



Effect of *Enterococcus Faecalis* Bacteria and Calcium Lactate on Concrete Properties and Self-Healing Capability

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Abstract: Concrete is one of the most durable and long lasting product in construction industry. Durable, high strength, and crack resistant concrete does not happen by accident. Therefore, established guidelines and procedures should be followed to achieve the desired strength and concrete properties. Many attempts were explored by researchers in producing good quality of concrete with minimum cracks. One of the potential methods of crack healing is through bacteria incorporation in concrete. This study was done with the aim to determine the effect of concrete properties and self-healing capability of bacteria and calcium lactate in concrete. Strain of bacteria that was used in this study was locally isolated from fresh urine. The novelty of this study is that the enrichment process in the bacteria culture and method of calcium lactate added into concrete to accelerate bacteria growth process. Bacterium was isolated, enriched and identified using PCR and DNA sequencing. Mechanical properties tests such as compressive, flexural and tensile strength test were conducted. Self-healing capabilities of the concrete were tested using UPV and stereomicroscope. SEM and EDX were conducted to verify and confirm the bacteria precipitation of calcium carbonate. It was found that 2.18 g/L of calcium lactate with 3% *Enterococcus faecalis* increase the compressive strength from 36 MPa (Control) to 39.6 MPa. Flexural strength test achieved 6.72 MPa from 4.78 MPa. This increase in mechanical properties was possibly due to calcium carbonate formed due to the bacteria ability to precipitate calcium carbonate through urease enzyme. This was confirmed through SEM and EDX. Formation of calcium carbonate is directly responsible for self-healing capabilities that was analyzed through UPV and stereomicroscope. The increased of concrete properties and crack healing process proof that addition of specific bacteria strain resulted to a more durable concrete.

Keywords: Bacteria identification, mechanical properties, durability, morphology

1. Introduction

Bacteria strain added in concrete as one of alternative to improve concrete properties and self-healing from cracks and had been studied by many researchers from all over the world (Dick *et al.*, 2006, Emilio *et al.*, 2008 Abo-El-Enein *et al.*, 2012, Amirreza *et al.*, 2014, and Leena *et al.*, 2014). Bacteria strains added in concrete that were used by researchers were isolated from various sources like soil, water, mud, springs and others and it were resulted to a

positive impact. One of the criteria of the bacteria is its survival in high alkalinity and an environment with near to zero oxygen (Jonkers and Erik, 2008). This condition is resembles as condition of concrete environment.

Understanding the important to explore other sources of bacteria, this study was focused in isolating bacteria from fresh urine. Literature already proved that high content of urea in the urine will be a good source to isolate ureolytic bacteria to be added in the concrete. Precipitation of calcium carbonate by bacteria is highly due to the production of urease enzyme from ureolytic bacteria. The present of ureolytic bacteria with urease enzyme will accelerate calcium carbonate production and indirectly will occupy concrete pores or any pores at the cracking area. Reduction of pores and crack in the concrete will significantly improve concrete properties. However, methods in which bacteria are added into concrete and type of bacteria use are varies. Therefore there is a need to study the effect of bacteria and calcium lactate addition in the concrete environment.

2. Experimental Program

2.1 Bacterium

A bacterium that was used throughout the study is a strain isolated from fresh urine. After isolation, the bacteria strain is enriched to suit concrete environment by purging flask that containing the bacteria with nitrogen gas and increasing the pH level with sodium hydroxide (NaOH). Survival of bacteria was monitored along this study. PCR and DNA sequencing was used to identify the bacteria strain. It is a popular technique to identify bacteria as it is not only fast but resulted toan accurate strain (Kit Boye *et al.*, 1999 and BS EN 12390-3, 2009).

2.2 Isolation and Enricment of Bacteria

A process of enrichment as in (Fig. 1) was adopted in isolating the respected strain of bacteria. Isolation process involved a serial dilution streaking plate, strain purification and gram staining. All media used were autoclave at 121° C for 15 minutes to ensure sterilization. The composition of enrichment media for the bacteria is as follow:

- i. Composition of control sample= Nutrient broth (25ml) +Urea 40% (10ml)
- ii. Composition of bacteria sample= Nutrient broth (25ml) + Urea 40% (10ml) + Urine (1ml)

The increase of pH during enrichment process is to ensure that the bacteria can survive in high alkaline condition in concrete. It is also attributes to high urease activity which leads to high carbonate precipitation rate. The pH of each sample was adjusted to an alkaline condition by adding sodium hydroxide until pH value reaches the range of 9-11.

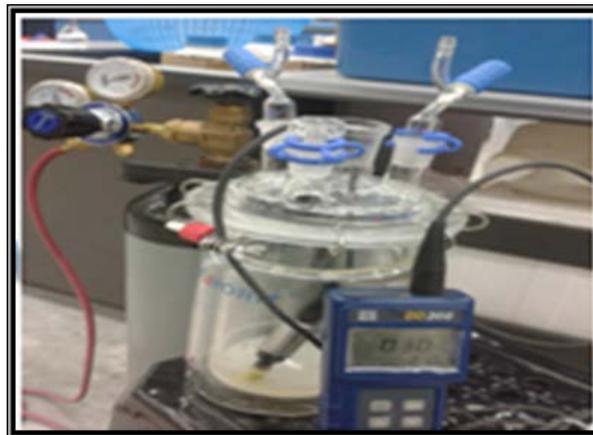


Fig. 1 - Enrichment flask containing bacteria

2.3 DNA Qualification and PCR of Bacteria

DNA quantification was quantified spectrophotometrically using a Nanodrop 2000 spectrophotometer (Fisher Scientific).

