Makara Journal of Technology

Volume 20 | Issue 3 Article 4

12-3-2016

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Recommended Citation
Sugiarta, Gede Yuda; Wiseso, Anggoro; Sari, Siska Yuliana; Kamila, Etri Dian; Geraldine, Vanessa; Christina, Diana; Hanifi, Muhammad; Satyapertiwi, Dwiantari; Hertanto, Robby; Bela, Budiman; Yohda, Masafumi; and Sahlan, Muhamad (2016) "Expression and the Functional Study of Fusion Proteins α-Amylase and Hemolysin- αas an Application in Biofilm Polysaccharide Degradation," *Makara Journal of Technology*: Vol. 20: Iss. 3, Article 4.
DOI: 10.7454/mst.v20i3.3067
Available at: https://scholarhub.ui.ac.id/mjt/vol20/iss3/4

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Makara J. Technol. 20/3 (2016), 127-131
doi: 10.7454/mst.v20i3.3067

Expression and the Functional Study of Fusion Proteins α-Amylase and Hemolysin- α as an Application in Biofilm Polysaccharide Degradation

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Abstract

Biofilm is an aggregate of consortium bacteria that adhere to each other on a surface. It is usually protected by the exopolysaccharide layer. Various invasive medical procedures, such as catheterization, endotracheal tube installation, and contact lens utilization, are vulnerable to biofilm infection. The National Institute of Health (NIH) estimates 65% of all microbial infections are caused by biofilm. Periplasmic α-amylase (MalS) is an enzyme that hydrolyzes α-1, 4-glicosidic bond in glycogen, starch, and others related polysaccharides in periplasmic space. Another protein called hemolysin-α (HlyA) is a secretion signal protein on C terminal of particular peptide in gram negative bacteria. We proposed a novel recombinant plasmid expressing α-amylase and hemolysin-α fusion in pSB1C3 which is cloned into *E.coli* to enable α-amylase excretion to extracellular for degrading biofilm polysaccharides content, as in starch agar. Microtiter assay was performed to analyze the reduction percentage of biofilm by adding recombinant *E.coli* into media. This system is more effective in degrading biofilm from gram positive bacteria i.e.: *Bacillus substilis* (30.21%) and *Staphylococcus aureus* (24.20%), and less effective degrading biofilm of gram negative i.e.: *Vibrio cholera* (5.30%), *Pseudomonas aeruginosa* (8.50%), *Klebsiella pneumonia* (6.75%) and *E. coli* (-0.6%). Gram positive bacteria have a thick layer of peptidoglycan, causing the enzyme to work more effectively in degrading polysaccharides.

Abstrak

Ekspresi dan Penelitian Fungsi Protein Fusi α-Amilase dan Hemolisin-α sebagai suatu Penerapan dalam Penurunan Polisakarida Biofilm. Biofilm adalah sekumpulan bakteria yang saling melekat satu sama lain pada suatu permukaan. Biofilm ini biasanya dilindungi oleh lapisan eksopolisakarida. Berbagai prosedur medis yang pro-aktif, seperti kateterisasi, instalasi alat bantuan pernafasan, dan penggunaan lensa kontak mata, rentan terhadap infeksi biofilm. NIH (*National Institute of Health* – Institusi Kesehatan Nasional) memperkirakan 65% dari semua infeksi mikroba disebabkan oleh biofilm. Enzim α-amilase periplasma (MalS) merupakan suatu enzim yang menghidrolisis α-1, ikatan 4-glikosidik dalam glikogen, zat tepung, dan lainnya terkait polisakarida pada ruang periplasma. Protein lainnya yang disebut hemolisin-α (HlyA) merupakan protein sinyal sekresi pada terminal C dari peptida tertentu dalam bakteria gram-negatif. C merupakan protein sinyal sekresi pada terminal C dari peptida tertentu dalam bakteria gram-negatif. Kami mengusulkan suatu plasmid rekombinan baru mengekspresikan fusi α-amilase and hemolisin-α dalam pSB1C3 yang diklon menjadi *E. coli* untuk memungkinkan ekskresi α-amilase ke luar sel tubuh (ekstraselular) untuk menurunkan isi polisakarida biofilm, seperti dalam agar zat tepung. Tes dengan tabung kecil dilakukan untuk menganalisis persentase pengurangan biofilm dengan menambahkan *E. coli* rekombinan ke dalam media. Sistem ini lebih efektif dalam menurunkan tingkat biofilm dari bakteria gram-positif, seperti *Bacillus substilis* (30.21%) dan *Staphylococcus aureus* (24.20%), dan kurang efektif menurunkan tingkat biofilm dari gram-negatif, *Vibrio cholera* (5.30%), *Pseudomonas aeruginosa* (8.50%), *Klebsiella pneumonia* (6.75%), dan *E. coli* (-0.6%). Bakteria gram-positif mempunyai lapisan peptidoglikan yang tebal, menyebabkan enzim untuk bekerja lebih efektif dalam menurunkan tingkat polisakarida.

