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ANTIBIOFILM ACTIVITY AND STABILITY ASSAY OF PIGMENT FROM MARINE BACTERIA *Bizionia paragorgiae*

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ABSTRACT

Biofilms are communities that contain one or multiple microorganisms that enclosed in extracellular polymeric substance (EPS) matrix Biofilm formation is one of important survival skills for bacteria to be able to grow in all surfaces, such as living tissues, medical devices, industrial water piping, shipping, and natural aquatic system. It is recognized as the important virulence factor and protection for bacteria from environment influences. *Bizionia paragorgiae*, which produced orange pigment, was examined in this research. In this research, antibacterial, antibiofilm activity, and stability assay were examined. There was no antibacterial activity indicated from this study against all tested Gram-negative and Gram-positive bacteria. Based on antibiofilm activity against four pathogenic bacteria, both destruction and inhibition assay reduced biofilm formation by up to 30-70%. According to the result revealed from stability assay, the pigment was more stable at its natural pH 5.83, at 25 °C, and stored in dark condition.

Keywords: marine bacteria, pigment, biofilm, antibiofilm, stability assay

INTRODUCTION

Biofilm is an important virulence factor, both for opportunistic and true pathogens. Bacterial biofilms can develop on many abiotic surfaces, such as plastic, glass, and metal, and on biotic surfaces, such as human, animal, and plants. The term of biofilm usually associates with various clinical diseases caused by bacteria, like caries, gingivitis, endocarditis, atopic dermatitis, and inflammatory bowel disease. Other negative impacts caused by biofilms are serious problems that take place in industry, public health and medicine, biofouling in marine transportation and pipes. Because the polymer extracellular matrix acts as the shield, the bacterial growth in biofilm is more resistant to antimicrobial agents and chemical biocides, leading to much effort for researches to discover new compound that has antibiofilm and antibacterial performance (**Thenmozhi *et al.*, 2009; Bernbom *et al.*, 2011**).

Pigments from marine bacteria are known to have antibacterial and antibiofilm activity. For example, the purple pigment violacein produced by *Chromobacterium violaceum* has antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Vibrio cholerae*, and *Klebsiella pneumoniae* (**Subramaniam *et al.*, 2014**). Carotenoid pigment from *Exiguobacterium* sp. has antibacterial activity against *Klebsiella* sp, *S. aureus*, and *Shigella*, except *Enterococcus* sp. (**Balraj *et al.*, 2014**). The yellow pigment produced by *Pseudomonas tunicate* possess the antibiofilm activity (**Bernbom *et al.*, 2011**).

Marine bacteria are great potential source of bacterial pigment, since they are abundant in the ocean that covers 70% of the Earth's surface. Marine microorganisms are different from terrestrial microorganism due to the influence of their environmental condition. Microorganisms living in the sea have to adapt with the high salinity, high pressure, and low nutrient in order to survive and grow, resulting in different survival mechanisms, genetic adaptation, and growth strategies. The adaptation influences the production of secondary metabolites, such as pigments, antibiotics, and enzymes (**Kim, 2015; Soliev *et al.*, 2011**).

Indonesia, as one of the largest maritime country in the world, has abundant amount of marine organisms. With the vast sea area and plentiful marine microorganisms, Indonesia offers a promising potential for discovering new marine bacterial pigment.

The objectives of this study were to discover the antibiofilm activity of marine bacterial pigment isolate *Bizionia paragorgiae* and determine the stability of the pigment in various pH, temperature, and in the presence or absence of light.

