**APPLICATION OF SEA SPONGE MICROSYMBIONTS AS A NEW BIOMATERIAL TO REDUCE CHROMIUM**

**HEAVY METAL TOXICITY**

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***Abstract****: The vulnerable marine environment is polluted by various types of heavy metals due to human and natural activities. One of the marine biota sponges can carry out heavy metal adsorption functions, so the life of the sponge must be well maintained. The application of sponge micro symbiont adsorption method aims to reduce the toxicity of chromium metal. Absorption stage: culture isolates code Sp1, Sp2, Sp3, Petrosia sponge (Strongylophore) Corticata symbionts, phenotypic and genotypic characterization, suspension symbionts were formed, interacted with 100 ppm of 1: 1 ratio chromium solution Interaction time variation 5, 10, 15, 20, 25 days, extracted. The remaining non-reduced chromium was analyzed using AAS. Observation of reduction, gas, pH, optical density parameters. The results of phenotypic characterization showed that 3 sponge symbiont isolates reacted with lactose, catalase, MR, VP and citrate. Genotypes of Pseudomonas stutzeri isolates, strain Sp1 are RCH2, Sp2 code SLG510A3-8, and Sp3 code GLB197. The reduction parameters reached a contact period of 5-15 days, the performance of the symbionts in the reduction of chromium metal toxicity in the range of 62.34 ppm to 70.47 ppm was equivalent to the reduction rate of 60.16 to 69.09% concentration.*

***Keywords****: chromium, microsymbiont, reduction, sponge, toxicity*

**1. INTRODUCTION**

Sponges are one of the marine biodiversity, often used as bioindicators for determining heavy metal status of pollution. The sponge life of filter feeder, makes the sponge as a unique animal that has many benefits and functions as well as mutual symbiotic potential with various microorganisms, such as bacteria, fungi and other microorganisms (Netty, *et al*., 2014). Another uniqueness inherent in the sponge besides the various colors is the ability to produce a substance that behaves like an enzyme, one of which is to protect itself from the threat of predatory animals and isolate themselves due to extreme environmental changes such as heavy metal pollution, poly aromatic hydrocarbons and turbidity due to strong underwater currents (Marzuki, *et al*., 2014; Ismet, *et al*., 2011).

Substances produced by sponges in response to changes in environmental habitat for the survival of the sponge, allegedly originated from microorganisms that nest in the body of the sponge. The development towards the application of micro symbionts as a new biomaterial to reduce heavy metal toxicity is the critical mind of scientists for the purpose of developing science and efforts to save the environment (Parama, *et al*., 2017; Alamri, 2012).

Heavy metal pollution that occurs both on land, sea and air is quite fragile, considering that there are quite a lot of heavy metal pollutants which in the end most of the pollutants come into the water and eventually enter the sea. Other sources of heavy metal pollution are transfortations of the sea, plastic waste and industrial activities including natural activities such as volcanic eruptions, volcanic lava flows (Lydia, *et al*., 2014). This heavy metal pollution load finally accumulates in the sea. This situation has a chain effect, where the resources from the sea (fish, seaweed, and other biota) are contaminated with heavy metals, ultimately affecting human health. This condition is a major problem in the future which requires systematic handling to minimize and reduce the increase in heavy metal pollution in the sea (Ismet, *et al*., 2011).

Development and application of sponge micro symbionts to reduce heavy metal toxicity is one of the efforts in saving the marine environment, and needs structural, systematic support, commitment, policy and alignment of all elements of humanity on earth, including universities in the scientific field.

