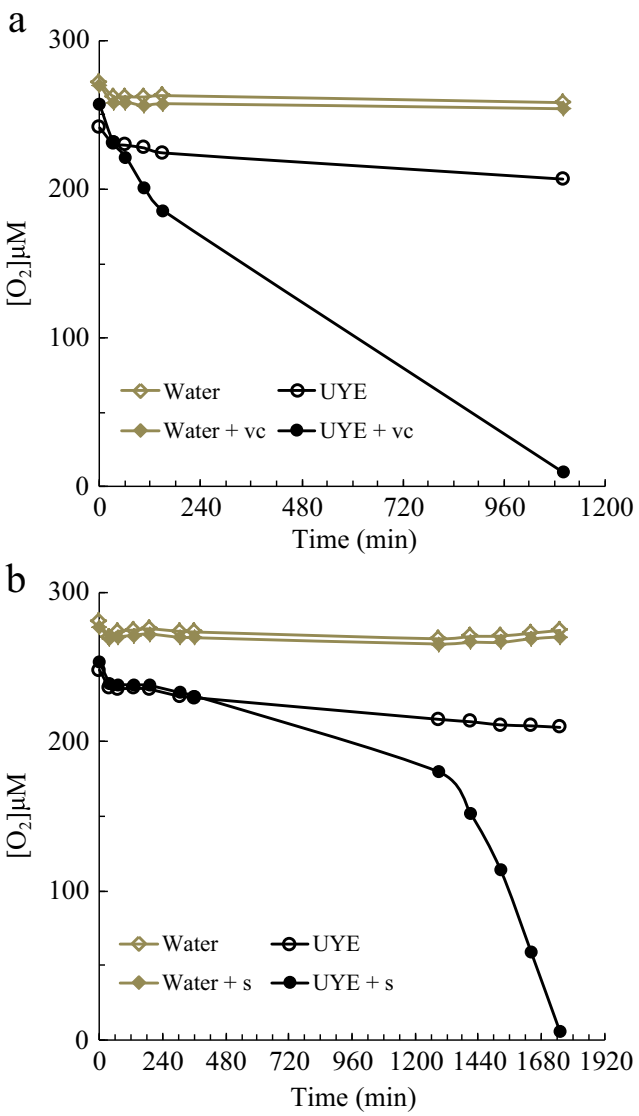


**Fig. 6** Oxygen consumption during bacterial growth (**a**) and spore germination (**b**) as time went on (*vc* vegetative cells, *s* spores)

These results are shown in Fig. 7. The concentration of the dissolved oxygen was quite stable in both the non-inoculated urea solution and the UYE medium (Fig. 7a, c). This is attributed to the fact that there is an equilibrium between the oxygen in the air and the oxygen in the solution in the open system. Without considerable consumption, the concentration of the dissolved oxygen remains stable. With the addition of the bacterial cells, the oxygen concentration in the urea solution was gradually decreased due to the respiration of the cells. Although oxygen concentration decreased, no decrease in rate of urea hydrolysis was observed, indicating that oxygen depletion did not affect urea hydrolysis (Fig. 7b). Similarly, in the UYE medium amended with the bacteria, oxygen depletion was fast. The rate of oxygen depletion was significantly higher in comparison to the urea solution as dissolved oxygen was almost completely consumed within 4 h. This was due to the bacterial growth triggered by yeast extract in the UYE medium. Nonetheless, no obvious difference was observed in the rate of conductivity increase (indication of urea decomposition rate) in the first 4 h and afterwards (Fig. 7d). This indicated that the amount of dissolved oxygen in the surroundings had no effect on bacterial ureolytic activity.

Furthermore, oxygen had also no influence on the revival of ureolytic activity of bacterial spore germination. As shown in Fig. 7e, dissolved oxygen was almost completely consumed due to spores germination and outgrowth in the first 24 h but did not hinder the revival of ureolytic activity. The conductivity in the UYE medium started to increase after spore