MATERIAL AND METHODS

Materials

Materials used in this study were crude extract of pigment from isolate *Bizionia paragorgiae*; Marine Agar, Marine Broth (Difco, Franklin Lakes, New Jersey); Brain Heart Infusion, Eosin Methylene Blue Agar, Yeast Extract, Tryptone, Bacteriological Agar (Oxoid, Basingstoke, Hampshire); NaCl, D-glucose, Methanol Absolute, Ethanol Absolute, and Crystal Violet (Merck, Darmstadt, Hesse). Pathogenic bacteria as the target for the assay were *Staphylococcus aureus* (ATCC 25923), Enterohemorrhagic *Escherichia coli* (EHEC), Enterotoxigenic *Escherichia coli* (ETEC), Enteropathogenic *Escherichia coli* (EPEC), *Bacillus licheniformis* (ATCC 12759), *Acinetobacter baumannii* (ATCC 19606), *Burkholderia cepacia* (ATCC 25416), *Escherichia coli* (ATCC 4157), *Pseudomonas aeruginosa*, *Salmonella enterica* (ATCC 51741), *Streptococcus pneumoniae* and *Staphylococcus haemolyticus*. This research was conducted according to the methods from **Alihosseini *et al.* (2008), Deil (2015)**, and **Vendy (2014)**, with slight modification.

Bacterial cultivation

The cryopreservation isolate of marine bacteria from previous study were used for this research study (**Deil 2015**). Isolate *Bizionia paragorgiae*, which was chosen because of its ability to produce orange pigment in relative high concentration, was a sea snail (*Babylonia lutosa*) associated bacteria from Binuangen, Banten. Isolate was inoculated on Marine Agar (MA) with continuous streak and then incubated at 28°C for minimum 48 hours. MA was used as the replica condition and nutrition of sea water, the natural habitat of marine bacteria.

After incubation, single colony was picked and inoculated on new MA medium to make sure that there was no contamination in the isolate. Four pathogenic bacteria used in this research were *S. aureus* (ATCC 25923), Enterohemorrhagic *E. coli* (EHEC), Enterotoxigenic *E. coli* (ETEC), and Enteropathogenic *E. coli* (EPEC). Isolates of the pathogens were obtained from the cryopreservation. Each isolate was grown on Luria Agar and incubated at 37°C for overnight. After one day of incubation, the isolates were refreshed on Brain Heart Infusion (BHI) Agar (BHI + 2% Agar Bacteriological) and incubated at 37°C for overnight. Test for the confirmation of the *E. coli* used in this study was carried out by growing each *E. coli* on Eosin Methylene Blue Agar.

Cell harvesting for pigment isolation

Marine Broth (MB) was used for cell harvesting. One loop of isolate from each Marine Agar plates was inoculated into 100 mL MB in 250 mL Erlenmeyer flasks. The flasks were then incubated in orbital shaker incubator at 28°C, 120 rpm, for 72 hours. Agitation was intended for aeration, homogenizing nutrient, suspending cells, and preventing cell adhesion to the wall of the flasks (**Perez *et al.*, 2014; Smith, 2009**).

After incubation, the turbidity was increase in each flask. To harvest the cells, all media was transferred into 50 mL conical tubes and centrifuged at 8500 rpm for 20 minutes. Supernatant was removed and the pellet was frozen at -20°C before being dried with freeze drying method under freeze dryer for 8 hours to remove the water.

Pigment extraction and drying

The dried pellet was added with 5 mL absolute methanol and mixed with vortex to homogenize the pellet. Mixed pellet was incubated in dark place for 60 minutes. Incubation was intended to break the cells and extract the pigment. Dark condition was used in order to prevent the degradation of the pigment because carotenoid pigments were light sensitive (**Kirti *et al.*, 2014**). After one-hour of incubation, the tubes were centrifuged at 8500 rpm for 10 minutes to separate the cells and the pigment. Successful extraction was indicated by the presence of colorless pellet.
The crude extract from methanol extraction was transferred to evaporation flask and concentrated with rotary evaporator at 52°C to remove the solvent until around 1% (v/v) of the initial volume was left. The concentrated pigment was then transferred into petri dishe and dried in vacuum oven at 42°C for minimum of 2 hours until the pigment dry. Finally, the dried pigment was collected by scraping the pigment and transferring it to microcentrifuge tube wrapped by aluminum foil to prevent light oxidation. The tube was then stored in refrigerator at 4°C.

