ČESKÉ VYSOKÉ UČENÍ TECHNICKÉ V PRAZE
Fakulta stavební
Katedra betonových a zděných konstrukcí

Beton se samohojícím účinkem založeným na biologické bázi

Bio-based self-healing concrete

Diplomová práce

Studijní program: Stavební inženýrství
Studijní obor: Konstrukce pozemních staveb

Vedoucí práce: Ing. Petr Bílý, Ph.D.

Bc. Hana Schreiberová

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ZADÁNÍ DIPLOMOVÉ PRÁCE

I. OSOBNÍ A STUDIJNÍ ÚDAJE

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Studijní program: Stavební inženýrství

Studijní obor: Konstrukce pozemních staveb

II. ÚDAJE K DIPLOMOVÉ PRÁCI

Název diplomové práce: Beton se samohojícím účinkem založeným na biologické bázi

Název diplomové práce anglicky: Bio-based self-healing concrete

Pokyny pro vypracování:
Zpracujte rešerši k tématu betonu se samohojícím účinkem založeným na biologické bázi (dále jen biobeton), která bude obsahovat:
- Vysvětlení různých principů samohojení betonu
- Přehled mikroorganismů vhodných pro vyvolání samohojícího efektu v betonu
- Přehled možných postupů pro aplikaci těchto mikroorganismů do betonu
- Souhrn dosavadních výzkumných prací zaměřených na biobeton a jejich výsledků
- Přehled dosavadních praktických aplikací biobetonu, pokud nějaké proběhly

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- Návrh, realizaci a vyhodnocení pilotní série zkoušek biobetonu

Seznam doporučené literatury:
Martin D. Hager et al: Self-Healing Materials. Springer, Switzerland, 2016. ISBN 978-3-319-32776-1.
Další literaturu vyhledejte v mezinárodních citačních databázích Scopus a Web of Science - časopisy Construction and Building Materials, Cement and Concrete Research, Cement and Concrete Composites a další.

Jméno vedoucího diplomové práce: Petr Bílý

Datum zadání diplomové práce: 2.10.2017 Termín odevzdání diplomové práce: 7.1.2018
Údaj uveďte v souladu s datem v časovém plánu příslušného ak. roku

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3.10.2017
Datum převzetí zadání

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Podpis studenta(ky)

SPECIFIKACE ZADÁNÍ

Jméno diplomanta: Hana Schreiberová

Název diplomové práce: Beton se samohojícím účinkem založeným na biologické bázi

Základní část: Betonové a zděné konstrukce podíl: 80 %

Formulace úkolů: Zpracujte rešerši k tématu betonu se samohojícím účinkem založeným na biologické bázi (dále jen biobeton), která bude obsahovat:

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Dále se pokuste provést návrh, realizaci a vyhodnocení pilotní série zkoušek biobetonu

Podpis vedoucího DP: [redacted] Datum: 2.10.2017

Případné další části diplomové práce (části a jejich podíl určí vedoucí DP):

2. Část: Kultivace mikroorganismů podíl: 20 %

Konzultant (jméno, katedra): Mgr. Pavla Ryparová - K124

Formulace úkolů:
Proveďte kultivaci vybraného typu mikroorganismů pro biobeton

Podpis konzultanta: [redacted] Datum: 14.12.2017

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I declare that I carried out this master thesis independently and only with the cited sources, literature and other professional sources.

In ….....….. on …..... Author signature [redacted]

I would like to express my deepest gratitude to my supervisor, Petr Bílý Ph.D., for all his help, valuable advice and comments he provided during writing this thesis. I would also like to thank Mgr. Pavla Ryparová, Ivana Loušová and everyone else in the chemical and microbiological laboratory for introducing me to a whole new field of study, practical help with experiments and especially for the patience they had with me. I am also indebted to Josef Fládr Ph.D. for his numerous suggestions and help with the experimental process. Last but not least, I would like to thank my family and friends for their endless support and patience throughout my studies.

Název práce: Beton se samohojícím účinkem založeným na biologické bázi

Autor: Bc. Hana Schreiberová

Katedra: Katedra betonových a zděných konstrukcí

Vedoucí práce: Ing. Petr Bílý, Ph.D.

Abstrakt: Práce se zabývá tématem betonu se samohojivým účinkem založeným na biologické bázi. Cílem zkoumané metody je zajištění trvanlivosti betonových konstrukcí, která je významně ohrožena vznikem a pozdějším rozšířením mikrotrhlin. Manuální sanace trhlin je časově a finančně velmi náročná, a proto výzkum možností jejich samovolného vyplňování je perspektivním tématem. Základním principem biologické metody je vyplňování trhlin pomocí uhličitanu vápenatého, který je produktem metabolismu specifických mikroorganismů, pokud mají k dispozici vhodné živiny a zdroj vápníku. První část práce tvoří rešerše dosavadních výzkumných prací. Popisují se zde různé principy metabolické produkce uhličitanu vápenatého, přehled vhodných mikroorganismů, živin a dalších komponentů a stanovují se potřebná opatření, která je potřeba dodržet k docílení efektivního procesu biologické samoregenerace betonu. Experimentální část této práce se věnuje kultivaci vybraného mikroorganismu – bakterie *Bacillus pseudofirmus*, určení její životaschopnosti a resistence v různých ztížených podmínkách, které simulují výrobní a finální prostředí uvnitř betonu, a výzkumu různých možností zapouzdření bakterií v ochranných obalech, konkrétně v polyvinylalkoholových nanovláknech a lehkém keramickém kamenivu.

Klíčová slova: beton, samoregenerace, sanace trhlin, bakterie, trvanlivost, zapouzdření

Title: Bio-based self-healing concrete

Author: Bc. Hana Schreiberová

Department: Department of Concrete and Masonry Structures

Supervisor: Ing. Petr Bílý, Ph.D.

Abstract: The thesis is focused on bio-based self-healing concrete. The aim of this method is to ensure durability of concrete structures, which is significantly affected by the microcracks formation. The manual remediation of cracks is time consuming and financially challenging, thus research of the self-healing potential is a promising topic. The basic principle of the biological method is to fill the cracks with calcium carbonate, which is the product of metabolism of specific microorganisms, when they are supplied with appropriate nutrients and a source of calcium. The first part of the thesis consists of a research of previous studies focused on the bio-based self-healing concrete. It describes various principles of metabolic production of calcium carbonate, gives an overview of suitable microorganisms, nutrients and other compounds, and determines the necessary measures which need to be followed in order to achieve an effective self-healing process. The experimental part of the thesis deals with cultivation of the selected microorganism – *Bacillus pseudofirmus,* determinates its survival and viability in various difficult conditions simulating the production and final environment of the cementitious material and examines different possibilities of encapsulation of bacteria in protective carries, namely polyvinyl alcohol nanofibres and lightweight ceramic aggregates.

Key words: concrete, self-healing, crack-sealing, bacteria, durability, encapsulation

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1 Introduction

"Concrete" is generally a name for a building material which consists of some kind of binder, filler and water. The most common ingredients are cement as binder, coarse and fine aggregate as filler, but many other options are possible. For example by substitution of cement with asphalt or polymers, we can achieve different types of concrete (asphalt concrete and polymer concrete) [1]. However, this thesis will be exclusively focused on traditional cement-based concrete.

Concrete, essentially artificial manmade stone, has many similar properties to natural stone. The benefits of this material such as high compressive strength, long service life, and good fire resistance, are unfortunately also accompanied by some negative properties. The main ones are low tensile strength, ductility, stability of volume and low ratio between strength and weight. Most of these drawbacks can be solved either during the process of designing the mixture (by addition of specific admixtures and/or additives), during curing period or by designing special structures (for example hollow core slabs). However, the weakest point is the lack of tensile strength, which is usually solved by an addition of reinforcement made from a material capable of transferring the tensile forces [2].

Concrete is a traditional material with many advantages. Besides the mechanical properties, which were already mentioned above, it is also relatively unpretentious from the point of view of production and formability, low costs and long-term experience which determines concrete to be one of the most used material in the world. In developed countries, the production of concrete reaches up to 1.5 – 3 tonnes per year per resident [3] and given the ever-increasing demand, it can be assumed that this value will continue to rise in the future. This trend, however, carries various problems. The production, transportation, building and subsequent care of concrete constructions are huge economical and ecological burden. Only the production of cement itself is very energy intensive. Burning lime and clay takes place at temperatures up to 1500 ° C and releases significant amounts of harmful emissions such as CO_2 and SO_2 [1]. From the economic point of view, not only the concrete production itself is problematic but also the need for maintenance and

repairs of concrete structures further in their life time. For example, it is estimated that only in the United States, annual maintenance of highway concrete bridges due to corrosion of reinforcement costs up to $ 4 billion [4].

As it can be seen from the specific properties of concrete and the widespread use of the material, it is clear that current development is focused on modifying traditional concrete into something more suitable for the concept of sustainable construction. In order to increase sustainability of concrete structures, it is advantageous, for example, to use industrial waste products (fly ash, slag etc.) that can not only partially replace the cement in the mix but even improve the properties of the concrete itself.

As it has been previously stated, maintenance costs, besides the energy intensity and the pollutants production, are the major drawback of this material. The process that affects durability of concrete structures the most is the formation of cracks. Water with dissolved substances penetrates through the cracks into the structure and causes corrosion of concrete as well as the reinforcement, resulting into defects or even collapse of the entire structure. Therefore, it is obvious that regular inspection of the structures and cracks reparation are crucial operations that need to be done. This can be, in general, done either by preventing the cracking or later sealing already formed cracks. Dealing with cracks through manual checks and repairs is not only laborious but also economically demanding and therefore it opens up the topic of possibility of a self-healing material. Concrete is known for the ability to naturally heal itself. When unhydrated cement particles in the cement matrix undergo delayed hydratation, already existing microcracks can be sealed and thus water tightness can be improved and chemically driven degradation can be reduced. However, this process is not efficient enough to ensure totally maintenance-free structure, so researchers have been investigating the possibility of improvement of the material's self-healing potential.

One of the principles that can be considered when searching for self-healing material is creation of secondary mineral structures that are compatible with the primary material. Therefore, they would be able to fill the formed cracks without affecting the mechanical and physical properties of the material. Many studies investigating this topic

have been carried out in recent years. Apart from exploring the possibility of using encapsulated chemicals to restore the properties of cracked concrete, they came up with a proposal that some kind of microorganisms could be able to produce minerals which would eventually seal the cracks.

This thesis will be focused on the investigation of this biological approach to self-healing concrete and apart from researching the already conducted experiments, the author will attempt to propose, realize and evaluate a set of experiments leading to the development of biological self-healing concrete based on the obtained informations from the previous studies. The main goal will be to examine the specific bacteria from genus Bacillus and evaluate its suitability for exploitation as biological healing agent in concrete.

2 Principles of structural material design

Structural materials perform a mechanical function in addition to any other function they have. However, it is not only the material properties that are essential for achieving the desired function. It is the combination of the material itself, the dimensions and geometry of the product. Material science as a discipline focuses on understanding, designing and creating materials which would have the desired properties and would lead, together with the right dimensions and geometry, to an ideal building material [5].

2.1 The damage prevention and the damage management concept

In the book Self Healing Materials: An alternative Approach to 20 Centuries of Material Science, current material design principles are divided into two different concepts: the damage prevention and the damage management [5].

The damage prevention concept operates with enhanced material properties to achieve stronger and more durable material. However, the cohesion between atoms of any material ultimately limits its properties and though we are able to use the cohesion to tune materials (high cohesion leads to stiff and strong material whereas low cohesion leads to flexible and weak material) we are not able to change all of the properties. For example strength (ability to sustain a high load without disintegrating i.e. creation of internal defects like nano- and microcracks which can grow into larger cracks) can be artificially enhanced but stiffness (the resistance of materials against extension) can hardly be tuned as the property depends on the strength of the interatomic bonds and their packing density which is not possible to change significantly. Although the concept of damage prevention has been successful and will be used in future material development, this principle cannot exclude the formation of damage which means the structures have to be regularly inspected to monitor damage and once any problem occurs it has to be taken care of [5].

The damage management concept is the base of development of self-healing materials. In contrast with the damage prevention concept, it calculates with formation of some damage but considers them non-threatening as long as the damage process is parallel to the “healing” process. Theoretically, such self-healing material would be infinitely

durable but many different conditions affect the efficiency of the healing process [5].

One of the limitations of the self-healing material is the number of possible healing cycles. As it can be seen in Figure 1a) after one healing action, the damage is cured almost completely. However, as the material a) is able to achieve only one healing process, newly created damage leads to a total failure. The schematic graph in Figure 1b) shows a material which is capable of multiple healing actions. Damages are partly removed after every cycle but when the material runs out of the available healing actions, complete failure takes place. The material with infinite lifetime is shown on the Figure 1c). This ideal self-healing material is capable of many healing cycles without any accumulations of damages [5].

Although the damage management concept is developing a material with special self-healing ability, it still has to meet all the requirements of classical structural material in order to perform its mechanical function. From this point of view, final products designed according to the damage prevention concept and the damage management concept can appear very similar [5].

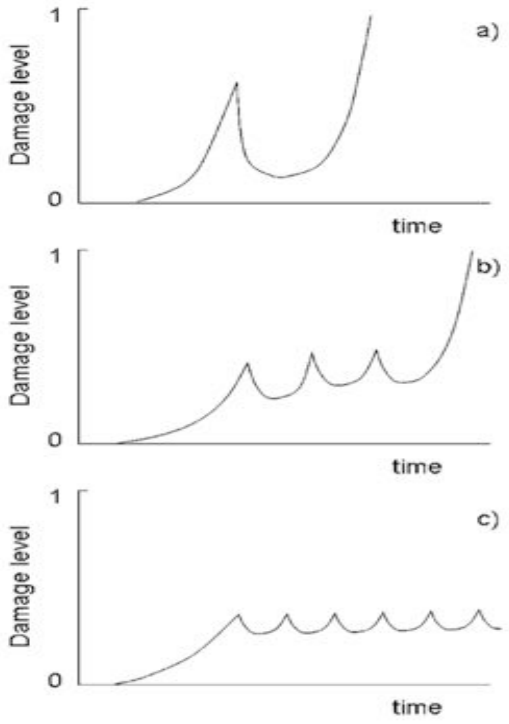

Figure 1: Schematic diagram of the damage development in three grades of self-healing material [5].

It is not surprising that for achieving truly self-healing material, it is necessary that damages disappear more or less spontaneously. Firstly, the damage of the material has to be detected in order to start the healing process. This could be done by some kind of special sensor but it would be much more convenient if the healing agent could do that itself [5].

Another problem which comes up is the transportation of the healing agent through rigid material to the destination of the formed damage. This requirement leads to an interesting clash. On one hand, the healing agent must be mobile to reach the damaged area but on the other hand, it has to create a rigid material to fix the damage and therefore to become immobile [5].

Also it is important to take into consideration the fact that the crack surfaces need to stay together or to be brought in close contact and stay in fixed position during healing process. Furthermore, fully separated parts cannot be healed unless they are brought together. These limitations imply that self-healing process could be more efficient for cycling loading as the applied load would be occasionally reduced and the crack surfaces would be brought closer together [5], in contrast to sustained loads.

3 Self-healing bio-based concrete

Many different methods are used for reparation of cracks in concrete structures to maintain their mechanical properties and durability. For many years there has been an idea that a special type of bacteria, resistant enough to survive a harsh environment of cement mortar, could induce the precipitation of calcium carbonate which would heal the cracks and reduce the negative effect of cracking [5]. Early age cracking in concrete is known to be the result of shrinkage of the setting concrete, temperature changes or loading of the structure. These microcracks do not immediately endanger the load-baring capacity of the structure but usually lead to problems with water tightness and therefore durability. Steel reinforcement, which is embedded in concrete, is covered by a layer of concrete which protects it from the negative effect of the outside environment. When this layer is damaged by cracking, steel reinforcement is exposed to water with aggressive compounds which can lead to corrosion of reinforcement, therefore to loss of durability [6].

As it is known, concrete has its natural autogenous healing capacity. When unhydrated cement particles in the cement matrix undergo delayed hydratation and calcium carbonate is created, already existing microcracks can be sealed and thus water tightness can be improved and chemically driven degradation can be reduced. To enhance this natural process and to obtain an “autonomous” healing capacity, bacterial treatment was suggested and investigated. In most performed experiments, bacteria with other agents were applied externally rather than incorporated directly into the material. However, in order to reduce cost and manual labour demands of crack sealing, it is necessary to incorporate the healing agent during mixing process to obtain a truly self-healing material [6]. Biological crack repair has been investigated in the past 16 years [4]. Addition of the biological healing agent directly into the material's matrix was firstly proposed by Jonkers et al. in 2008. They used an aerobic bacteria which produced $CaCO_3$ when supplied with a specific substrate [4].

3.1 Principles of precipitation of $CaCO_3$

As stated, cement mortar is highly alkaline and dry environment which seems to be inhospitable for any living forms. However, even inside rocks, in desserts and other extreme environments, active bacteria can be found [5]. Bacteria are able to survive under these difficult conditions thanks to their ability to produce spores, a dormant state which is characterized by no metabolism, high chemical and mechanical resistance [7]. Spores are viable for up to hundred years and they germinate i.e. become active when the conditions are hospitable again. Thanks to the high resistance of spore-forming bacteria, it is apparent that they are a suitable candidate to be taken into consideration when searching for a biological treatment of concrete. Bacteria are able to stay in a dormant state in the cement mortar until cracking occurs and water penetrating through the cracks brings the spores to active state so the precipitation of $CaCO_3$ can take place [5]. Different types of metabolical activity, which leads to $CaCO_3$ precipitation are known. Typical bacterial metabolic pathways that increase the carbonate ion concentration and related calcium carbonate saturation in solution are the hydrolysis of urea, oxidation of organic compounds using oxygen under aerobic conditions and oxidation of organic compounds using nitrate under anaerobic conditions [6].

3.1.1 Enzymatic hydrolysis of urea

Urease-active bacteria precipitate microbial $CaCO_3$ through urease catalyzed urea hydrolysis [8]. To attain the self-healing biological concrete it is needed to add specific compounds besides bacterial spores: the nutrients for bacterial spores (usually yeast extract) and the deposition agents (urea and Ca source) [8].

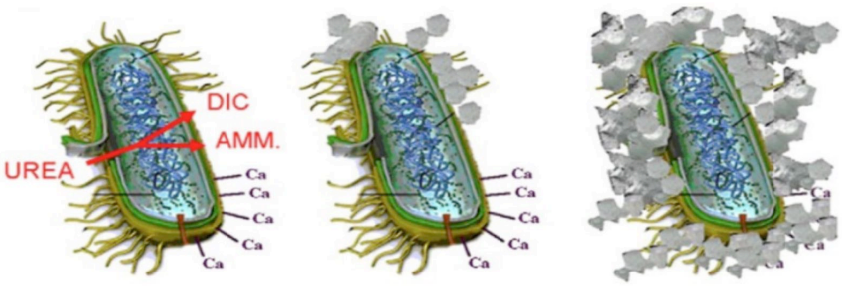

Figure 2: Schematic overview of ureolytic carbonate precipitation occurring at the microbial cell wall. DIC: Dissolved Inorganic Carbon; AMM: Ammonia [9]

The healing process starts when cracking occurs. Water gets into cracks and release the nutrients, which are embedded in the crack zone so they are available for the bacterial spores which start to germinate and recover ureolytic activity. In this urease-mediated process the reaction of urea ($CO(NH_2)_2$) and water yields CO_2 and ammonia (NH_3). Due to the high pK value (acid dissociation constant – a quantitative measure of the strength of an acid in solution [10]) of the NH_3/NH^+_4 system (about 9.2) the reaction results in a pH increase and concomitant shift in the carbonate equilibrium (CO_2 to HCO^{-3} and CO_3^{-2}) which results in the precipitation of calcium carbonate ($CaCO_3$) when sufficient amount of calcium ions (Ca^{2+}) is present [5]. Schematic overview if ureolytic precipitation can be seen in Figure 2. Calcium carbonate precipitation is influenced by the concentration of bacteria, which should be higher than 10^6 cells/ml according to Gent university studies, and also by the concentration of both urea and calcium ions Ca^{2+} (recommended concentration of both is 0.5 M) [01]. To achieve a more economical way of producing ureolytic bacterial suspension, as pure cultures are rather expensive, Gent University developed CERUP (cyclic enriched ureolytic powder) which has about 40 times lower production cost than pure cultures [6].

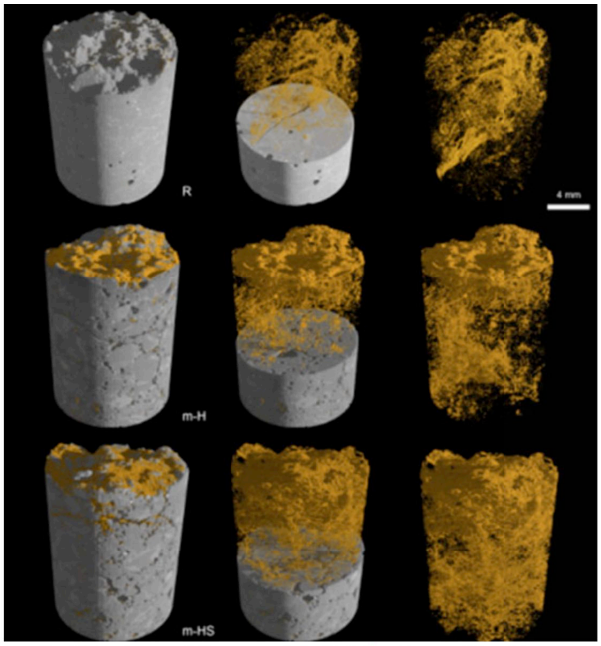

Figure 3: 3D view of the spacial distribution of healing products (in yellow) in reference specimens (R) - top picture, inspecimens with pure hydrogel (m-H) - middle picture and in specimens with bacteria-loaded hydrogel (m-HS) - bottom picture [6].

