Eco. Env. & Cons. 26 (April Suppl. Issue) : 2020; pp. (S106-S110)
Copyright@ EM International
ISSN 0971–765X

New isolation of hydrocarbonoclastic bacteria from local Limestone mining as promising concrete strength enhancer

Enny Zulaika[1]*, M. Andry Prio Utomo[1], Nur Hidayatul Alami[1], Endry Nugroho Prasetyo[1], Tri Wulan[2] and Agoes Soegianto[3]*

[1]Department of Biology, Faculty of Sciences, Institut Teknologi Sepuluh Nopember, Sukolilo, Surabaya 60111, Indonesia
[2]Department of Civil Engineering, Faculty of Civil, Environmental and Geo- Engineering, ITS, Sukolilo, Surabaya 60111, Indonesia
[3]Department of Biology, Faculty of Science and Technology, Universitas Airlangga, Kampus C, Mulyorejo, Surabaya, Indonesia

(Received 27 September, 2019; Accepted 10 January, 2020)

ABSTRACT

A small group of bacteria which are able to precipitates calcium carbonates ($CaCO_3$) are known as hydrocarbonoclastic bacteria. Since $CaCO_3$ is a main raw material for cement production, hydrocarbonoclastic bacteria are reliable to be applied as concrete additive for strengthen concrete structures. The objectives of present study were to isolate hydrocarbonoclastic bacteria strains that are able to precipitate $CaCO_3$ and examine its potential as concrete reinforcement materials. Isolates identification through phenotypic methods was conducted following Bergey's Manual of Determinative Bacteriology protocols. Meanwhile, concrete was prepared following SNI 7656:2012 with bacteria cell introduction. Concrete compressive strength test was conducted using compression testing method. Although 15 isolates were able to precipitate $CaCO_3$, only 8 isolates give positive result for urease test. One of the isolates identified as *Bacillus* JB2 has the highest ability to precipitates $CaCO_3$ with amount of 3.28 mg/mL. This strain could successfully increase concrete maximum load up to 26,673.48 kg and compressive strength up to 33.30 Mpa compared to control concrete. Our result suggest that *Bacillus* JB2 is suitable to be applied as concrete additives to strengthen concrete structures.

Key words: *Bacillus, $CaCO_3$, Concrete, Hydrocarbonoclastic bacteria.*

Introduction

Concrete is the main component of building construction which widely used, easy to cast, high compressive strength and resistant to fire (Wang *et al*., 2016; Achal *et al*., 2011). Cement is the main binder content of concrete which contains 77-78% of calcium carbonates ($CaCO_3$) (Van Damme, 2018; Muhammad *et al*., 2018). $CaCO_3$ is inorganic compound which can be precipitated through micobial mineral precipitation (biodeposition) pathway (Chahal *et al*., 2011). Bacteria group which are capable to induce $CaCO_3$ mineralization processes named hydrocarbonoclastic bacteria (Krishnapriya *et al*., 2015).

Recently construction technology using microorganism applications has been proposed, one of this tehnique is biocement which can be produced from

$CaCO_3$ or dolomite in the temperature of 20-60 ° C with 10% lower energy than the energy need edin conventional process, so that the cost is relatively lower and environmentally friendly (Ivanov *et al*., 2015). Concrete with the addition of hydrocarbonoclastic bacteria can increase the compressive strength of concrete, so that it can strengthen infrastructure construction. Bacteria which are added to concrete raw materials will produce endospores that can resist up to 50 years, so that the age of the concrete will be longer (Wang *et al*,. 2016). The objective of this study was to discover the hydrocarbonoclastic bacteria strain which is able to form $CaCO_3$ as concrete reinforcement.

Materials and Methods

Bacteria Isolation and Purification

Bacterial isolation was carried out in local limestone quarries in Gresik, Bangkalan and Tuban, East Java, Indonesia. Lime material samples were put into sterile vials. Inoculation was conducted using pour plate method on selective CCP-agar media containing (per liter) 20 g urea, 2.12 g $NaHCO_3$; 10 g NH_4Cl; 3 gr Nutrient Broth; 30 mM $CaCl_2$; 20 g agar, pH was set 8.5 (Wei *et al*., 2015). Bacteria culture then incubated at room temperature for 7 days, the growing colonies are hydrocarbonoclastic bacteria isolates. The isolate was purified until pure culture was obtained using 16 streaks method.

