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Degradation performance and microencapsulation of hydrolytic bacterial consortium formulated as bioremediation agent of liquid biomedical waste

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Abstract. This study aimed to develop bioremediation agent with bacterial cells as components for the treatment of biomedical wastes from two hospitals in Semarang City (Central Java), i.e. Roemani Muhammadiyah (coded R1) and Wongsonegoro (coded R2). Single isolates and consortium of indigenous hydrolytic bacteria characterized as multiple hydrolytic enzyme producers with low- to non-pathogenic properties obtained from previous study were tested for their degradation performance. The degradation performance test is necessary to formulate components of bacterial consortium as bioremediation agent. The tests were conducted on the selected bacteria as single isolate and as consortium. The six bacteria tested as single colonies and as consortium were *Bacillus velezensis* R1.3, *B. amyloliquefaciens* R1.6, *B. amyloliquefaciens* R1.14, *B. velezensis* R1.16, *B. licheniformis* R2.5, and *B. amyloliquefaciens* R2.9. Degradation performance on biomedical waste mainly containing organic matters was assessed based on water pollution parameters on 4.0-L samples. They included a control, 6 samples treated with bacteria as single colonies, a sample treated with bacteria as indigenous consortium and a sample treated with bacteria as mixed consortium. Parameters of wastewater pollution measured included COD (Chemical Oxygen Demand), BOD (Biological Oxygen Demand), TSS (Total Soluble Solid), NH_3, and PO_4. Next, encapsulation of a bacterial consortium as the best condition for degradation was also carried out using maltodextrin to allow storage and preservation of the bioremediation agent for longer period. The encapsulated product was visualized in SEM images to evaluate its quality. The results showed that a consortium comprising 4 indigenous bacterial isolates from R1 hospital could decrease BOD of biomedical wastewater by 85% and TSS by 43%. Those from R2 showed performance in reducing PO_4 by 21%. This study demonstrated that compared to single isolates and mixed bacterial consortium tested, the indigenous hydrolytic bacterial consortium showed better ability in improving BOD and TSS of liquid biomedical waste of R1 hospital.


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1. Introduction

The quantity of hospital biomedical waste, which is infectious and contaminating other waste, is on the rise. This is due to the increasing number of hospitals and centers health services as the producer of waste. The increasing amount of biomedical wastes is one of the world's health problems requiring an immediate solution [1-3]. In the operation of incinerator, there are fire risks, injuries and toxicological effects on the operator. In Philippines, hospital incinerators have been replaced by autoclaves and microwaves. Such replacement has reduced the production of 3.8 g of ITEQ dioxin and furans each year [4-5].

In a number of developing countries including Indonesia, the handling of liquid biomedical waste in city hospitals is still inadequate [6]. Not only in Indonesia, in many countries, the handling of biomedical waste still relies use of methods that are not environmentally friendly such as incineration, or expensive such as autoclaves and WWTP or wastewater treatment plant [5].

Bioremediation is remediation of polluted sites using microbial process [7]. It is a biological mechanism of recycling wastes in to another form that can use and reused by other organisms. The main principle of bioremediation is degrading and transforming pollutants metabolizing them in enzymatic ways [8]. The non-incineration method is the method recommended by the World Health Organization in 2017 for the management of biomedical waste in Indonesia. Based on these recommendations, bioremediation as a non-incineration method is considered appropriate for the handling of biomedical waste because it is environmentally friendly and more economical [3,6].

The group of bacteria producing hydrolytic enzymes that break down organic matter plays an important role in degradation of biomedical waste whose main component is organic matter. Hydrolytic bacteria producing a number of hydrolytic enzymes could serve as potential candidates for remediating and reducing the organic load of wastewaters [9]. Based on the results preliminary research, hospital biomedical waste proved to be a source of bacteria hydrolytic. A total of 26 biomedical waste indigenous bacteria isolates from two hospitals in Semarang City successfully cultured and each one is able to produce one or more hydrolytic enzymes [1]. Five of the 26 hydrolytic bacterial isolates were identified as members of the *Bacillus genus* [10].