Antibacterial activity assay

To analyze the antibacterial activity of the pigment, one loop of the pathogenic bacteria was inoculated in BHI + 1% glucose (BHIG) at 37°C for overnight. The bacteria were then diluted with BHIG until reaching the absorbance value of 0.132 at OD=600 nm (McFarland 0.5). The assay was performed using agar well diffusion method. After dilution, 100 µL of test-bacteria suspension were uniformly streaked continuously in three directions on BHI Agar plates using sterile cotton swap. Each plate was divided into a few sections and wells were made using cork borer. Dried crude pigment was diluted in methanol with concentration of 1% (w/v) and 50 µL of the diluted pigment were added to each well. The plates were then incubated at 37°C for overnight. Zone of inhibitions were observed as positive result and compared with standard antibiotic like Ampicillin (100 µg/mL) as positive control and methanol as negative control.

Biofilm inhibition and destructive activity assay

Dried crude pigment was diluted in methanol with concentration of 1% (w/v). To assay the inhibition activity, 96-well microplates were coated with adherent buffer and various concentrations of diluted pigment (5%, 10%, 20% of loaded pathogens volume) before being dried overnight. The leftover adherent buffer was discarded and air-dried for one hour. The microplate wells were loaded with 200 µL of pathogenic bacteria inoculated in BHIG with absorbance value of 0.132 at OD=600 nm (McFarland 0.5) and incubated at 37°C, overnight. Sterile BHIG was used as negative control. Planktonic cells and media were discarded and adherent cells were rinsed once with water, and air-dried for one hour. Adherent biofilm cells were stained with 200 µL 0.4% crystal violet for 30 minutes. Crystal violet was distained twice with water and then air-dried. To solubilize the crystal violet, 200 µL of absolute ethanol was added to each well, resuspended, and 100 µL ethanol from each well ware transferred to a new microplate. Absorbance was determined at 595 nm with microplate reader Biorad 680 Microplate Reader.
To assay the destructive activity, planktonic cells and media were discarded after overnight incubation. Then, diluted pigment with various concentrations was added to each well, incubated at 37°C for 60 minutes, and assayed. Adherent buffer used in this assay was artificial saliva with the composition from **Wang *et al.* (2011)** with slight modification (0.059 g $MgCl_2.6H_2O$, 0.166 g $CaCl_2.2H_2O$, 0.625 g KCl, 0.326 g KH_2PO_4, 0.804 K_2HPO_4, and 10 g carboxymethyl cellulose in 1000 mL distilled water).
Percentage of antibiofilm activity was calculated using formula:

$$\%\ Activity = \frac{OD\ positive\ control - OD\ sample}{OD\ positive\ control} \times 100\%$$

Pigment stability in various pH, temperature, and the presenece of light

Pigment was treated at different pH, temperature, and light exposure respectively. To assay the stability of the pigment in various pH, sample was adjusted to pH 2.00, 7.00, 13.00 using HCl 0.01 M, NaOH 0.01 M, and 0.1 M for acidic and alkaline condition. For thermal stability, sample was incubated at 25, 55, and 85 °C for 15 minutes using water bath shaker. The pigment was stored both in dark and bright condition for 4 days and monitored daily. Color changes of the pigment were quantified at 448 nm using VIS spectrophotometer (**Woo *et al.*, 2011**).

RESULTS AND DISCUSSION

RESULTS

Antibiofilm activity

In order to evaluate whether the pigment was able to reduce biofilm formation, antibiofilm assay was performed against four pathogenic bacteria (*S. aureus*, EHEC, ETEC, and EPEC). The pigment was found to be a promising antibiofilm compound as it could inhibit and destruct biofilm formation at its lowest concentration. As shown in figure 1, overall the biofilm reduction was obtained by up to 30-72%, both for destruction and inhibition assay. The highest reduction of biofilm formation was achieved at concentration 20% of sample with the amount of 50-72%. The antibiofilm activity increased as the concentration of the sample raised. It could be seen that the antibiofilm activity was dependent on the concentration used in the assay.