Qualitative Urease Test

The urease activity from selected isolates were tested qualitatively on urea broth media containing (per 100 mL): 0.9 gr urea base broth, 5 mL 40% urea solution, and pure water. Isolates were inoculated in 5 mL of urea broth, following incubation at room temperature for 1-2 days. The color changes from pale yellow into cherrish pink indicates isolates which produces urease (Chahal *et al*., 2012).

Bacteria Identification and $CaCO_3$ Production

Selected isolates were identified based their biochemical characteristics following Bergey's manual of determinative bacteriology (Holt *et al*., 1994), including cell shape, cell wall properties (Gram staining), endospore, motility, oxygen demand test, and catalase test (Harley and Prescott, 2002).

Isolates were grown on CCP-broth medium, following incubated for 7 days at room temperature under shaking condition (130 rpm). The formed $CaCO_3$ crystals then filtered using Whatman filter paper. The trapped materials were dried in the oven (60°C for 3 hours), and the weight of $CaCO_3$ was calculated by the formula below:

$$Wc = Wfc - Wf$$

Note:
Wc = $CaCO_3$ Precipitant Weight
Wfc = The weight of filter paper and precipitant
Wf = The dry weight of filter paper (Hammad *et al*., 2013)

Concrete Preparation and Viability Test

The bacteria starter that were used is in the logarithmic phase based on the growth curve (Sharma *et al*., 2011), before added into the concrete raw material, bacteria cell density was directy calculated using a haemocytometer. The raw material of concrete (per kg/m^3) is formulated following SNI 7656: 2012 which consists of cement, sand, coarse aggregate and water, then added with bacterial starter. Concretes were casted in cylindrical shape caster with a diameter of 10 cm and a height of 20 cm (Krishnapriya *et al*., 2015). Concrete without bacteria cell addition was used as a control.

Bacterial viability test was used to determine the bacteria survival after being used as raw material for concrete. Concrete specimens were taken before casted and after the curing process for 28 days. The mashed concrete samples were aseptically transferred into physiological solution (0.9% NaCl), then inoculated on the CCP-agar medium, the growing colonies indicates isolates viability in concrete raw material.

Compressive Strength Test

Concrete compressive strength test using compression testing method, both for bacteria added concrete and control concrete. The value of compressive strength of concrete was measured following National Indonesian Standard 2847-2013 below:

$$\sigma = p/A$$

Note:
σ = Compressive strength (MPa)
p = Maximum load (N)
A= Surface area (mm)

Results and Discussion

Bacteria Isolation and Purification

The results of isolation and purification of hydrocarbonoclastic bacteria was successfully obtained 15 isolates, colonies which grew on the CCP agar were able to formed $CaCO_3$ crystals around the growth area (Fig.1).

CCP media is a media that used to detect $CaCO_3$ minerals precipitation, it contains urea and $CaCl_2$ as precursors of carbonate ions and calcium ions formation (Kim *et al*., 2016). The $CaCO_3$ precipitation zone around the bacteria colonies is formed because the growing isolates produce urease so that the $CaCO_3$ precipitation will be induced (Hammad *et al*., 2013).

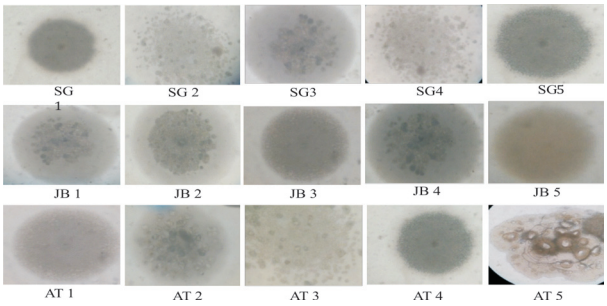

Fig. 1. $CaCO_3$ Forming Bacteria from Limestone Area in East Java.

Qualitative Urease Activity

Qualitative urease test was conducted to differentiate urease producing bacteria (Atlas, 2010). Based on the urease activity test, among 15 isolates which produced $CaCO_3$, only 8 isolates were able to produce urease which can be characterized by the color changes on Christensen-Urea broth medium from pale yellow into cherrish pink (Fig. 2).

Urea in the medium will be hydrolyzed by isolates urease into ammonia and carbamate. In the other stage it will be spontaneously hydrolyzed and produce ammonia and carbonic acid. Ammonia is alkaline compound, so it will increasing the pH of the medium. The pH changes in the medium into alkali generated the color changes phenol red indicator into cherrish pink (Siddique and Chahal, 2011).