The quality of wastewater is determined by parameters such as pH, COD, BOD, nitrate, TSS, TDS, and PO_4, which could describe the quality of wastewater [11]. It means that the test of the bacterial bioremediation ability in wastewater could be done by measuring the ability of these bacteria to improve the pollution parameters of existing wastewater [3]. Tests for bacterial bioremediation ability in the form of single isolates and consortia have been carried out. Generally, the ability of bacterial bioremediation in the form of a consortium is higher than in the form of a single isolate [12].

The development of bioremediation agent from a group of bacteria in liquid culture has the disadvantage of decreasing the viability and performance of the bacteria during the storage period, and the need for continuous rejuvenation of the culture [13]. Encapsulation could overcome such problems by providing protection on bacterial cells in dry forms. The use of encapsulating materials such as biopolymers or semipermeable membranes will facilitate the use and packaging and increase the shelf life of bacteria used as starters [14-15]. Encapsulant could be selected from a variety of polymers based on the characteristics of the microcapsules. The commonly used encapsulant substance is maltodextrin. Maltodextrin is starch derivative compounds showing high viscosity, yet low solubility in water. It is widely used as micro-carrier because of its low cost [16]. This study aimed to test degradation performance of ingle isolates and consortia of indigenous and non-indigenous hydrolytic bacteria characterized as multiple hydrolytic enzyme producers with low- to non-pathogenic properties. The test is necessary to formulate components of bacterial consortium as bioremediation agent of liquid biomedical waste of Semarang hospitals. Next, encapsulation of the best bacterial formulae for degradation was also carried out using maltodextrin to allow storage and preservation of the bioremediation agent for longer period.

2. Materials and methods

2.1. Materials

Main chemicals used for BOD test include mineral-free water, $MgSO_47H_2O$, $KHPO_4$, $CaCl_2$, $Na_2HPO_47H_2O$, and NH_4Cl. Chemicals required for COD test were phenanthroline mono-hydrate ferro-ammonium sulphate (FAS), $K_2Cr_2O_7$), ferroine indicator, potassium hydrogen phthalate (KHP), sulphamic acid, $HgSO_4$, and $FeSO_4.7H_2O$. TSS determination procedure needs glass-fiber filter type E-D Scientific Specialities grade 161 (VWR brand grade 161) with particle retention of 1,1 µm recommended for use in TSS/TDS testing in water and wastewater and filter with size of 0,45 µm. Reagents needed to determine NH3 level were 2% of $Na_2Fe(CN)_5NO.2H_2O$, NaOH, NaOCl), phenol (C_6H_5OH), buffer Na_3PO_4, NH_4Cl, and $CHCl_3$. For PO_4 test, chemicals needed were H_2SO_4, $K(SbO)C_4H_4O_6.1/2H_2O$, $(NH_4)_6Mo7O_{24}.4H_2O$, $C_6H_8O_6$, and KH_2PO_4 (all from Merck, Germany). Materials needed for the formation of microcapsules include: bacterial consortium consisting of obtained from previous study *Bacillus velezensis* R1.3, B. *amyloliquefaciens* R1.6, B. *amyloliquefaciens* R1.14, B. *velezensis* R1.16, B. *licheniformis* R2.5, and B. *amyloliquefaciens* R2.9 [1], maltodextrin (from CV Multi Kimia Raya, Indonesia) as micro-carrier, aquadest, and nutrient broth medium (Sigma-Aldrich, UK).

2.2. Methods

All works related with hospital wastewater were handled by wearing anti-infection protection kit (Dupont). The bacterial samples used in this study were isolated from primary reservoir of liquid biomedical waste of B- and C- class hospitals, (Roemani Muhammadiyah Hospital, R1) and (RSUD KRT (Rumah Sakit Umum Daerah Kanjeng Raden Tumenggung) Wongsonegoro Hospital, R2) at Semarang, Central Java.