**2. MATERIALS AND METHODS**

**Material**

Set catalog of standard biochemical tests 15 specific reagents, sterile seawater, NA media, marine agar (MA), 25% glycerol, 4% formalin, aqubides, ddH2O, 20% chelex, a pair of universal primary sequences of 16S rRNA E. coli gene: FP-U1(5'-CCAGCAGCCGCGGT AATACG-3') at nucleotides 518-537, and RP-U2 (5'-ATCGG(C/T)TACCTTGTTACG ACTTC-3') nucleotides 1513-1491, DNA template, Taq DNA polymerase (Perkin Elmer, Norwalk, Conn), PCR mixture, Triton X-100, Tris, EDTA, HCl, KCl, MgCl2, paraffin, tripophosphate deoxynucleoside, HCl pa, Cr2O3 pa, 96% alcohol. Applied Biosystem (ABI) 9700 type Thermal Cycler, Spectronic-20D + Shimadzu, AAS type AA240FS Variance, Shaker, incubator, glass set, incubator, laminary air flow, micropipette, mortar, round ose, oven, tweezers, pipettes, tube racks, horn spoon, micro pipette, test tube, analytic scales, glass decks, Erlenmeyer, handskun, spirits, mortar, pH indicator, counter colonies, matches, masks, microscopes, glass objects, water baths. Sponge *Petrosia (Strongylophore) Corticata*. The sponge was obtained from the beach of Kodingareng Keke part of the Spermonde archipelago, the sampling point S : 050 06 '06.76 "E : 1190 17' 10.66" ", salinity 29.3 ‰, temperature 28 0C.

**Experiment  
*Sample Preparation***

Phenotypic and genotypic characterization of the three sponge isolates were carried out, each using biochemical test methods and PCR applied biosystem. Selected isolates were multiplied by culture method on Nutrient Agar media, then isolates were made into suspension by adding 2 ml of 0.9% physiological NaCl, shaken. Add the suspension to the 100 mL Erlenmeyer, enough volume to use physiological 0.9% NaCl. Each pipette is 10 ml of suspension into 5 pieces, then incubated for 1 x 24 hours. Enter 10 ml of 100 ppm chromium [Cr(III)] solution. Enter into the sheaker incubator with each contact time (5, 10. 15, 20, 25) days for each pial. The sample is filtered using whattman 41 filter paper, the filtrate is obtained, acidified with hydrochloric acid (pH 3-4), then concentrated (Akinde, *et al*., 2012).

***Determination of metal content in the sample***

Determination of Cr(III) absorbance, measured using AAS at λmax: 357.9 nm. Determination of Cr(III) concentration was not absorbed, it was done by plotting absorbance into series calibration curves of 1 ppm, 10 ppm, 20 ppm, 30 ppm and 50 ppm. Absorption value is obtained in the range of calibration values of the standard solution so that the metal concentration of Cr(III) in the sample can be calculated using the regression line equation (Marzuki, 2016).

**Characterization  
*Fenotype Analysis***

The biochemical test method was chosen as the basis for the phenotypic analysis of sponge isolates, aiming to see the tendency of reactions to several specific reagents in biochemical tests.

***Polymerase Chain Reaction (PCR) Analysis***

Molecular biology methods are used to make copies of segments of sponge isolate DNA sequences exponentially strengthened to produce thousands to millions of copies of DNA isolate segments to determine species and isolates (genotypes). The type of PCR used is Applied Biosystem (ABI) 9700 Thermal Cycler (Marzuki, *et al.*, 2015).

***Spectrophotometry (Spectronic-20D +) Analysis***

Determination of optical density of chromium reduction media by symbiont suspension was measured every 5 days of contact. Optical density is one of the parameters for chromium metal reduction by sponge symbiont. Spectronic-20D + Shimadzu λ: 400-700 nm

***Atomic Absorption Spectrophotometer (AAS) Analysis***

Used to determine the chromium concentration that is not reduced after the contact period is reached. The basis of determination of concentration based on the absorbance measured by AAS type AA240FS Variance according to cathode lamp at λmax: 357.9 nm.