The polymerase chain reaction(PCR) condition was a 10X PCR Buffer, 25 mm MgCl₂, 2.5 mm dNTPs, 10 μmol each primer (27F; 1492R), 5U Taq DNA polymerase. Universal 16SrDNA PCR was performed with forward and reverse primers, which are 27 (forward primer) 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492 (reverse primer) 5'-

GGGTTACCTTGTTACGACTT-3'. After 35 cycles consisting of denaturation at 95° C (30 Seconds), Annealing at 50 ° C (30 Seconds) and extension at 72 ° C (1 min and 30 seconds), the PCR product is analyzed on an agarose gel. A band indicating a fragment was shown by agarose gel electrophoresis. The results were blasted to National Centre for Biotechnology Information (NCBI) database.

2.4 Preparation of Concrete Test Sample

Concrete samples were prepared with Ordinary Portland Cement (OPC), fine aggregate (sand), coarse aggregate, water, bacteria liquid culture and calcium lactate solution. Table 1 shows the applied mixing proportions used in this study. This study was divided into two phases where the first phase is evaluating the bio-concrete compressive and flexural strength. The first phase uses concrete cube of 150 mm × 150 mm × 150 mm and prism of 500 mm × 100 mm × 100 mm. The second phase is evaluating the self-healing of the bio-concrete. This phase consists of prism embedded with 2R8 steel reinforcement and cube with steel plate of 150 mm × 0.3 mm × 20mm used to create standardized crack. The verification of self-healing was conducted with Scanning Electron Microscope (SEM) and Energy Dispersive Spectroscopy (EDX).

Table 1 - Mixing proportion of bio-concrete.

Materials	Weightage (kg)
Cement	63
Fine aggregate (sand)	111.6
Coarse aggregate	183
Water (w/c 0.5)	33.2
Enterococcus faecalis	1
Calcium lactate	0.22 g/L
	1.09 g/L
	2.18 g/L

2.5 Compressive and Flexural Strength

Bio-concrete properties studies including compressive and flexural strength were the first phase of this study. Compressive strength test is conducted using a standardized cube. The test was conducted in triplicates as is accordance to BS EN 12390-3, (2009). Flexural strength test was conducted using standard prism size. The test is conducted based on BS EN 12390-5, (2009).

2.6 Self-Healing of Bio-concrete

Second phase of this study is to determine the bio-concrete self-healing capability. This is done with prism and cube. The reinforced prism is fabricated and after 28 days of dry curing, crack is created by 3-point bending. The cracks on all prism samples were created simultaneously by applying load on the prisms at the same time. This causes the prisms to crack but not break due to the reinforcement within the prism (Wang *et al.*, 2012). Apart from that, cubes were fabricated and steel plate was used in fresh bio-concrete cubes. The steel plate was then removed after 24 hours. The cracks created on Prisms and cubes were analyzed on the 20, 40, 70 and 100 days using Stereomicroscope and Ultrasonic Pulse Velocity (UPV) (Wiktor and Jonkers, 2011, Wang *et al.*, 2012, Tittelboom *et al.*, 2012, Quiviger *et al.*, 2012, Varenayam *et al.*, 2013, Wiktor *et al.*, 2013, Guadalupe *et al.*, 2014 and Zhong and Yao, 2018). Preparation of both prism and cubes for self-healing is as in Fig. 2.



Fig. 2 - Prism and cubes for phase 2

2.7 Verification of Bio-concrete Morphology through SEM and EDX

Scanning Electron Microscope (SEM) and Energy Dispersive Spectroscopy (EDX) are used to observe and analyze bio-concrete morphology. Small portions were cut from 150mm × 150mm × 150mm cube to perform SEM and EDX. Method to prepare samples for SEM and EDX was similar as Faiz *et al.*,(2014). Both SEM and EDX were conducted based on DD ISO/TS 10798, (2011).

3 Experimental Results and Discussions

3.1 Bacteria Identification

Bacteria identification was conducted through DNA extraction, PCR and analyzing through BLAST software. The absorbance measurement that was taken to calculate DNA purity was at 260nm and 280nm. DNA samples were considered free from protein contamination if the A_{260} to A_{280} ratio was between 1.8 and 2.0. Table 2 presents results of protein ration as compare to control experiment.

Table 2 - Quantification result

No.	Sample ID	Nucleic Acid Conc.	Unit	260/280	260/230
1	U	159.7	ng/μl	1.84	2.12

A PCR product of about 600 kb in size was successfully amplified from the sample. The process and timing for each cycle are as in Table 3 and the agarose gel product is shown Fig. 3.