Selection of indigenous, non-pathogenic, hydrolytic bacterial isolates had previously been carried out in previous study 1-2,6]. Six single isolates of *Bacillus velezensis* R1.3, *B. amyloliquefaciens* R1.6, *B. amyloliquefaciens* R1.14, *B. velezensis* R1.16, *B. licheniformis* R2.5, and *B. amyloliquefaciens* R2.9 were known to be able to hydrolyse lipid, protein, amylum and cellulose substrates [1]. Synergism interaction among them had also been previously confirmed [17] These strains previously stored in glycerol at 80ºC were sub-cultured in 100-mL of NB media of each to reach OD = 1. The same was also carried out for a control, indigenous- and non-indigenous-mixed-consortia resulting in total 36 of 100-mL starters because all were prepared in duplicates for 2 hospitals (R1 and R2). Each starter (100-mL with OD=1) was then mixed with each of 36 samples of 4.0-L untreated hospital wastewater in each of 36 5.0-L plastic vessels [a control, 5 single isolates and 2 consortia coded A-I in duplicate]. Composition of bacteria in each starter is always equal, i.e. in a 3-isolate mix, the ratio of each of different strain = 1:1:1; in a 2-isolate mix = 1:1. The resulted mixtures were all kept in the dark place for 5 days prior to performance analysis to allow degradation process. During the storage, the vessels were left closed without aeration, nor stirring.

2.2.1. Degradation performance test

The initial screening of bacterial isolates for bioremediation studies was conducted with medium containing untreated hospital wastewater. The wastewater characterization test was done before and after bacterial administration. Wastewater parameters encompass all water pollutant parameters such as pH, BOD_5, COD, TSS, and PO_4, adopted from Standard Methods for the Examination of Water and Wastewater [18]. All wastewater quality analysis procedures of in this part referred to Standar Nasional Indonesia – SNI, which is equal to International Standard Methods for the Examination of Water and Wastewater [19]. The procedures to conduct BOD_5 and COD tests were according to SNI 6989.72:2009 and SNI 06-6989.15-2004. Determination of TSS was done by following instruction of SNI 06-6989.3-2004. As references of procedure for NH_3 and PO_4 determination were SNI 19-7119.1-2005 and SNI 06-6989.31-2005. All of these tests were conducted at Regional Health Laboratory, Semarang City, Central Java, Indonesia.

2.2.2. Microencapsulation

Microencapsulation was carried out on the consortium of selected bacteria showing the best performance in improving water pollution parameter. Maltodextrin was used as micro-encapsulating agent through freeze drying process following previously reported method [20]. Common microencapsulation composition was used, but with modification as follows (considering double-size of the developed freeze dryer machine): 2 g of bacterial biomass (obtained by centrifugation at 150 RPM), 1 g of maltodextrin and 500 mL ddH_2O. The obtained mixture was homogenized using shaker and then placed in freezer to allow dormancy phase of bacterial cells. The homogenized liquid was finally transferred into freeze dryer, separated into 4 racks, which were arranged vertically.

The steps taken in microencapsulation process are shown in Figure 1. The initial steps of encapsulation include cultivation of bacterial cells of consortium using 50-ml NB medium in 250-ml Erlenmeyer flasks at 37°C on a rotary shaker at 150 rpm (Figure 1. A). This step was followed by transferring LB medium containing to centrifugation flask (Figure 1. B). Centrifugation was conducted at 150 rpm (Figure 1 C) and the result is shown in Figure 1.D. The freeze-drying process using a freeze dryer prototype of Faculty of Agriculture (Universitas Gadjah Mada), from 2 lower dryer racks resulted properly dried microcapsules (Figure 1.E), while using that from 2 upper racks resulted in slightly wet microcapsules (Figure 1.F). The properly dried microcapsules seen in Figure 1.E were then morphologically analysed sing SEM.