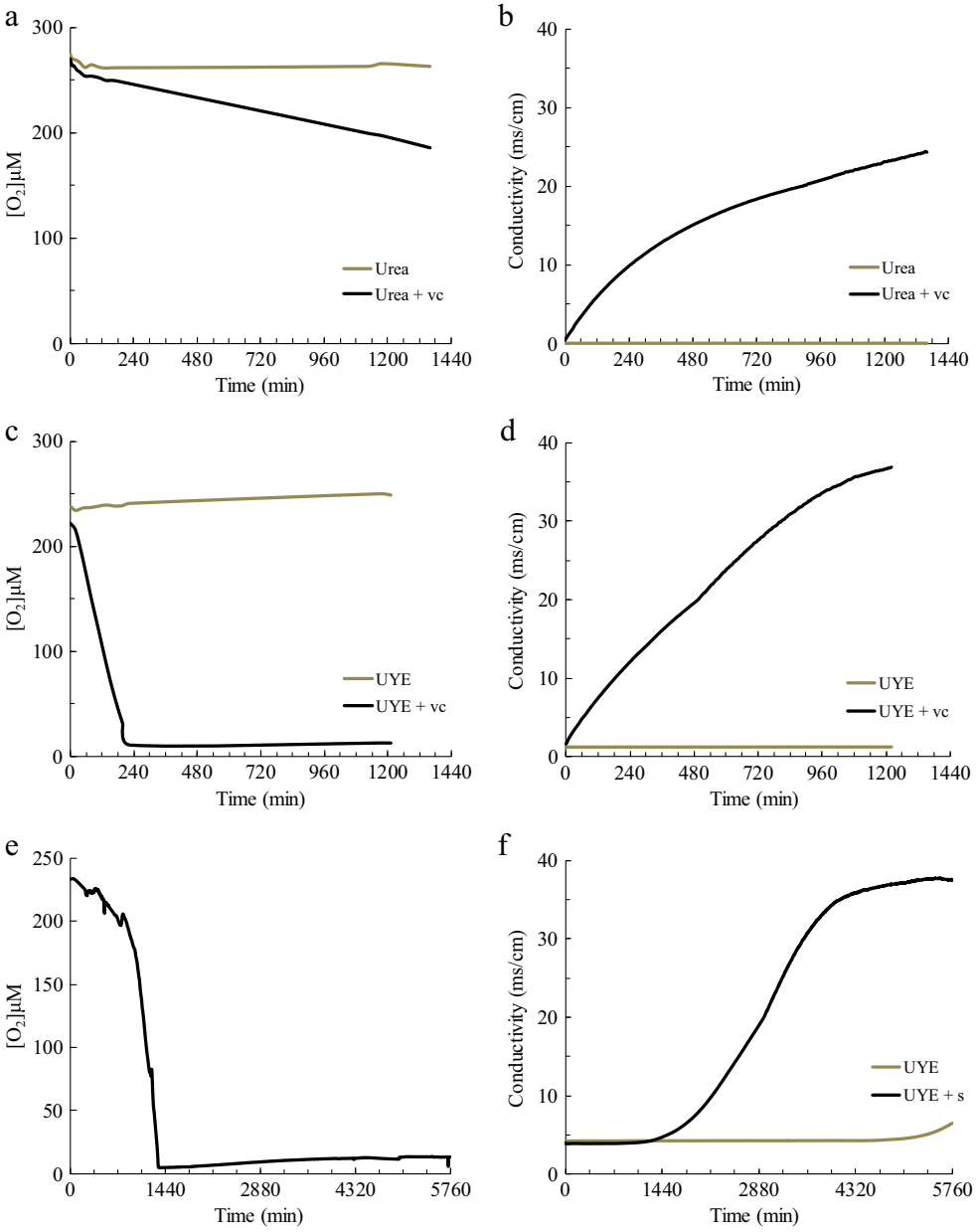


**Fig. 7** Evolution of oxygen concentration (**a**, **c,** and **e**) and conductivity (**b**, **d,** and **f**) during urea decomposition by bacterial cells and spores in the open system (**a**, **b** cells in the urea solution; **c**, **d** cells in the UYE medium; **e**, **f** spores in the UYE medium; +*vc* and +*s* inoculated with vegetative cells or spores)

germination from 24 h on (Fig. 7f). Within 3 days, the urea was completely decomposed. This implied that revival of ureolytic activity only occurred after spore germination and can therefore be attributed to urease production by vegetative cells. Ureolytic activity itself does not require oxygen.

### Influence of temperature

Figure 8 shows bacterial growth and spore germination at 10 °C in growth medium of pH 9. It can be seen that low temperature greatly slowed down the bacterial growth and spore germination. The lag phase during bacterial growth was about 48 h at 10 °C (Fig. 8a) and only 6 h at 28 °C (Fig. 1a). The growth rate in the log phase was also considerably lower. More significant influence was seen on spores germination. As shown in Fig. 8b, the outgrowth of the spores only started after 6 days; while for the spores at 28 °C, outgrowth occurred after only 12 h (Fig. 1b). The germination time was greatly prolonged from 12 h at 28 °C to 6 days at 10 °C.