Keywords: Alpha amylase, Biofilm, Hemolysin-α, Recombinant plasmid

1. Introduction

Biofilms are dense packed microbial cells that grow on surface and covered with secreted polymers [1]. The National Institute of Health (NIH) estimates 65% of all microbial infections are caused by biofilm. Biofilm causes 80% of chronic inflammation and it leads to device-related infection (DRI) [2]. According to the National Allergy and Infectious Disease (NAID) 2010, two million cases of medical device-related infection caused by biofilm infection [3]. Gram positive and gram negative bacteria, such as *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus viridians*, *Escherichia coli*, *Psedomonas aeruginosa*, *Klebsiella pneumonia*, and *Proteus mirabilis*, have been successfully isolated from various medical devices [4]. Biofilm is difficult to destroy because the bacteria inside the biofilm are highly resistant to antibiotics. Pipes, catheters, nasogastric tubes, and other medical devices that have been colonized by biofilms are usually replaced to remove the contaminant [5].

Synthetic biology is a new field with growing trends of novel solutions for old problems. We proposed a novel solution for biofilm infection by reprogramming bacteria using synthetic biology principles. The engineered *E. coli* is capable to produce α-amylase and secrete the enzyme to degrade biofilm in medical devices.

Alpha-amylase is an essential enzyme for *α-glucan* metabolism. It is also capable to catalyze the hydrolysis of α-1, 4-glicosidic bond in glycogen, starch, and some others related polysaccharides. *MalS* is a 1967 bp gene responsible for expression of α-amylase in *E. coli* strain K12 [6-8]. HlyA is a signal peptide found in C-terminal signal sequence of alpha-hemolysin. Gram negative bacteria use HlyA protein tag to secrete proteins via Type I secretion pathway. Fusion of the HlyA signal peptide with the target protein caused the secretion of target protein to extracellular medium in a single step. HlyA interacts with the cytoplasmic region of the preformed HlyB-D complex. After the binding of the HlyA secretion signal by the HlyB-D complex, HlyD induces the TolC interaction and exports the target protein to the extracellular space [9]. A former study which was conducted by Bisi DC & Lample DJ shows that anti-bovine serum albumin (BSA) scFv fused with HlyA tag has been successfully secreted into extracellular compartment [10].

2. Methods

Biofilm-producing bacteria are obtained from Clinical Microbiology Laboratory, the Faculty of Medicine Universitas Indonesia, consisting of *E. coli*, *Pseudomonas aeruginosa*, *Vibrio cholera*, *Klebsiella pneumonia*, *Staphylococcus aureus*, and *Bacillus substillis*. We used pSB1C3 plasmid as the expression vector. The plasmid carries chloramphenicol resistance gene and pUC19-derived pMB1 origin of replication. The pSB1C3 has terminators in the downstream of its multiple cloning sites to hinder transcription [11]. We used a strong constitutive promoter, whose activity is not governed by any transcription factors. Universal primers used to amplify all the genes were VF2 (5'-tgccacctgacgtctaagaa-3') and VR (5'-attaccgcctttgagtgagc-3').

The promoter, α-amylase, and hemolysin-α gene sequences are obtained from the International Genetically Engineered Machine (IGEM) standard registry of biological parts. The experiment was conducted in a biosafety level two (BSL-2) laboratory at the Institute of Human Virology and Cancer Biology (IHVCB), Universitas Indonesia.

Determining restriction sites and the primer design. The restriction sites used in this experiment were EcoRI, NotI, XbaI, DpnI, and PstI. The codon optimization was performed in order to enable *E. coli* cells to express the genes of interest.