Figure 1. Steps of preparation, mixing and product obtained from microencapsulation process of selected bacterial consortium. A. Cultivation of bacterial cells. B. Transfer of LB medium containing to centrifugation flask. C. Centrifugation at 150 rpm. D. Result of centrifugation step. E. Dried microcapsules generated from freeze-drying process using a freeze dryer prototype of UGM, collected from 2 lower dryer racks. F. Wet microcapsules collected from 2 upper dryer racks. SEM imaged of properly dried microcapsules.

2.2.3. SEM analysis

Microcapsules resulted from this step were subjected to SEM (Scanning Electron Microscopy) for cell surface characterization [21]. The products were collected in a sterile glass bottle, and obtained

powdery particles were observed morphologically using SEM (JEOL JSM-6380 LA, Japan) following instructions from the manufacturer in 150x and 500x magnification at 5 kV.

3. Results

3.1. Degradation test

In this study degradation performance tests of selected non-pathogenic bacterial as single (indigenous or non-indigenous), as well as indigenous and non-indigenous-mixed consortium from both hospitals (R1 and R2) were conducted in duplicate. Average of the results were summarised in Table 1. Based on data of results displayed in Table 1, several key findings could be highlighted as follows: Overall, performance of indigenous hydrolytic bacteria from R1 hospital (A-I) was better than that of R2 hospital (J-R) in decreasing liquid waste parameter values (with # symbol) from control.

Table 1. Test results of decreasing liquid waste parameters by selected isolates and bacterial consortium.

Bacterial Degradation Performance on Biomedical Wastewater of Roemani Muhammadiyah Hospital (R1)						
Sample code	*Treatment*	*TSS**	*NH_3**	*PO_4**	*BOD**	*COD**
A	Control (without bacterial treatment)	61	61	14	97	207
B	Single R1.3 isolate	48#	80	23	141	296
C	Single R1.6 isolate	82	89	23	177	381
D	Single R1.14 isolate	75	87	27	14#	305
E	Single R1.16 isolate	109	56#	24	165	350
F	Single R2.5 isolate	42#	85	19	108	230
G	Single R2.9 isolate	46#	80	19	116	242
H	Consortium from R1 + R2 hospitals (isolates: R1.3, R1.6, R1.14, R1.16, R and R2.9)	47#	86	23	97	207
I	Consortium from R1 hospital only (4 isolates: R1.3, R1.6, R1.14, and R1.1	35#	85	24	15#	320
J	Control (without bacterial treatment)	111	64	38	95	201
K	Single R1.3 isolate	139	88	43	158	335
L	Single R1.6 isolate	128	82	45	154	332
M	Single R1.14 isolate	159	75	30#	173	366
N	Single R1.16 isolate	116	93	27#	161	335
O	Single R2.5 isolate	125	82	27#	153	326
P	Single R2.9 isolate	79#	89	30#	138	292
Q	Consortium from R1 + R2 hospitals (isolates: R1.3, R1.6, R1.14, R1.16, R and R2.9)	129	90	39	164	347
R	Consortium from R1 hospital only (4 isolates: R1.3, R1.6, R1.14, and R1.1	133	90	30#	178	371

*Average of duplicate test values; #: Lower value than that of control

The indigenous consortium bacteria (I) could reduce both TSS parameters by 39% and BOD5 by 85% whereas mixed consortium bacteria could only reduce TSS values by 21%, but not the BOD_5 one (H). Single R1.14 isolate has the best ability in terms of decreasing BOD_5 value of wastewater sample by 86%, however, it could not improve TSS value. The ability of the bacterial consortium (I) to reduce TSS parameter value was the highest of all. Yet, actual values of TSS and BOD of I are believed to be even lower than what reported here. It is because the degradation test of samples was only performed up-to 5 days in BOD_5 test, which means that only about 68% of total organic waste was degraded by bacteria. This is based widely accepted understanding that keeping dissolved oxygen available at particular temperature, up to 99 % of total BOD could be exerted by bacteria within 20 days (BOD_{20}), 90 % within 10 days (BOD_{10}), and 68 % within 5 days (BOD_5) [22].