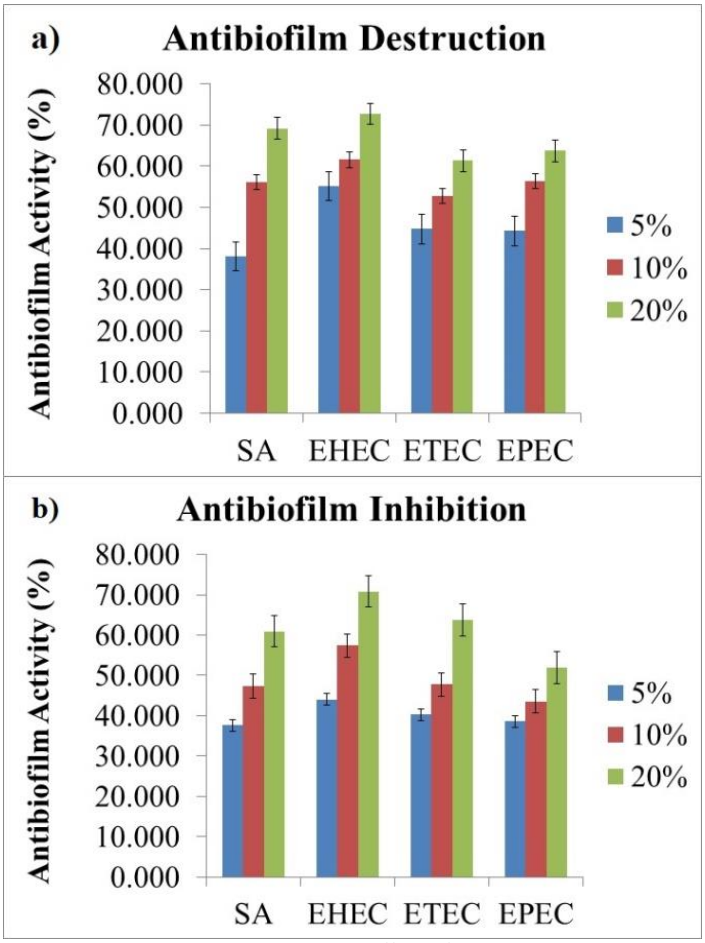

Figure 1 Destructive (a) and inhibitory (b) effects of the pigment against *S. aureus* (SA), EHEC, ETEC, and EPEC

Antibacterial activity

To check whether the pigment had antibacterial activity, the pigment was tested to several pathogenic bacteria. There were no clear zones of inhibition around the well, indicating that the pigment did not have any inhibitory activity against both Gram-positive bacteria: *S. aureus*, *B. licheniformis*, *S. pneumoniae*, *S. haemolyticus*; and Gram-negative bacteria: EHEC, ETEC, EPEC, *A. baumannii*, *B. cepacia*, *E. coli*, *P. aeruginosa*, *S. enterica*.

Stability assay

The influence of pH, heat treatment, and light exposure to the degradation of sample was shown in figure 2. Temperature, pH and light exposure have affected the stability of the pigment. From the result, it could be seen that pH had a great influence in stability of the pigment. Stability of the pigment decreased under acidic and alkaline condition. The

pigment was stable most at its natural pH and slightly stable at neutral pH. There was no significant pigment degradation due to temperature effect. Dark condition was better to store the pigment since the color decreased gradually in the presence of light on the second day. According to the result, the stability of the pigment was dependent upon pH, temperature and light exposure.