In addition to growth and germination, bacterial ureolytic activity was also greatly affected by temperature. The higher the temperature was, the higher the urease activity obtained. Low temperature significantly slowed down urea decomposition. As shown in Fig. 9, when using $10^8$ cells/mL, urea was completely decomposed after 4, 6, and 13 h at 28, 20, and 10 °C, respectively. When the bacterial concentration was decreased to $10^7$ cells/mL, about 100 and 70% of the urea were decomposed after 24 h at 28 and 20 °C, respectively. However, the amount of urea decomposed at 10 °C was only about 30%. It was also noted that this negative effect was more pronounced when using a relatively lower bacterial concentration. That is, high bacterial concentration may counteract the negative effect of the low temperature on ureolytic activity.

The results of revival of ureolytic activity of germinating spores at 10 and 20 °C are shown in Fig. 10. Same test series were also performed at 28 °C, and the results are shown in Fig. 3b. Spores at 20 °C had a similar revival behavior as those at 28 °C. In the first 3 days, 90 ~ 100% of the urea in the media with 20 and 5 g/L yeast extract was decomposed, which was similar as that at 28 °C. At lower concentrations of yeast extract (2 and 0.2 g/L), the decomposed urea was less than that at 28 °C. Yet the urea in the media with yeast extract was almost completely decomposed within 7 days (Fig. 10a). Spores in the media without yeast extract, resulting in only minor spore germination, decomposed 15% (3 g/L) and 45% (9 g/L) of the urea after 7 and 28 days, respectively.

The revival of ureolytic activity was much slower at 10 °C. As shown in Fig. 10b, in the media with 20 g/L YE, about 3 ~ 4 g/L urea was decomposed in the first 3 days. A great increase of ureolytic activity occurred between the 3rd and 7th day; 15 ~ 17 g/L urea was decomposed at the 7th day. For the media with 5 and 2 g/L YE, the major revival of ureolytic activity occurred between the 7th to 14th day and between the 14th to 21th day, respectively. Urea was completely hydrolyzed after 21 days in the media with 20, 5, and 2 g/L YE. However, due to only minor spore germination in the media with 0.2 and 0 g/L YE, no noticeable decomposition of urea within 28 days occurred. Low temperature greatly slowed down the revival of ureolytic activity which can be attributed to slower germination of spores and related production of urease enzyme by vegetative cells.

## Discussion

### Vegetative cells vs. spores

It can be seen the lag phase during spores germination and outgrowth was always longer than that during cells growth under the same pH values. The lag phase was around 3 h

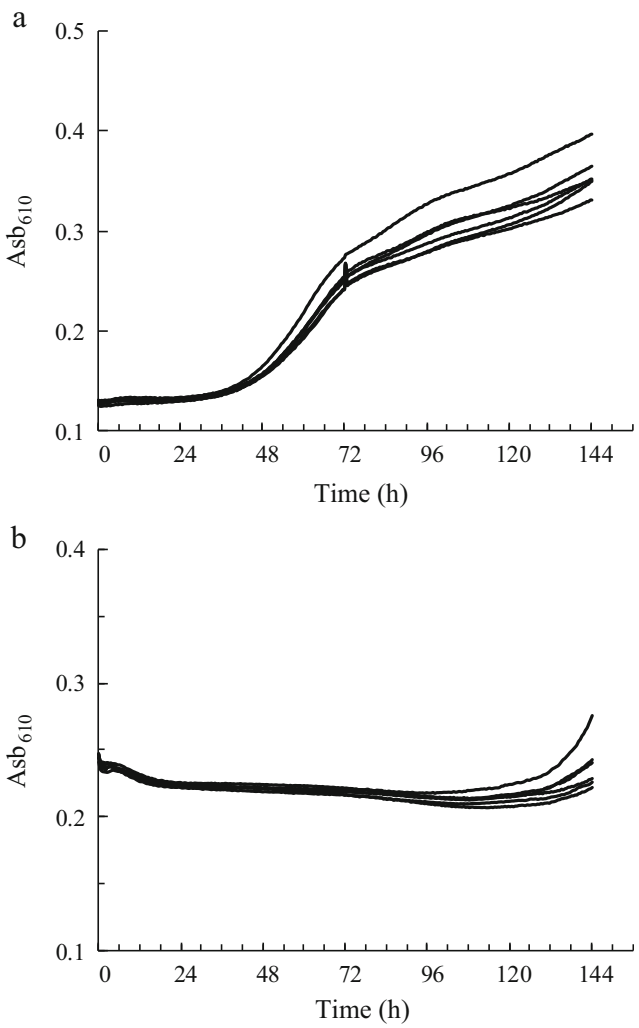


**Fig. 8** Optical density ($Abs_{610}$) evolution during bacterial growth (**a**) and spores germination (**b**) at a temperature of 10 °C (6 curves represent 6 replicates in each chart)