Vector plasmid preparation. Plasmids were digested with restriction enzyme. The pSB1C3 with red fluorescent protein (RFP) was digested with EcoRI and the gene of interest was inserted into the vector. Plasmids containing the desired gene from "iGEM kit" were transformed into *E. coli* Top10. The *E. coli* was then cultured and the plasmids were isolated using Qiagen® plasmid isolation kit. Obtained plasmids were then digested and ligated into pSB1C3 using the standard cloning protocol.

Competent cells. The bacteria were cultured on *Luria Bertani* (LB) broth and incubated overnight. Overnight cultures were then added into 30 mL fresh media LB and incubated for 1.5 hours (OD 600 nm). The culture was put on ice for 30 minutes, then centrifuged 3500 rpm for 10 minutes at 4 °C. Supernatant was discarded. 6 mL $MgCl_2$ 100 mM was added and the cultures were incubated for one hour at 4 °C, then it was centrifuged again on 3500 rpm for 10 minutes. The supernatant was discarded and 0.2 mL $MgCl_2$ 100 mM was added. The competent cells were ready to be used [12].

Transformation of plasmid into *E. coli* Top10. The competent cells were thawed and labeled for each concentration. 1 µL of DNA and 50 µL of competent cells were added into each tube before chilled in ice for 30 minutes. The cells were heat-shocked by placing into 42 °C water bath for 1 minute. The tubes were immediately transferred back to ice and incubated for 5 minutes. 200 µL of SOC media was added per tube and incubated at 37 °C for 2 hours. 20 µL from each tube was added into the appropriate plate [12].

Verification of DNA plasmids. Verification of DNA plasmids was done by isolating the plasmids from *E. coli* culture. The isolated plasmids were then visualized

using the electrophoresis method to prove that the desired genes were successfully inserted to the plasmids. The electrophoresis was using 0.8% agarose gel in 100 mL 0.5× TAE with 100 mV voltages and run for 30 minutes.

Secretion test of alpha-amylase (MalS) on starch agar. Starch (1.5 g) was dissolved with 200 mL ddH_2O and mixed with 300 mL LB agar evenly by heating it in an oven. The wild type *E. coli*, recombinant *E. coli* containing plasmid for MalS and HlyA, and *E. coli* containing empty plasmids were then incubated for 18 hours and 24 hours. The agar was then washed using iodine. In this experiment, we compared the activity of α-amylase under the control of a strong promoter, α-amylase without a promoter and wild type *E.coli* as the negative control.

Anti-biofilm activity assay. Biofilm-producing bacteria were incubated in 50 mL LB Broth for 48 hours at 37 °C in a 200 rpm dynamic incubator. The cultures were then diluted using fresh broth with 1:100 ratio. The diluted cultures were placed into 96-well plate with each well containing 200 µl of culture, and further incubated for 20-28 hours in a 37 °C static incubator. The LB media were then discarded, leaving biofilm on the bottom of the well plate. Recombinant *E. coli* was then added into the 96-well plate and incubated for 6 hours. Then, the plates were washed gently and stained with 0.5% crystal violet, aspirated using 2% acetic acid in 96% ethanol (v/v). The absorbance was quantified in 595 nm ELISA Reader [13].

Micro titer assay for efficacy of biofilm removal. The micro titer assay was performed according to Molobela, *et al.* (2010) to quantify the biofilm removal by adding α-amylase [14]. The percentage of reduction in Eq. (1) was used as a measurement to evaluate the efficacy of this enzyme.

$$\frac{(C-B)-(T-B)}{(C-B)} x\,100\% \qquad (1)$$

Where B denotes the average absorbance per well for blank (no biofilm, no treatment); C denotes the average absorbance per well for control wells (biofilm, no treatment), and T denotes the average absorbance per well for treated wells (biofilm and treatment).

3. Results and Discussion

The resulting red and white colonies were screened to select the *E. coli* which carries the desired inserts (Figure 1). The white colonies indicate interested plasmids were successfully inserted into pSB1C3 plasmid backbone because the expression of RFP was interrupted with the expression of desired genes. After that, the cultures from selected colonies are visualized on electrophoresis to confirm the length of plasmid. Alpha amylase-hemolysin-α-fusion gene has a length of 2298 bp. The resulting electrophoresis band was located near 2500 bp, confirming that α-amylase and hemolysin-α have been fused (Figure 2).

The starch agar assay using classic iodine-polysaccharide interaction was performed to prove that recombinant *E. coli* cells were able to secrete α-amylase and degrade the starch. The degraded starch will be shown as a clear zone. The clear zones indicate that iodine molecules can no longer bind to the polysaccharides since those have been degraded into their monomer. The longer the incubation time, the more vivid a clear zone appears to be, as shown at the figure below (Figures 3, 4).