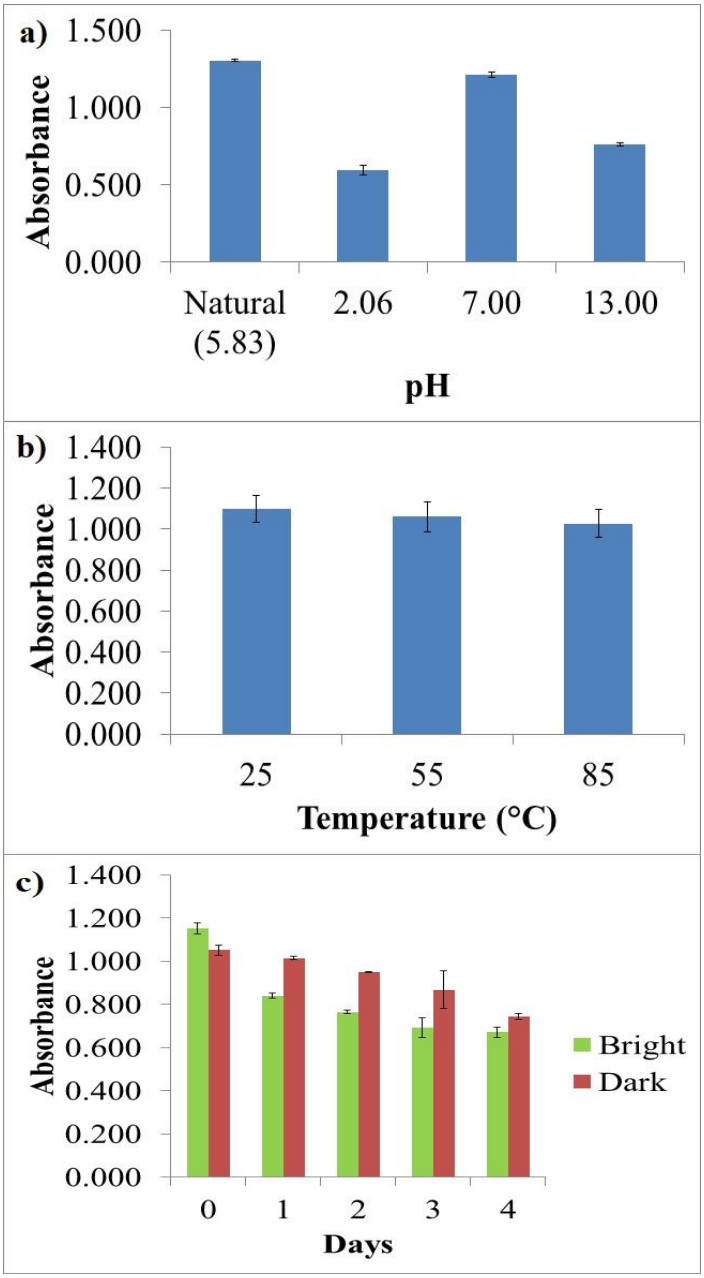

Figure 2 Effect of different parameters on stability of the pigment a) pH; b) temperature; c) light exposure

DISCUSSION

Marine organisms have a promising potential as the source of new antibiofilm compounds. Several bacteria isolated from marine biota, such as corals and sponges, have the ability to generate antibiofilm compound against pathogenic bacteria. The most common sponge-associated bacteria known to have antibiofilm compound are *Bacillus* sp. *B. licheniformis*, isolated from orange orange-colored sponge *Spongia officinalis*, has antibiofilm activity against both Gram-positive and Gram-negative bacteria, such as *S. aureus*, *Bacillus amyloliquefaciens*, *E. coli*, *Pseudomonas fluorescens*, and *Listeria monocytogenes* (**Sayem *et al.*, 2011**). According to **Thenmozhi *et al.* (2009),** bacteria associated with coral *Acropora digitifera*, such as *Bacillus horikoshii* and *Bacillus pumilus*, show antibiofilm activity against *Streptococcus pyogenes*.

In this research, orange pigment from marine bacteria isolate *Bizionia paragorgiae* was able to destruct and inhibit biofilm development from four pathogenic bacteria up to 70%. Literature on microorganisms which own carotenoid pigment that can actively inhibit and disrupt biofilm is very limited. Nevertheless, there are some information about usnic acid, a dibenzofuran-derivate yellow pigment produced by several lichens, *Usnea* and *Cladonia*. This pigment can actively prevent biofilm attachment of *Marinobacter hydrocarbonoclasticus* which is marine biofilm forming bacteria (**Salta *et al.*, 2013**).

Initial attachment of bacteria on surfaces is one of the important stages in biofilm development. Cell-to-cell interaction is also a necessary key of biofilm growth. Due to the ability of the pigment to prevent and destroy biofilm, it may be possible that the pigment has the capability to disrupt the influence of cell-surface and cell-to-cell interaction. It may also be possible that the pigment can modify the physicochemical and structure of EPS matrix of the biofilm. Polysaccharides produced by *B. licheniformis* (**Sayem *et al.*, 2011**), *E. coli* group II capsular polysaccharide, and *Vibrio* sp. QY101 are able to reduce cell-to-cell and cell-surface contacts (**Jiang *et al.*, 2011**).