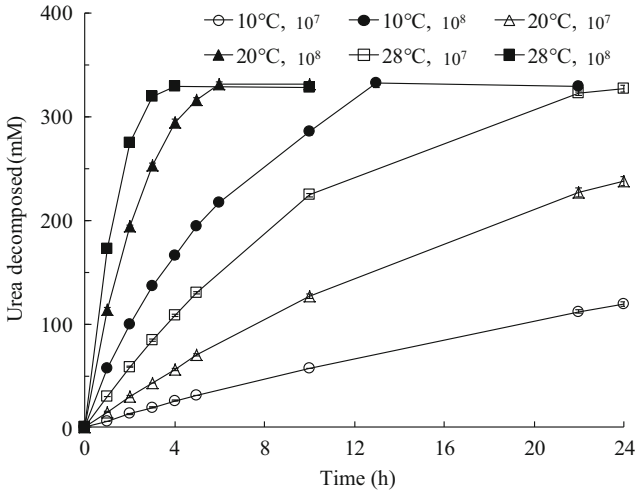


**Fig. 9** Urea decomposition under different temperatures (10, 20, and 28 °C) by different amounts of bacteria ($10^7$ and $10^8$ cells/mL)

during cells growth and 6 ~ 12 h during spores germination and outgrowth when the pH of the medium was 7 to 9. Higher pH enlarged this difference. With the pH increased to 10 ~ 12, the lag phase during spores germination lasted at least 20 h longer than that during cells growth. This is due to the fact that spores first germinate into active cells and then start to grow (with the presence of nutrients). This germination occurred in the "lag phase." The germination time prolonged with an increased pH. Meanwhile, the germination was greatly prolonged under a low temperature. The lag phase during bacterial growth was about 48 h, but the outgrowth of the spores only started after 6 days (Fig. 8b). Regarding the ureolytic activity, urea decomposition immediately started after the addition of vegetative cells (Fig. 7d). While for spores, in the first 24 h, spores germinated into cells, then followed by the bacterial urea hydrolysis (Fig. 7f). Overall, it can be seen that spores need more time than vegetative cells to grow and to decompose urea due to the fact that spores are dormant and need time to first become active (germinate), while vegetative cells are always in active state. This is ideal for the self-healing application. The bacteria should stay dormant inside the concrete until the cracks appear; by that time, the bacteria become active and induce $CaCO_3$ precipitation to heal the crack.

### Alkali tolerance of *B. sphaericus*

*B. sphaericus* has a good alkali tolerance. It can grow and germinate in a broad range of alkaline pH values from 8 to 11. The optimal pH range for bacterial growth and spores germination is 7 ~ 9. Under higher alkaline conditions (pH 10 ~ 11), bacteria can still grow or germinate, but at a much retarded rate. Nonetheless, this indicates that the embedded spores in cracks in concrete can germinate when pH drops below 12 due to carbonation of the crack fluids. The pH value can drop to 8 ~ 11 depending on the carbonation rate. Therefore, theoretically, it will take around 3 days for the spores in the crack zone to germinate if other conditions are optimal, for instance, sufficient nutrient, water, and oxygen are available (Wang et al. 2015). Furthermore, in the pH range of 8 ~ 11, bacteria had a remarkable ureolytic activity, which ensures the decomposition of urea and the precipitation of $CaCO_3$. Overall, it can be concluded that the appropriate working pH of *B. sphaericus* is 7 ~ 11. Above pH 11, the bacteria have limited capacity to precipitate $CaCO_3$. This implies that bacterial spores will keep dormant after being embedded in concrete matrix (pH > 12), and only start to become active after cracks appear and crack surface pH drops.