The healing potential of enzymatic hydrolysis of urea is considerable. Wang et al. in their study used *Bacillus sphaericus* spores encapsulated in hydrogel accompanied with hydrogel encapsulated nutrients and other agents, which were added to the mortar during mixing process, to investigate healing effect on cracked mortar specimens. Cracks were introduced by tensile test in 28 days and then the specimens were subjected to wet and dry cycles for 4 weeks. The specimens with bio-hydrogels healed cracks up to 0.5 mm whereas the specimens with non-bio hydrogels only 0 – 0.3 mm [11]. The difference between the healing product distribution of biological and non-biological hydrogel can be seen in Figure 3.

However, it is important to mention that the massive amounts of ammonia produced during the ureolytic process drastically increase the risk of reinforcement corrosion and degradation of concrete matrix particularly when further oxidized by bacteria to yield nitric acid [4]. Therefore other metabolical pathways of $CaCO_3$ precipitation were later preferred.

3.1.2 Oxidation of organic carbon

Bacteria are catalysts of degradation of organic compounds which leads to precipitation of calcium carbonate and carbon dioxide production. Bio-based concrete based on carbon oxidation (as developed in Delft University) needs apart from the suitable alkaliphilic bacterial spores of the genus *Bacillus* also organic compounds as "food" for bacteria (yeast, pepton) and Ca source [6]. The process of water releasing of nutrients and germination of bacterial spore is identical to hydrolysis of urea process but the precipitation of calcium carbonate ($CaCO_3$) is due to metabolic conversion of Ca-source according to the following reaction [4]:

$$\text{substrate (Ca source)} + O_2 \rightarrow CaCO_3 + CO_2 + H_2O \quad (1)$$

Also the yield of calcium carbonate-based minerals will even increase when produced CO_2 molecules react with portlandite ($Ca(OH)_2$) minerals, which are quantitatively important hydration products of cement particles, according to [4]:

$$CO_2 + Ca(OH)_2 \rightarrow CaCO_3 + H_2O \quad (2)$$

This phenomenon has been later utilized in an experiment performed by Quian et al. who investigated improvement of $CaCO_3$ precipitation by providing a part of the carbon dioxide extra by yeast fermenting glucose [12].

Jonkers et al. performed an experiment in which bacterial spores, yeast extract and calcium lactate were embedded in expanded clay particles and added into the cement mortar during mixing process. The results presented that the applied two-component healing agent can be successfully applied to promote and enhance the self-healing capacity of concrete. In the specimens with incorporated bacteria significantly larger (0.46 mm) cracks were healed after 100 days of curing whereas in the non-bacterial specimens only maximum 0.18 mm wide cracks were healed as it can be seen in Figure 4 [13].

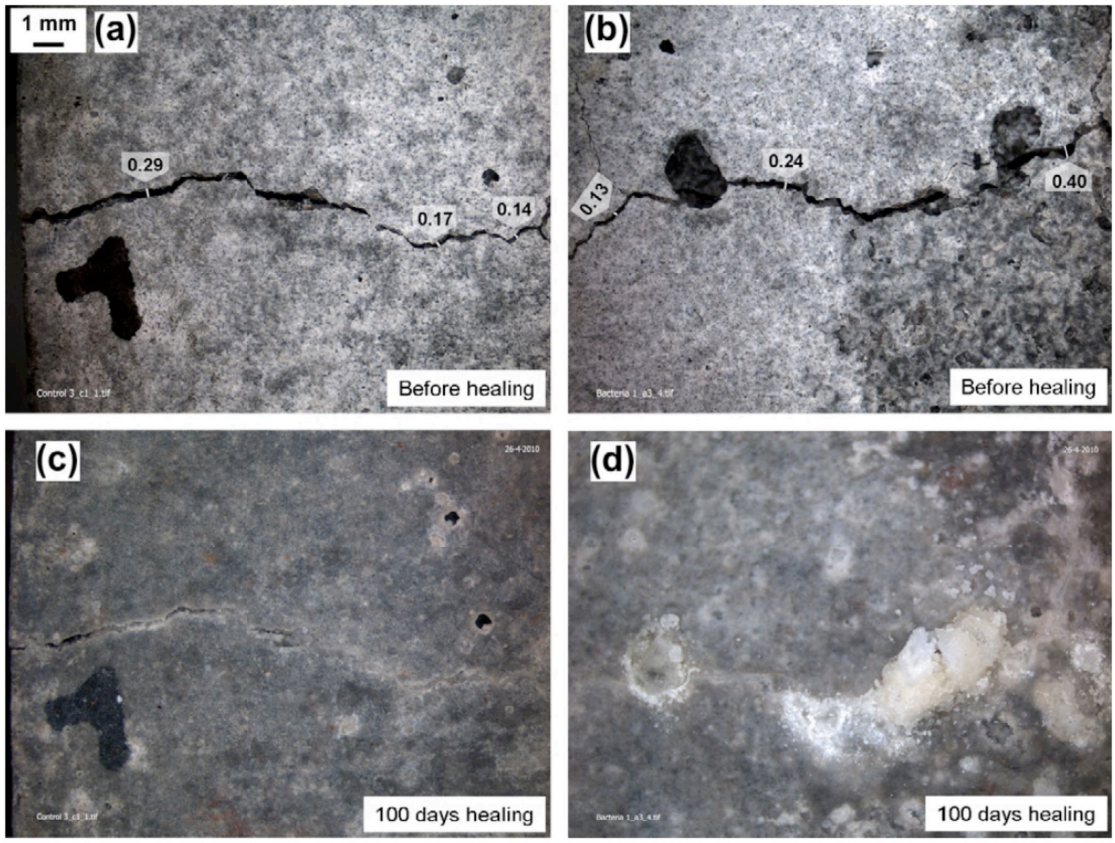

Figure 4: Stereomicroscopic images of crack-healing process in control mortar specimen before (a) and after 100 days healing (c), in bio-chemical agent-based specimen before (b) and after 100 days healing (d) [13]

3.1.3 Anoxic oxidation of organic carbon

Both of the previously mentioned metabolical pathways, oxidation of organic carbon and hydrolysis of urea, need oxygen to begin and maintain metabolical activity of bacteria. However, as oxygen rather badly dissolves in water, there is a shortage in deeper parts of cracks therefore healing efficiency is significantly lowered. Moreover, anoxic oxidation of carbon is more efficient in precipitation of $CaCO_3$ than aerobic oxidation of organic carbon and unlike urea hydrolysis, it does not produce any toxic by-product [6]. Anoxic oxidation of organic carbon consumes nitrate (NO_3^-) or nitrite (NO_2^-) ions as electron acceptor. The process is called nitrate reduction and has been proposed for soil consolidation and Ca^{2+} removal from industrial wastewater. Nitrate reduction can be expected to dominate in the presence of NO_3^- and organic carbon, under oxygen-limited conditions, and generate carbonate (CO_3^{2-}) and bicarbonate (HCO_3^-) ions, which are necessary for $CaCO_3$ precipitation [6]. Experiments have shown that precipitation rates of $CaCO_3$ achieved by hydrolysis of urea are 100 – 1000 times higher than through denitrification. However, this only applies to "optimal" environment and, as we stated in previous chapters, concrete is very harsh and inhospitable environment. Thus the process of denitrification could be more advantageous than urea hydrolysis as sufficient quantity of oxygen and nutrients cannot be ensured [6].

For an effective $CaCO_3$ precipitation through denitrification, similar components to other metabolic pathways are needed. Appropriate bacteria for enzymatic oxidation, nitrite or/and nitrate as electron acceptor and source of Ca (calcium nitrate and/or calcium formate) are crucial. As with other pathways, encapsulation of the healing agents can significantly increase the healing potential as it protects the healing agent from mechanical forces and high pH of concrete [6].

Belie et al. proposed and investigated two resilient strains, *Pseudomonas aeruginosa* and *Diaphorobacter nitroreducens*, as potential candidates for soil and concrete applications. Results showed that both of the strains can be considered as potential candidates due to their ability to precipitate $CaCO_3$ in minimal nutrient environment. Also both of the strains were capable of repetitive $CaCO_3$ precipitation [14]. These starvation-resilient bacteria were able to survive when nutrient diffusion was limited

and create new cells so repetitive precipitation was possible [6]. This ability is especially beneficial for healing of already healed cracks. The experiment also revealed that both of the strains were able to survive, with the aid of protective carrier, the alkaline pH of concrete matrix. Furthermore, in contrast with bacterial agents from genus *Bacillus (Bacillus sphaericus, Bacillus cohnii)*, the strains presented in this study did not require nutrients (urea, yeast extract, calcium lactate) which can negatively affect mechanical properties of concrete and they are more expensive than admixtures used for anoxic oxidation (Ca-formate and Ca-nitrate) [14].

Different protective carries for non-anexic bacteria were investigated. Expanded clay particles and granular activated carbon particles appeared to be appropriate carriers as they did not significantly affect setting or strength properties of mortar. However, diatomaceous earth together with needed nutrients significantly decreased setting time of mortar and therefore it proved to be inappropriate material for protective carries [6].

The healing potential of non-axenic resilient strain *D. nitroreducens* incorporated into concrete mixture was recently investigated. The bacterial cells were impregnated to expanded clay particles and together with dissolved nutrients added into concrete mixture during mixing. Mortar specimens were after 28 days submitted to tensile test to create multiple cracks. After cracking, specimens were immersed in tap water for 28 days and then examined with stereomicroscope. Results showed that cracks up to 0.35 mm were completely healed and also prisms with bio-healing agent absorbed 51% less water than the reference prisms in the first 24h. However, in later research it will be necessary to determine whether the healing effect was result of autogenous or autonomous healing process in order to state the efficiency of anoxic oxidation [6].

In conclusion, biological concrete based on non-axenic bacterial strains, in an optimized amount and with appropriate nutrients, could achieve similar results to axenic bacteria but with less costs so further development should proceed [6], [15], [9].

3.2 Materials

As stated before, this study will concentrate on an internal application of bacteria in order to attain a truly self-healing material. Thus, all the components, bacterial agent and other relevant agents, must be added into the concrete matrix during casting.

3.2.1 Bacterial agent

Calcite-producing bacteria for development of self-healing concrete must be spore-forming alkaphilic (to be capable of survival in a highly alkaline environment of concrete with pH around 12.5) and have no negative effect on health and environment. These types of bacteria can be found in natural soils, alkalic lakes or certain natural stones [16]. In past studies many different bacteria of different dosages were tested: *Bacillus cohnii* [4][16], *Bacillus halodurans* [16], *Bacillus pseudofirmus* [16], *Bacillus sphaericus* [8][17][18][11] [19], *Bacillus musilaginous* [20], *Bacillus alkalinitrilicus* [13]. Photomicrographic picture of vegetative cells and spores can be seen in Figure 5 and Figure 6.

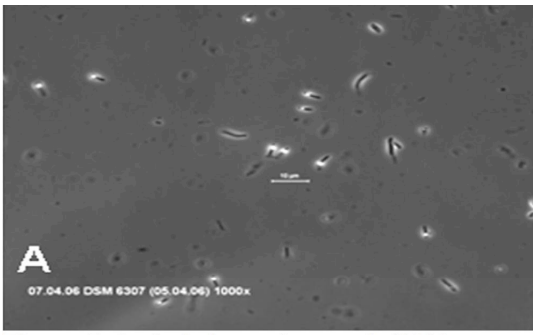

Figure 5: Optical photomicrograph (1000 x magnification) of *B. cohnii* culture showing vegetative cells with intracellular spores (bright spheres) (A) [4]

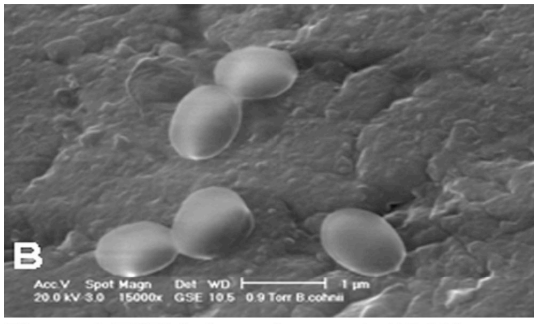

Figure 6: ESEM photomicrograph (15,000x magnification) of isolated *B. cohnii* spores (B) [4]

3.2.2 Nutrients and deposition agents

Nutrients and deposition agents are added into the concrete mixture so bacterial spores can germinate and produce biominerals. However, these compounds can, positively or negatively, affect the mechanical properties of concrete so their composition and amount need to be tested.

Yeast extract (possibly with peptone) is used as a nutrient; it works as a stimulator of bacterial growth. However, according to results of Wang et al. when the dosage of yeast extract is higher than 0.85% (to cement weight) it could delay hydration and decrease hydration degree. The inhibition effect from organic compounds is mainly because they are easily absorbed on the surface of mineral particles, thereby screening the contact of cement with water, and hence decrease cement hydration [8].

Deposition agents are essential for microbial precipitation of $CaCO_3$. All metabolical pathways need appropriate Ca source to achieve bio-precipitation and therefore crack-healing. Furthermore, urease-active bacteria need an addition of urea and for metabolical pathway based on anoxic oxidation of organic carbon, source of nitrate is essential. Healing agents with different Ca source were investigated in previous studies. Furthermore, a study carried out by Wang et al. (an experiment with ureolytic bacteria) showed that specific types of amino acids or carbohydrates in an adequate dosage (maximum 0.5% per cement weight according to Wang et al.) could enhance the plasticizing effect and accelerate hardening of the cement paste and thus compensate the negative effect of yeast extract on the hydration degree [8].

In an experiment performed by Qian et al. specimens with calcium formate (J), calcium lactate (R) or calcium nitrate (N), and non-ureolytic bacterial spores (B) or non-bacterial control specimens (C), were tested (see Figure 8 to Figure 10) [21].

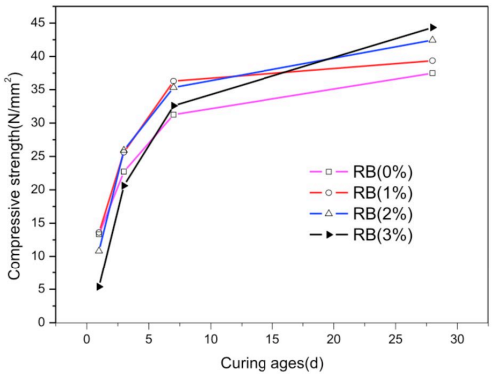

Figure 8: Compressive strength development of cement mortar samples with bacteria spores powder and calcium lactate [21]

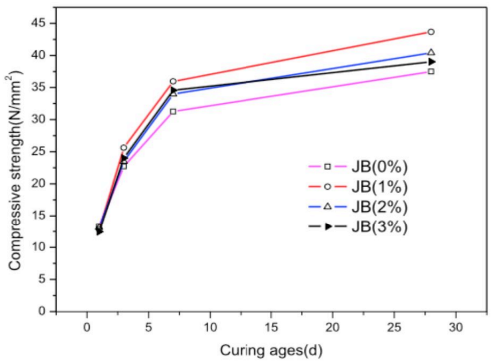

Figure 7: Compressive strength development of cement mortar samples with bacteria spores powder and calcium formate [21]

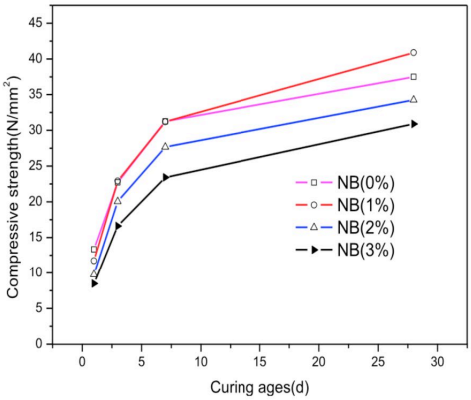

Figure 10: Compressive strength development of cement mortar samples with bacteria spores powder and calcium nitrate [21]

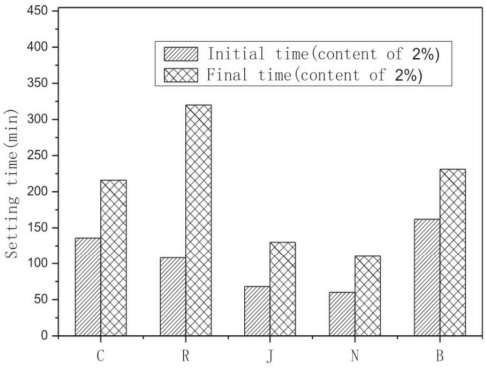

Figure 9: Initial (from addition of water to the beginning of losing plasticity) and final (from addition of water to complete lost of plasticity) setting time of cement mortar with each component of self-healing agents [21]

Results showed that calcium formate (dosage 1% to cement weight) accelerated hydration (Figure 9) of cement paste and enhance compressive strength of cement specimen (Figure 7). Calcium nitrate accelerated hydration (Figure 9) of cement paste as well as calcium formate. Compressive strength at the dosage of 1% to cement weight was nearly the same as for the control specimen without healing agent but at the dosage of 3% significant loss of compressive strength was reported (Figure 10) [21]. Calcium lactate delayed hydration (Figure 9) of cement paste in this experiment but when compared to calcium acetate in an experiment performed by Jonkers et al., calcium lactate had no effect or even slightly increased compressive strength in contrast to calcium acetate which lowered compressive strength significantly [4]. Experiment (with non-ureolytic bacteria) performed by Xu et al. compared another two organic calcium sources: calcium lactate and calcium glutamate. Results of this experiment showed that specimens with calcium lactate reached higher compressive and flexural strength but calcium source conversion and $CaCO_3$ precipitation by calcium glutamate proved to be more efficient. Thus, recovery ratio of flexural modulus was higher with calcium glutamate than calcium lactate [22].

An experiment carried out by Jonkers et al. [23] (with non-ureolytic bacteria) used and tested different organic compounds. Na-asparate, Na-glutamate, Na-polyacrylate, Na-gluconate, Na-citric acid and Na-ascorbic acid (0.5% of cement weight) were firstly dissolved in the mixing water and then used for preparation of cement specimens.

Results showed that concrete to which polyacrylic acid and citric acid was added suffered significant strength loss, while gluconate- and ascorbic acid amended concrete did not develop any strength during the 28 days curing period as it can be seen in Table 1.

Type of concrete:	Tensile strength (N/mm^2):	Compressive strength (N/mm^2):
Control	7.78 ± 0.38	31.92 ± 1.98
S. pasteurii	7.45 ± 0.45	34.78 ± 1.52
Na-aspartate	7.33 ± 0.37	33.69 ± 1.89
Na-glutamate	7.16 ± 0.19	28.52 ± 3.56
Na-polyacrylate	6.42 ± 0.47	20.53 ± 4.50
Na-citrate	3.48 ± 1.72	12.68 ± 1.82
Na-gluconate	0	0
Na-ascorbate	0	0

Table 1: Fexural tensile and compressive strength characteristics of control, bacteria and organic carbon amended concrete specimens after the 28 days curing period [23]

As stated in previous chapter, in the process of decomposition of specific substrate by non-ureolytic bacteria to calcium carbonate, CO_2 is released. Carbon dioxide than reacts with portlandite minerals and produces more $CaCO_3$. This process could potentially increase the healing effect of bacterial treatment but the decomposition speed of substrate is slow and the quantity of CO_2 from substrate is limited [20].

The experiment carried out by Quian et al. in 2016 proposed an improvement of self-healing effectiveness by providing a part of the carbon dioxide extra by yeast fermenting glucose. The results presented in this study showed that addition of glucose to self-healing agent could improve the efficiency of the microbiological self-healing method. The best results were achieved by specimens containing both bacteria with nutrients and glucose in the test of flexural strength after 28 days of healing (Figure 12), the area repair rate of section surface (Figure 11) as well as water permeability test [20].

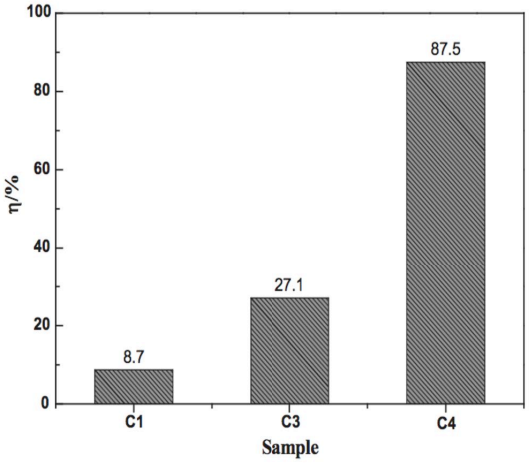

Figure 11: Area repair rate of section surface after 28 days of healing in control specimen (C1), specimen with bacteria only (C3) and specimen with bacteria and glucose (C4) [20]

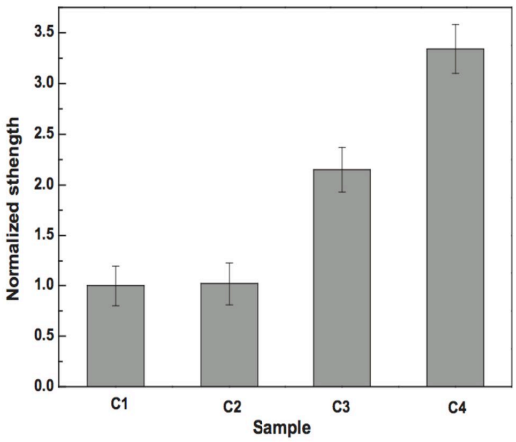

Figure 12: Comparison of flexural strength in control specimen (C1), specimen with glucose only (C2), specimen with bacteria only (C3) and specimen with bacteria and glucose (C4) [20]

3.3 Encapsulation or immobilization of bacterial spores

Jonkers et al. in 2008 performed an experiment in which cement specimens with and without incorporated healing agent (non-ureolytic bacteria with organic compounds and other agents) were prepared to investigate viability of incorporated bacteria, pore size distribution of ageing specimens, effect of agent additions on strength and self-healing properties [4]. This study brought an important discovery that influenced further development of microbiological self-healing method. Jonkers et al. found out that the functionality of bacterial mineral production of the incorporated healing agent is limited to young concrete (Figure 13). Massive production of larger-sized precipitates was observed in 7 days but not in 28 days cured cement stone specimens [4]. Mercury porosimetry showed that larger-sized pores (0.1–1 μm) which were present in the young cement specimens were replaced by smaller pores (0.01–0.1 μm) in 28 days of curing so the decrease of the functionality is likely to be linked to this change of pore sizes. Pores in the young cement are large enough to accommodate bacterial spores (typically 0.8–1 μm) while in the aged specimens bacterial spores are crushed and thus it results in loss of viability and decrease of mineral-forming capacity [4].