Mostly, extracts of secondary metabolite from plants performed both antibiofilm and antimicrobial effect. For example, compounds extracted from *Laurus nobilis*, such as alginate, fucoidan, essential oil, and fatty oil, can prevent formation of biofilm and inhibit growth of *Staphylococcus epidermidis* (**Chmit *et al.*, 2014**). Some compounds extracted from ginger (*Zingiber officinale*) have both antibiofilm and antimicrobial effect against *P. aeruginosa* (**Nikolic *et al.*, 2014**). Meanwhile, the pigment used in this research showed only antibiofilm activity. There was no antibacterial effect against several pathogenic bacteria demonstrated from this study. Almost similar results have been reported by **Sayem *et al.* (2011)** and **Thenmozhi *et al.* (2009),** where sponge-associated bacteria and nine coral-associated bacteria showed absence of antibacterial activity.

Carotenoid is a group of pigment that gives yellow-red color. Basic structure of carotenoid consists of 40 carbon atoms arranged by eight isoprene units. From the result, carotenoid was degraded due to pH, temperature, and the presence of light. Carotenoid is not stabile in acidic condition. The loss of the yellow color in acidic condition is due to the production of ion-pairs, which dissociate to form a carotenoid carbocation. Carbocation is positively charge species which is reactive and unstable. The reaction of carotenoid carbocation can be seen in figure 4. Another carotenoid, canthaxanthin, was reported to be degraded by sulfuric acid (**Boon *et al.*, 2010**).

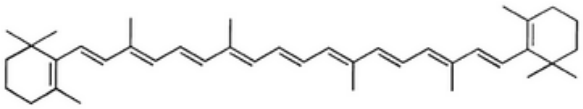

Figure 3 Basic structure of carotenoid (**Krinsky *et al.*, 2004**)

$$Car + AH \leftrightarrow (CarH^{+}\cdots A^{-}) \leftrightarrow CarH^{+} + A^{-}$$

Figure 4 Carbocation reaction of carotenoid (**Boon *et al.*, 2010**)

Effat *et al.* (2014) reported that carotenoids from tomato peel are degraded in acidic media, but more stable in alkaline condition. Meanwhile, results from **Amin *et al.* (2015)** showed that carotenoids from carrot were not stable both in acidic and alkaline condition. It may happen due to the differences of the functional group and the structure of the carotenoids. Carotenoids from tomato consist mostly of lycopene, which is acyclic and linier. Meanwhile, carotenoids from carrot consist of highly amount of carboxylic acid.

Carotenoid is quit heat-stabile. Only slight degradation occurs during thermal treatment which is caused by rearrangement of double-bond configuration. This rearrangement induces slight shift in the maximum absorbance. According to **Oliveira *et al.* (2010)**, degradation of carotenoid due to heat starts from temperature of 40°C. **Amin *et al.* (2015)** have reported that increasing temperature and duration of treatment promotes destruction of carotenoid. Maximum stability is obtained at 20°C and carotenoids will completely degrade at 120°C. The presence of light degrades carotenoids because photooxidation will produce carotenoid radical cations. β-carotene molecules will be excited and at the same time the excited molecules will return to ground state. At this moment, they might be attacked by radical by-products created during the reaction (**Boon *et al.*, 2010**; **Stevens & Verhe, 2004**).

One of methods used to stabilize carotenoids is encapsulation. It is able to protect pigment from degradation caused by physical and chemical treatment. Coating in an appropriate matrix can longer pigment shelf life, increase pigment stability in various pH, light, and prevent color degradation. Coating materials such as liposome and cyclodextrin are

potential to be used. Multilamellar liposomes from soy lecithin will increase the efficiency of β-carotene. Using food-grade starch-based materials like cyclodextrin will increase the solubility of carotenoids in water and protect them from degradation due to light and oxygen (**Rahman, 2007**; **Aguilera & Lillford, 2008**).