The result shows that the supernatant of the culture is able to degrade the starch. This infers that the α-amylase and hemolysin-α secretion tag recombinant protein has been expressed and secreted into the medium. However, we cannot absolutely conclude that the clear zone was formed because the enzymes were excreted by recombinant *E. coli*. This is because the wild type *E. coli* also has functional α-amylase in its periplasmic region, but not exported into extracellular media. If the bacteria are to

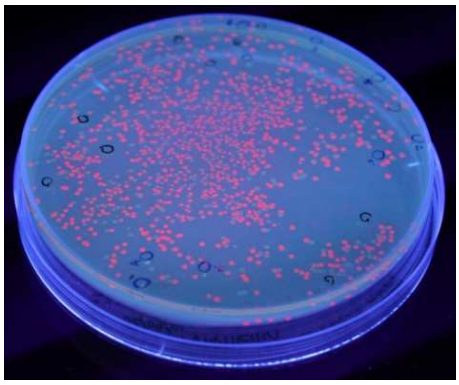

Figure 1. Red and White Colony Selection under UV (White Colony Denoted by Encircled Label)

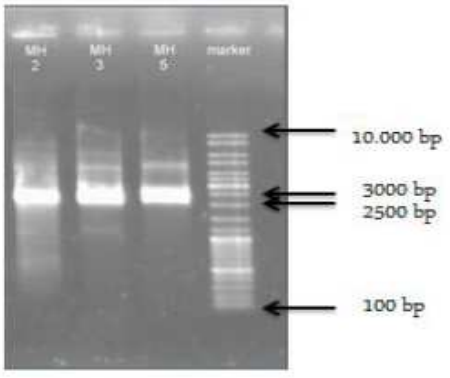

Figure 2. The Electrophoresis Pattern of Single Restriction of MalS-HlyA Fusion by Enzyme EcoRI

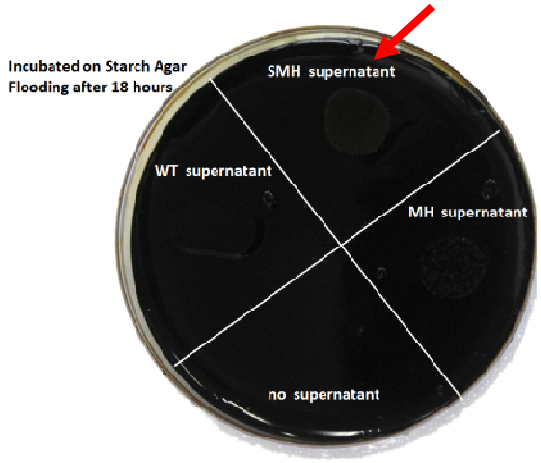

Figure 3. Incubation of 18 Hours of *E. coli* Expressing Recombinant Plasmid after Iodine Flooding Treatment; WT (Wild Type), SMH (Strong Promoter-MalS-HlyA), MH (MalS-HlyA). Red Arrow Pointed the Clear Zone

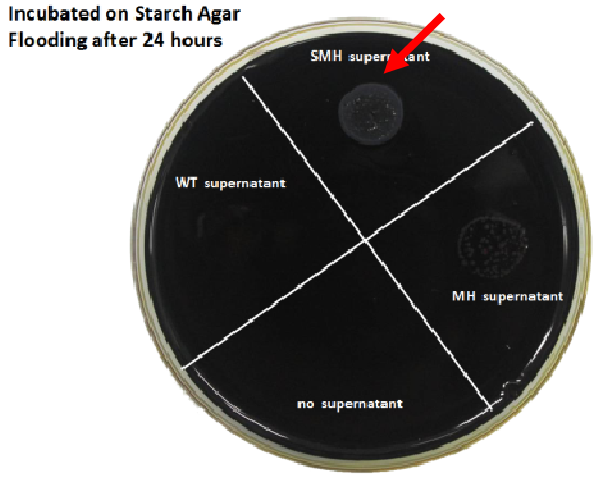

Figure 4. Incubation of 24 hours of *E. coli* Expressing Recombinant Plasmid after Iodine Flooding Treatment. The Clear Zone Appears more Vivid than Incubation of 18 Hours. Red Arrow Pointed the Clear Zone

be lysed, a clear zone may be formed because of the activity of periplasmic α-amylase. However, over expression of α-amylase under the control of a strong promoter as we did in our experiment can degrade polysaccharides more efficiently.