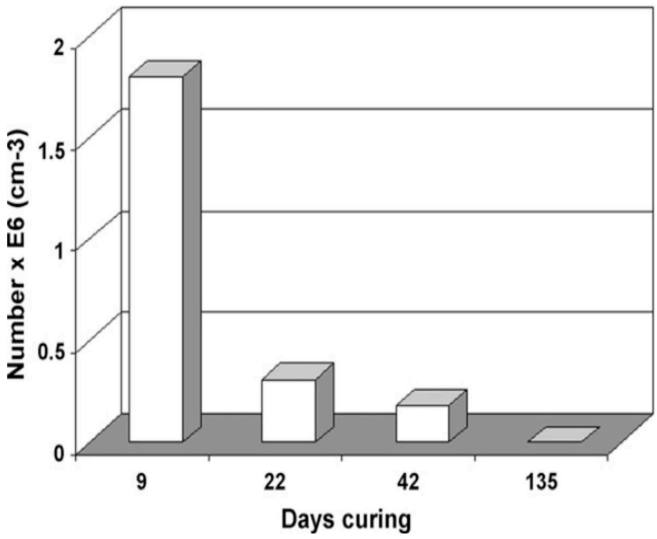

Figure 13: Most-probable-number estimate of viable bacterial spores (*B. cohnii*) incorporated in aged stone specimens [4]

From this observation it is possible to conclude that for maintaining viability of bacterial spores and efficient bio-mineral precipitation of $CaCO_3$, it is crucial to either ensure sufficient porosity of the concrete matrix or protect bacterial spores by encapsulation or immobilization in a protective carrier prior to addition to the concrete mixture [4]. Furthermore, the carrier of bacteria could provide the needed protection from mechanical forces during mixing process as well as from the harsh high alkaline environment of concrete matrix and thereby increase the effectiveness of self-healing potential [6].

The other agents that are necessary for precipitation of $CaCO_3$ do not need to be immobilized and can be added directly into the concrete matrix, under the assumption that they have no negative effect on the properties of the material. However, as stated before, yeast extract and other organic compounds can negatively influence the properties, thus encapsulation should be considered [6].

3.3.1 Silica fume as a protective carrier for non-ureolytic bacteria

In an experiment carried out by Xu at al. in 2013 [22], silica fume together with air entraining agent in powder form was added to the cement mortar in order to alleviate the

high alkalinity of the material and to create isolated micropores in which bacterial spores could be accommodated and stay viable. In Table 2 we can see various healing methods that were investigated by Xu et al. [22].

Series	Group No.	Composition description	Healing conditions
Control	1	Without healing agent	In water
Nutrients only external healing	2	Without healing agent	In L-Ca media
	3	Without healing agent	In G-Ca media
External applied healing	4	Without healing agent	In L-Ca media + bacteria
	5	Without healing agent	In G-Ca media + bacteria
Nutrients only self-healing	6	With L-Ca	In water
	7	With G-Ca	In water
Two-component self-healing	8	With L-Ca + bacteria healing agent	In water
	9	With G-Ca + bacteria healing agent	In water

Table 2: Overview of the different treatments during healing. (L-Ca media consist of calcium lactate and yeast extract, G-Ca media consist of calcium glutamate and yeast extract) [22].

Results showed that although external application of bacteria and nutrients is the most effective way of healing (group no.4 and 5), two-component healing agent with calcium glutamate (group no.9), which was incorporated directly to the mixture, improved the self-healing potential significantly in comparison with control specimens (group no.1) as it can be seen from Figure 14 [22].

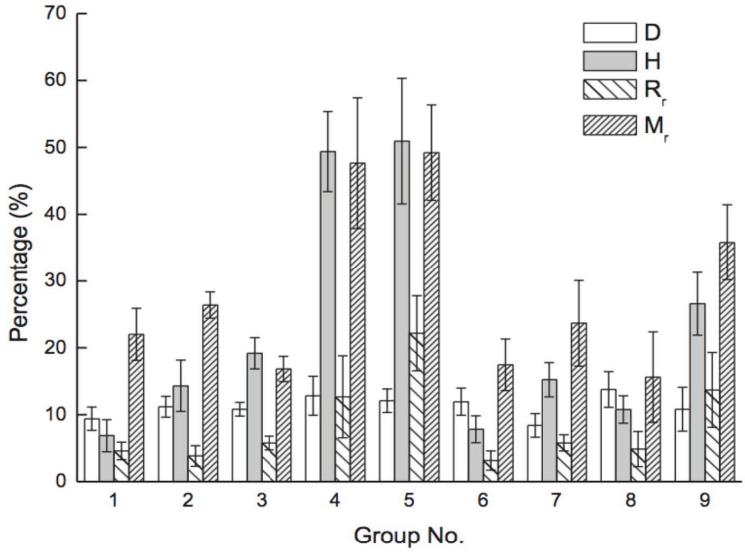

Figure 14: Damage degree (D), healing ratio (H), recovery ratio of flexural strength (R) and modulus (M) results [22].

3.3.2 Expanded clay particles as a protective carrier for non-ureolytic bacteria

Wiktor et al. in their study introduced another idea that could increase the functionality of the self-healing agent. Immobilization of the healing agent in porous expanded clay particles could create a reservoir of healing agent and constitute a protective matrix as well as a structural element of concrete. In this experiment, light weight aggregates (Liapor) were impregnated with both non-ureolytic bacterial spores and nutrients and added into the concrete mixture [13]. In Figure 15 we can see SEM images with incorporated bacteria.

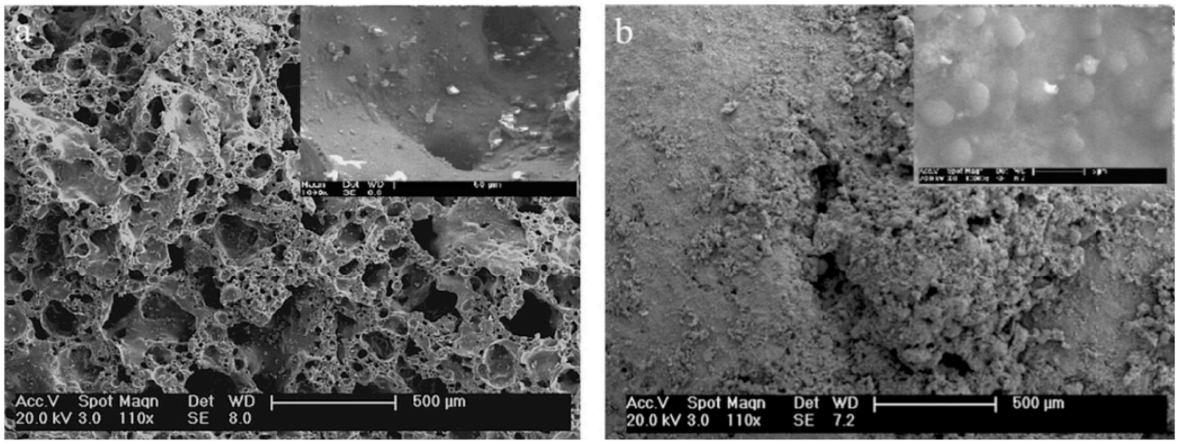

Figure 15: SEM images of ceramsite carrier (a) and ceramsite with bacteria (b), SEM images of the inside of ceramsite and the surface of ceramsite with bacteria (insert) [20]

The proposed principle is rather simple. When crack occurs, the weak lightweight capsule breaks so the healing agent can be activated and starts to precipitate the $CaCO_3$ which fills the crack [24]. Schematic diagram can be seen in Figure 16. Results of this experiment showed that the researchers assumption had been right and the functionality (mineral deposition on cracks surfaces) truly increased from 7 days (according to previous study with bacterial spores directly added into the concrete matrix) to 100 days [13].

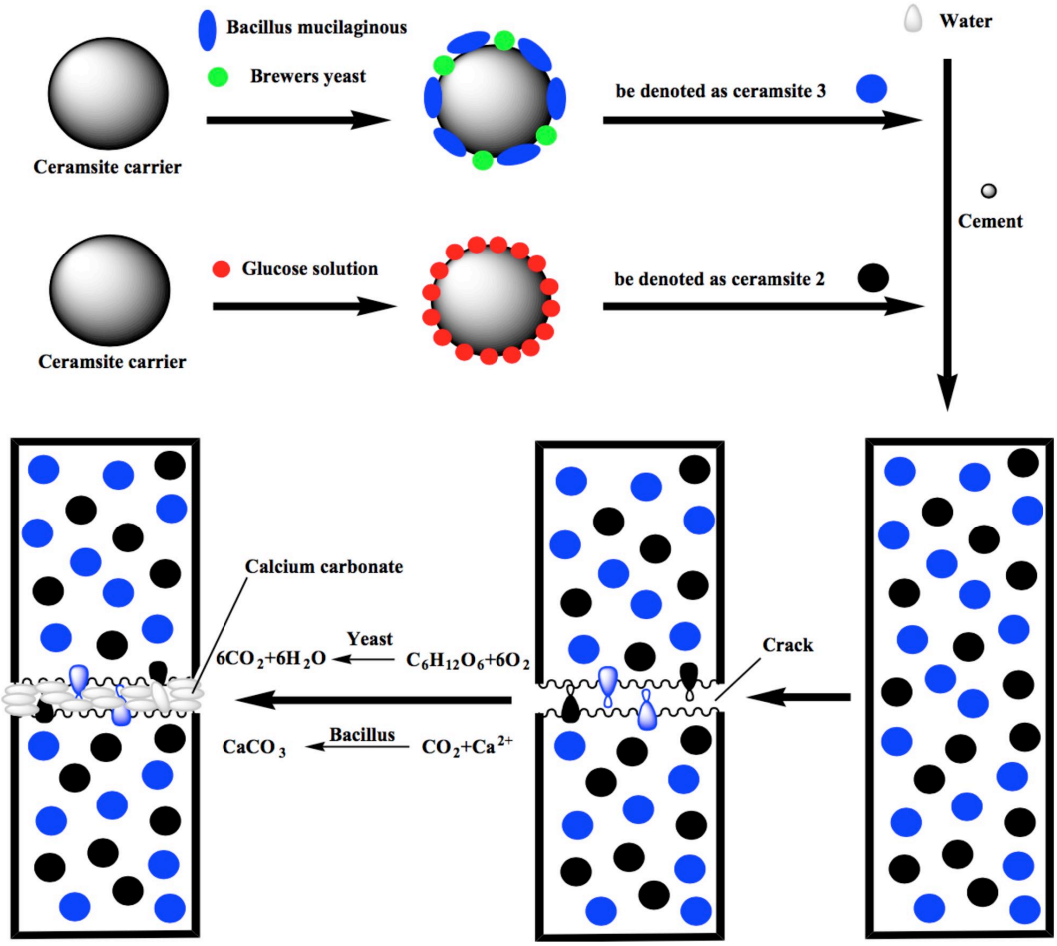

Figure 16: Schematic diagram for the self-healing cementitious materials. Experiment investigating the effect of glucose to the self-healing potential [20].

Later on, many other experiments which used porous expanded clay particles as a protective matrix were carried out. One of the main issues of this method that came up in these studies was reduction of compressive strength. As it can be expected, addition of lightweight aggregates into concrete matrix decreases the bulk density and tends to increase the air content which leads to loss of the compressive strength. However, as stated by Tziviloglou et al., this material could be used where a lightweight structure is needed or as an external layer on a normal weight structure and benefit from its better crack sealing capacity, which can improve durability of the structure at the same time [24].

3.3.3 Condensed powder system as a carrier of non-ureolytic bacteria

As stated before, the main disadvantage of using light weight aggregates as protective carriers is its porosity which decreases the compressive strength of concrete mixture and limits the range of use of the material. Therefore this problem could be solved by reducing volume of the protective carrier while keeping the amount of the healing agent the same.

Researchers proposed an idea of roller-compacting of powders (Figure 17), mostly composed of the healing agent, which would eventually be milled into flakes. These flakes would dissolve in water which would penetrate the material through cracks and release the healing agent. However, solubility in water is a problem during wet mixing stage of the concrete matrix because premature activation of the bacterial spores and nutrients could occur. A possible solution could be an application of a protective barrier around the flake which would stop it from premature dissolving during mixing process but break when crack forms and allow the water in. The coating material can be inorganic (e.g. cement paste or geopolymer) or organic (e.g. calcium cross-linked polyvinyl alcohol alginate or lactic acid derivatives) [6].

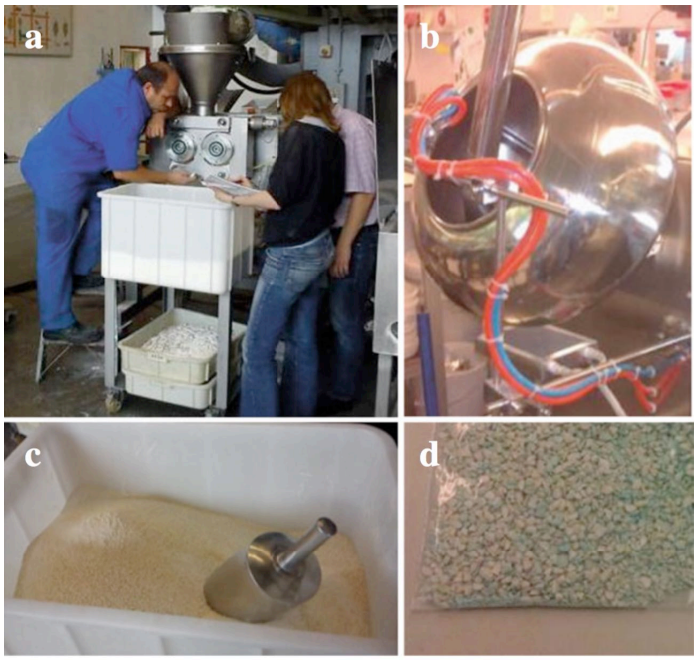

Figure 17: Roller compaction of powders (a), coating of particles (b), uncoated powders (c) and coated powders (d) [6]

In an experiment which examined the potential of flakes with the healing agent, significant decrease of volume has been achieved (from 30% by volume of lightweight aggregates to 1% of flakes) with equal healing capacity. Addition of flakes had insignificant influence on fresh mixture consistency and strength development after 7 days. Decrease of compressive strength was detected only in the young concrete up to 7 days. This could be due to partial loss of soluble core material near the particle surface, which could be amplified by increased surface area. This problem could be possibly solved by either increasing the particle size or shape them into more spherical shape [6].

3.3.4 Diatomaceous earth as a protective carrier for ureolytic bacteria

Diatomaceous earth (DE) is a naturally occurring, chalk-like, earthy, very-fine-grained, siliceous sedimentary rock that consists of fossilized remains of diatoms, a type of hard-shelled algae [25]. The material is light in weight, highly porous, chemically stable and inert and so it could be a suitable carrier for bacterial spores [18]. Diatomaceous earth is commercially used in filters (filtration aid in chemistry, to filtrate water and other liquids, syrups or honey), as very mild abrasive (toothpaste, cosmetics, metal polishes), as an insecticide or as the barrier material in some fire resistant safes.

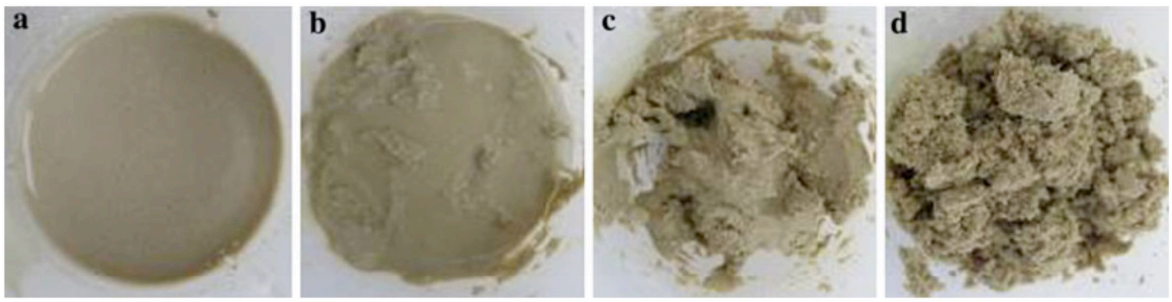

Figure 18: Digital photos of the mixture of DE and bacterial suspension at different concentrations of DE: 40% (a), 50% (b), 60% (c) and 70% (d) [18]

Wang et al. [18] used DE as a carrier for the ureolytic bacteria (Figure 18). Results of this experiment showed that in neutral pH and moderate alkaline environment, both free bacterial cells and cells immobilized in DE reached high ureolytic activity and there was no significant difference between them. However, when free and immobilized bacteria

were tested in high pH cement slurry, ureolytic activity of free bacteria greatly decreased and the immobilized bacteria kept much higher activity. The possible mechanism is that DE particles have a strong capacity to sorb bacterial cells on the surfaces due to their high specific surface area. After sorption, DE provided a kind of microenvironment around the bacteria, in which the local pH was less aggressive than that in the whole cementitious environment and thus bacteria could still decompose urea. Also ureolytic activity, therefore protective effect, increased with increasing amount of DE (the highest activity was obtained with dosage of 60 or 70 % diatomaceous earth in the mixture with bacterial suspension) as it is shown in Figure 19 [18].

In conclusion it could be assumed that addition of diatomaceous earth into concrete matrix could not only provide the pore space for bacterial cells to survive, but also create a more hospitable environment which could result in more effective precipitation of bio-minerals [18]. Furthermore, DE has no negative effect on the mechanical properties of mortar and may even show pozzolanic activity [6].

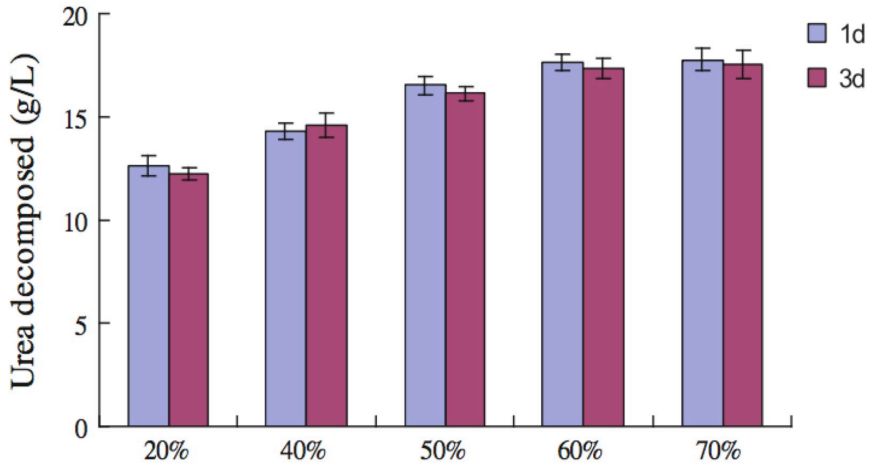

Figure 19: Ureolytic activity of immobilized bacteria in high-pH cement slurry when using different concentrations of DE [18]

3.3.5 Silica gel and polyurethane as a protective carrier for ureolytic bacteria

Wang et al. came up with a proposal that silica gel and polyurethane could be used as a protective carrier for ureolytic bacteria. Silica gel is a popular carrier for microorganisms,

like bacterial cells, yeast and algae, because it has good properties of mechanical, thermal and photochemical stability, biological inertness (not a food source for bacteria), and suitable matrix porosity for the transmission of molecules and ions. It had been previously successfully used in an experiment of external application of bacterial treatment in which silica gel with bacterial suspension had been injected directly to the cracks. In contrast, Wang et al. used silica gel for internal self-healing. Silica sol (colloidal dispersion of silicic acid in water) has been encapsulated together with bacterial, nutrients and other agents into glass tubes. These glass tubes, which can be seen in Figure 20, would break when a crack occurs and releases the healing agent while silica sol would react with Ca^{2+} from the concrete matrix and created silica gel in which bacteria would be immobilized [17].

(a) Silica sol as carrier (b) PU as carrier

Figure 20: Glass tubes filled with healing agents. Length of 40 mm and inner diameter of 3 mm. [17]

Polyurethane (PU) had been previously used for external healing by Wang et al. in 2001. PU with bacterial cells had been cut into pieces and placed into cracks. In a study from Wang et al., glass tubes were filled with bacteria and PU prepolymer and added directly into the concrete mixture. The principle of immobilization was rather similar to silica gel. Cracking would break the glass tube so PU foam would form and immobilize the bacteria at the same time [17].

The experiment with polyurethane and silica gel as protective carriers was successful. After immobilization, bacterial ureolytic activity could still be kept (from 30% to 70%) and after one week, the amount of decomposed urea was almost the same as in the

first week, thus there was no decrease of ureolytic activity. It was found that specimens with polyurethane immobilized bacteria had significantly more self-healing efficiency as it is shown in Figure 22. Furthermore, the results of strength regain of cracked specimens showed that the specimens with PU reached the highest value (see Figure 21). However, it seems, that the presence of bacteria did not affect the strength regain as there was not a significant difference between the specimens with living or dead bacteria, which were not able to decompose urea [17].