Table 3 - PCR parameter

Stage	Temp. (°C)	Time (min)	No. of cycles
Initial denaturation	95	5:00	1
Denaturation	95	0:30	35
Annealing	50	0:30	35
Extension	72	1:30	35
Final extension	72	7:00	1
End	4	-	-

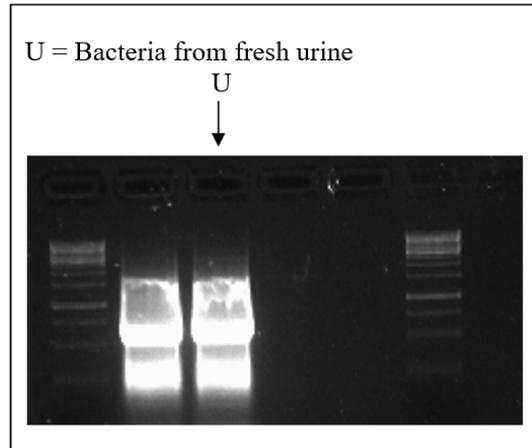


Fig. 3 - Agarose gel

After performing PCR reaction and getting the PCR product. A purification step is done using the Qiagen PCR Purification Kit. Identification of the isolate was performed and the DNA of isolated strain will be identified. The basic unit of DNA structure comprise of nucleotide, phosphate, deoxyribose sugar and a nitrogen base. Each deoxyribose sugar bonds covalently in a repeating pattern with two phosphates, One of the bond is to 5' (five primer) another bond is to 3' carbon on deoxyribose. This specifies the order and direction of each strand. The nitrogen bases, purines and pyrimidines attach by covalent bonds at the 1' position of the sugar. Pairing of purines and pyrimidines is dictated by the formation of hydrogen bonds. Thus DNA sequence, is the pairing of purine Adenine (A) pairs with pyrimidine Thymine (T) and the purine Guanine (G) pairs with pyrimidine Cytosine (C) (Talaro and Chess, 2012). The DNA sequence is then compared to similar or to any other DNA sequences by uploading the sequence to BLAST programs. BLAST is a program that is supported by the National Center for Biotechnology Information (NCBI). The result of BLAST software is shown as a phylogenetic tree as shown in Fig. 4.

3.2 Compressive and Flexural Strength

Effect of *Enterococcus faecalis* with calcium lactate on compressive strength at the age of 7, 14 and 28th day was studied and it was found that inclusion of *Enterococcus faecalis* in concrete significantly increases the compressive strength. Results of compressive strength by using *Enterococcus faecalis* with different concentrations of calcium lactate are presented in Fig. 5. The highest increment of strength is 39.6 MPa which was achieved with addition of 2.18 g/L calcium lactate. The increment of strength by 0.22 g/L is 37.8 MPa which is an increase of 2.2% from bio-concrete (*E. faecalis*). Whereas, addition of 1.09 g/L achieved strength of 38.5 MPa. Thus, increasing strength by 4.1%. Lastly, the addition of 2.18 g/L has allowed concrete to increased in strength by 7%, which is 39.6 MPa. While compared to control, the increments are 5%, 6.9% and 10% with 0.22 g/L, 1.09 g/L and 2.18 g/L respectively. Addition of calcium lactate has provided additional calcium ions to increase the amount of precipitated calcium carbonate. Hence, higher compressive strength is achieved. Trend of results obtained from this research are similar to Ramachandran *et al.*, (2005) which both studies resulted to an improvement of compressive strength with the addition bacteria in concrete. Where, a 15.4% increment of strength was reported. Comparatively to this study, the difference in increment of strength is by 8.4%. This is due to the difference in bacteria used. As different bacteria produce calcium carbonate in different pace. The *shewanella* species was reported to be aided increment of compressive strength in mortar of up to 25% after 28 days (Ramachandran *et al.*, 2001).

Apart from that, flexural strength test have similar results as compressive strength test. Flexural strength of concrete increased with the addition of *Enterococcus faecalis* and calcium lactate. Flexural strength was conducted at the age of 28 days. Fig. 6 shows the results of Flexural strength test. Addition of 0.22 g/L calcium lactate achieved an increment of flexural strength by 14.2% compared to *Enterococcus faecalis*. The highest increment of flexural strength is by using 2.18 g/L calcium lactate which increased strength by 31.25% compared to *Enterococcus faecalis*. This is followed by 1.09 g/L which increased the strength by 29.49%. Increment of flexural strength compared to control are 22.38%, 38.70% and 40.59% with 0.22 g/L, 1.09 g/L and 2.18 g/L respectively. Overall, increased of flexural strength with the addition of calcium lactate is higher compared to bio-concrete. Similar trends of improvement of flexural strength with addition of bacteria in concrete were reported by previous studies (Chattopadhyay *et al.*, 2011, Majumdar *et al.*, 2012 and Parmar *et al.*, 2013). Previous study proved that the use of bioremediase in mortar beam have recorded to an increase of 33% with 3 µg/g bioremediase compared to control (Majumdar *et al.*, 2012). An addition of calcium lactate acts as a food source for the bacteria to further increased the precipitation therefore increasing the strength of concrete properties. This is verified with SEM and EDX.