CONCLUSION

Pigment from marine bacteria *Bizionia paragorgiae* has the ability to effectively inhibit and destruct biofilm formation of four pathogenic bacteria. This pigment is potential to be explored as the novel source of natural antibiofilm compound. There was no antibacterial activity shown in this research. Stability of the pigment depends on pH, temperature, and light exposure. The best condition to store the pigment is at its natural pH, at 25 °C, and without light exposure. Further exploration and research are needed to purify the pigment, determine quorum-sensing inhibition, and confirm anti-adherence activity of the pigment toward cell-cell and cell-surface interactions.

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Conflict of interest : The authors declare that there is no conflict of interest.

REFERENCES

AGUILERA, J.M., LILLFORD, P.J. 2008. Food Materials Science Principles and Practice. New York: Springer, 592 p. ISBN 978-0-387-71947-4. http://dx.doi.org/10.1007/978-0-387-71947-4

ALIHOSSEINI, F., JU, K.S., LANGO, J., HAMMOCK, B.D., SUN, G. 2008. Antibacterial colorants: characterization of prodiginines and their applications on textile materials. *Biotechnology progress*, 24(3), 742–747. https://doi.org/10.1021/bp070481r

AMIN, A.A., SALAMA, M.F., SELIEM, E.I., MAHMOUD, K.F. 2015. Encapsulation of nano carotenoids; evaluation of stability and safety. *Int J Curr Micriobiol App Sci*, 4(2):1017-1029.

BALRAJ, J., PANNERSELVAM, K., JAYARAMAN, A. 2014. Isolation of pigmented marine bacteria *Exiguibacterium* sp. from peninsular region of India and a study on biological activity of purified pigment. *International Journal of Scientific & Technology Research* 3(3):375-384.

BERNBOM, N., NG, Y.Y., KJELLEBERG, S., HARDER, T., GRAM, L. 2011. Marine bacteria from Danish coastal waters show antifouling activity against marine fouling bacterium *Pseudomoalteromonas* sp. strain S91 and zoospores of the green alga *Ulva australis* independent of bacteriocidal activity. *Appl Environ Microbiol* 77(24):8557-8567. https://doi.org/10.1128/aem.06038-11

BOON, C.S., MCCLEMENTS, J., WEISS, J., DECKER, E.A. 2010. Factors influencing the chemical stability of carotenoids in foods. *Critical Review in Food Science and Nutrition* 50:515-532. https://doi.org/10.1080/10408390802565889

CHMIT, M., KANAAN, H., HABIB, J., ABBASS, M., MCHEIK, A., CHOKR, A. 2014. Antibacterial and antibiofilm activities of polysaccharides, essential oil, and fatty oil extracted from *Laurus nobilis* growing in Lebanon. *Asian Pac J Trop Med* 7(1):546-552. https://doi.org/10.1016/s1995-7645(14)60288-1

DEIL. A.L.Y. 2015. Isolation and characterization of pigment from marine bacteria [thesis]. Jakarta: Fakultas Teknobiologi, Universitas Katolik Indonesia Atma Jaya.

EFFAT, M.R., EL-KADY, A.T., EL-BIALY, A.R. 2014. Characterization of carotenoids (lyco-red) extracted from tomato peels and its uses as natural colorants and antioxidants of ice cream. *Annals of Agriculture Science* 59(1):53-61. https://doi.org/10.1016/j.aoas.2014.06.008

JIANG, P., LI, J., HAN, F., DUAN, G., LU, X., GU, Y., YU, W. 2011. Antibiofilm activity of an exopolysaccharide from marine bacterium *Vibrio* sp. QY101. *Plos One* 6(4):1-11. https://doi.org/10.1371/journal.pone.0018514

KIM, S.K. 2015. Springer Handbook of Marine Biotechnology. Berlin: Springer, 1249p. ISBN 978-3-642-53971-8. https://doi.org/10.1007/978-3-642-53971-8

KIRTI, K., AMITA, S., PRITI, S., KUMAR, A.M., JYOTI, S. 2014. Colorful world of microbes: carotenoids and their applications. *Advances in Biology* 2014:1-14. https://doi.org/10.1155/2014/837891