### Ca tolerance

*B. sphaericus* is a carbonate-precipitating bacterium. It produces urease, which catalyzes urea hydrolysis resulting in the formation of carbonate and ammonium ions. In the presence of Ca ions, $CaCO_3$ can therefore be formed due to the precipitation reaction of calcium and carbonate ions. Bacterial urease activity directly determines the productivity of biogenic $CaCO_3$, which is the main crack healing material in the bacteria-based self-healing system. The amount and rate of urea that can be decomposed were influenced by the amount

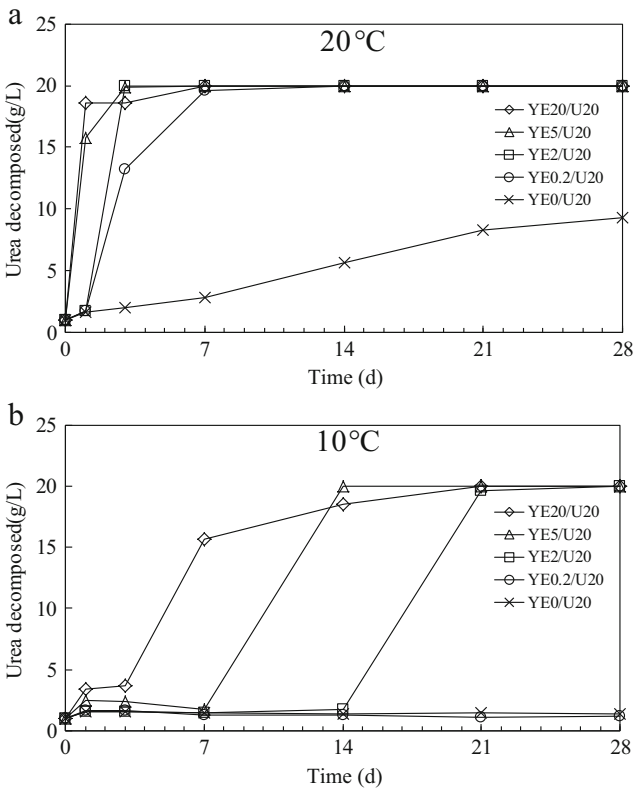


**Fig. 10** Revival of ureolytic activity indicated by urea decomposition at 20 (**a**) and 10 °C (**b**) at different yeast extract concentrations

of bacteria, urea, and Ca source. The more bacteria, the more urease present, and hence higher rate of urea decomposition. In this study, it was found that the concentration of bacteria should be at least $10^7$ cells/mL in order to obtain a significant rate of urea decomposition and hence $CaCO_3$ precipitation. This indicated that the bacterial concentration in the crack zone should be as high as possible, at least $10^7$ vegetative cells per milliliter, for a good healing efficiency. In this system, urea is the source of carbonate. The more urea is supplied, the more $CaCO_3$ can be formed, provided that sufficient amount of calcium ions is available. However, it is shown that there was an upper limit for the amount of urea that can be used for a certain amount of bacteria. For instance, the upper limit for $10^7$ and $2 \times 10^7$ cells/mL was 1.5 and 2 M urea, respectively. Above this limit, the amount of urea decomposition was decreased and thus appears inhibitory. The reason could be due to too high urea molecule transportation over the cell membrane into the cell at elevated urea concentrations, inhibiting other cellular processes. Therefore, a certain amount of bacteria can only metabolize a certain amount of urea hydrolysis.

Calcium ion concentration had also a profound effect on biogenic $CaCO_3$ precipitation activity. On the one hand, $Ca^{2+}$ is essential for the formation of $CaCO_3$. On the other hand, $Ca^{2+}$ influences bacterial ureolytic activity as demonstrated in this study. Too much $Ca^{2+}$ will become toxic to bacteria since they only need limited amount to regulate their life activities. It can be concluded in this research that *B. sphaericus* had a good Ca tolerance, especially at higher bacterial cell densities. With a bacterial concentration of $10^8$ cells/mL, no significant influence was observed on bacterial ureolytic activity at the presence of 0.9 M $Ca^{2+}$. With a decreased bacterial concentration ($10^7$ cells/mL), significant urea decomposition was still obtained at 0.5 M $Ca^{2+}$. Available $Ca^{2+}$ in crack zone is mainly from $Ca(OH)_2$. The solubility of $Ca(OH)_2$ is around 1.6 g/L (at 20 °C), which indicates that the maximum $Ca^{2+}$ concentration in the crack zone is around 0.02 M. Therefore, in the developed bacteria-based self-healing system, an additional Ca source, for instance Ca-nitrate was supplemented (Wang et al. 2012b, 2014a, 2014b). In addition, high Ca tolerance indicates a potentially high carbonate productivity, which ensures sufficient crack healing efficiency.