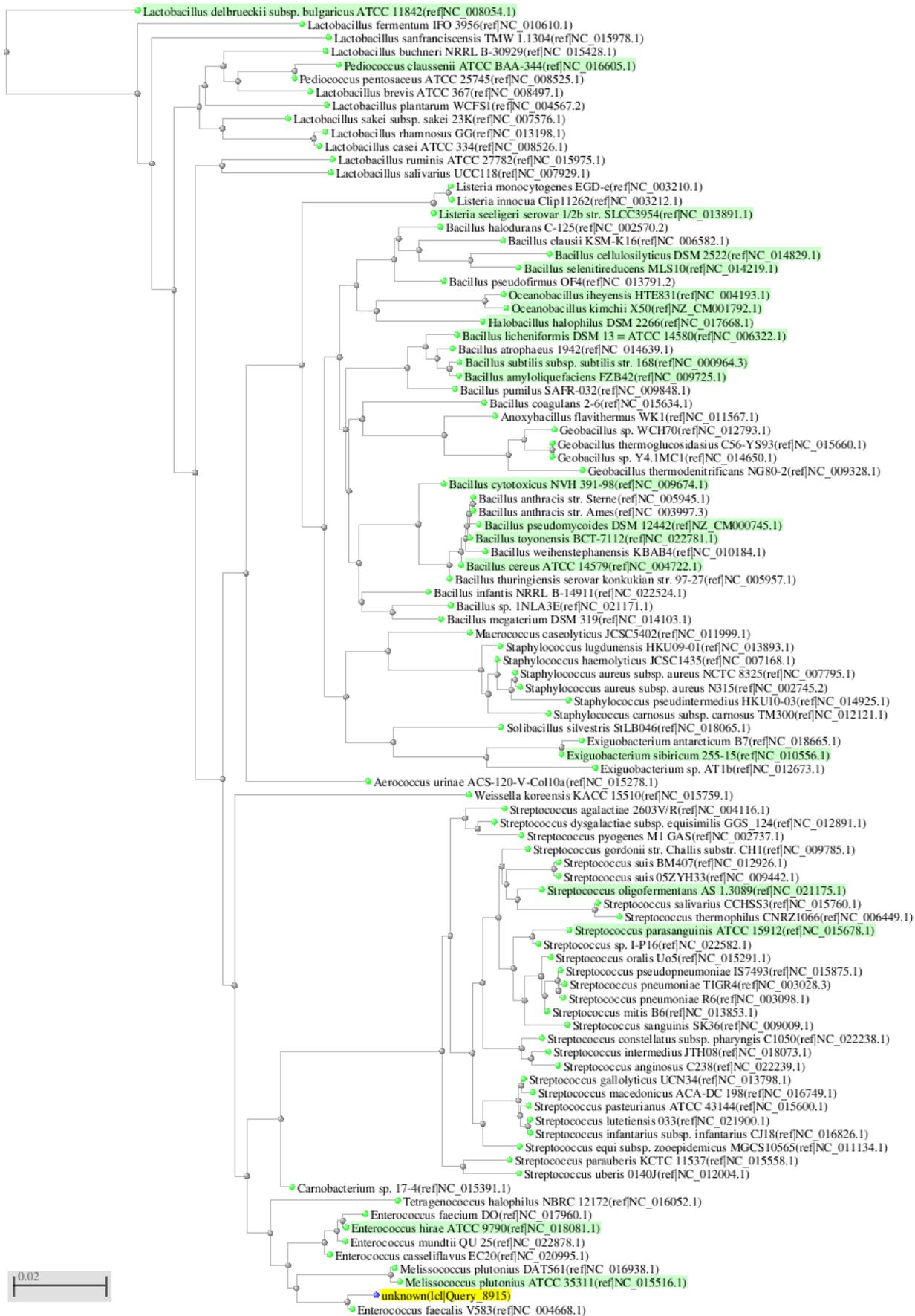


Fig. 4 - Phylogenetic tree of enterococcus faecalis

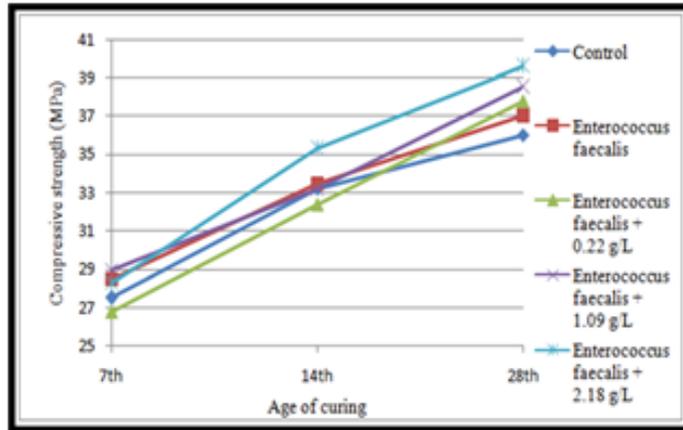


Fig. 5 - Compressive Strength of bio-concrete and calcium lactate

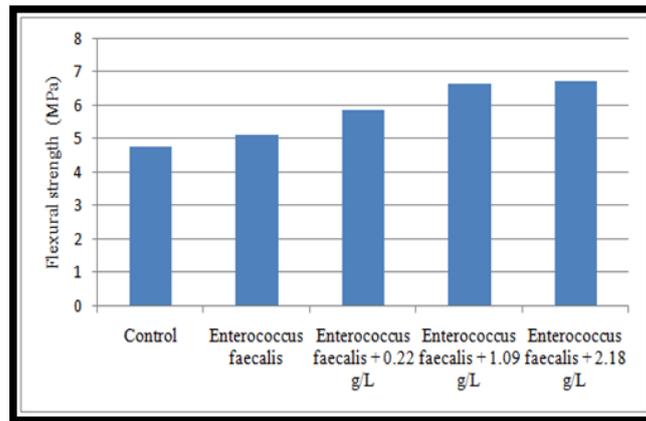


Fig. 6 - Results of flexural strength test for specimens containing bacteria and calcium lactate

3.3 Self-Healing of Bacteria

Second phase of this study involved self-healing of bio-concrete with added calcium lactate. Ultrasonic Pulse Velocity (UPV) and Stereomicroscope were used to verify self-healing and data is collected after crack is formed at the age of 20, 40, 70 and 100 days. The self-healing is divided to crack with metal plate and crack with three-point bending. Fig. 7 shows the crack with metal plate results for UPV and Fig. 8 shows the crack with three point bending results for UPV.