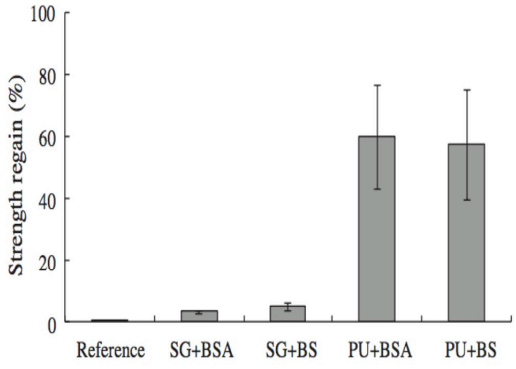

Figure 21: Strength regain of cracked specimens (R – reference, SG – silicagel, PU – polyurethane, BS – deposition medium + living bacteria, BSA – deposition medium + dead bacteria) [17]

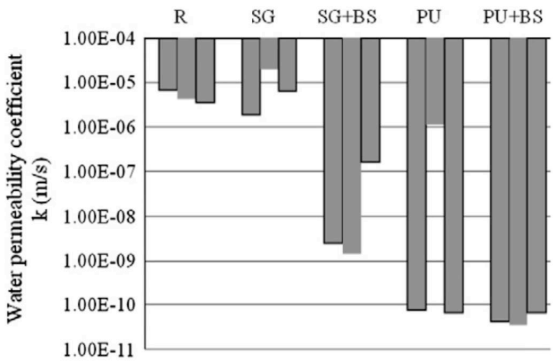

Figure 22: Water permeability of cracked cylinders after being repaired by the different incorporated healing agents. (R – reference, SG – silicagel, PU – polyurethane, BS – deposition medium + living bacteria, BSA – deposition medium + dead bacteria) [17]

3.3.6 Hydrogel as a protective carrier for ureolytic bacteria

One of the key factors for achieving an efficient self-healing concrete is sufficient water supply which is a basic element for bacterial activity. In natural conditions, sufficient water supply is difficult to secure, therefore researchers proposed an application of hydrogels as a carrier of healing agents. Hydrogel, a network of polymer chains with water as the dispersion medium, is hydrophilic and highly absorbent gel which can retain a large amount of water or aqueous solution in the network without dissolving. Therefore, hydrogel could create not only a protective barrier for bacterial spores but also a water reservoir which would supply water for spore germination and bacterial activity [11].

Wang et al. in 2014 examined hydrogels as protective carriers in their study. Pure hydrogel, hydrogel with bacterial spores, hydrogel with nutrients and hydrogel with both

spores and nutrients were formed in a gel-like sheets and further tested. Results of the experiment showed that bacterial spores were successfully encapsulated and did not lose viability after encapsulation and also that no bacterial precipitation would be formed before cracking (ureolytic activity was recovered after removing from high alkaline environment to less aggressive one). Also crack healing efficiency in the specimens with incorporated hydrogels (with or without the healing agents) was tested. It was detected that the specimens with bio-hydrogels had more improved self-healing efficiency. Regarding crack filling ability, bio-hydrogels filled much wider cracks (0.5 mm) than non-bio hydrogels (maximum 0.3 mm) as it can be seen in Figure 23, and also reached lower water permeability [11]. However, when specimens with bio-hydrogels were tested in compressive strength, drastic loss was detected and hydrogels were found not useable for the purpose. In further studies, more compatible hydrogels were developed and examined [6].

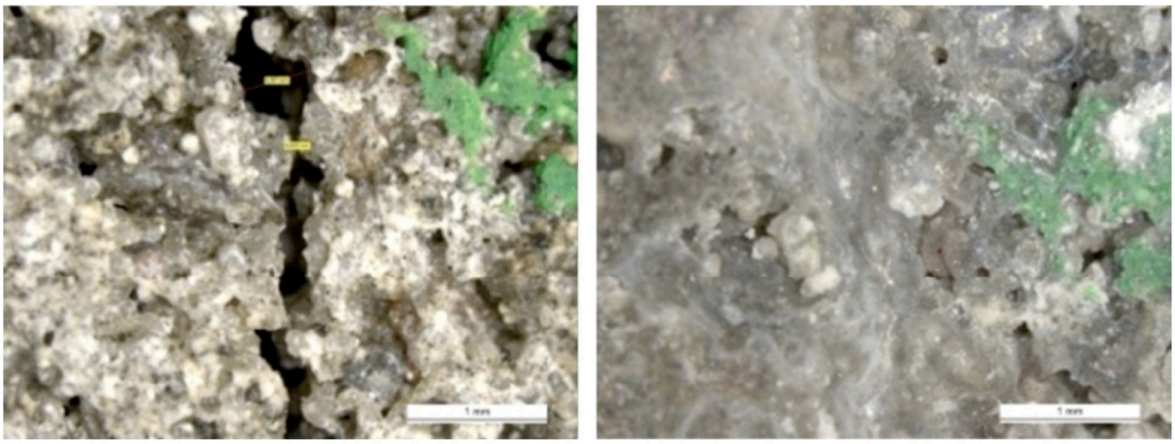

Figure 23: Maximum crack width healed in the specimen with hydrogel encapsulated both bacterial spores and the bio-reagents. (initial 0,57 mm; final 0,00 mm) [11]

Mignon et al. proposed pH-sensitive superabsorbent polymers as a potential candidate material for self-healing concrete. Superabsorbents (SAPs) possess lower cross-linking density than conventional hydrogels, therefore they have a much higher absorbent capacity and could be more beneficial as a water reservoir for bacterial activity [26].

In a different study, Wang et al. in 2015 investigated an application of a new type

of alginate-based hydrogel as a protective bacterial carrier. Material, originating from cell walls of brown algae, proved to be appropriate as a bacterial carrier. Spores stayed viable after encapsulation, special hydrogel fulfilled the role of water reservoir and in contrast to regular hydrogel, alginate-based hydrogel caused only an acceptable decrease of compressive strength (16 – 23%) which could be compensated by special admixtures to concrete mixture [19].

3.3.7 Microencapsulated spores of ureolytic bacteria

Polymeric microcapsules containing bacterial spores and/or nutrients have been already patented in 2014 [27]. The principle is similar to other types of encapsulation: microcapsules protect the healing agent from mechanical forces during mixing, create a space where bacterial spores can be accommodated and prevent premature precipitation of $CaCO_3$. Microcapules need to be able to withstand the mixing process but must be easily broken when crack occurs so the spores can germinate and start the precipitation. Therefore the coating material, apart from being resistant to the harsh alkaline environment of concrete matrix, needs to be flexible in water but in low humidity become fragile [8]. An example of crack healing process with microencapsulated bacteria can be seen in Figure 24.

Wang et al. [8] carried out an experiment with microencapsulated bacterial spores. Bacterial spores (10^9 cells/g of microcapsule weight) were incorporated in melamine based microcapsules according to patented encapsulation process and added to the cement mortar. Nutrients (yeast extract and deposition agents urea and Ca source) were incorporated directly in the mixture.

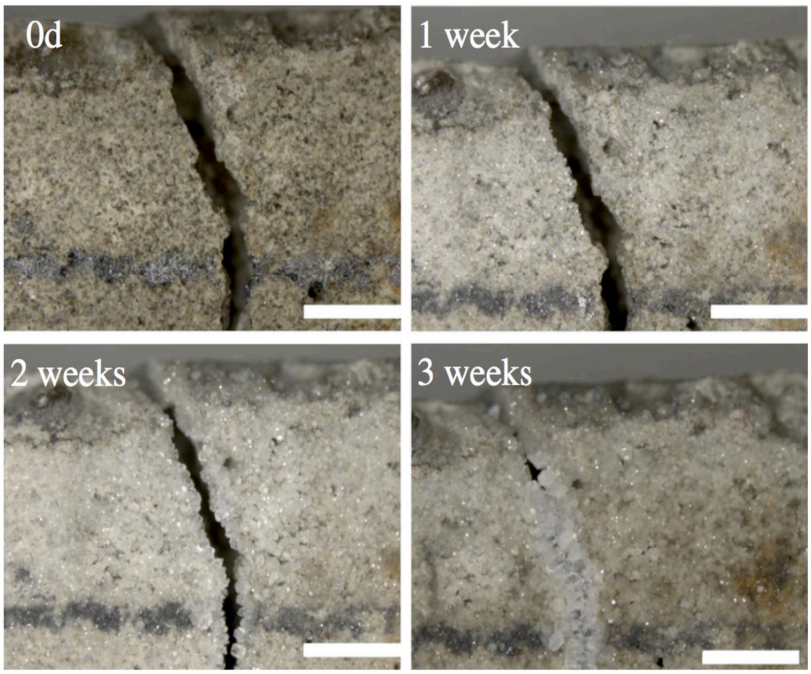

Figure 24: An example of crack healing process - specimen with 5% of microcapsules (of cement by weight) impregnated with nutrients and bacterial spores. [8]

The performed tests showed that the spores were still viable (able to decompose urea) after immobilisation and capsules broke only due to cracking. Another investigated aspect was influence on the mechanical properties of mortar. Microcapsules did not significantly affect volume density but caused loss of compressive and tensile strength (Figure 26). This loss was probably caused by large size pores (1–2 μm) which were created by microcapsules. On the other hand, microcapsules with bacteria had a positive effect on water absorption which decreased by more than 40% after healing (Figure 25), probably due to decrease of open porosity. Also different dosages of microcapsules were tested: 0%, 1%, 2%, 3%, 4% and 5% of cement by weight. Study showed that addition higher than 3% had great negative effect on mechanical properties of cement mortar. Furthermore, self-healing efficiency was not significantly different for 3% and 5% of microcapsules. Possible explanation could be a waterproofing effect of inert substance which is encapsulated together with bacterial spores in capsules. This barrier could slow down the penetration of water into matrix which has to dissolve nutrients so spores would germinate and start the healing activity [8].

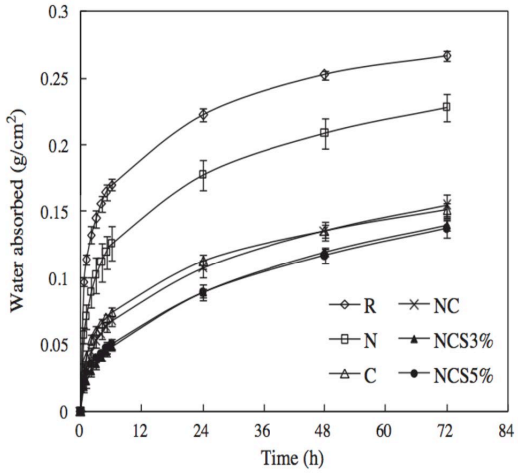

Figure 25: Water absorption of the specimens with and without microcapsules. (R - control, N – only nutrients, C – only microcapsule emulsion, NC – nutrients + microcapsule emulsion + bacterial spores) [8]

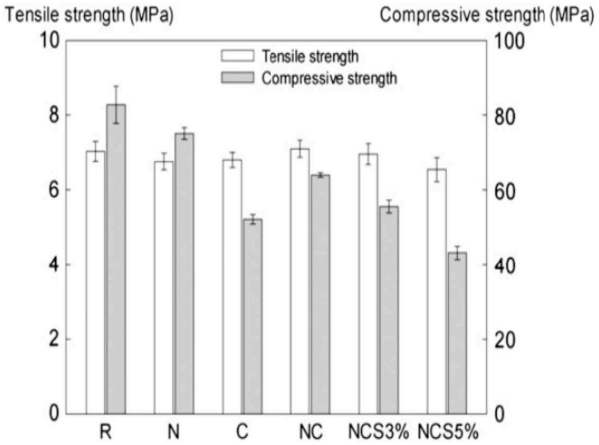

Figure 26: Mechanical properties of the specimens at the age of 3 months. Mechanical properties of the specimens at the age of 3 months. (R - control, N – only nutrients, C – only microcapsule emulsion, NC – nutrients + microcapsule emulsion + bacterial spores) [8]

3.4 Experiments on properties of bio-based self-healing cementitious materials

Many experiments with bio-based precipitation of $CaCO_3$ for crack-healing in concrete have been carried out in recent years. They investigated not only the healing efficiency of different bacteria, nutrients and other compounds, but also effect on mechanical properties of concrete, water permeability after healing and other factors affecting crack repairing capacity.

3.4.1 Types of specimens

To investigate the healing potential of bio-based concrete in laboratory conditions, experiments were carried out typically on small prismatic mortar specimens but also on mortar cylinders [20][17]. For the purpose of obtaining rather large cracks but without losing the integrity of the specimens, some experiments used reinforced specimens. For example prismatic mortar specimens were modified with hole in the middle in which steel wires were placed [28][24], mortar was reinforced with basalt fibers [22], with polyvinyl

alcohol fibers [29] or wrapped with adhesive tape [30]. Usually not only specimens with bio-based healing agent were prepared but also control specimens without the healing agent in order to compare the results.

To determine the self-healing efficiency on more realistic specimens, one research has been carried out on concrete beam 150 x 250 x 3000 mm with incorporated ureolytic mixed self-protected bacterial cultures (CERUP). To create multiple cracks, four point bending was performed on the concrete beam. After cracking and curing (spraying water for 1 minute four times a day over 6 weeks) water permeability and crack filling were investigated. Results were significantly worse than on small prismatic specimens with the same healing agent. The average crack filling ratio was around 24 % (for the beam without the healing agent 20%) whereas for small prismatic specimens it reached 40 %. Also the recovery of water permeability after curing was insignificant [6].

3.4.2 Material characterization: fresh-state properties

Some researchers investigated influence of the healing agent on fresh-state properties of the mortar. For example Jonkers et al. in 2016 prepared mortar with expanded clay particles (LWA – lightweight aggregates) impregnated with healing agent (bacterial spores, calcium lactate and yeast extract) and immediately after mixing three fresh-state properties were tested: consistency, bulk density and air content [24].

Results of experiments with non-bacterial LWA showed that replacement of sand with LWA led to decrease of bulk density and increase of air content, but it did hardly affected consistency. On the other hand, experiments with bacterial LWA showed that the healing agents affected all the mentioned properties and led to a lighter and more flowable mixture [24].

Quian et al.in 2016 investigated three different types of bacteria-based self-healing agents (different Ca sources) on rheology and hydration kinetics. In agreement with previous research, addition of the healing agent improved fluidity of mortar (Figure 28). Also setting time of cement paste was measured by the technique for determining the time of setting of hydraulic cement using Vicat needle. Results revealed that bacteria spore powder had only a slight retarding effect and acceleration/retarding was more influenced

by addition of other agents. Furthermore, the rate of heat evolution and the total heat evolution during 72 h of cement hydration were measured (Figure 27). The results were consistent with the setting time results – bacterial spores had only slight effect on cement hydration but calcium formate and nitrate accelerated the process whereas the delay of cement hydration could be influenced by calcium lactate [21].

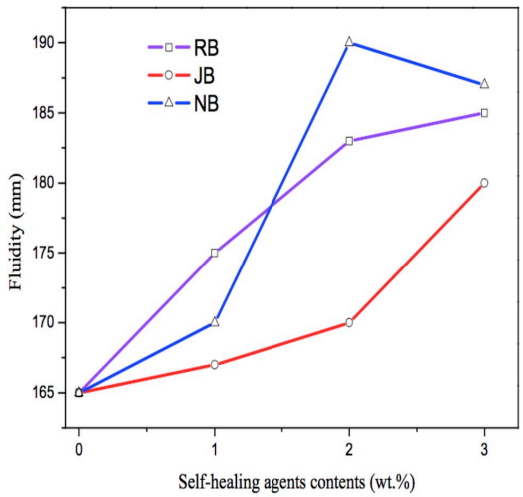

Figure 28: Results of the fluidity test of cement mortar. (RB - calcium lactate and bacterial spore powder, JB - calcium formate and bacterial spore powder, NB - calcium nitrate and bacteria spore powder) [21]

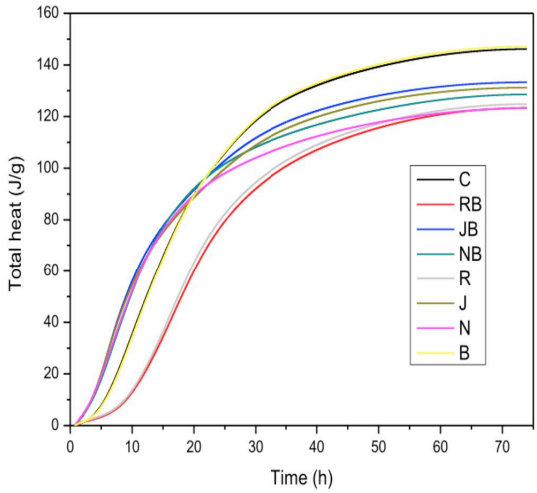

Figure 27: Total heat during the hydration of cement pastes with and without healing agent. (C - control specimens, B - bacterial spore powder, R - calcium lactate, J - calcium formate, N - calcium nitrate) [21]

3.4.3 Material characterization: hardened-state properties

In order to develop self-healing concrete which could be commercially used, it is important to ensure that mechanical properties of the material will not be negatively affected by the healing agents. One of the observed properties is porosity. On one hand, the development of pore diameter size influences the strength properties of the material but on the other hand, it can create a natural protective capsule in which bacterial spores can survive, when they are added directly into the mixture without any prior encapsulation in protective matrix.

Pore size distribution was in previous studies determined by mercury intrusion porosimetry (MIP) [4]. However, the accuracy of this method is reduced when the healing agent is protected by microcapsules. Wang et al. came across conflicting results of water

absorption and porosity. Lower water permeability of specimens with microcapsules would indicate decreased open porosity but the MIP showed different results. However, porosity of these specimens was the same or even higher than of the specimens without the protective carrier. This conflict was probably caused by high pressure during mercury intrusion which had broken the microcapsules and so more space was released. Also the connection between porosity and strength is not completely direct in matrix with microcapsules. Although microcapsules stay intact during water absorption and water saturation and take up some pore space, they cannot withstand mechanical forces during strength test and they need to be regarded as a weak point of the material [8].

To investigate the influence of the healing agents, specimens were usually subjected to tensile and compressive tests in 3, 7 and 28 days and the pore size distribution test [04, 09]. Results showed that strength is not primarily affected by the bacteria itself but rather by addition of other agents [5][6][4][22][24][20][16][23][21] or protective carriers [24][8][18][11][19][26].

3.4.4 Damage introduction and curing

Next step of determining the possibility of application of bacteria in self-healing material is, after investigation of the effect of the healing agent on material's mechanical properties, to examine the actual healing potential of the biological approach to self-healing materials. In laboratory studies it can be achieved by creating an artificial cracks in concrete specimens and subjecting them to curing under different conditions in order to simulate real environment as accurately as possible.

In previous studies, cracking was introduced to the specimens usually after 28 days of curing by strength tests. In order to achieve larger cracks without losing the integrity, the mortar specimens were reinforced as stated in chapter 3.4.1. In some experiments different diameter nails were embedded into formed microcracks in order to obtain various widths of cracks [30].

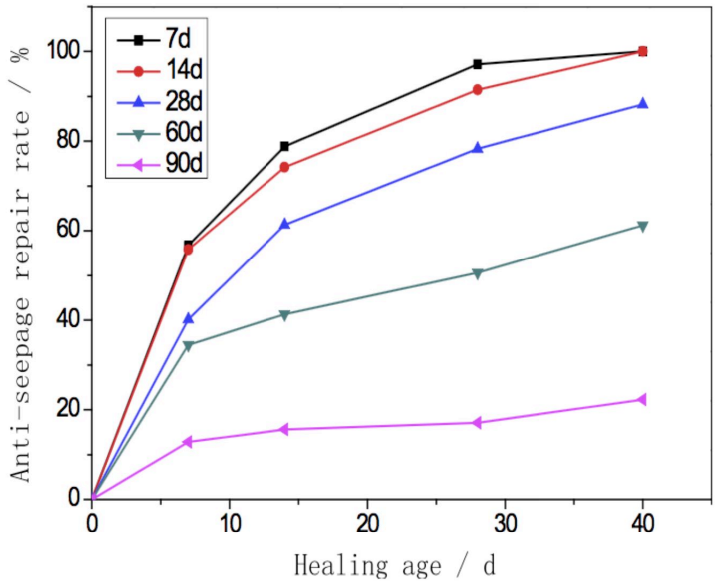

Figure 29: Crack healing ratio of specimens in different cracking age. [30]

Quian at al. in their work which studied factors affecting crack repair capacity of bacteria-based self-healing concrete investigated the effect of different cracking age. Cracks were introduced to the specimens in 7, 14, 28, 60, 90 days. Results showed that the healing ratio significantly decreased after 60 days as it can be seen in Figure 29 [30]. However, it is important to take into account that the study was performed on specimens with the healing agent directly added into the mixture without any protective carrier so it is possible that this loss of healing potential could be a consequence of the decreased porosity and mechanical damage of the spores.

As it is apparent from the principle of bacterial $CaCO_3$ precipitation, water is a crucial catalyst of the process. In laboratory work, different ways of curing were used. Wet curing (in room 25°C, 90% RH), wet and dry cycles and immersion in water. Although the healing ratio of specimen under wet curing was very small, wet and dry cycles and immersion in water reached significantly higher values than non-bacterial specimens [30]. Furthermore, more realistic wet and dry cycles (external pump either supplying or removing water in 12 h cycles) proved to be more efficient than water immersion as it is shown in Figure 30. Possible explanation could be that higher oxygen concentration leads

to increased bacterial activity and amount of $CaCO_3$ precipitation [24]. The efficiency of the crack healing was monitored since the first day up to 100th day.