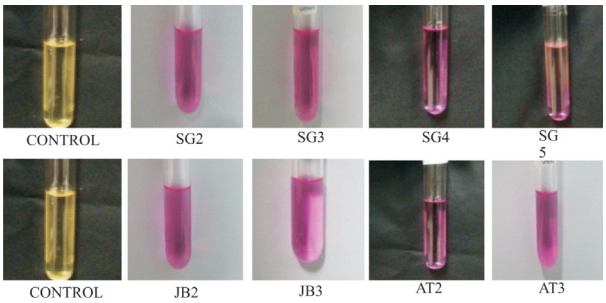

Fig. 2. The Results of Qualitative Urea Test

Isolate Identification and $CaCO_3$ Production

Following Bergey's Manual Determinative of Bacteriology, isolates SG3, SG4, JB2, JB3, AT2, AT3 belong to genera *Bacillus*, SG2 isolates was *Sulfido bacillus* and SG5 isolates was *Escherichia*. According to Holt *et al*., (1994), the characteristics of *Bacillus* are: rod shaped cell, Gram positive, motile, endospores forming, aerobic/ facultative anaerobic, and positive catalase reaction. *Sulfido bacillus* has rod shaped cell, Gram positive, non-motile, endospores forming, aerobic/facultative anaerobic, and positive catalase reaction. *Escherichia* has rod shaped cell, Gram negative, motile, non-endospores forming, aerobic/facultative anaerobes, and positive catalase reaction. Isolates which grew in CCP-broth media formed $CaCO_3$. Based on our observation *Bacillus* JB2 has the highest value of $CaCO_3$ precipitate compared to other isolates (Table 1).

Table 1. $CaCO_3$ Production.

Isolate	Genus	$CaCO_3$ (mg/ml)
SG 2	*Sulfidobacillus*	0.96
SG 3	*Bacillus*	1.12
SG 4	*Bacillus*	2.64
SG 5	*Escherichia*	2.68
JB 2	*Bacillus*	3.28
JB 3	*Bacillus*	2.80
AT 2	*Bacillus*	2.80
AT 3	*Bacillus*	0.48

The bacteria ability to form $CaCO_3$ as their tolerant in the high urea and calcium environment. It will produce urease to hydrolyze urea and release carbonate ions (CO_3^{2}), then it will bind to calcium ions (Ca^{2+}) from $CaCl_2$ to generates $CaCO_3$ and H_2O (Chaparro-Acuna *et al*., 2017).

The formed $CaCO_3$ crystals can be used as additives in concrete raw materials which has role to strengthen materials in the raw material (Krishnapriya *et al*., 2015). Beside of concrete Furthermore, for concrete production application this present study used *Bacillus* JB2 since its able to produce the highest $CaCO_3$ amounts (3.28 mg/mL) compared to other isolates.

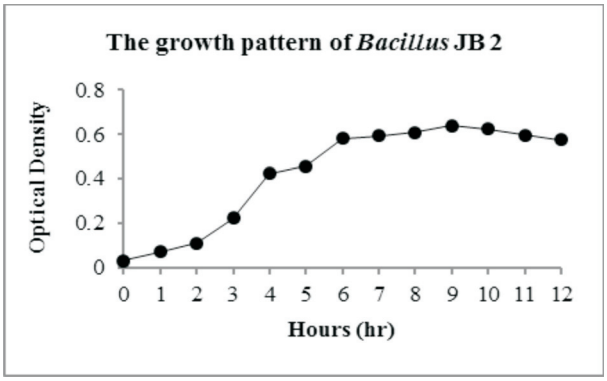

Fig. 3. The growth pattern of *Bacillus* JB 2.

Concrete Preparation and Viability Test

Based on the isolates growth pattern (Fig. 3), the logarithmic phase and optimal growth of *Bacillus* JB2 are after incubation for 10 hours, the cell density was calculated from 2.80 to 4.96 x 10^6. The addition of bacteria for concrete preparation was carried out using 10 hours incubated culture.

According to SNI 03-3976-1995, concrete must be in moist for at least 7 days, the quality could be better if the curing stage is carried out for 28 days. Concrete which induced by *Bacillus* JB2 is shown in Fig. 4.