KRINSKY, N.I., MAYNE, K.T., SIES, H. 2004. Carotenoids in Health and Disease. Boca Raton: CRC. 2p. ISBN 9780367393892. https://doi.org/10.1201/9780203026649

NIKOLIC, M., VASIC, S., DURDEVIC, J., STEFANOVIC, O., COMIC, L. 2014. Antibacterial and anti-biofilm activity of ginger (*Zingiber officinale* (Roscoe)) ethanolic extract. *Kragujevac J Sci* 36:129-136. https://doi.org/10.5937/kgjsci1436129n

OLIVEIRA, R.G., CARVALHO, M.J.L., NUTTI, R.M., CARVALHO, L.V.J., FUKUDA, W.G. 2010. Assesment and degradation study of total carotenoid and beta-carotene in bitter yellow cassava (*Manihot esculenta* Crantz) varieties. *African Journal of Food Science* 4(4):148-155.

PEREZ, R.A., RICCARDI, K., ALTANKOV, G., GINEBRA, M.P. 2014. Dynamic cell culture on calcium phosphate microcarriers for bone tissue engineering applications. *J Tissue Eng* 5:1-10. https://doi.org/10.1177/2041731414543965

RAHMAN, M.S. 2007. Handbook of Food Preservation. Boca Raton: CRC. ISBN 9781574446067. https://doi.org/10.1201%2F9781420017373

SAYEM, S.M.A. *et al*. 2011. Anti-biofilm activity of an exopolysaccharide from a sponge-associated strain of *Bacillus licheniformis*. *Microbial Cell Factories* 10(74):1-12. https://doi.org/10.1186/1475-2859-10-74

SALTA, M., WHARTON, J.A., DENNINGTON, S.P., STOODLEY, P., STOKES, K.R. 2013. Anti-biofilm performance of three natural products against initial bacterial attachment. *Int J Mol Sci* 14(11):21757-21780. https://doi.org/10.3390/ijms141121757

SMITH, J.E. 2009. Biotechnology. Ed 5. Cambridge: Cambridge University. ISBN: 9780511802751. https://doi.org/10.1017/CBO9780511802751

SOLIEV, A.B., HOSOKAWA, K., ENOMOTO, K. 2011. Bioactive pigments from marine bacteria: applications and physiological roles. *Evidence-Based Complementary and Alternative Medicine*. 2011:1-17. https://doi.org/10.1155/2011/670349

STEVENS, C.V., VERHE, R.G. 2004. Renewable Bioresources Scope and Modification forNon-Food Applications. West Sussex: Wiley. ISBN: 978-0-470-85446-4.

SUBRAMANIAM, S., RAVI, V., SIVASUBRAMANIAN, A. 2014. Synergistic antimicrobial profiling of violacein with commercial antibiotics against pathogenic micro-organisms. *Pharm Biol*. 52(1):86-90. https://doi.org/10.3109/13880209.2013.815634

THENMOZHI, R., NITHYANAND, P., RATHNA, J., PANDIAN, S.K. 2009. Antibiofilm activity of coral-associated bacteria against different clinical M serotypes of *Streptococcus pyogenes*. *FEMS Immunol Med Microbiol* 57(2009):284-294. https://doi.org/10.1111/j.1574-695x.2009.00613.x

VENDY, V. 2014. Antiacne activity of marine sponge *Thalysia vulpina* extract *in vitro* [thesis]. Jakarta: Fakultas Teknobiologi, Universitas Katolik Indonesia Atma Jaya.

WANG, X., MIHAILOVA, B., KLOCKE, A., HEIDRICH, S., BISMAYER, U. 2011. Effect of artificial saliva on the apatite structure of eroded enamel. *International Journal of Spectroscopy* 2011:1-9. https://doi.org/10.1155/2011/236496

WOO, K.K., NGOU, F.H., NGO, L.S., SOONG, W.K., TANG, P.Y. 2011. Stability of betalain pigment from red dragon fruit (*Hylocereus polyrhizus*). *American Journal of Food Technology* 6(2):140-148. https://doi.org/10.3923/ajft.2011.140.148