In case of R1 hospital, in overall, even at its best performance in reducing BOD and TSS, the indigenous consortium has not been able to overcome the values of other pollution parameters, NH_3, PO_4 and COD. Only R1.16 isolate could decrease NH_3 parameter. The rest of treatment seemed to even worsened the values of PO_4 and COD. As described in the method, as starter we used 100 mL liquid containing each sample (A - I) in media with OD = 1 to be directly added 4.0 L of raw wastewater from primary tank. The use of NB media used as a starter medium is likely contribute to the increasing levels of NH_3, PO_4 and COD. If using dry starter, additional burden of medium to the waste could be eliminated. Consequently, the ability of an indigenous consortium (I) to reduce the value of TSS and BOD_5 parameters could be better than reported. Else, the results could mean that more specific enzymes of bacteria other than hydrolytic ones are required to digest NH_3, PO_4 and COD molecules in biomedical waste.

In R2 hospital seen on Table 1, isolate R1.6, despite its properties as non-pathogenic hydrolytic enzyme producer, did not contribute to degradation. Instead, it seemed to decrease the consortium's ability to exert organic waste. In addition, isolate R1.6 only (L) even caused higher TSS and BOD_5 values than untreated (control) wastes. In this case, the ability of the indigenous consortium (R) to reduce the value of TSS and BOD_5 parameters could be also expected to increase if isolates R1.6 (L) were excluded from the consortium. Interestingly the indigenous consortium (R) along with several single isolates (M-P) could improve decrease of PO_4 values.

Biochemical oxygen demand (BOD) is one of the most important and widely used parameters for characterizing the organic pollution of water and wastewater. It is estimated by determining the amount of oxygen required by aerobic microorganisms for degrading organic matters in wastewater. Conventional BOD method is the well-known BOD_5 which needs 5-day incubation at 20°C in the dark [23]. Above all, being indigenous and low pathogenic and with ability to produce hydrolytic enzyme and reduce BOD_5 and TSS values, the obtained consortium (I) could be used as component bioremediation agent. To preserve bacterial cells of the consortium from damages, microencapsulation process was then conducted.

3.2. Microencapsulation by freeze-drying

In this study, the freeze-drying process consisted of two cycles, i.e. primary freezing and secondary freezing as stated in previous literature [24]. The works to obtain the freeze-drying results of the selected bacterial consortium (C), encapsulation was carried out at the Faculty of Agriculture, Universitas Gadjah Mada (UGM) and Universitas Katolik Soegijapranata, Semarang. The selected strains used as a consortium were *Bacillus velezensis* R1.3, *B. amyloliquefaciens* R1.6, *B. amyloliquefaciens* R1.14, *B. velezensis* R1.16. Two different free drying instruments were used in each laboratory. Freeze drying process in Food Laboratory of Unika Soegijapranatha used freeze dryer, while that in Faculty of Agriculture, Universitas Gadjah Mada used a newly developed freeze dryer instrument (an unpublished prototype).

The results of capsule morphology test with SEM are shown in Figure 2 & 3. Based on the results of the SEM analysis, it could be seen that the product of bacterial consortium encapsulation obtained was not completely perfect. Capsules, despite their smooth shape were not fully homogeneous in size or not fully round. Improvement of the encapsulation process needs to be done at the next step of research to obtain capsules containing whole and homogeneous capsules.

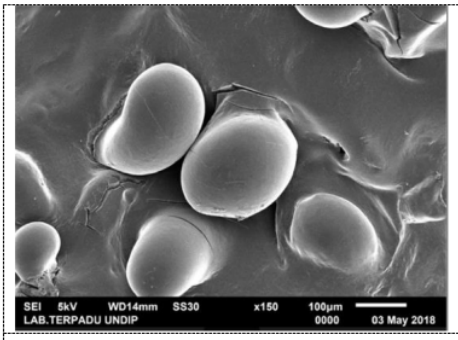

Figure 2. SEM image of the encapsulation products of isolates and bacterial consortium obtained in this study. The image of microcapsules using maltodextrin as micro-carrier were obtained in 150x magnification.