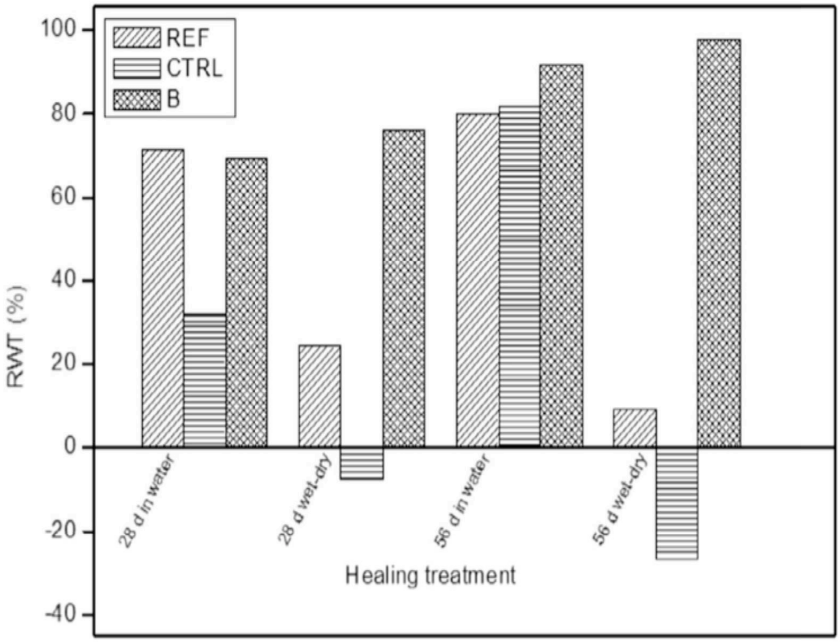

Figure 30: RWT (recovery of water-tightness) in specimens subjected to 28 and 56 days healing treatment. (REF – reference, CTRL – control with non-bacterial ceramsite, B – bacterial ceramsite) [24]

Also external healing was applied in some studies. Xu et al. used two component healing agent incubated in distilled water in which each specimen was immersed. The external treatment achieved the best results. The cracks were healed completely in contrast to two-component healing agent incorporated in the material cured in water in which cracks were filled only partially [22]. However, concrete treated externally with the healing agent cannot be considered as a truly self-healing material.

3.4.5 The healing potential

The basic requirement for successful crack healing process is viability of bacterial spores after direct incorporation into the material or encapsulation in a protective carrier. Jonkers et al. [4] investigated viability of incorporated spores (2,4 x 10^8 *B. cohii* spores per cm^{-3} of cement stone) in aged cement stone specimens. This was done by application of a standard microbiological dilution-to-extinction method, i.e. the most-probable-number (MPN)

technique. Results showed rapid decrease of viability from day 9 to day 135. This loss of functionality appeared to be linked to continuing decrease in matrix pore diameter sizes, which lead to mechanical damage of bacterial spores as they had no protective carrier.

In a study which investigated microencapsulation of bacterial spores, Wang et al. demonstrated that bacterial spores are able to germinate into vegetative cells and revive ureolytic activity (Figure 31) after encapsulation and therefore proved microcapsules to be appropriate form of protection [8].

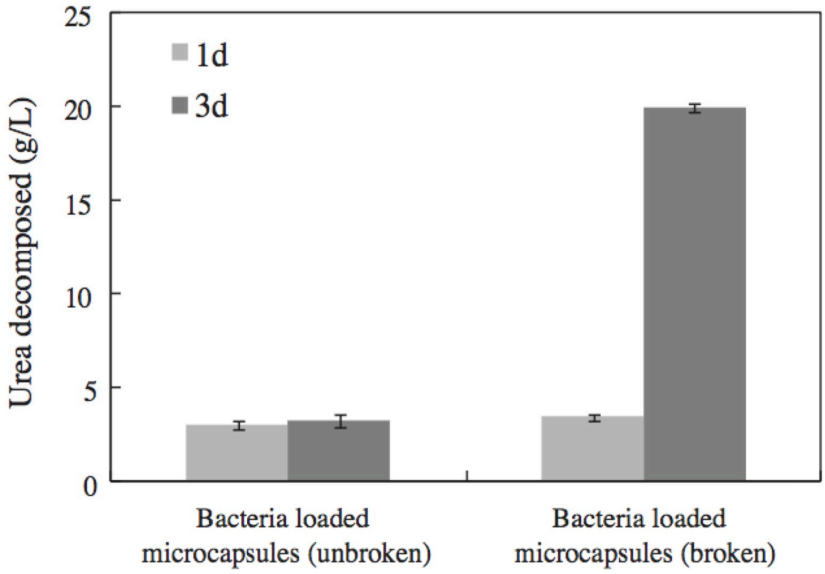

Figure 31: Urea decomposed in YU (yeast extract and urea) medium after the addition of the bio-microcapsules [8].

Microscopic investigation of the cracked specimen after curing was carried out in almost all of the previous studies. Specimens were regularly examined through stereomicroscopic inspection and photographic imaging for quantification of crack-healing in time [13]. For further analysis in many studies the precipitates which had formed on the crack surface were investigated through Environmental Scanning Electron Microscope (ESEM) for examination of the morphology of precipitates and Fourier-Transform Infrared spectrometer was used for identification of manually removed precipitates from the crack

surfaces [24]. From the result of these analyses researchers determined that crystal forms were indeed formations of $CaCO_3$ (Figure 32).

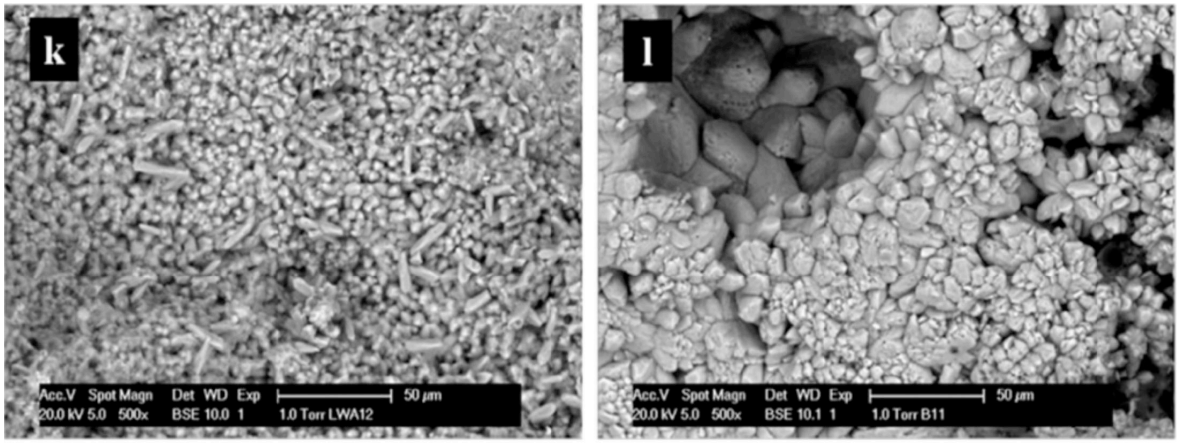

Figure 32: ESEM images of precipitates that were found on the surface of the crack after wet-dry cycles for 56 days. (k - specimen with non-bacterial LWA, b - specimens with bacterial LWA) [24]

For estimating the healing potential of the internal bacterial treatment, the maximal sealed crack width was observed. Jonkers et al. reported that in specimens with two-component healing agent, which consisted of calcium lactate and bacterial spores both embedded in expanded clay particles, cracks were healed up to 0.46 mm, which was more than twice the size than in control specimens (0.18 mm) after 100 days of immersion in water [13]. Furthermore, in mortar specimens with *Bacillus shaericus* and nutrients both encapsulated in melamine based microcapsules, cracks up to 0.96 mm were sealed which was about 4 times wider than non-bacterial series (0.25mm) [8]. However, with different crack width the healing efficiency changes so it is convenient to determine and compare percentage of crack healing closer. Either by measuring the crack in regular intervals [13] or by area repair rate [30].

When Quian et al. in their study investigated the influence of the width of cracks to healing efficiency of bacterial spores without any protective carrier, they determined that the area repair rate significantly decreased with crack width. The repair effect for average crack width of 0.1 – 0.3 mm was very good, crack repair rate reached 85% or more after 20 days repairing. For average crack width of 0.3 – 0.5 mm, the crack repair effect was also good and crack repair rate reached 50 – 70% after 20 days of repairing. However, the

repair ability of microbial repair agent was limited for specimen with crack width up to 0.8 mm, and the corresponding crack repair rate was lower than 30% (Figure 33, Figure 34) [30].

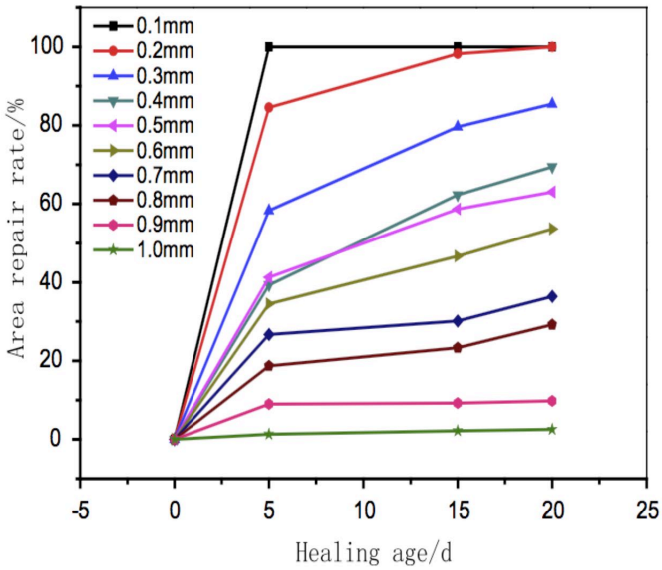

Figure 33: The repair rate of specimens with different crack width after different repair time. [30]

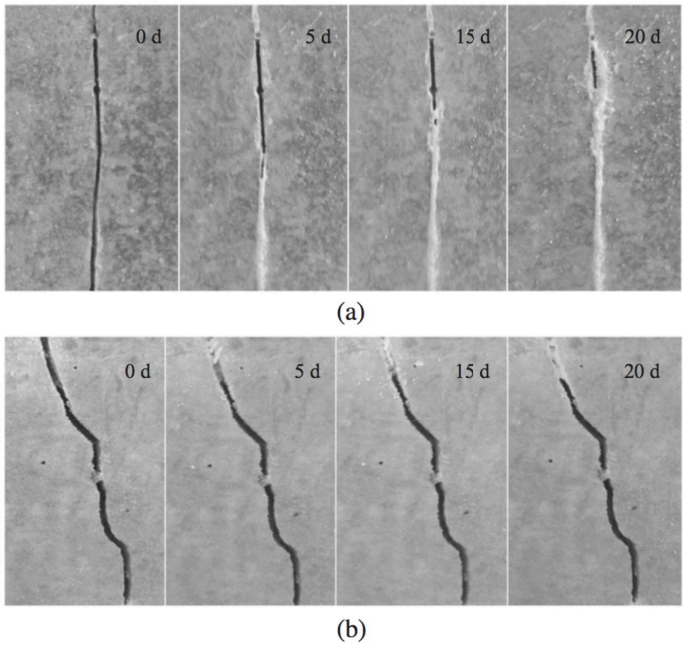

Figure 34: Surface images of specimens with different crack width after different repair time: (a) specimen with an average crack width of 0.3 mm; (b) specimen with an average crack width of 0.8 mm [30]

To link the visual stereomicroscopical observations with functional property, water permeability test was used in many laboratory work [24]. Recovery of water-tightness, along side with the crack filling product investigation, is a good indicator of healing potential and was used for comparison of different dosages, compositions or protective carriers.

For correct determination of the healing potential of bacterial spores it is necessary to investigate whether the crack sealing is only result of natural self-healing ability of concrete or it can be attributed to the biological treatment. Apart from simultaneously testing non-bacterial specimens, many studies used oxygen consumption measurements for estimating bacterial metabolic activity. The active bacteria cells convert nutrients to $CaCO_3$ by using oxygen. Therefore, by measuring oxygen concentration on the surface of specimens immersed in water/other solution, it is possible to evaluate bacterial activity in mortar [28]. For example in an experiment carried out by M. Guadalupe Sierra-Beltran et a1. [29], cracked specimens were submerged in solution with pH 11 for 18 hours and oxygen concentration towards surfaces (in the 2.5 mm above the samples) was measured. Results showed that the O_2 profiles of mortar specimen which contained bacterial LWA (Mix1B) significantly decreased in the diffuse boundary layer (0.6 mm), whereas the O_2 profiles of the control non-bacterial specimen (Mix 1) stayed almost constant (see Figure 35). This meant that 3 months after casting, impregnated bacteria in LWA were viable and able to metabolize [29].

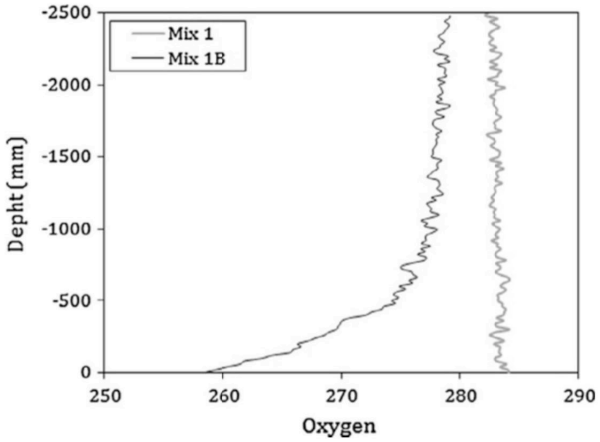

Figure 35: Measured oxygen concentration microprofiles towards surfaces of submerged control (Mix 1) and bio-based samples (Mix 1B) [29]

3.5 Field application

3.5.1 Irrigation canal in Ecuador

The first application of self-healing concrete with alkaliphilic spore-forming bacteria and reinforcement took place in the Andean mountains in Ecuador in July 2014. For over a century, an 24-kilometers-long irrigation canal has been used by local farmers to transport water from glaciers to fields. However, as the walls and bottom of the canal were until not long ago made out of compressed soil, about 70 % of the water was lost due to evaporation and infiltration into the soil. To reduce this loss, canal was lined with concrete without any steel reinforcement. Unfortunately, only a year after the reconstruction, the concrete linings started to crack and the water was leaking through the cracks [31].

To solve this problem, Delft University of Technology together with Foundation Imagine and local Catholic University of Santiago de Guayaquil came up with the idea of application of self-healing biological concrete to improve the durability and functionality of the irrigation canal. The proposed concrete mix consisted of gravel, sand, cement, lightweight aggregates (LWA) impregnated with the healing agent and natural fibers. The effort was to make the mixture from local sources as much as possible. Therefore, Abaca, a fibre indigenous to Ecuador which has been already studied and applied as a reinforcement for mortar used in structures under seismic loads, was chosen as natural fibre reinforcement. The theory was that the fibers would increase the tensile strength of the concrete and also it would control crack width. The bio-based healing agent would sealed those possible cracks. As stated before, the narrower the crack is, the more efficient is the bio-based healing [31].

The proposed mixture was priorly tested in laboratory. The compressive strength reached 30 MPa with the healing agent and 26 MPa for the control specimens. Also artificial cracks created by three-point bending test were cured under similar conditions to real irrigation canal and after 6 weeks sealing could be observed (Figure 36) [31].

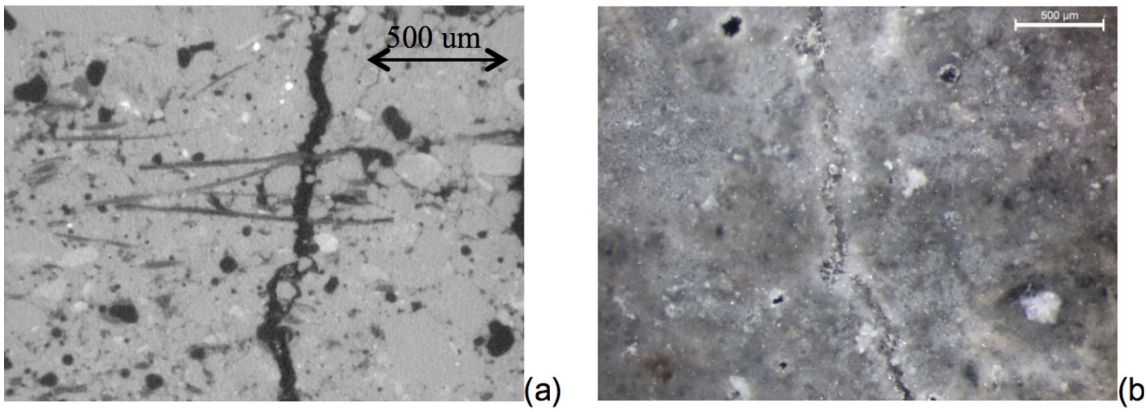

Figure 36: CT showing Abaca fibres bridging the crack (a) and crack sealing (b) [31]

After laboratory testing, the field application could take place. The concrete was prepared in situ with local materials. In field work, superplasticiser was added into the mixture to create more workable mix. Small part of the of the irrigation canal was cleaned and three meters of bacterial concrete and three meters of non-bacterial concrete were cast into wooden framework (Figure 37). After 3 days, the framework was removed and the canal was reopened for water. The canal was regularly observed since then and the last inspection after 5 months from casting did not reveal any cracking or deterioration [31].

However, the description of the experiment does not provide very detailed information. The article does not mention the cause of the problems with the original concrete linings. A possible explanation of the cracking might be, that the original linings were not properly divided into expansion units. In that case, however, the length of 3 meters of the new bacterial and non-bacterial concrete linings would not be sufficient to determine the effectivity of the new material, as the length of an appropriate expansion unit would be around 6 meters. Furthermore, the authors do not compare the condition of the biological and non-biological concrete linings. That would correspond to the theory that the original concrete linings were not properly divided into expansion units, thus the new experimental linings (both biological and non-biological) did not reveal any cracking or deterioration, as they were shorter than the appropriate expansion unit.

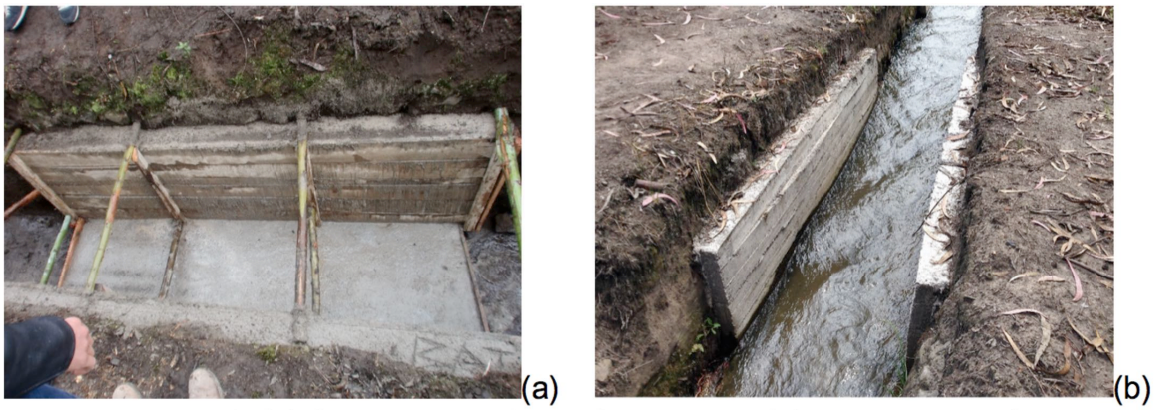

Figure 37: Canal section 24h after casting (a), canal section 5 months after casting (b). [23]

3.5.2 Outdoor condensed powder system application

Another example of field application is given in [6], unfortunately with no particular details. The healing agents in a form of flakes were added into commercially available repair mortar. A concrete wall was divided into parts. On one half, layer of concrete was removed until steel reinforcement was visible and on the other half concrete was removed behind the reinforcement and the reinforcement was cleaned. Then the repair mortar with and without the healing agent was sprayed on both parts and observed (Figure 38) [6].

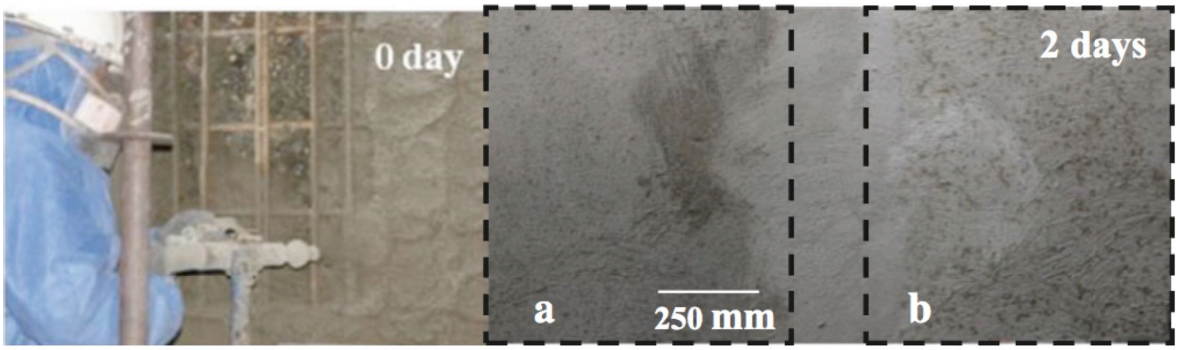

Figure 38: Left: Spray application of commercial ready-mix mortar complemented with healing agent flakes. Right: During hardening, locations of healing agent particles are indicated by formation of speckles (b) compared with control (a) [6]

3.6 External repair systems

To maintain durability of structures it is necessary to control and improve their condition regularly. However, current repair systems are often based on environmental unfriendly materials such as epoxy systems, acrylic resins or silicone-based polymers. Researches

have proposed bio-based treatment in order to improve traditional repair systems efficiency and possibly to reduce its ecological impact.

Systems with ureolytic bacteria have been investigated in laboratory and even applied on site in recent years. An application of bio-treatment for surface protection was first used in 1993 on the limestone of the Saint Medard Church in Thouras in France. Surface bio-protection reached similar or even better results than traditional treatment but number of aspects should be pointed out. Firstly, it is necessary to explore the microbial community inhabiting the matrix in order to choose appropriate culture medium. Furthermore, the bio-treatment should not negatively affect the building material and the process should be repeatable and the used bacterial strains should not be pathogenic. It is necessary to mention that number of researchers did not recommend surface application of biological treatment as the spore germination and uncontrolled growth could pose a potential risk to the environment. On the other hand, others disagree with this statement and claim that with carefully chosen and controlled treatment the risk should be reduced to minimum. Other possible application of biological repair systems is external crack repair. Experiments have been carried out either with bacteria and nutrients only, with an addition of sand, with bacteria immobilized in polyurethane foam or silica sol-gel, manually applied into the cracks. When compared with traditional treatments, biological remediation reached similar or better results [6].