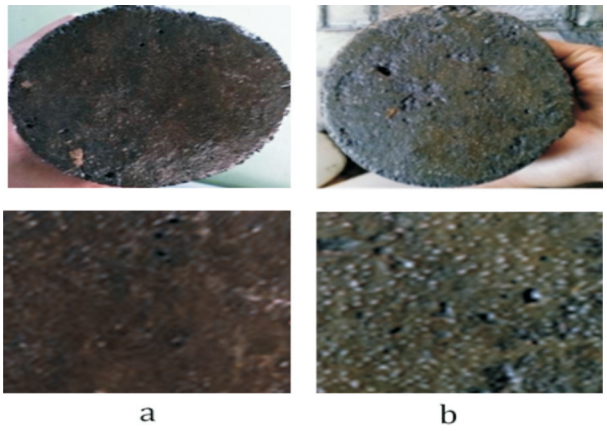

Fig. 4. The morphology of concrete : (a) Control (b) Concrete with *Bacillus* JB2 addition.

Based on Fig. 4, the morphology of the control concrete and concrete which added by *Bacillus* JB2 isolates posses relatively the same external morphology, so that the addition of *Bacillus* JB2 as a hydrocarbonoclastic bacteria did not affect the external morphology of the concrete.

The bacterial viability test was carried out to determine the viability of hydrocarbonoclastic bacteria after being embedded in concrete. Bacterial colonies that grew on CCP-agar media show their ability to resist against harsh environment inside the concrete (Fig. 5).

Based on Fig. 5 above, *Bacillus* JB2 is able to resist in concrete samples before the casting process or after 28 days curing. Our data revelas that *Bacillus* JB2 is viable in concrete material and possess a great potential to be used as biocement.

Concrete Compressive Strength

Compressive strength is one of the main properties of concrete, the results of compressive strength tests on control concrete and applicative concrete which have been added by *Bacillus* JB2 are served in Table 2.

Based on Analysis of Variance (ANOVA), there are differences in the concrete compressive strength between control concrete and concrete which is added by *Bacillus* JB2, the LSD test (P value 5%) shows a significant difference. Based on Table 2, concrete with the addition of *Bacillus* JB2 has higher score in maximum load and compressive strength than control concrete. Increased strength of concrete is mainly caused by $CaCO_3$ crystals which formed by bacteria that can fill the micro pores inside concrete, so that the size of the pores will shrink and maximize the compressive strength of concrete sample (Chahal *et al*., 2012). The formation of $CaCO_3$ by bacteria due to hydrolysis mechanisms of urea which generates ammonia ions and carbonate ions. Ammonia will increase the pH of the medium, then the environment becomes alkaline. That is favorable condition and support the binding of carbonate ions and calcium ions to generating $CaCO_3$ crystals (Wei *et al*., 2015).

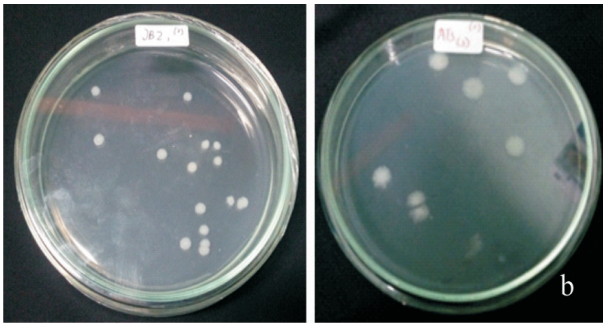

Fig. 5. Bacteria viability: a) viability before casting process; b) viability after 28 days curing.

Table 2. Maximum Load and Compressive Strength.

Treatment	Cell Density (sel/mm^3)	Maximum Load (Kg)	Compressive Strength (Mpa)
Control (without isolate)	0	24,383.03	30.44[a]
Bacillus JB 2 (1)	3.38 x 10^6	26,520.20	33.11[b]
Bacillus JB 2 (2)	2.80 x 10^6	26,672.48	33.30[b]
Bacillus JB 2 (3)	4.94 x 10^6	26,727.44	33.36[b]

Note: the number besides different letter showing significants difference (P value 5%).

Conclusion

Eight isolates of hydrocarbonoclastic bacteria are able to produce and form $CaCO_3$ crystals. Concrete with the addition of bacteria cells has a maximum load and compressive strength of concrete higher than control concrete. Therefore, *Bacillus* JB 2 bacteria can be applied as additives to strengthen concrete structures.