**3. RESULTS AND DISCUSSION**

**Isolation of micro symbiont sponges**

Isolation of sponges *Petrosia (Strongylophore) Corticata* using streak plate (culture) method produced three (3) kinds of isolates code Sp1, Sp2, and Sp3, with the characteristics as in Table 1, as follows:

**Table 1. Results of Isolation and Gram Staining of Symbionts of Sponge *Petrosia (Strongylophore) Corticata***

|  |  |  |
| --- | --- | --- |
| **Symbiont code** | **Isolate Morphology** | **Conclusion** |
| Sp1 | round shape, bluish cream color, dispersed clustered, stem shape, fixed color with safranin, endospores less clear, insoluble with alkaline 1% KOH | Gram positive |
| Sp2 | jagged rod shape, brown color, separate spread of fixed colors with safranin reagent, there is endospora, insoluble with alkaline 1% KOH | Gram positive |
| Sp3 | round shape, brown color, separate spread, color changes with safranin reagent, no endospores, soluble in 1% KOH alkali | Gram negative |

Three symbionts from isolation of *Petrosia (Strongylophore) Corticata sponge*, two of them (Sp1 and Sp2) are gram-positive symbionts, determined based on the reaction with safranin which does not change color and does not dissolve with 1% KOH reaction, while Sp3 symbionts are group gram negative (Marzuki, *et al*., 2016).

**Analysis of Sponge Microscopy Phenotype**

The principle of micro symbionts phenotype analysis is symbiont reaction with specific reagents to see the nature of acid-base symbionts, the availability of enzymes and the ability to ferment and can respond to environmental changes that occur, such as heavy metal pollution. The phenotypic characteristics of the sponge of symbiont *Petrosia (Strongylophore) Corticata*, can be seen in Table 2, as follows:

**Table 2. Analysis of Symbiont Phenotypes of Sponge**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Number | Code | | Symbiont Sponge *Petrosia (Strongylophore) Corticata* | | |
| Sp1 | Sp2 | Sp3 |
| 1 | TSIA | Stlant | Base | Base | Base |
| 2 | Butt | acid | acid | acid |
| 3 | H2S | - | - | - |
| 4 | Gas | - | - | - |
| 5 | SIM | Indol | - | - | - |
| 6 | Motirety | - | - | - |
| 7 | H2S | - | - | - |
| 8 | MR/VP | MR | + | + | + |
| 9 | VP | - | + | - |
| 10 |  | Citric | + | - | - |
| 11 |  | Urea | - | - | - |
| 12 |  | Glucose | - | - | - |
| 13 |  | Lactose | + | + | + |
| 14 |  | Sucrose | - | - | - |
| 15 |  | Mannitol | - | - | - |
| 16 |  | catalase | + | + | + |

The results of phenotypic analysis of three types of symbionts showed a positive reaction to the red methyl (MR) reagent indicating that symbionts can carry out fermentation activities, and the resulting fermentation reaction is acidic. The reaction with the positive results of the Voges-Proskauer (VP) reagent is shown by Sp2, meaning that the symbiont is capable of carrying out the fermentation reaction. In the reaction with catalase reagents, the three symbionts show positive results, meaning that the symbionts in their activities require hydrogen and produce H2O2 gas which is potentially toxic to itself. There is also a positive reaction with lactose which shows that symbionts can react to breakdown of carbohydrates into simple sugars and the results of positive reactions with citrate are shown by the Sp1 sample, indicating that this symbiont is capable of fermenting reactions using carbon as a source of energy (Liu, *et al*., 2017; Marzuki, *et al*., 2016).

**Micro symbiont Sponge Genotype Analysis**

Molecular molecule 16S rRNA gene characterization *Petrosia (Strongylophore) Corticata* is carried out by identifying the sequence of DNA gene molecules through the Polymerase Chain Reaction (PCR) method. The DNA gene sequencing results obtained in the form of chromatogram form data, then the gene molecule is sorted and stretched to see the bank gene homologous series. The chromatogram obtained is processed using the Bio Edit program software version 7.2.5. The data that has been processed by the Bio Edit program is used as a basic data to be reprocessed in multiple alignment sequences of isolate gene molecules on the DNA molecule database that is in Gene Bank. The 16S rRNA gene molecule is universal in bacteria, in general it can be compared with the sample RNA sequence (Liu, *et al*., 2017; Marzuki, *et al*., 2016).