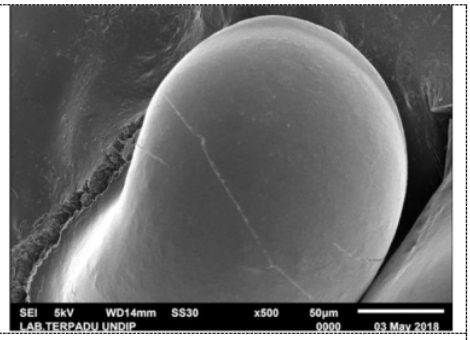

Figure 3. SEM image of the encapsulation products of isolates and bacterial consortium obtained in this study. The image of microcapsules using maltodextrin as micro-carrier were obtained in 500x magnification.

Based on the results of the previous report, a consortium of 4 non-pathogenic hydrolytic indigenous bacteria which interacted antagonists with pathogenic bacteria was obtained. The consortium with 4 isolates decreased the TSS_5 value by 39%, which was believed to be because bacteria had degraded organic matter in the form of macromolecules. Furthermore, decreasing the BOD_5 parameter by the same consortium of 85% indicates the degradation of organic waste. Although it has not been able to improve the value of NH_3, PO_4 and COD, the selection process has proven to be a process that can illustrate that liquid biomedical waste is rich in hydrolytic bacteria, and is proven to be useful for obtaining non-pathogenic groups, which can degrade the main ingredients of waste, organic ingredients at the same time able to interact antagonistically with pathogenic hydrolytic bacteria at once.

Evaluation steps that need to be done after the bioremediation test as the final stage of the selection of bio-remediated agents in the previous study:

(a) Reducing the concentration of medium starter (NB) which is rich in organic matter or replace the use of a starter medium of bacterial consortium with the use of microcapsules to reduce the burden of organic waste material which must be degraded by bacteria. You could also centrifuge the culture, and transfer the pellet without NB included.
(b) Increasing the time of treatment with bacteria more than 5 days because it is estimated that in 5 days only 68% of bacteria degrade waste.

In this study, a prototype of a bioremediation agent is developed in the form of a bacterial consortium, consisting of 4 selected hydrolytic indigenous bacterial isolates from the genus Bacillus. Members of its consortium were previously found synergized with one another but interacted antagonist with pathogenic hydrolytic bacteria, and now proven to be able to improve hospital liquid biomedical waste parameters. The consortium of hydrolytic bacteria obtained has been encapsulated, and has been designated as a prototype of liquid biomedical waste bioremediation agent with specialization:

1) Improving biomedical waste turbidity (contributing to improving flow smoothness, preventing clogging and increasing biomedical waste clarity),
2) Improving BOD value (contributing to reducing material toxicity organic and eliminates foul odour of waste), and suppressing the proliferation of pathogenic bacteria (contributing to reducing the pathological hazard of waste).

This study has demonstrated that a consortium comprising 4 indigenous bacterial isolates from R1 hospital could decrease BOD_5 of biomedical wastewater by 85% and TSS by 39%. In case of R2 hospital, single R1.16, R2.5 and R2.9 isolates as well as its indigenous consortium (consisting of R2.5 and R2.9) consistently showed ability in decreasing PO_4 parameter of the waste sample.

4. Conclusion

In case of R1 hospital study, compared to single isolates and mixed bacterial consortium tested, the indigenous hydrolytic bacterial consortium showed better ability in improving BOD and TSS of liquid biomedical waste of R1 hospital. Through this study, the bioremediation agent prototype for biomedical waste in the form of an indigenous bacterial consortium that has been obtained could be encapsulated with maltodextrin as micro-carrier. However, optimization to result in fully-shaped microcapsules is suggested.

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