Acknowledgements

This research was funded by Direktorat Riset dan Pengabdian Kepada Masyarakat (DRPM), Republic of Indonesia (Scheme PDUPT).

References

Achal, V., Mukherjee, A. and Reddy, M.S. 2011. Microbial concrete- a way to enhance the durability of building structure. *J. Mater. Civ. Eng.* 23: 730-734. https://doi.org/10.1061/(ASCE)MT.1943-5533.0000159

Atlas, R.M. 2010. *Handbook of Microbiological Media.* 4th edition. CRC Press Taylor and Francis Group, Boca Raton.

Chahal, N., Rajor, A. and Siddique, R. 2011. Calcium carbonate precipitation by different bacterial strains. *African Journal Biotechnology.* 10: 8359 - 8372. https://doi.org/10.5897/AJB11.345

Chaparro-Acuna, S.P., Becerra-Jimenez, M.L., Martinez-Zambrano, J.J. and Rojas-Sarmiento, H.A. 2017. Soil bacteria that precipitate calcium carbonate: mechanism and applications of the process. *Acta Agronomica.* 67 (2): 277-288. https://doi.org/10.15446/acag.v67n2.66109

Hammad, I.A., Talkhan, F.N. and Zoheir, A.E. 2013. Urease activity and induction of calcium carbonate precipitation by *Sporosarcina pasteurii* NCIMB 8841. *Journal of Applied Sciences Research.* 9(3): 1525-1533.

Harley, J.P. and Prescott, L.M. 2002. *Laboratory Exercise in Microbiology.* 5th edition. Mc Graw Hill, USA.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T. and Williams, S.T. 1994. *Bergey's Manual of Determinative Bacteriology*. 9th edition. Lippincot Williams & Wilkins, Philadelphia.

Ivanov, V., Chu, J. and Stabnikov, V. 2015. Basics of construction microbial biotechnology. In : *Biotechnologies and Biomimetics for Civil Engineering*, Springer International Publishing, Switzerland : 21-39. https://doi.org/10.1007/978-3-319-09287-4_2

Kim, H.J., Eom, H.J., Park, C., Jung, J., Shin, B., Kim, W., Chung, N., Choi, I.G. and Park, W. 2016. Calcium carbonate precipitation by *Bacillus* and *Sporosarcina* strains isolated from concrete and analysis of the bacterial community of concrete. *J. Microbiol. Biotechnol.* 26 (3) : 540-548. https://doi.org/10.4014/jmb.1511.11008

Krishnapriya, S., Babu, D.L.V. and Arulraj, P.G. 2015. Isolation and identification of bacteria to improve the strenght of concrete. *Microbiological Research.* 174 : 48-55. https://doi.org/10.1016/j.micres.2015.03.009

Muhammad, U.L., Musa, M.Y., Yusuf, U. and Nasir, A.B. 2018. Limestone as solid mineral to develop national economy. *American Journal of Physical Chemistry*. 7 (2): 23-28. https://doi.org/10.11648/j.ajpc.20180702.13

Sharma, B.S., Riyaz, Z.S., Mrugesh, H.T. and Thivakaran, A.G. 2013. Phosphate solubilizing microbes: Sustainable approach for managing phosphorus deficiency in agricultural soils. *Review Springer Plus*. 2 : 5-7. https://doi.org/10.1186/2193-1801-2-587

Siddique, R. and Chahal, N.K. 2011. Effect of ureolytic bacteria on concrete properties. *Construction and Building Materials.* 25 : 3791-3801. https://doi.org/10.1016/j.conbuildmat.2011.04.010

VanDamme, H. 2018. Concrete material science: past, present, and future innovations. *Cement and Concrete Research*. 112 : 5-24. https://doi.org/10.1016/j.cemconres.2018.05.002

Wang, J., Ersan, Y.C., Boon, N. and DeBelie, N. 2016. Application of microorganisms in concrete: a promising sustainable strategy to improve concrete durability. *Appl. Microbiol. Biotechnol.* 100 (7) : 2993-3007. https://doi.org/10.1007/s00253-016-7370-6

Wei, S., Cui, H., Jiang, Z., Liu, H., He, H. and Fang, N. 2015. Biomenarilization processes of calcite induced by bacteria isolated from marine sediments. *Brazilian Journal of Microbiology*. 46 (2) : 455-464. https://doi.org/10.1590/S1517-838246220140533