## Oxygen dependence

This study demonstrated that oxygen is needed for bacterial growth and spores germination. However, oxygen has no influence on bacterial ureolytic activity; that is, with or without oxygen, urea can be decomposed by the active vegetative cells. This is attributed to the fact that urease-driven urea decomposition is independent on oxygen concentration. Therefore, it implies that after cracking, spores first need oxygen to germinate and become active cells which produce urease enzyme, where after ureolytic activity is independent of oxygen. In previous research, it was found that most of the biogenic precipitation was concentrated on the surface layer of cracks and particularly at the crack-mouth where oxygen is available, while limited amount was precipitated in the deep part of the crack where oxygen becomes rapidly depleted (Wang et al. 2014c). Spores in the deep part of the crack inside the concrete matrix might therefore experience oxygen limitation, which hampers spore germination and therefore urease production resulting in limiting amounts of $CaCO_3$ precipitation. This might be a bottleneck problem for the aerobic bacteria-based self-healing system. A possible solution is to supplement nitrate to the system. Denitrification is a common property for *B. sphaericus* (Verbaendert et al. 2011). Nitrate can function as an electron acceptor under a limited oxygen conditions. The potential positive effect of nitrate addition to stimulate $CaCO_3$ precipitation needs to be further studied in the future research. Another option is to incorporate an oxygen generator into the system as was suggested by Zhang et al. (2016). In that study, different types of peroxides were applied and it was found that bacterial spore germination and carbonate precipitation were indeed enhanced. Currently, this research is still in the proof-of-concept stage. A challenge in that system is how incorporation of peroxides into the concrete matrix can be tuned in such a way that crack formation triggers the generation of oxygen release.

## Low-temperature adaptability

Bacterial ureolysis is an enzyme-controlled reaction, which was greatly affected by temperature. Temperature affects bacterial activity, enzymatic activity and therefore reaction rate, and hence the rate of formation of biogenic $CaCO_3$ and crack healing efficiency. Urease activity was stable between 15 and 25 °C, and an increase in temperature (until 60 °C) results in increased urease activity (Whiffin 2004). This is also demonstrated in this study: ureolytic activity was similar at a temperature of 20 and 28 °C. In practice, temperature varies from below 0 to over 30 °C in concrete constructions. This implies that at higher temperature, bacteria-based self-healing is significant while at low temperature the process may be inhibited. It was found in this research that *B. sphaericus* can still germinate and retain considerable ureolytic activity at a lower temperature of 10 °C; only the germination time was much longer (6 days) and the urea decomposition rate was slowed down. However, this retardation can be compensated by using a higher bacterial concentration what can be obtained by supplementing additional yeast extract. The higher the concentration of yeast extract, the shorter the retardation.

Yeast extract has an overall positive effect on germination of spores and formation of bio-precipitation. Without yeast

extract, spore germination is only moderate (but without significant outgrowth) resulting in limited precipitation of $CaCO_3$ (Wang 2013). Therefore, particularly under low-temperature conditions, enhanced addition of yeast extract is required. To assure a significant self-healing, a considerable amount of yeast extract (≥2 g/L) should be used. Overall, it can be concluded that *B. sphaericus* is a suitable bacterium for bacteria-based self-healing featuring aspects of good alkaline tolerance capacity, high Ca tolerance, high carbonate productivity, and temperature adaptability.

It should be noted that except for a proper bacterial source, another constituent, the bacterial carrier, is also of crucial importance in a bacteria-based self-healing system. In order to protect bacterial spores during concrete mixing and hardening, encapsulation of bacteria beforehand is preferable. A suitable carrier can protect bacteria and has no hindering effect on bacterial precipitation upon cracking, with no/limited negative effect on the cementitious matrix. Porous carriers were among the first to be used for immobilization of bacteria (Jonkers et al. 2010; Wang et al. 2012b). The immobilization can be realized easily by submersion or vacuum impregnation. Upon cracking, the bacteria immobilized in porous carriers easily reach oxygen, water, nutrients, etc., which facilitates the biogenic precipitation. However, the leakage of the bio-agents from the porous carrier into the matrix during the mixing process cannot be ignored. The loss or the release of the bio-agents in the early stage may greatly weaken the self-healing efficiency. The use of completely impermeable microcapsules to encapsulate bacteria can solve the leakage problem (Wang et al. 2014a). The microcapsules were alkali resistant and humidity sensitive. They were flexible under high humidity (in water) and brittle at low humidity, so they can withstand the mixing process and are easily broken upon cracking. The only problem with the microcapsules is that they have negative effect on the mechanical properties of the concrete. The dosage of the microcapsules should be less than 3 m% by cement weight, which restricts the amount of the healing agent that can be added. Glass tubes are a good option to carry a large volume of healing agents for a sufficient crack healing (Wang et al. 2012a). However, one big disadvantage is that they are too fragile to be mixed together with the concrete. So far, one very promising bacterial carrier is bio-compatible hydrogels. The bacteria spores can be encapsulated in the hydrogel matrix with very limited leakage (<1%). The added value of hydrogel is that it has a high water absorption and retention capacity. The absorbed water can be used for spores germination and bacterial activity when cracks appear, which is extremely important for a bacteria-based self-healing system (Wang et al. 2014b). Yet, due to its negative effect on the strength, modification on the hydrogel properties to improve the compatibility with the cementitious matrix is utmost important and is the focus of ongoing research.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