Liquid repair systems based on oxidation of organic carbon have been investigated and developed at the Technical University of Delft. Large in-situ experiment has been carried out in a parking garage. The parking deck suffered from cracking which lead to heavy leaking, although some cracks had been treated with traditional repair systems. In the experiment, area of around 2000 m^2 has been treated with biological liquid treatments. After first application, leakage test has been carried out. The results showed that cracks still leaked and also stereomicroscopy showed no significant difference between areas with and without biological treatment. However, there were doubts about proper penetration with the healing liquid during the first treatment application. The second treatment (Figure 39) was performed with bacteria adapted to cold temperatures and cracks were manually impregnated until the liquid was dripping through the structure to the level below. Results

showed that the cracks leaked significantly less after 7 weeks and also microscopic observation indicated bacterial activity. Thus, liquid repair systems based on oxidation of organic carbon could be a faster and economically advantageous alternative to traditional repair systems [6].

Figure 39: Second leakage test. [6]

Overlay application or patch repair of concrete structures is difficult because of lack of compatibility between the concrete substrate and the repair material. Especially, volume changes of the materials are different (due to their different age that results in differential shrinkage) which leads to tensile strains and therefore cracking or interface delamination.

In recent years, a special type of strain-hardening cement-based composite, called Engineered Cementitious Composite (ECC), has been studied and applied as an overlay or patch repair. This material with randomly distributed polymer fibers has large strain capacity and therefore it is more resistant to the mechanical forces during volume changes. Also the addition of fibers helps to control drying shrinkage and service load-related cracking. At Delft University of Technology researchers suggested that ECC with a biological healing agent could be more durable repair material as the cracks from

restrained shrinkage or other mechanical forces could be sealed [29].

In laboratory work, ECC with an addition of lightweight aggregates impregnated with the healing agent was tested (Figure 40). Result showed that the biological ECC had reduced delamination (Figure 41) from the concrete substrate and also the bio-mixtures showed slightly better recovery of flexural strength and deflection capacity from the non-biological mixtures [29].

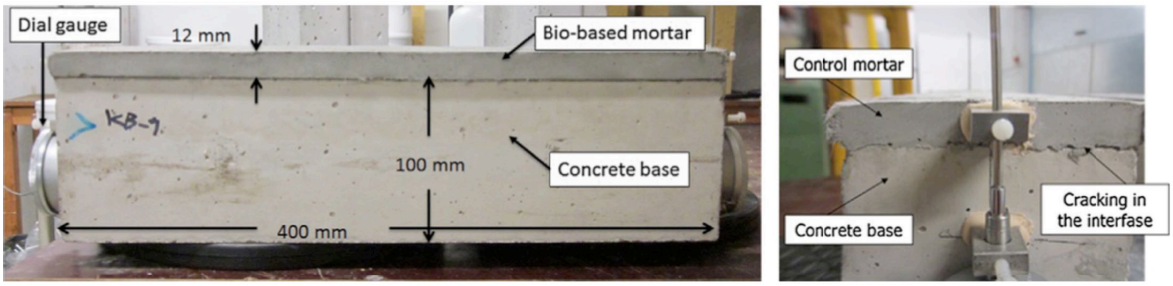

Figure 40: Layered repair system setup under restrained shrinkage [29].

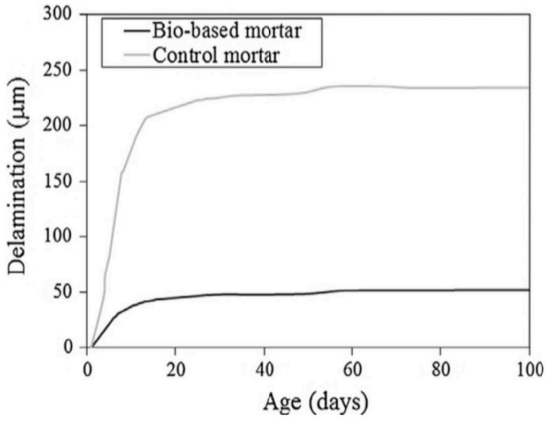

Figure 41: Specimen delamination heights at different ages [29]

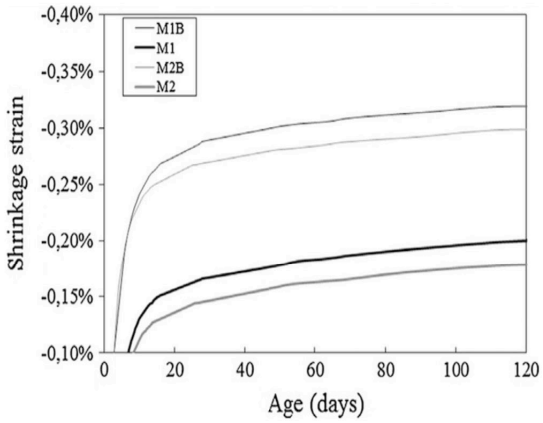

Figure 42: Free shrinkage strain of repair mortars at different ages. (B - bacterial) [29]

The first application of ECC with a biological healing agent was carried out in 2013 in Netherlands. In an outside parking garage, the cover of reinforcement of a concrete wall was damaged and the steel reinforcement was exposed to the weather effects. ECC with a biological agent was applied until it completely covered the reinforcement and observed

(Figure 43). After 1.5 years, the patch repair was undamaged with no sings of deterioration. In the next years, the biological patch repair has been successfully used under different conditions all over the Netherlands as experiments showed that the material significantly reduced both delamination and shrinkage cracking [6].

Figure 43: Self-healing mortar applied in a tunnel in the Netherlands: (a) surface preparation and (b) monitoring for shrinkage and possible delamination. [6]

3.7 Summary

The literature review has determined many requirements which must be met in order to achieve truly self-healing concrete through biological treatment. These findings will be exploited in further research of the author. The most important ones are:

- Bacteria used for biological treatment must be able to form spores – resistant dormant state in which bacteria can survive highly alkaline and dry environment of concrete.
- Nutrients (usually yeast extract) and deposition agents (Ca source, nitrate source and/or urea depending on a specific metabolical pathway) are essential for germination of spores and $CaCO_3$ precipitation. However, these compounds can, positively or negatively, affect the mechanical properties of concrete so their composition and amount need to be tested.
- As functionality of bacterial mineral production of incorporated bacteria is limited only to young concrete (likely caused by decrease of porosity and mechanical destruction of bacterial spores), encapsulation or immobilization of bacterial spores into protective carries is crucial in order to obtain an effective self-healing material.
- To investigate the healing potential of bio-based concrete in laboratory, it is necessary to create an environment which is similar to real outside conditions as much as possible (for example testing larger specimens and curing by wet and dry cycles instead of full immersion in water).
- When estimating the bacterial treatment efficiency, it is necessary to separate the crack-healing caused by metabolical activity of bacteria and by natural autogenous healing capacity of concrete (either by comparison of crack-healing in non-biological mixture under the same conditions or by other techniques like oxygen consumption measurements).
- The repair efficiency significantly decreases with increasing width of cracks. Therefore by controlling the width (for example by addition of reinforcement fibres) it is possible to achieve more efficient self-healing concrete.

4 Experimental work

The main aim of this work was to develop, describe and verify a method of self-healing bacterial process based on the information gathered from the already performed experiments. The first step was to select an appropriate type of bacteria, to perform its cultivation and to determine the properties which could influence the efficiency of the self-healing process. In the next stage we inspected the possibility of an practical application. As it was already stated in the theoretical part of this thesis, encapsulation of bacterial spores is crucial for the functionality of the process. Therefore we further inspected the possible options of protection for application into cementitious matrix.

4.1 Materials and methods

4.1.1 Selection and cultivation of bacteria

As it was previously mentioned in the chapter 3.2 Materials, there are several requirements that must be met by the selected bacteria. Not only the bacteria must be able to withstand the mechanical forces during the mixing process but also to be alkali-resistant enough to survive in the harsh highly alkaline environment which concrete is. Suitable candidates, alkali-resistant spore-forming bacteria, can be found in the genus *Bacillus*. For the purpose of this work, alkaliphilic and alkalitolerant aerobic endospore-forming bacteria *Bacillus pseudofirmus* was selected as this type was successfully used in previous experiments [4] [5][16].

Bacteria were cultured in two different media: culture medium for the growth of bacteria and mineral alkaline medium to enhance sporulation. Culture medium consisted of 1 g lamb extract, 2 g yeast extract, 5 g pepton, 5 g NaCl, 0.42 g $NaHCO_3$ and 0.53 g Na_2CO_3 per liter of distilled water (see Figure 44). Bacteria was cultured either in liquid or solid media, which was prepared by mixing the liquid media with agar and poured into Petri dishes to solidify. These solid media were used for the conservation of bacteria and obtaining a specific growth form of the bacteria – colonies. Alkaline medium enhancing sporulation contained 0.2 g NH_4Cl, 0.02 g KH_2PO_4, 0.225 g $CaCl_2$, 0.2 g KCl, 0.2 g $MgCl_2 . 6H_2O$, 0.01 g $MnSO_4 . 2H_2O$, 0.1 g yeast extract, 5.16 g citric acid tridosium salt,

4.2 g $NaHCO_3$ and 5,3 g Na_2CO_3 per liter of distilled water (see Figure 45). Final pH of the both media was around 10.

Liquid cultures of the bacteria were stored in room temperature (25°C ± 2°C) in Erlenmeyer flasks (to ensure the supply of oxygen while keeping the sterile environment) on a shaker tables at 160 rpm.

Figure 44: Culture medium in Erlenmeyer flasks.

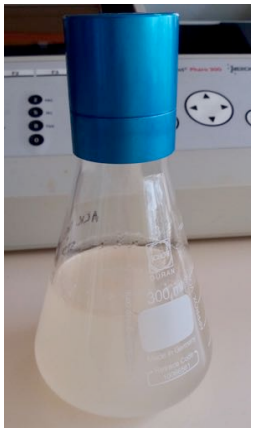

Figure 45: Alkaline mineral medium in an Erlenmeyer flask.

4.1.2 Sporulation of bacteria

The production of a resistant, dormant and non-reproductive state of bacteria – bacterial spores is usually triggered by a lack of nutrients or other adverse environment change [7]. Therefore to obtain bacterial spores, bacteria were cultured in the mineral alkaline medium, which is not as rich in nutrients as the culture medium, and it was additionally heated in a drying room at 80°C for 30 minutes after cultivation. This process should ensure complete sporulation of bacteria in the medium. Bacterial spectrum of mineral alkaline medium with *Bacillus pseudofirmus* was measured on spectrophotometer (for description see chapter 4.1.3) before and after the heating process. Finally, cultures containing spores were washed by centrifugation and the cell pellets (Figure 65) were kept in a fridge at 5°C for further use.

4.1.3 Survival and viability of bacteria under different conditions

To determine the potential of biological self-healing method it is necessary to inspect whether the selected bacteria are able to withstand other than optimal conditions which may occur either during the production process or in the final field application.

One of the possible methods for determining the viability of bacteria is graphical representation of time-dependent cell concentration which is called bacterial curve. The most commonly used measurement is based on optical density (absorbance) measuring using a device called spectrophotometer (Spectroquant Pharo 300, Merck or Opsys MR, Dynex). The principle of determining the bacterial concentration, by Beer-Lambert law, is to irradiate the measured sample with light of a certain wavelength. Some light beams scatter outside the optical path and the detector then records the amount of light that passed through the sample without distraction. Using this absorbance value, we can measure the change in cell concentration.

At first, bacterial curve of both media (culture medium and mineral alkaline medium) with *Bacillus pseudofirmus* was measured to represent viability of the bacteria in optimal conditions. Pure and sterilized culture and mineral alkaline media were inoculated with 0.1 ml of 4 days old cultures and then measured on spectrophotometer for 51 hours.

One of the common problems in the Central European region is freeze-thaw cycles during winter time. To inspect the influence of this phenomenon, we exhibited *Bacillus pseudofirmus* to artificial freeze-thaw cycles (20 cycles from -10°C to 10°C). Several different samples exposed to freeze-thaw cycles were prepared. The first group of samples named "BP-Spores" was prepared by centrifugation of alkaline mineral medium which was preliminary exposed to 80°C in a drying room for 30 minutes to enhance sporulation of bacteria. The second group of samples named "BP-O/N" was prepared by centrifugation from over-night culture of *Bacillus pseudofirmus* in alkaline mineral medium but without the heat treatment.

After the end of freeze-thaw cycles, sterilized pure culture and alkaline mineral media were inoculated with bacteria (20 ml medium with 1 pellet of *Bacillus pseudofirmus* in Erlenmayer flasks) and bacterial growth was measured by spectrophotometer.

4.1.4 The biological healing agent

Bacillus pseudofirmus is capable of metabolic activity which results in precipitation of $CaCO_3$ (see chapter 3.1.2 Oxidation of organic carbon), when supplied with two basic components – source of Ca and nutrients. These compounds, however, must not negatively affect the properties of the final cementitious material so they have to be chosen carefully. Our biological healing agent was composed of 1 g yeast extract and 80 g calcium lactate per liter of distilled water (CLY solution - Figure 46) and bacterial spores *Bacillus pseudofirmus*. This composition was based on a experiment carried by V. Wiktor and H. Jonkers [13].

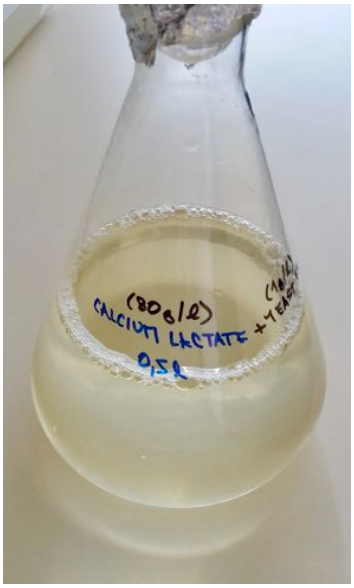

Figure 46: Calcium lactate and yeast solution.

4.1.5 Encapsulation of bacteria in nanofibre textile

Fibres that have a width of less than 100 nm are generally defined as nanofibres [32]. The use of nanoparticles and nanofibres to produce specialized nanofabrics became a subject of interest in the 1980s and in recent years, dramatic increases in global funding have accelerated research efforts in nanotechnology, including nanofabrics research. Due to its special properties such as small fibre diameter, high surface area, small and controllable pore size, nanofibres have begun to be used in many different areas (filtration, wound dressing, composites, tissue engineering, biomedical devices, membrane, sound absorption etc.) [33].

The encapsulation of biological material (bacteria and viruses) in polyvinyl alcohol (PVA) nanofibres has been already researched by W. Salalha et al. in 2006 [34]. The experiment showed that a range of organisms can be efficiently encapsulated (Figure 47) and stay viable, thus it could be a promising method suitable for our purpose.

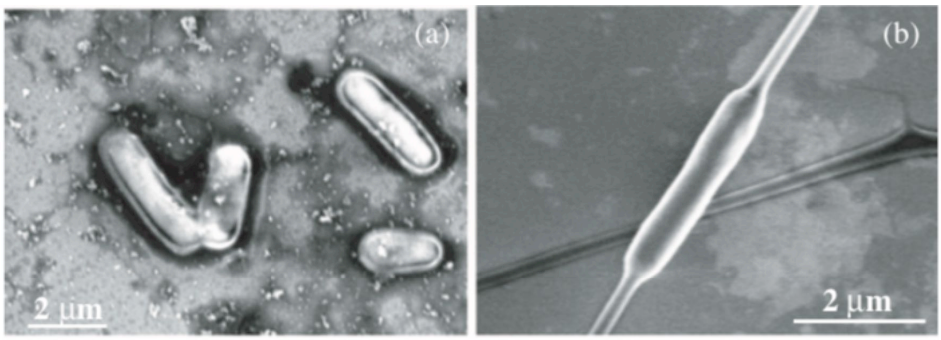

Figure 47: HRSEM micrographs of (a) individual E.coli cells and (b) embedded E.coli cells in electrospun PVA nanofibres in experiment carried by W. Salalha. [34]

In this work, the nanofabric was produced by the roller electrospinning process (developed by Jirsak et al. in 2003) using Nanospider by the Elmarco Company in Liberec, Czech Republic. The roller spinning device consists of a slowly rotating roller partially immersed in the polymer solution and a collector electrode. A high voltage source is connected to the solution. Collector electrode is usually grounded. The roller is covered by the polymer solution which is always fresh due to rotation of the roller. Many Taylor cones (a shape of liquid observed in electrospinning and electrospraying) are simultaneously created on the roller surface producing nanofibers (Figure 48). The nanofibers are then transported towards the collector electrode. A supporting textile or nontextile sheet moves usually along the collector electrode and is covered by nanofiber layer (called spunbond) so that the production process is continuous [33].

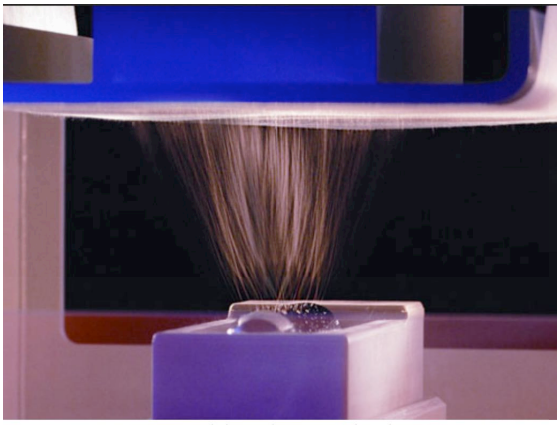

Figure 48: Nanospider,electrospinning [35]

a) Influence of the stabilization process on bacterial spores

PVA nanofibres are soluble in water and this property is inconvenient for our purpose, which is direct addition to cementitious material. To overcome this problem, there are several methods how to increase stability of PVA nanofibres. In this work, we used exposure to high temperatures and glutaraldehyde (GA) vapour.

To examine the influence of high temperatures to bacterial spores of *Bacillus pseudofirmus,* we prepared three different specimens. Bacterial spores were washed by centrifugation from untreated alkaline mineral medium, from alkaline mineral medium exposed to 80°C for 30 minutes and from alkaline mineral medium exposed to 140°C for 10 minutes. These spores were then applied in the amount of 0.1 ml on sterile filter papers and placed to desiccator with glutaraldehyde for two hours. After exposition to GA vapour, bacterial filter papers were placed in 100 ml of culture medium in BOD (biochemical oxygen demand) sensors which determine the amount of dissolved oxygen needed by aerobic biological microorganisms in water (Figure 49), thus show bacterial activity in examined samples. These values were then the basis for creation of bacterial curves.

Figure 49: Sterilized flasks with culture media and BOD sensors.

b) Samples of PVA nanofabric with encapsulated bacteria

To determine the potential of encapsulation of *Bacillus pseudofirmus* into PVA nanofibres, samples (double layer circles with a diameter of about 4.4 cm – see Figure 50) with different composition and concentration of PVA were prepared. The base of all series is 10% or 16% PVA. Series 10PVA and 10PVAB also contain H_3PO_4 and glyoxal, which improve the efficiency of the stabilization process. Letter "B" in sample name then indicates bacterial variant – an addition of pellets with bacterial spores to the basic solution. For the composition of samples and the method of stabilization see Table 3.

Samples	**Composition**					
	16% PVA	10% PVA	H_2O	H_3PO_4	Glyoxal	Bacterial spores
10P		●	●			
16PVA	●		●	●	●	
10PB		●	●			●
10PVAB		●	●	●	●	●

Table 3: Composition of PVA nanofabric samples

Samples were either stabilized by exposure to high temperature (140°C at least 20 minutes), placed in desiccators with glutaraldehyde for two hours or left unstabilized. After the stabilization process, all of the samples were placed into liquid and solid media (culture media or calcium lactate with yeast extract media) to examine the survival and viability potential via visual inspections and bacterial curves based on the values acquired from a spectrophotometer or BOD sensors. For description of the experiment see Table 4.

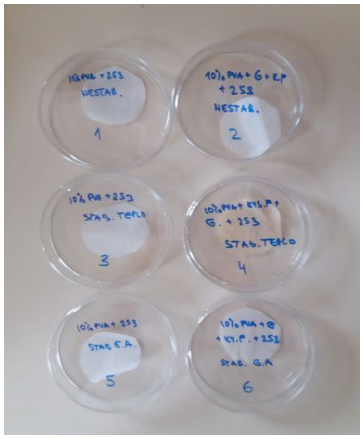

Figure 50: Samples of PVA nanofibres prepared for measurement of oxygen demand with BOD sensors.

Composition	Stabilization	Sample num.	Visual Culture, solid -	Visual CLY, solid -	Spectrophotometer CLY, liquid 20 ml	BOD sensors Culture, liquid 100 ml
10P	Temperature	1	●	●		
		2	●	●		
		3	●	●		
	GA	1	●	●		
		2	●	●		
		3	●	●		
	Unstabilized	1	●	●		
		2	●	●		
		3	●	●		
16PVA	Temperature	1	●	●		
		2	●	●		
		3	●	●		
	GA	1	●	●		
		2	●	●		
		3	●	●		
	Unstabilized	1	●	●		
		2	●	●		
		3	●	●		
10PB	Temperature	1	●	●	●	●
		2	●	●		
		3	●	●		
	GA	1	●	●	●	●
		2	●	●		
		3	●	●		
	Unstabilized	1	●	●	●	●
		2	●	●		
		3	●	●		
10PVAB	Temperature	1	●	●	●	●
		2	●	●		
		3	●	●		
	GA	1	●	●	●	●
		2	●	●		
		3	●	●		
	Unstabilized	1	●	●	●	●
		2	●	●		
		3	●	●		

Table 4: Nanofabric samples; type of stabilization, media and evaluation method.