The results of sequencing of the isolates were opened through the bio edit program, then the sequence of bacterial DNA samples were entered into the BLAST (Basic Local Alignment Search Tool) program, (http://blast.ncbi.nlm.nih.gov/Blast.Cgi), sequences identified with the DNA database Gen Bank on that site. The results of the sample sequence alignment with the Gen Bank sequence show the similarity of the high homologous series, which can be seen in Table 3, as follows:

**Table 3. Results of BLAST (Basic Local Alignment Search Tool) Symbiont Sponge *Petrosia (Strongylophore) Corticata***

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Number | Symbiont  code | Sample Sequence | Sequence *Gen Bank* | Identity | Difference | Not DNA | Species |
| 1 | Sp1 | 17-972 | 608.723-609.890  (955) | 934/955 (97,80%) | 4/955  (0,42%) | 17/955 (1.78%) | *Pseudomonas stutzeri* strain RCH2 |
| 2 | Sp2 | 11-985 | 524.589-525.563  (974) | 927/974  (95,17%) | 17/974  (1,75%) | 30/974 (3,08%) | *Pseudomonas stutzeri* strain SLG510A3-8 |
| 3 | Sp3 | 21-935 | 574.123-575.079  (956) | 922/956 (96,44) | 6/956 (0,63%) | 28/956 (2,93%) | *Pseudomonas stutzeri* strain GLB197 |

The final result of the genotype analysis is that sponge symbiont species are determined based on the suitability of the homologous sequence of the sample sequence with the gen bank sequence. Species of three spongy symbionts *Petrosia (Strongylophore) Corticata* analyzed are Pseudomonas group, meaning that they generally have the same relative characteristics, although there are special characteristics that these three symbionts have as identifiers and are the differentiator of the three in the activity and reaction mechanism that will be occupied. This difference can be seen in the differences in the strains of each symbiont (Liu, *et al*., 2017).

***Heavy Metal Reduction Parameters***

In general, all microorganism activity occurs, based on fermentation reactions, although the mechanisms that are used can vary, due to differences in phenotypes and symbiont genotypes. Parameter of reaction of microorganism fermentation, including activity in reducing heavy metal toxicity can be seen in Table 4, as follows:

**Table 4. Parameters Value of Heavy Metal Reduction Cr (III) Based on Contact Time**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Number** | **Symbiont code** | **Parameters reduction** | **Contact time (days)** | | | | | |
| **0** | **5** | **10** | **15** | **20** | **25** |
| 1 | Sp1 | pH | 7 | 6 | 7 | 7 | 7 | 7 |
| gas bubble | - | √ | √ | - | - | - |
| Optical Density (OD) | 0.008 | 0.039 | 0.078 | 0.082 | 0.086 | 0.087 |
| 2 | Sp2 | pH | 7 | 6 | 6 | 7 | 7 | 7 |
| gas bubble | - | √ | √ |  | - | - |
| Optical Density (OD) | 0.007 | 0.041 | 0.081 | 0.087 | 0.093 | 0.101 |
| 3 | Sp3 | pH | 7 | 6 | 7 | 7 | 7 | 7 |
| gas bubble | - | √ | √ | - | - | - |
| Optical Density (OD) | 0.007 | 0.052 | 0.090 | 0.093 | 0.096 | 0.097 |

The parameter value of chromium reduction by sponge symbiont of *Petrosia (Strongylophore) Corticata*, illustrates that the maximum reduction of chromium heavy metal toxicity occurs in 5 to 10 days of contact. Parameters of decreasing pH value, formation of gas bubbles and increasing optical density are evidence that sponge symbiont fermentation reactions reduce toxicity of heavy metal chromium at contact periods of 5 to 10 days (Ziarati, *et al*., 2018; Shama, *et al*., 2010).