## References

Bachmeier KL, Williams AE, Warmington JR, Bang SS (2002) Urease activity in microbiologically-induced calcite precipitation. J Biotechnol 93:171–181

Benini S, Gessa C, Ciurli S (1996) *Bacillus pasteurii* urease: a heteropolymeric enzyme with a binuclear nickel active site. Soil Biol Biochem 28(6):819–821. doi:10.1016/0038-0717(96)00017-x

Boquet E, Boronat A, Ramoscor A (1973) Production of calcite (calcium-carbonate) crystals by soil bacteria is a general phenomenon. Nature 246(5434):527–529. doi:10.1038/246527a0

De Muynck W, Cox K, De Belle N, Verstraete W (2008a) Bacterial carbonate precipitation as an alternative surface treatment for concrete. Constr Build Mater 22(5):875–885. doi:10.1016/j.conbuildmat.2006.12.011

De Muynck W, Debrouwer D, De Belie N, Verstraete W (2008b) Bacterial carbonate precipitation improves the durability of cementitious materials. Cement Concrete Res 38(7):1005–1014. doi:10.1016/j.cemconres.2008.03.005

DeJong JT, Fritzges MB, Nusslein K (2006) Microbially induced cementation to control sand response to undrained shear. J Geotech Geoenviron 132(11):1381–1392. doi:10.1061/(asce)1090-0241(2006)132:11(1381)

DeJong JT, Mortensen BM, Martinez BC, Nelson DC (2010) Bio-mediated soil improvement. Ecol Eng 36(2):197–210. doi:10.1016/j.ecoleng.2008.12.029

Dick J, De Windt W, De Graef B, Saveyn H, Van der Meeren P, De Belie N, Verstraete W (2006) Bio-deposition of a calcium carbonate layer on degraded limestone by *Bacillus* species. Biodegradation 17(4):357–367. doi:10.1007/s10532-005-9006-x

Harkes MP, van Paassen LA, Booster JL, Whiffin VS, van Loosdrecht MCM (2010) Fixation and distribution of bacterial activity in sand to induce carbonate precipitation for ground reinforcement. Ecol Eng 36(2):112–117. doi:10.1016/j.ecoleng.2009.01.004

Ivanov VM, Figurovskaya VN, Barbalat YA, Ershova NI (2005) Chromaticity characteristics of $NH_2Hg_2I_3$ and $I^{-2}$: molecular iodine as a test form alternative to Nessler's reagent. J Anal Chem 60(7):629–632. doi:10.1007/s10809-005-0150-6

Jimenez-Lopez C, Jroundi F, Pascolini C, Rodriguez-Navarro C, Pinar-Larrubia G, Rodriguez-Gallego M, Gonzalez-Munoz MT (2008) Consolidation of quarry calcarenite by calcium carbonate precipitation induced by bacteria activated among the microbiota inhabiting the stone. Int Biodeter Biodegr 62(4):352–363. doi:10.1016/j.ibiod.2008.03.002

Jimenez-Lopez C, Rodriguez-Navarro C, Pinar G, Carrillo-Rosua FJ, Rodriguez-Gallego M, Gonzalez-Munoz MT (2007) Consolidation of degraded ornamental porous limestone stone by calcium carbonate precipitation induced by the microbiota inhabiting the stone. Chemosphere 68(10):1929–1936. doi:10.1016/j.chemosphere.2007.02.044

Jonkers HM, Schlangen E (2007) Self-healing of cracked concrete: a bacterial approach, vol 1–3

Jonkers HM, Schlangen E (2009) A two component bacteria-based self-healing concrete. Concr Repair Rehab Retrofit II:215–220

Jonkers HM, Thijssen A, Muyzer G, Copuroglu O, Schlangen E (2010) Application of bacteria as self-healing agent for the development of sustainable concrete. Ecol Eng 36(2):230–235. doi:10.1016/j.ecoleng.2008.12.036

Kalfon A, Largetthiery I, Charles JF, Debarjac H (1983) Growth, sporulation and larvicidal activity of *Bacillus sphaericus*. Eur J of Appl Microbio Biotechnol 18(3):168–173. doi:10.1007/bf00498040