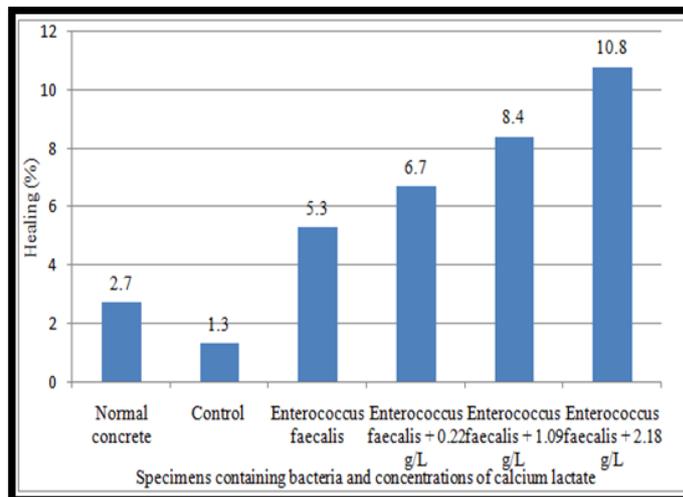


Fig. 7 - Percentage of healing of bacteria and calcium lactate in concrete by UPV

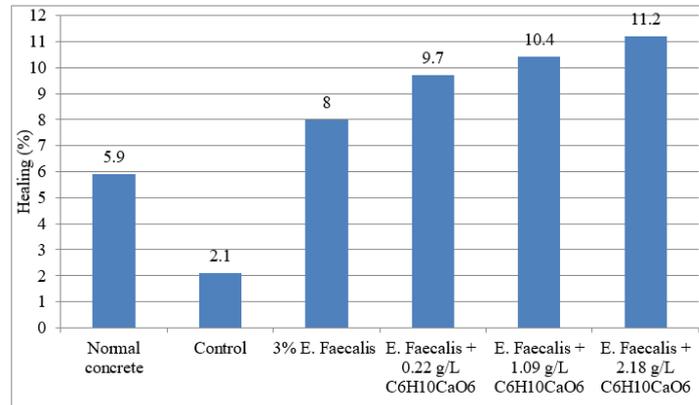


Fig. 8 - Percentage of healing for crack by 3-point bending

Percentage of healing obtained in this study are 2.7%, 1.3%, 5.3%, 6.7%, 8.4% and 10.8% for normal concrete (without crack), control (with crack), 3% *E. Faecalis*, *E. Faecalis* with 0.22 g/L, 1.09 g/L, 2.18 g/L respectively. The highest increment of percentage is by 2.18 g/L of calcium lactate which is 9.5% higher than the control (with crack) and 8.1% higher than normal concrete (without crack). On the other hand, the lowest increment is from concrete with *E. Faecalis*, which improved healing by 4% compared to control (with crack) and 2.6% compared with normal concrete (without crack).

Self-healing observation through stereomicroscope was conducted with prism (three-point bending). The results were taken on the 20, 40, 70 and 100 days after crack is created. Results show an increasing trend in percentage of healing with bio-concrete containing calcium lactate. Fig 8 shows increment trend with addition of 0.22 g/L, 1.09 g/L and 2.18 g/L of calcium lactate in bio-concrete which improved healing of concrete by 9.7%, 10.4% and 11.2% respectively. The highest difference in increment is with concrete sample containing bacteria and 2.18 g/L calcium lactate of up to 9.1% compared to control and 5.3% compared to normal concrete. This improvement of healing by calcium lactate addition has proven that bacteria utilize additional calcium added into bio-concrete. Result from compressive and flexural strength in concrete properties obtained in this study has shown similar trends which indicates that the addition of calcium lactate in concrete has positive results on the mechanical and healing capabilities of concrete.

Stereomicroscope data collected for bio-concrete containing *Enterococcus faecalis* with different addition of calcium lactate is presented in Table 4. An increase in self-healing percentage by 22.9%, 25% and 30.9% for bio-concrete containing 0.22 g/L, 1.09 g/L and 2.18 g/L of calcium lactate respectively. The data obtained proved that the highest percentage of healing and difference compared to control is by adding 2.18 g/L of calcium lactate. Bacteria in the concrete uses calcium lactate added in producing calcium carbonate. Calcium carbonate produced fills in the voids and resulted to strengthen the concrete. Results from this study are in line with study by Jonker and Erik, (2008).