**Performance of Chromium Heavy Metal Reduction by Symbiont Sponge**

Decrease in chromium metal concentration after contact with sponge symbiont suspension is interpreted as weakening of metal toxicity, as a result of sponge symbiont work. The performance of sponge symbionts decreases chromium concentration based on certain contact periods can be seen in Table 5, as follows:

**Table 5. Reduction Performance Concentration of Heavy Metals Cr (III) by Symbionts Sponge *Petrosia (Strongylophore) Corticata* Based on Contact Time**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Number** | **Symbiont code** | **Contact Time (days)** | **Average Absorbance** | **Average Level of Cr (III) (ppm)** | **Percent (%) Absorption** |
| 1 | Sp1 | 0 (early) | 0.1171 | 102.03 | - |
| 5 | 0.0603 | 39.69 | 61.10 |
| 10 | 0.0547 | 35.96 | 64.76 |
| 15 | 0.0543 | 35.87 | 64.65 |
| 20 | 0.0542 | 35.93 | 64.48 |
| 25 | 0.0543 | 35.91 | 64.46 |
| 2 | Sp2 | 0 (early) | 0.1181 | 104.04 | - |
| 5 | 0.063 | 41.45 | 60.16 |
| 10 | 0.0593 | 39.04 | 62.48 |
| 15 | 0.0537 | 35.31 | 66.06 |
| 20 | 0.0532 | 34.95 | 66.41 |
| 25 | 0.0533 | 34.95 | 66.41 |
| 3 | Sp3 | 0 (early) | 0.1170 | 102.02 | - |
| 5 | 0.0493 | 32.46 | 68.18 |
| 10 | 0.0487 | 31.86 | 68.77 |
| 15 | 0.0482 | 31.54 | 69.08 |
| 20 | 0.0483 | 31.56 | 69.10 |
| 25 | 0.0482 | 31.55 | 69.09 |

The description of the reduction value of chromium concentration according to Table 5, by adsorption of symbiont cells for fermentation reaction mechanism. Decrease in chromium concentration occurs significantly during contact periods of 5-10 days. The decrease in slope concentration after the 10th day of contact continues to stagnate after 15 to 25 days. The performance of sponge symbionts *Petrosia (Strongylophore) Corticata* reduces the toxic properties of chromium metal significantly occurring during 5 to 10 days of contact (Table 5) is estimated to be based in the data in Table 2 (phenotypic characteristics) and Table 4 (reduction parameters). Reduction of chromium metal toxicity by sponge symbiont stopped after a 15-day contact period caused by several factors: (1) one of the reduction products is H2O2 which is toxic to the symbiont, so the cells cannot divide even die, (2) the reduction in the pH of the reducing medium not in accordance with the conditions of the symbiont cell needs to defend themselves (Zhou*, et al*., 2017).

**Picture 1.** Chromium Uptake Concentration by Symbiont Sponge Based on Contact Time

Figure 1, above shows that the performance of sponge symbiont cells reduces the toxicity of maximal chromium metal occurs in the range of contact periods of 5 to10 days, although it is known that 5 to 10 days of cell microorganisms in the growth and cell division phase, but this condition does not work because it is hampered by metal reduction products by symbionts and inhibits the performance of symbiont cells to carry out further fermentation reactions. Chromium metal adsorption by enzymes that are owned by symbionts through fermentation reaction pathways. One of the reaction products is peroxide poison gas, which causes the growth of symbiont cells to become blocked and eventually die (Vaezzadeh, *et al*., 2017; Pawar, *et al*., 2017).

**4. CONCLUSION**

Sponges of symbiont *Petrosia (Strongylophore) Corticata* can reduce the toxicity of heavy metal chromium in the range of 62.34 ppm to 70.47 ppm achieved in the contact period of 5 to 25 days. Significant performance of symbiont reducing chromium metal toxicity occurs during 5-10 days of contact. The selection of potential sponge symbionts as biomaterials for reducing heavy metal toxicity can be determined based on the characteristics of the symbiont phenotype.

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