Okwadha GDO, Li J (2010) Optimum conditions for microbial carbonate precipitation. Chemosphere 81(9):1143–1148. doi:10.1016/j.chemosphere.2010.09.066

Pandey R, Ter Beek A, Vischer NOE, Smelt J, Brul S, Manders EMM (2013) Live cell imaging of germination and outgrowth of individual *Bacillus subtilis* spores; the effect of heat stress quantitatively analyzed with SporeTracker. PLoS One 8(3). doi:10.1371/journal.pone.0058972

Qian CX, Wang J, Wang RX, Cheng L (2009) Corrosion protection of cement-based building materials by surface deposition of $CaCO_3$ by *Bacillus pasteurii*. Mat Sci Eng C-Bio S 29(4):1273–1280. doi:10.1016/j.msec.2008.10.025

Rodriguez-Navarro C, Rodriguez-Gallego M, Ben Chekroun K, Gonzalez-Munoz MT (2003) Conservation of ornamental stone by *Myxococcus xanthus*-induced carbonate biomineralization. Appl Environ Microb 69(4):2182–2193. doi:10.1128/aem.69.4.2182-2193.2003

Setlow P (1994) Mechanisms which contribute to the long-term survival of spores of *Bacillus* species. J Appl Bacteriol 76:S49–S60. doi:10.1111/j.1365-2672.1994.tb04357.x

van Melis CCJ, Groot MNN, Tempelaars MH, Moezelaar R, Abee T (2011) Characterization of germination and outgrowth of sorbic acid-stressed *Bacillus cereus* ATCC 14579 spores: phenotype and transcriptome analysis. Food Microbiol 28(2):275–283. doi:10.1016/j.fm.2010.04.005

Verbaendert I, Boon N, De Vos P, Heylen K (2011) Denitrification is a common feature among members of the genus *Bacillus*. Syst Appl Microbiol 34(5):385–391. doi:10.1016/j.syapm.2011.02.003

Wang J (2013) Self-healing concrete by means of immobilized carbonate precipitating bacteria. Ghent PhD thesis, Ghent University

Wang J, De Belie N, Verstraete W (2012b) Diatomaceous earth as a protective vehicle for bacteria applied for self-healing concrete. J Ind Microbiol Biot 39(4):567–577. doi:10.1007/s10295-011-1037-1

Wang J, Dewanckele J, Cnudde V, Van Vlierberghe S, Verstraete W, De Belie N (2014c) X-ray computed tomography proof of bacterial-based self-healing in concrete. Cement Concrete Comp 53:289–304. doi:10.1016/j.cemconcomp.2014.07.014

Wang J, Mignon A, Snoeck D, Wiktor V, Van Vliergerghe S, Boon N, De Belie N (2015) Application of modified-alginate encapsulated carbonate producing bacteria in concrete: a promising strategy for crack self-healing. Front Microbiol 6. doi:10.3389/fmicb.2015.01088

Wang J, Snoeck D, Van Vlierberghe S, Verstraete W, De Belie N (2014b) Application of hydrogel encapsulated carbonate precipitating bacteria for approaching a realistic self-healing in concrete. Constr Build Mater 68:110–119. doi:10.1016/j.conbuildmat.2014.06.018

Wang J, Soens H, Verstraete W, De Belie N (2014a) Self-healing concrete by use of microencapsulated bacterial spores. Cement Concrete Res 56:139–152. doi:10.1016/j.cemconres.2013.11.009

Wang J, Van Tittelboom K, De Belie N, Verstraete W (2012a) Use of silica gel or polyurethane immobilized bacteria for self-healing concrete. Constr Build Mater 26(1):532–540. doi:10.1016/j.conbuildmat.2011.06.054

Whiffin VS (2004) Microbial $CaCO_3$ precipitation for the production of biocement. School of Biological Sciences and Biotechnology, Murdoch University, Perth

Wiktor V, Jonkers HM (2011) Quantification of crack-healing in novel bacteria-based self-healing concrete. Cement Concrete Comp 33(7):763–770. doi:10.1016/j.cemconcomp.2011.03.012

Zhang JL, Wang CG, Wang QL, Feng JL, Pan W, Zheng XC, Liu B, Han NX, Xing F, Deng X (2016) A binary concrete crack self-healing system containing oxygen-releasing tablet and bacteria and its $Ca^{2+}$-precipitation performance. Appl Microbiol Biot 100(24):10295–10306. doi:10.1007/s00253-016-7741-z