Table 4 - Stereomicroscope results for prism

Batch	Days				Percentage of healing (%)
	20 (mm)	40 (mm)	70 (mm)	100 (mm)	
Control	0.045	0.041	0.041	0.038	15.6
Enterococcus faecalis 3%	0.029	0.024	0.023	0.023	20.7
Enterococcus faecalis + 0.22 g/L	0.035	0.031	0.030	0.027	22.9
Enterococcus faecalis + 1.09 g/L	0.040	0.036	0.036	0.035	25.0
Enterococcus faecalis + 2.18 g/L	0.042	0.034	0.030	0.029	30.9

3.4 Verification through SEM and EDX

Morphology tests such as Scanning Electron Microscope (SEM) and Electron Diffraction X-ray (EDX) are conducted as verifications. Small samples are prepared to conduct SEM and EDX. Both of which were conducted after samples reach maturity of 28 days. Fig. 9-14 shows the SEM results for this study.

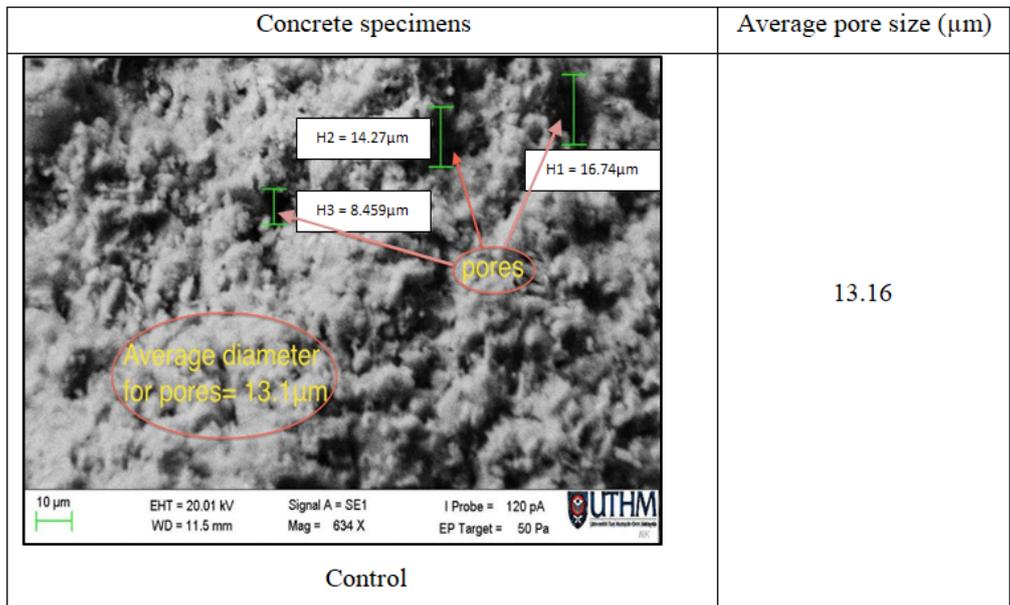


Fig. 9 - Microstructure of normal concrete

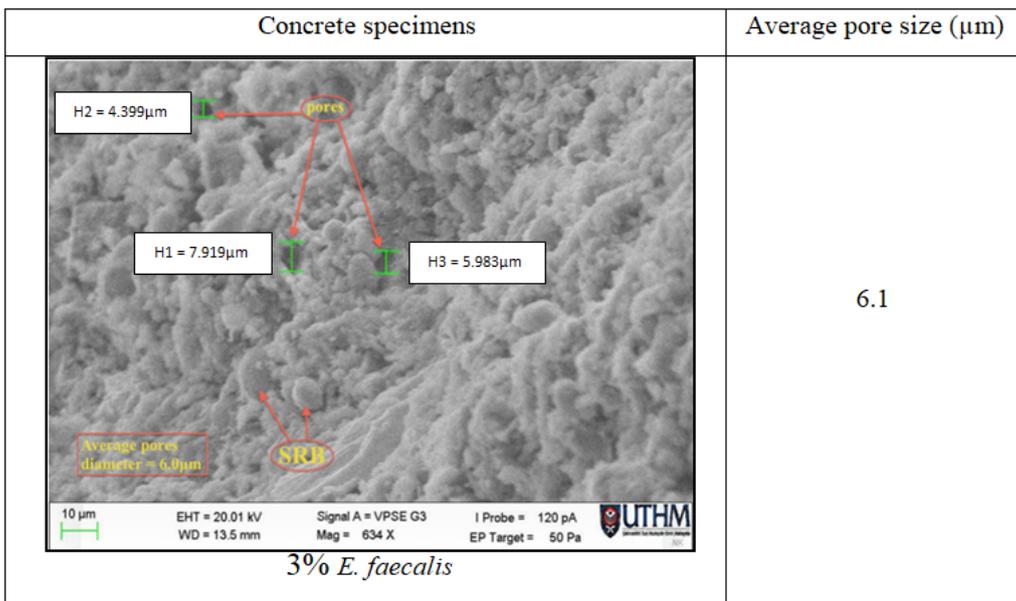


Fig. 10 - Concrete sample with *E. faecalis*

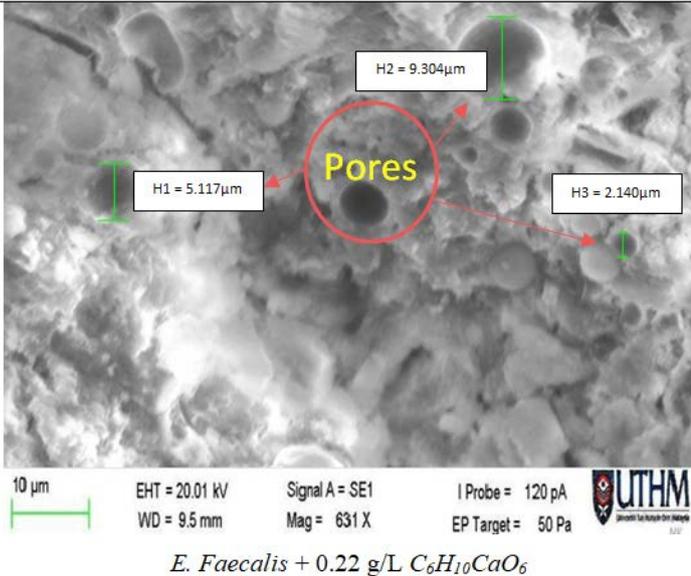
Concrete specimens	Average pore size (μm)
 <p data-bbox="475 788 837 817"><i>E. Faecalis</i> + 0.22 g/L $C_6H_{10}CaO_6$</p>	<p data-bbox="1136 376 1187 405">5.52</p>

Fig. 11 - Sample with *E. Faecalis* and 0.22 g/L calcium lactate

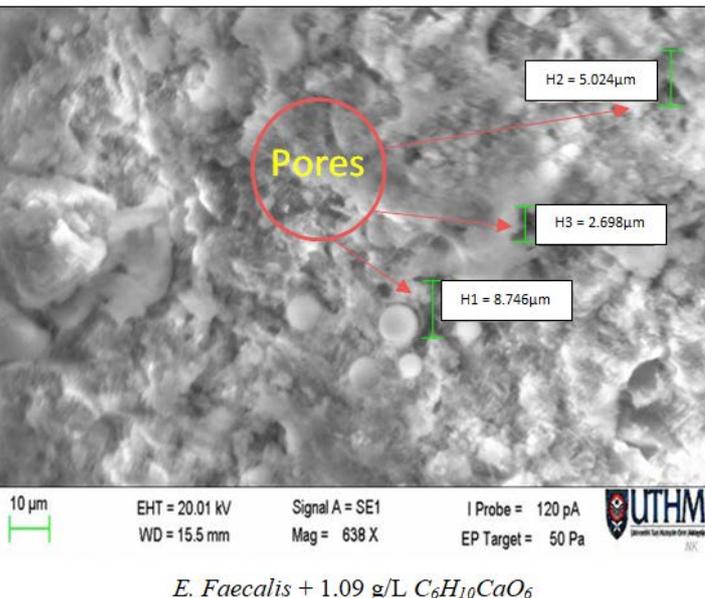
Concrete specimens	Average pore size (μm)
 <p data-bbox="475 1574 837 1603"><i>E. Faecalis</i> + 1.09 g/L $C_6H_{10}CaO_6$</p>	<p data-bbox="1136 1227 1187 1256">5.49</p>