The reduction of biofilm quantified by micro titer assay shows surprising results as in Figure 5. Biofilm removal in gram positive bacteria is noticeably greater than in gram negative bacteria. In Figure 6, *Bacillus substilis* and *Staphylococcus aureus* biofilm show 30.21% and 24.20% of degradation respectively, higher than those of gram negative bacteria, such as *Vibrio cholerae* (5.3%), *Pseudomonas aeruginosa* (8.50%), *Klebsiella pneumonia* (6.75%), and *E.coli* (-0.6%).

Gram positive bacteria have a thick layer of peptidoglycan. Peptidoglycan consists of glycan backbone of muramic acid and glucosamine (both N-acetylated), and also other cell wall polymers, such as teichoic acid, polysaccharides, and peptidoglycolipids [15]. In contrast, gram negative bacteria have thinner peptidoglycan. It is suspected that more substrate for alpha amylase available causes an increase in an alpha amylase activity. Direct inoculation of *E. coli* on to *E. coli* cells does not cause biofilm degradation. In Figure 6, *E. coli* shows a negative percentage which means that *E. coli* cells undergo an overgrowth to form biofilm. However, future research should be done to purify and further characterize the α-amylase protein.

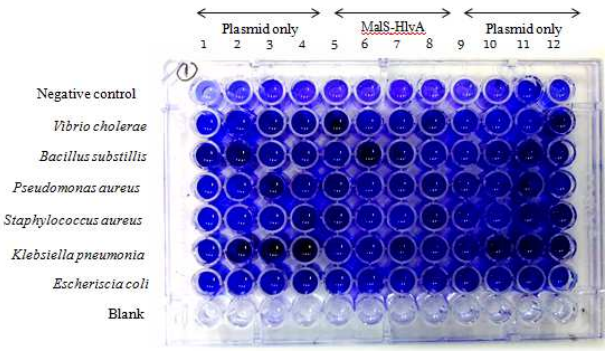

Figure 5. Biofilm Staining. The Darker Blue Color Indicates more Biofilm Residing The Well

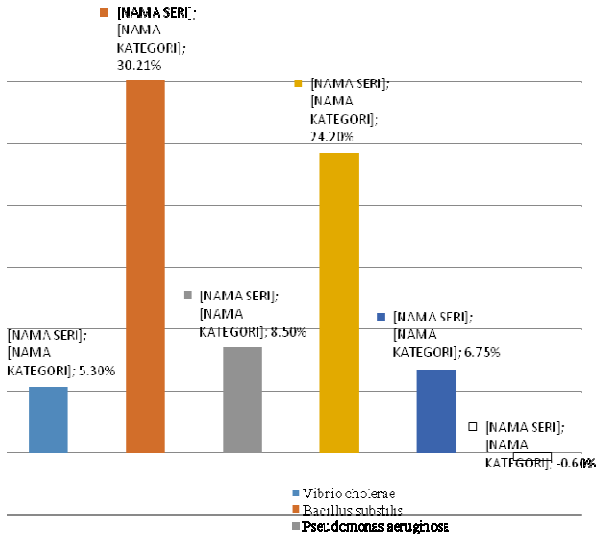

Figure 6. Percentage of Biofilm Reduction

4. Conclusions

Recombinant plasmid expressing protein fusion of α-amylase and hemolysin-α is able to be cloned in *E. coli* cells and works functionally. The protein fusion works more effectively in biofilm degradation of gram positive bacteria than gram negative bacteria. However, protein extraction should be performed to obtain the recombinant protein. For further applications, a comprehensive study is needed to assess the effectivity of recombinant protein erradicating biofilm in various medical devices.

Acknowledgements

This work was supported by the International Genetically Engineered Machine (iGEM) UI 2014 advisors and instructors: drg. Endang W. Bachtiar. M.Biomed, Ph.D, Dr. Drs. Abinawanto, S.Si, drh. Yulianty, M.Biomed, Nada Fitria, S.Si, M. Biomed, and the chief of biosafety and biosecurity of the Faculty of Medicine, Universitas Indonesia, Ms. Aroem Naroeni, D.E.A, Ph.D., and also supported by Tokyo University of Agriculture and Technology in the Short Visit Research Program that was awarded to RH.

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