4.1.6 Encapsulation of bacteria in light weight aggregates

Light weight aggregates (LWA) proved to be a suitable carrier for bacterial spores and/or accompanying compounds in previous experiments (see chapter 3.3.2 Expanded clay particles as a protective carrier for non-ureolytic bacteria). In our experiment, the healing agent was incorporated into expanded clay particles Liapor 4/8 mm.

Before impregnation, LWA was dried on open glass plates (2 hours in a drying chamber) and then covered and sterilized in 165°C for 2,5 hours. Sterilized LWA was divided into three series – bacteria with yeast (BY), bacteria with calcium lactate (BCY) and bacteria with calcium lactate and yeast gradually dried in humidity (BCYH). For the composition of particular solution see Table 5.

LWA type	**yeast [g]**	**calcium lactate [g]**	***Bacillus pseudofirmus***
BY	0,2	0	4 cell pellets
BCY	0,2	16	4 cell pellets
BCYH	0,2	16	4 cell pellets

Table 5: Composition of LWA impregnation solution; amount per 200 ml of distilled water.

a) Impregnation process

Process of impregnation was identical in all cases. Dried and sterilized LWA was placed into vacuum pump and embedded into solution. Vacuum pump was turned on for 5 minutes and then the treated LWA was left to soak up for 20 minutes in atmospheric pressure. After this procedure, surplus solution was poured out and impregnated LWA was placed onto filter papers and let to dry out.

b) Drying process

The drying process was carried out under different conditions. Our preliminary examinations of the LWA impregnation with non-bacterial medium (only calcium lactate and yeast) showed that calcium lactate has a pronounced tendency to crystallize on the surface of the particles and thus there is a risk of insufficient penetration of the particles while drying at room temperature and humidity. To examine the influence of temperature and humidity on the efficiency of drying process, two of the series (BY and BCY) were placed into desiccator with silicagel (see Figure 51) and left at room temperature until

further use, but the BCYH series was at first placed into desiccator with 75% humidity at 5°C for 24 hours and then placed into desiccator with 55% humidity and left at room temperature. Finally, after two days, BCYH was also placed into desiccator with silicagel and left there for further use. All of the series were regularly examined under a microscope to determine presence of nutrients.

Figure 51: BY and BCY in desiccator with silicagel.

c) Survival and viability of impregnated bacteria

For determination of efficiency of the healing agent composition and impregnation and drying process, impregnated LWA was subjected to measurement of bacterial activity via BOD sensors. To simulate future conditions of the bacterial LWA in cementitious material, all the series were cultured in media (see Table 6) which would demonstrate real behaviour and supply of nutritions. Approximately 30 ml of bacterial LWA was immersed in sterilized media and oxygen decrease was measured with BOD sensors. LWA with embedded yeast and bacteria (BY) was cultured in 100 ml of distilled water with 8 g calcium lactate (to simulate direct addition of the Ca source into the material's matrix). LWA with addition of calcium lactate (BCY and BCYH) was cultured in 100 ml of sterilized tap water.

LWA type	Approximate volume [ml]	Type of medium
BY	30	100 ml calcium lactate solution
BCY	30	100 ml sterilized tap water
BCYH	30	100 ml sterilized tap water

Table 6: Composition of media for oxygen demand measurements of bacterial LWA.

d) Bacterial LWA exposure to freeze-thaw cycles

An exposure to freeze-thaw cycles procedure, which was already performed on bacterial spores without any encapsulation, was repeated with impregnated LWA to determine the survival and viability of encapsulated bacteria in the typical conditions which can occur in the Central European region.

Three group of samples BY, BCY and BCYH (see Table 5) were subjected to artificial freeze-thaw cycles (5 cycles between -10°C and +20°C). After the end of the procedure, the samples were immersed in media and subjected to measurements of oxygen demand with BOD sensors. Approximately 30 ml of the series BY was immersed in 100 ml of calcium lactate solution (80 g/l) and the series BCY and BCYH were immersed in 100 ml of sterile tap water (see Table 6).

4.2 Results and discussion

4.2.1 Sporulation of bacteria

Bacterial spectrum of mineral alkaline media with *Bacillus pseudofirmus* measured with spectrophotometer shows that there is not a significant difference between untreated culture and culture which was exposed to 80°C for 30 minutes (see Figure 53). This result might show that mineral alkaline medium itself ensure sufficient sporulation of bacteria and additional treatment with temperature is not necessary. For microscopic photograph of stained bacteria after exposure to 80°C (see Figure 52).

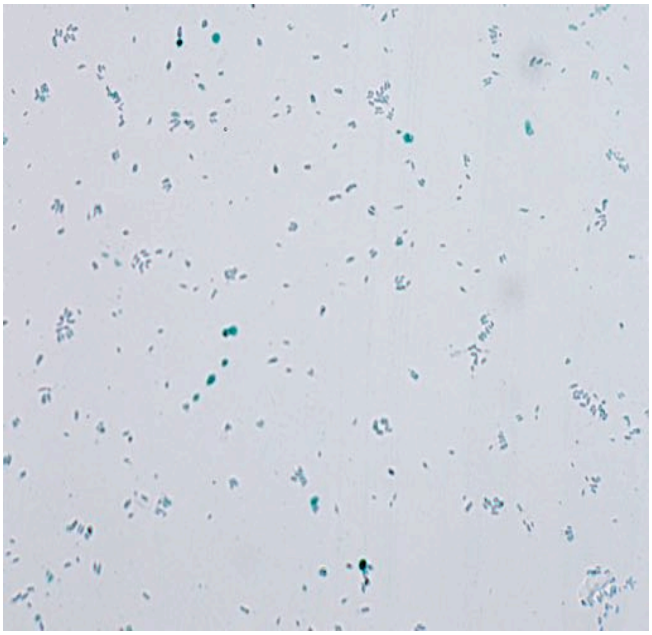

Figure 52: Visualization using the Ziehl–Neelsen stain. Active bacteria (red) and spores (green), after exposure to 80°C.

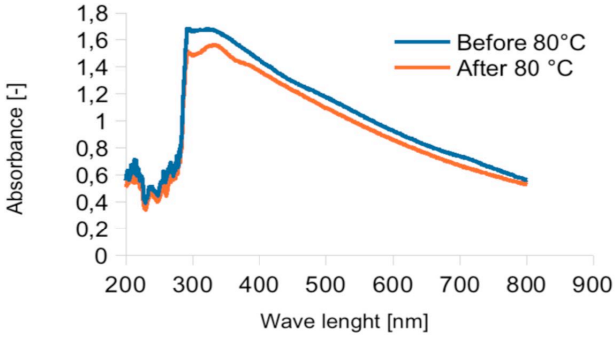

Figure 53: Bacterial spectrum of *Bacillus pseudofirmus* in alkaline medium before and after the exposure to 80°C.

4.2.2 Survival and viability of bacteria under different conditions

Bacterial curves of *Bacillus pseudofirmus* in optimal conditions were determined via spectrophotometer in culture and alkaline mineral media (see Figure 54 and Figure 55). Results show that the bacteria are viable and capable of growth in both of them. In both media, the lag phase (period, where bacteria adapt themselves to the growth conditions) lasted around 4 hours and the exponential phase (phase of cell reproduction) lasted from 4 hours to approximately 27 hours. After the end of this phase, the stationary phase (period, where growth rate and death rate are equal, possibly caused by limited amount of

nutritions) lasted to the end of the measurements. Also the results of comparison of bacterial growth rate (see Formula 3 where R is the growth rate, t is time and *absorbance* was measured with spectrophotometer with 630 nm wavelength) in different media are in agreement with the assumptions.

$$R = \frac{(\log_2(absorbance_2) - \log_2(absorbance_1))}{(t_2 - t_1)} \tag{3}$$

	Culture medium	**Mineral alkaline medium**
t_1 [hrs]	5	
t_2 [hrs]	7	
$\log_2(absorbance_1)$	-2,806	-2,796
$\log_2(absorbance_2)$	-1,916	-2,238
R [-]	0,445	0,279

Table 7: The growth rate of *Bacillus pseudofirmus* in culture and mineral alkaline media.

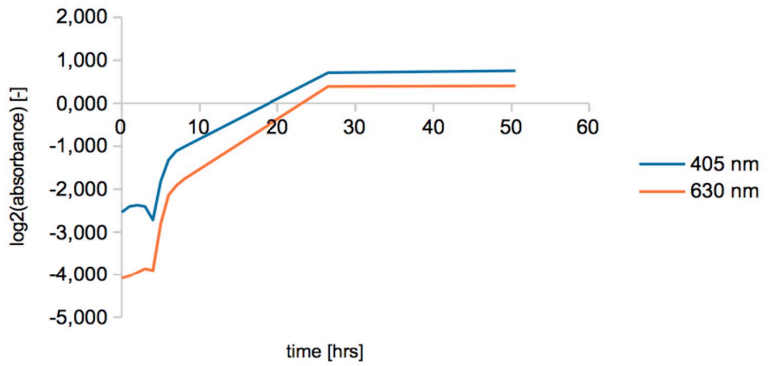

Figure 54: Bacterial curve of *Bacillus pseudofirmus* in culture medium, optimal conditions.

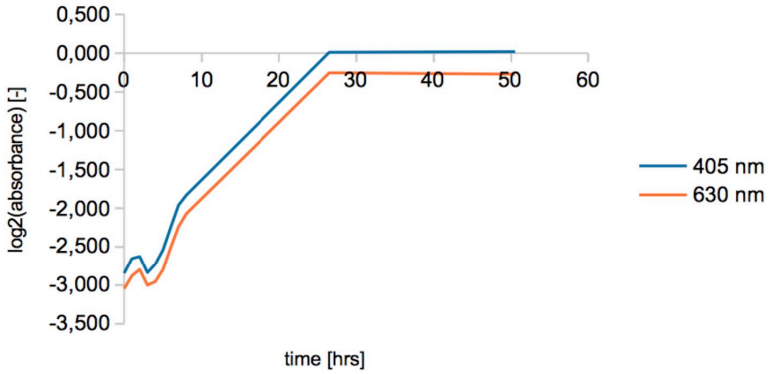

Figure 55: Bacterial curve of *Bacillus pseudofirmus* in mineral alkaline medium, optimal conditions.

The growth rate reaches higher values (see Table 7) in the culture medium than in the mineral alkaline medium, as the culture medium is significantly richer in nutrients.

Bacterial curves of *Bacillus pseudofirmus* exposed to freeze-thaw cycles were estimated with spectrophotometer. Results show that both groups of samples (BP-O/N and BP-Spores, see page 60) are able to withstand the freeze-thaw cycles. Samples which were cultured and measured in culture media show significantly better ability to reproduce than samples which where cultured and measured in alkaline mineral media. This result is in agreement with our expectation as the culture medium is richer in nutritions, thus bacterial spores have better conditions for restoring their activity and their subsequent growth (see Figure 56 and Figure 57).

The lag phase of culture media samples lasted around 5 hours whereas in alkaline media samples it was around 8 hours. Also the growth rate (see Figure 56 and Figure 57) was significantly higher in samples with culture media as it had been expected. A comparison (see Figure 59) of bacterial curves before (BP culture m. - before cycles) and after (BP-O/N culture m. and BP-Spores culture m.) the freeze-thaw cycles shows a slight decrease of the growth rate of samples exposed to freeze-thaw cycles when cultured in culture medium. However, the growth rate of samples cultured in mineral alkaline medium before freeze-thaw cycles (BP alkaline m. - before cycles) is substantially higher then after the cycles (BP-O/N alkaline m. and BP-Spores alkaline m.) as it can be seen in Figure 58.

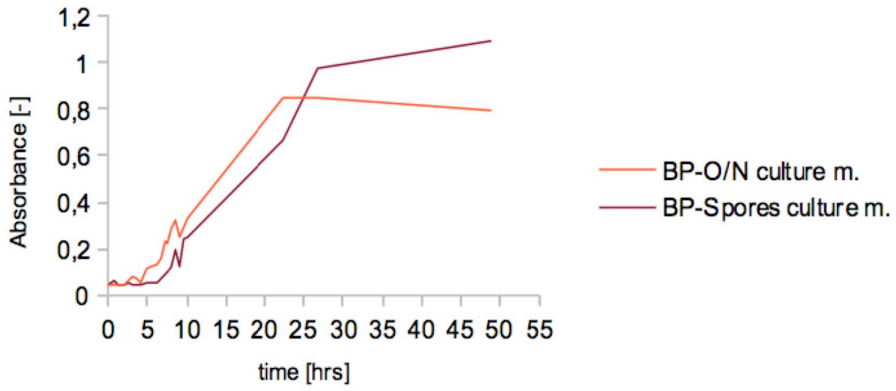

Figure 56: Bacterial curve of *Bacillus pseudofirmus* after freeze-thaw cycles in culture medium.

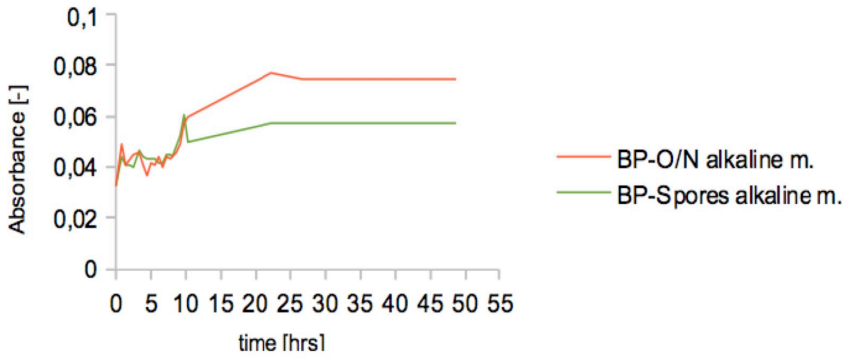

Figure 57: Bacterial curve of *Bacillus pseudofirmus* after freeze-thaw cycles in alkaline mineral medium.

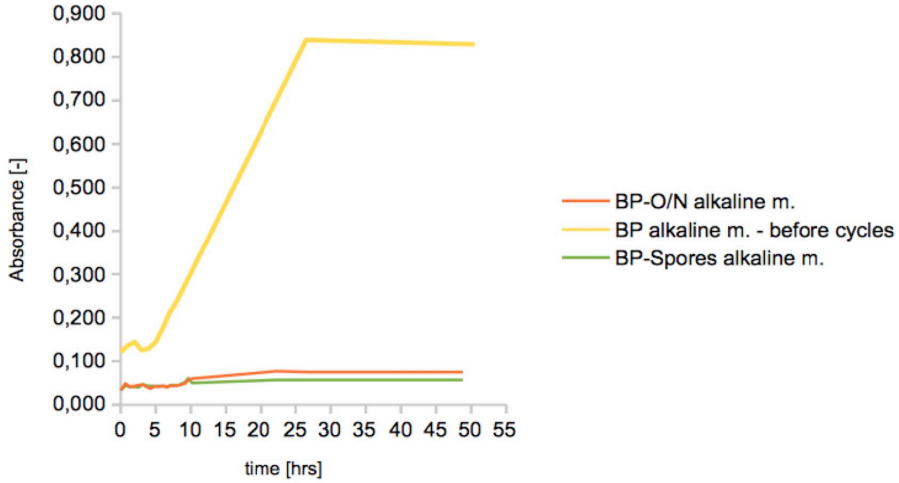

Figure 58: Comparison of bacterial curves of *Bacillus pseudofirmus* before and after freeze-thaw cycles in alkaline mineral medium.

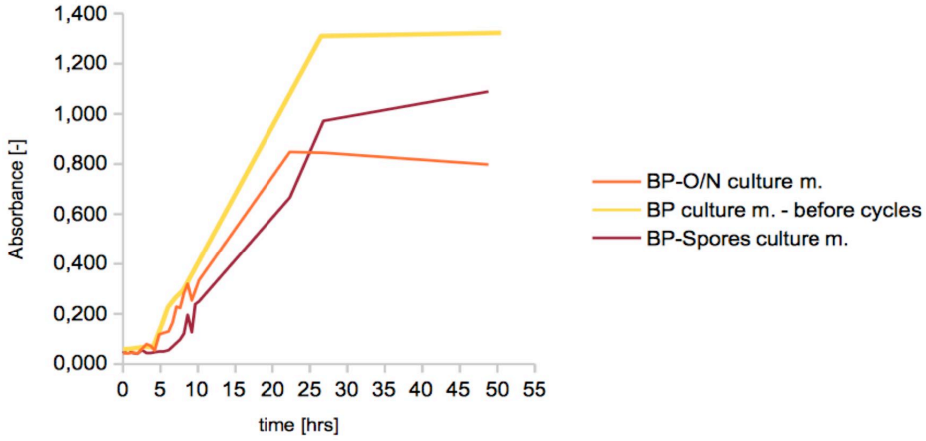

Figure 59: Comparison of bacterial curves of *Bacillus pseudofirmus* before and after freeze-thaw cycles in culture medium.

4.2.4 Encapsulation of bacteria in nanofiber textile

a) Influence of the stabilization process on bacterial spores

The oxygen demand measurements show that *Bacillus pseudofirmus* has a potential to withstand both of the used stabilization processes. All of the samples restored metabolic activity after an exposure to GA vapour for two hours (see Figure 60). However, the results also show that an exposure to high temperature (140°C) could lead to decrease of viability of bacteria when compared to exposure to 80°C and untreated bacteria (25°C).

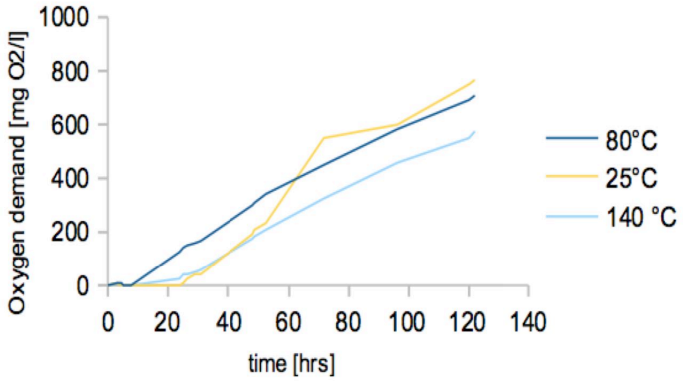

Figure 60: Viability of bacteria exposed to GA vapour and high temperatures.

b) Samples of PVA nanofabric with encapsulated bacteria

At first, the experiment showed that nanofibres without the addition of H_3PO_4 and glyoxal are almost impossible to be stabilized by glutaraldehyde (GA) vapour or temperature, therefore completely unstabilized samples had almost identical water solubility as the samples without the specific addition (series 10P and 10PB). Thus stabilization appeared only in series 16PVA and 10PVAB when stabilized by GA vapour or temperature.

Measurements of oxygen demand of nanofabric samples with encapsulated bacterial spores in culture medium show metabolic activity in all series. However, in our conditions and due to the method of production of PVA nanofibres, it was impossible to keep complete sterility of the environment and the samples, therefore it is difficult to distinguish the activity of *Bacillus pseudofirmus* from unintentional contamination.

Generally, samples which were stabilized by temperature show a lower value of

metabolic activity which is in agreement with the previous experiment investigating the influence of the stabilization process to bacterial viability. Results show that series 10PVAB stabilized by GA vapour had the latest start (see Figure 62) of metabolic activity but reached the highest values (see Figure 61) first. This result could indicate that stabilization by GA vapour is the most effective method, as the bacterial spores probably stayed captured within the fibres the longest when measured immersed in culture media but also stayed protected before the immersion (when compared to unstabilized samples).

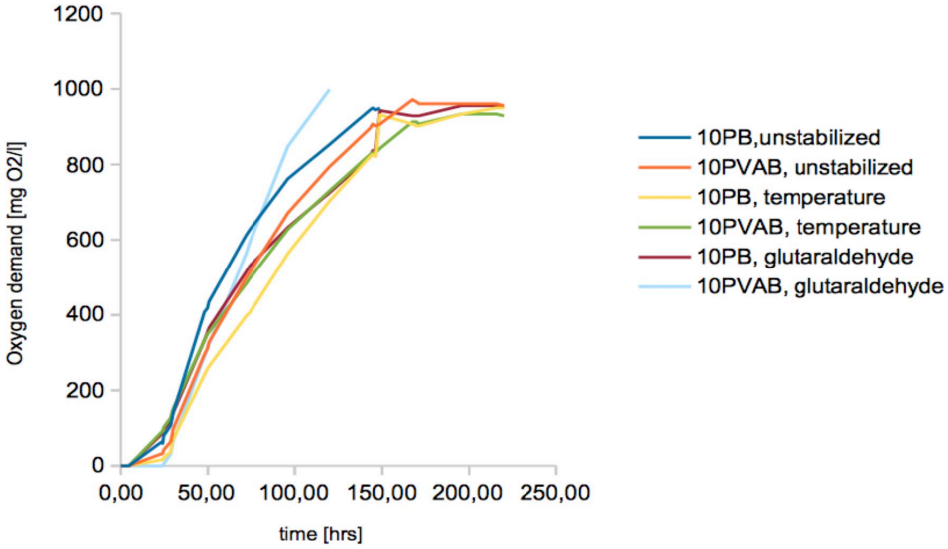

Figure 61: Oxygen demand of nanofabrics with encapsulated bacteria in culture media.