Fig. 12 - Concrete sample with *E. Faecalis* and 1.09g/L calcium lactate

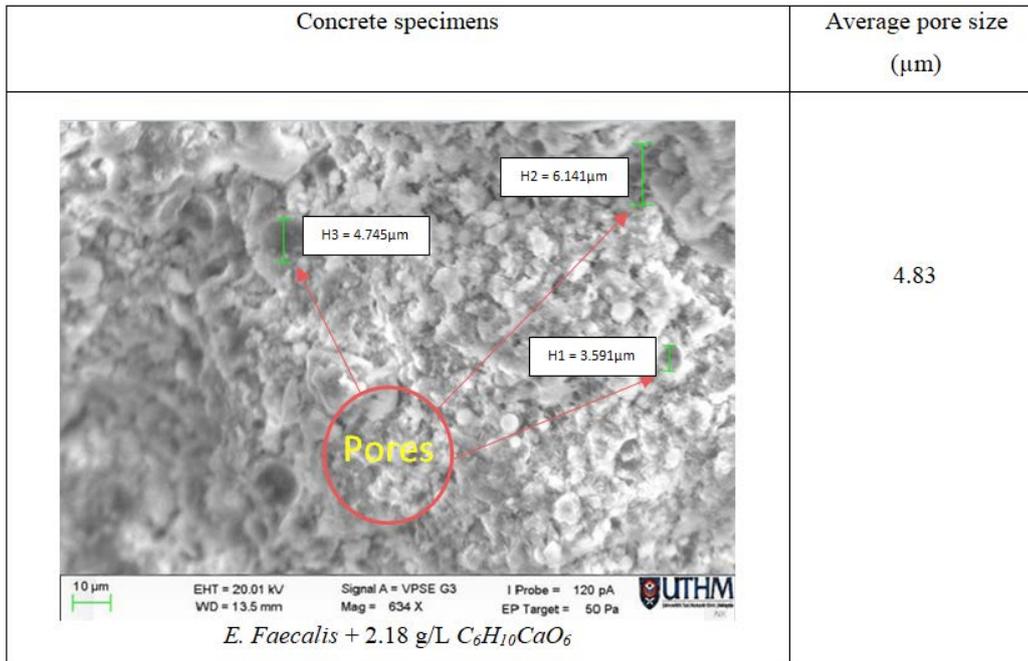


Fig. 13 - Microstructure of *E. Faecalis* and 2.18g/L calcium lactate

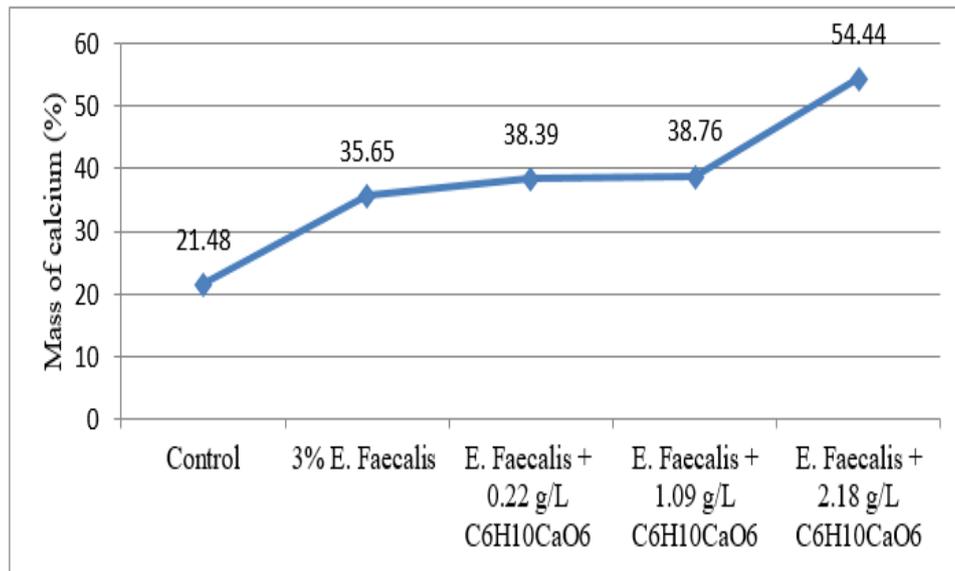


Fig. 14 - Comparison of calcium mass in control and *E. Faecalis* sample with calcium lactate

Concentrations of calcium lactate was added into concrete with 3% *Enterococcus faecalis* and the microstructure of these concrete are analyze using SEM. The pore size difference between specimens with bacteria added in concentration of 0.22 g/L, 1.09 g/L and 2.18 g/L compare to control are 58.05%, 58.28% and 63.29% respectively. The pores filled the concrete which improves concrete mechanical properties. Filling or concrete pores resulted to increase compressive and flexural strength. The results also have shown that addition of calcium lactate in bio-concrete improve concrete properties. *Enterococcus faecalis* produces urease enzyme which catalyzes hydrolysis of urea to CO₂ and ammonia and finally resulting in an increase of pH and carbonate concentration in the bacterial environment (Dick *et al.*, 2006, Chahal *et al.*, 2010, Aiko *et al.*, 2011, Wang *et al.*, 2012 and Tittelboom *et al.*, 2012). The production of calcium carbonate is facilitated with the addition of calcium ions in the form of calcium lactate through the bacteria enzymatic pathway (Chahal *et al.*, 2010).

Fig. 14 shows the result of EDX for bio-concrete specimens containing calcium lactate of 0.22 g/L, 1.09 g/L and 2.18 g/L respectively. Addition of calcium lactate with concentration of 0.22 g/L, 1.09 g/L and 2.18 g/L resulted to

an increase of calcium ion with 38.39%, 38.76% and 54.44% respectively. An increase of calcium mass signifies the precipitation of calcium carbonate by bacteria.

4.0 Discussions

Enterococcus faecalis from genus bacillus was successfully isolated from urine source. Results of the compressive and flexural strengths showed that with the addition of bacteria, the concrete has achieved higher strengths. While, adding calcium lactate has further improved the concrete properties. This is due to the calcite deposition by the bacteria in concrete, which reduces the concrete pores thus increasing the strengths. This is proven with SEM and EDX. The calcium lactate added acts as a food source for the bacteria. *Enterococcus faecalis* bacteria with ureolytic enzyme had ability to hydrolysis of urea to CO₂ and ammonia (Chahal *et al.*, 2010). This study also results to an increase of pH and concentration of carbonate in the bacterial environment. An increment of pH is vital for survival of the bacteria as concrete is naturally high in alkaline. Based on Mayur and Jayeshkumar, (2013), there are two pathways for precipitation of calcium carbonate by bacteria. The first pathway usually involves sulphur cycle and particular sulphate reduction. This pathway is usually carried out by sulphate reducing bacteria under anoxic condition. Second pathway involves nitrogen cycle which is usually carried out by ureolytic bacteria. In this study, the addition of calcium lactate benefits the nitrogen pathway as calcium ions are taken in to combine with carbonate and resulted to calcium carbonate. Self-healing of concrete is analyzed through UPV and Stereomicroscope. Cracks were formed in concrete through using metal strip and applying 3-point bending and were studied throughout 100 days after formation of crack. Results showed that, addition of calcium lactate in concrete containing *Enterococcus faecalis* has significant improvement in healing. This increased in healing is due to microbial activity between the calcium lactate and bacteria which increased the precipitation of calcium carbonate or is known as calcite deposition in healing of concrete micro-cracks.

5.0 Conclusions

This study concluded that the metabolic processes of calcium carbonate production by bacteria can be influenced by adequate amount of calcium lactate. This study also shows that addition of calcium lactate in bio-concrete has profound effects on the concrete properties and self-healing. Different amount of calcium lactate added influence the production of calcium carbonate in the concrete.

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