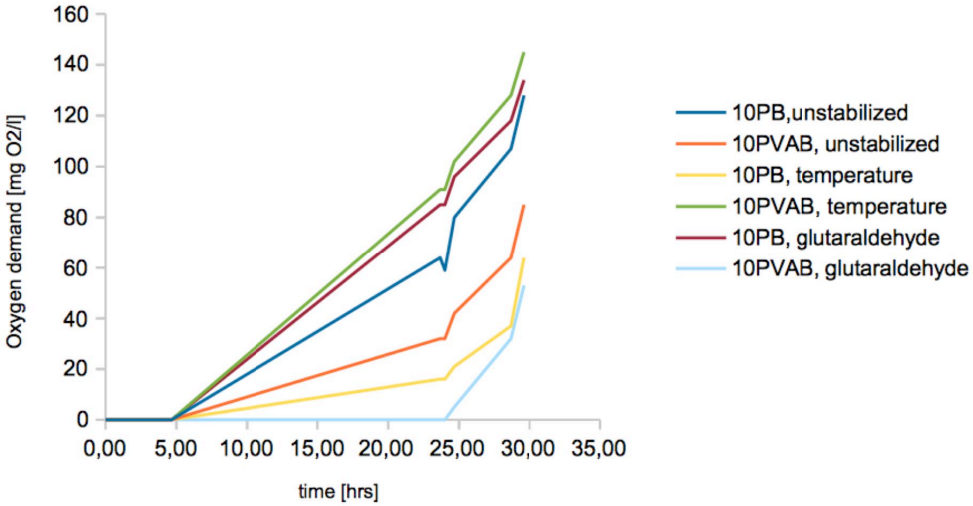

Figure 62: Oxygen demand of nanofabrics with encapsulated bacteria in culture media, the first 30 hours of measurements.

Another measurement was focused on the potential of encapsulated bacteria in nanofibres when cultured in the healing agent solution (calcium and yeast solution), which is necessary for the calcite production and therefore crack-healing potential of the biological method of self-healing concrete. To simulate final conditions, bacterial nanofabric samples (10PB and 10PVAB) were immersed in calcium and yeast solution and bacterial concentration was measured with spectrophotometer.

As it was already mentioned, it is difficult to distinguish the activity of *Bacillus pseudofirmus* from unintentional contamination and also stabilization of samples without the addition of H_3PO_4 and glyoxal proved to be almost impossible. Thus one of the investigated series (10PB) kept its water solubility though it was exposed to GA vapour and temperature treatment.

Bacterial curves based on the spectrophotometer measurements show that in the series 10PVAB (samples with successful stabilization) results are in agreement with previous experiments focused on the influence of a stabilization method and oxygen demand of bacterial samples. Samples of 10PVAB exposed to GA vapour reached the highest value compared to the temperature treatment and unstabilized samples (see Figure 63). Therefore, it could indicate that the stabilization process by GA vapour is more effective than stabilization by temperature, it is less harmful to bacterial spores than the temperature exposer and also it possibly captures and protects bacterial spores better than nanofibres without any stabilization.

Results based on the measurements of the series 10PB do not match previous experiments. The series 10PB stabilized by temperature exposure reached the highest values while the unstabilized series and GA vapour stabilization series had very similar concentration increase (see Figure 64). However, as it has been already mentioned, series 10PB stayed completely soluble in water after the stabilization procedures, thus it is difficult to draw any clear conclusions from these results.

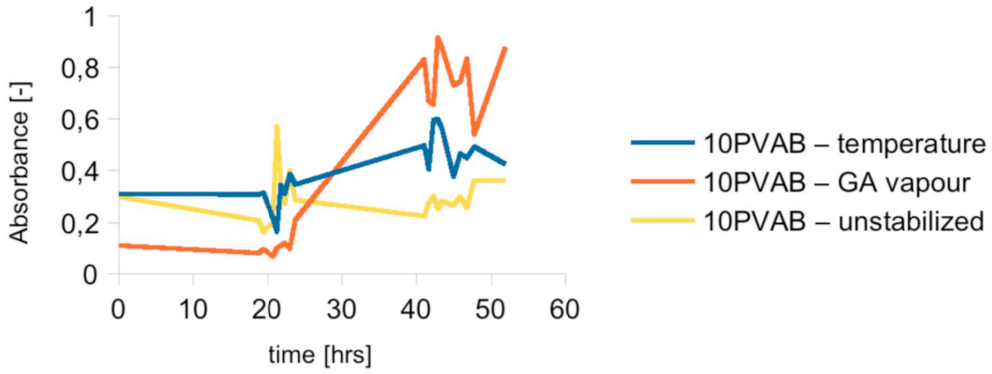

Figure 63: Bacterial curves of nanofabrics with encapsulated bacterial spores 10PVAB in calcium and yeast solution.

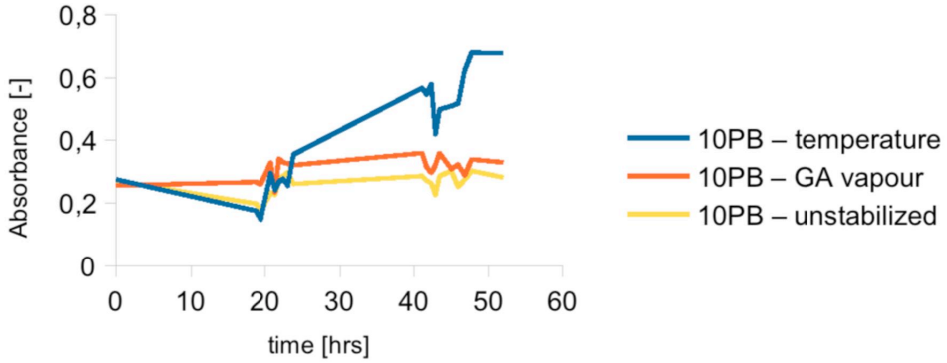

Figure 64: Bacterial curves of nanofabrics with encapsulated bacterial spores 10PB in calcium and yeast solution.

Evaluation of results obtained from placing all of the nanofabric samples on solid media (either culture or calcium lactate and yeast solution) is difficult due to the possible contamination of the samples. However, some bacterial activity is noticeable, especially in samples in solid culture media. For examples see Figure 65 - Figure 73.

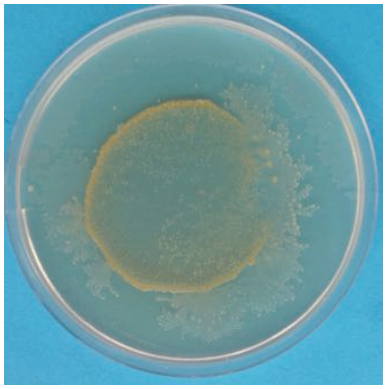

Figure 65: 10PB in culture medium, GA vapour stabilization.

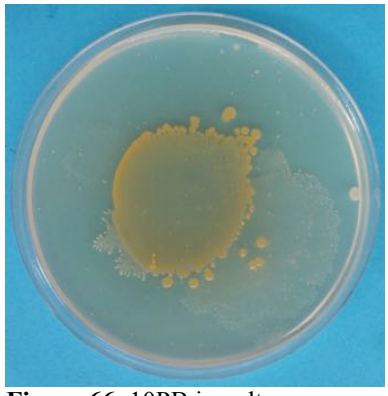

Figure 66: 10PB in culture medium, temperature stabilization.

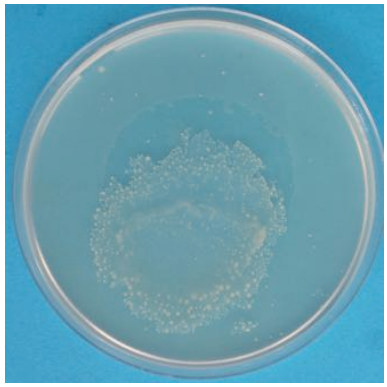

Figure 67: 10P in culture medium, temperature stabilization.

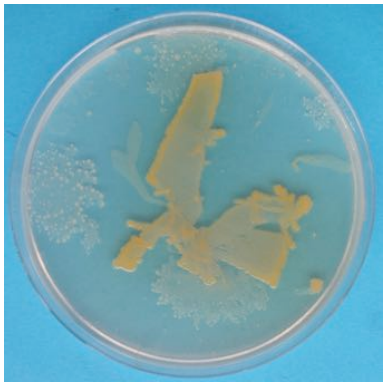

Figure 68: 10PVAB in culture medium, temperature stabilization.

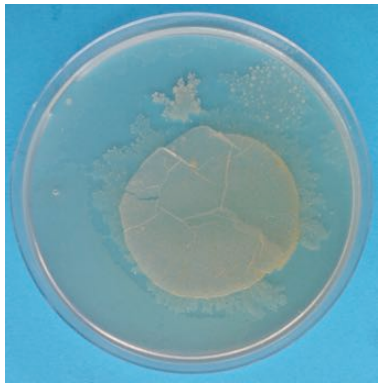

Figure 69: 10PVAB in culture medium, GA vapour stabilization.

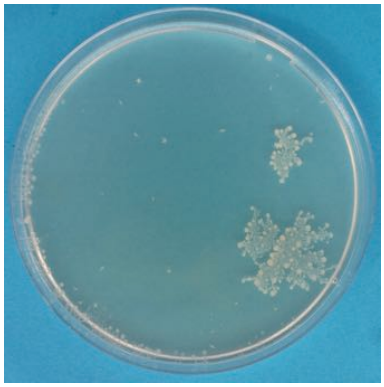

Figure 70: 10P in culture medium, unstabilized.

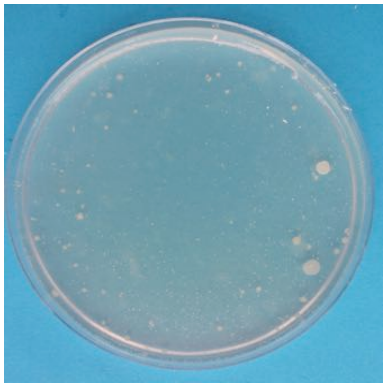

Figure 71: 10P in CLY solution, GA vapour stabilization.

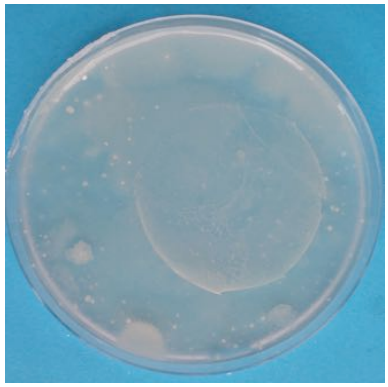

Figure 72: 10PB in CLY medium, GA vapour stabilization.

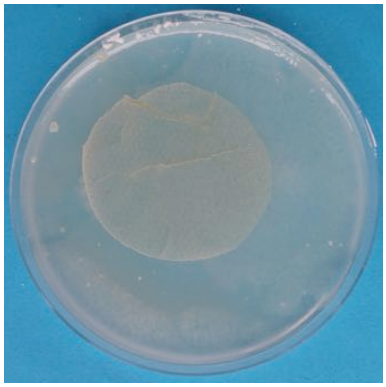

Figure 73: 10PVAB in CLY, temperature stabilization.

4.2.5 Encapsulation of bacteria in light weight aggregates

At first, impregnated light weight aggregates were subjected to visual investigation after 7 days of drying. Series BY (LWA with bacterial spores and yeast) did not differ from untreated LWA. Both series with the calcium lactate addition (BCY and BCYH) had white crystalline coating on the surface of particles, possibly crystallized calcium lactate (see Figure 75 and Figure 74). Series BCYH, which was dried gradually and initially kept in cold environment, was covered by the white coating slightly more than the series BCY, which was dried in room humidity and temperature (see Figure 76).

Furthermore, one aggregate grain from each series was selected, cut in half and inspected under a microscope (Olympus BX41 Fluorescence Microscope, magnification 10x). Aggregates from all of the series contained some yellow particles (see Figure 77, Figure 79, Figure 81). These particles were not present in untreated LWA (Figure 80), therefore it could indicate a successful impregnation. As all of the series were impregnated with yeast and bacteria, the yellow particles could be the result of their presence. In aggregates from series with the addition of calcium lactate (BCY and BCYH), copious amounts of white to transparent crystals (Figure 78, Figure 81) were present along with the yellow particles. These crystals were present neither in untreated LWA nor in LWA impregnated only with bacteria and yeast (series BY), thus it could indicate a successful impregnation with calcium lactate.

Figure 75: LWA - series BCY after 7 days of drying.

Figure 74: LWA - series BY after 7 days of drying

Figure 76: LWA - series BCYH after 7 days of drying.

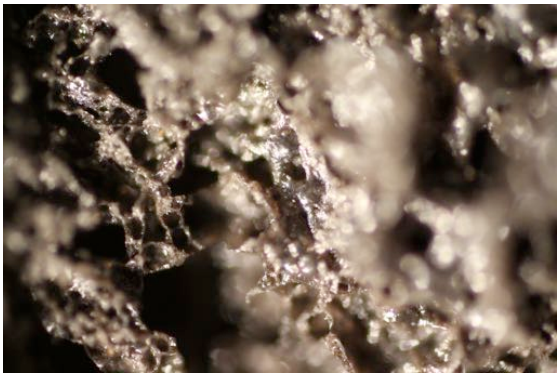

Figure 77: Cross-section of BY after 7 days of drying.

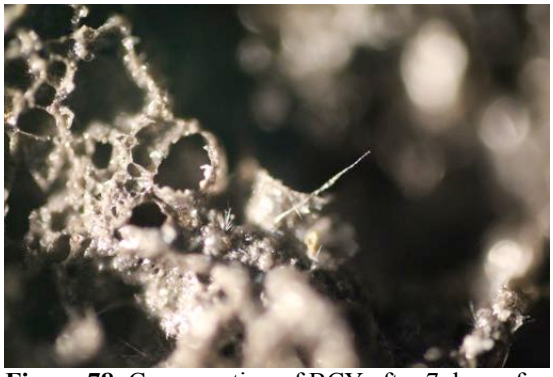

Figure 78: Cross-section of BCY after 7 days of drying.

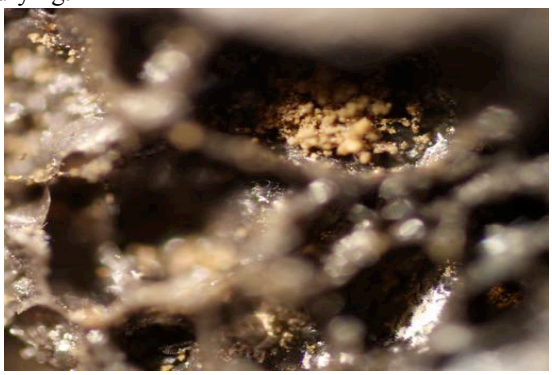

Figure 79: Cross-section of BCY after 7 days of drying.

Figure 80: Cross-section of untreated LWA.

Figure 81: Cross-section of BCYH after 7 days of drying.

a) Survival and viability of impregnated bacteria

Bacterial curves of impregnated LWA were derived from the oxygen demand measured with BOD sensors. Results show that in all of the series spores of *Bacillus pseudofirmus* probably became active and their metabolic activity was restored. However, the beginning of oxygen demand, thus metabolic activity, occurred relatively late after immersion of LWA into media. The metabolic activity of the series BY became noticeable after approximately 65 hours from the immersion, the activity of the series BCYH after 160 hours from the immersion and the activity of the series BCY after 190 hours from the immersion. The metabolic activity of the series BY reached the highest values while the series BCY and BCYH reached similar values, despite the earlier beginning of the BCYH series.

A possible explanation of the differences between the metabolic activity of the LWA series could be a different nutrition supply. The series BY was immersed in 100 ml calcium lactate solution (80 g/l), therefore the quantity of the Ca source was ensured. However, the series BCY and BCYH were immersed in sterilized tap water, thus the only Ca source available was incorporated inside the LWA particles in the crystallized form, as it could be seen under the microscope. The results show that the gradual drying, which was applied on the series BCYH, does not have a great influence on the final bacterial activity. The series BCYH became active approximately 30 hours earlier than the BCY series, but they both reached identical values of oxygen demand in their peaks.

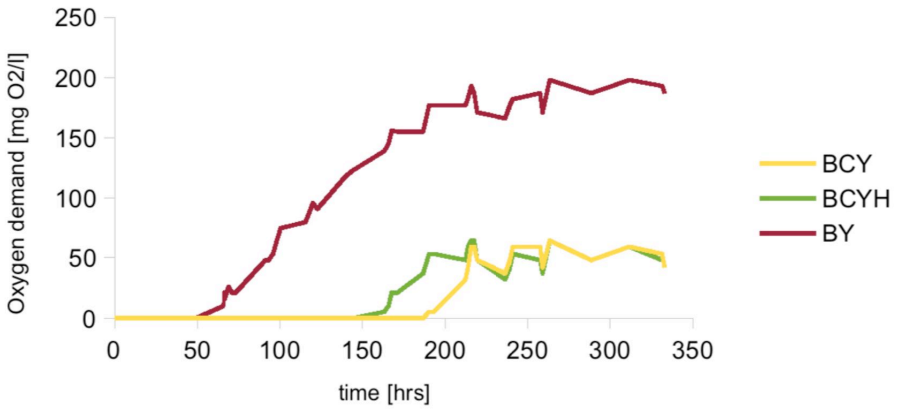

Figure 82: Measurements of oxygen demand of impregnated bacterial LWA in tap water/calcium lactate solution.

b) Bacterial LWA exposure to freeze-thaw cycles

After exposure of impregnated LWA to freeze-thaw cycles (5 cycles between -10°C and 20°C), bacterial curves were determined from oxygen demand measured with BOD sensors. As expected, metabolic activity became noticeable after a relatively long time. The series BY started its activity after approximately 70 hours from immersion in the medium, the series BCYH and BCY after approximately 140 hours from immersion. The early beginning of the metabolic activity in the BY series after the freeze-thaw cycles is almost equal as in the measurements before the cycles. Also in this case, the possible explanation could be the difference in nutrition supply. The series BY was immersed in 100 ml calcium lactate solution, whereas the series BCYH and BCY were immersed in sterilized tap water. Therefor the series BY had ensured supply of Ca source, but the series BCYH and BCY were dependent on the amount of Ca which was incorporated inside the particles.

In general, the results show that incorporated bacteria *Bacillus pseudofirmus* in LWA can survive the freeze-thaw cycles and restore its metabolic activity. In contrast with the measurements before the cycles, the series BCY reached similar values as the BY series, whereas the series BCYH (impregnated LWA with gradual drying) showed the least activity. These results could indicate that after the exposure to freeze-thaw cycles, the difference between an incorporation of the Ca source inside the particles and a direct addition into the materials matrix does not have a great influence on the final bacterial activity.

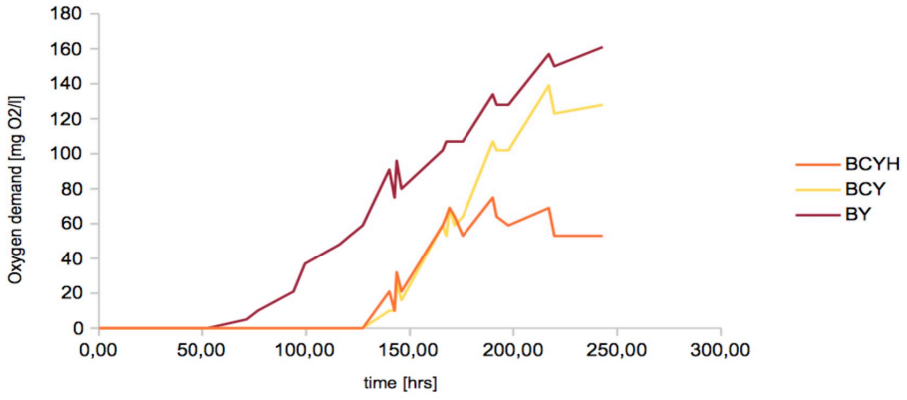

Figure 83: Measurements of oxygen demand of impregnated bacterial LWA in tap water/calcium lactate solution after exposure to freeze-thaw cycles.

5 Conclusions

The main assignment of this diploma thesis was to research the topic of biologically based self-healing concrete, to prepare suitable bacterial culture and to attempt to prepare and evaluate our own self-healing biological concrete specimens.

Research showed that the phenomenon of self-healing concrete has been investigated for many years as the topic could promise economic and environmental improvement of one of the most used building material. The biological approach specifically has been studied by many researchers in the past approximately 10 years and results showed that it could have the potential to be used in common practice.

However, the topic of the self-healing biological concrete is rather extensive. The type of bacteria, the composition of nutritions and Ca sources, protection of the bacteria and the production process itself influence the efficiency of the self-healing process and properties of the final material and therefore they have to be chosen carefully.

In our experimental work the bacteria (*Bacillus pseudofirmus*) and other healing agents (calcium lactate and yeast extract) were chosen based on the previous studies. Survival and viability measurements of bacteria under freeze-thaw cycles indicated that *Bacillus pseudofirmus* could be a suitable candidate for the self-healing biological concrete in the conditions of the Central European region.

The next step in our experiment was to determine the efficiency of encapsulation of bacteria in different carriers. Lightweight aggregates, which have been successfully used in previous studies, proved to be a suitable carrier in our experiment as well. However, the results indicated that the impregnation process of the particles with the complete healing agent was not efficient enough. Thus one the of the subjects of further research could be an improvement of the impregnation process or investigation of the possibility of a direct addition of parts of the healing agent into the concrete mixture.

The method of bacteria encapsulation in PVA nanofibres has not been found in previous studies focused on self-healing concrete. In our experimental work, we tried to determine the influence of the stabilization methods and the production process to the bacteria. Results showed that the most suitable stabilization method is probably the

exposure to glutaraldehyde vapour. However, it was difficult to draw any firm conclusions from the experiments with bacterial nanofibres, as it was impossible to ensure complete sterility in our conditions. Thus samples showed some bacterial activity but it was difficult to distinguish the activity of *Bacillus pseudofirmus* from the possible contamination.

This diploma thesis provides solid basis for future research of the topic of biological approach to self-healing concrete. The conducted experiments provided valuable information about the behaviour of the selected bacteria, the chosen encapsulation methods and revealed specific problems that may arise. As the topic is rather extensive, future studies could be focused on the production of concrete samples with the bacterial healing agent and determination of self-healing efficiency and influence of the biological method to the material properties. The research of different types of healing agents, protective carriers and production processes could also lead to further improvements of the biological approach to the self-healing materials in general.

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