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Antimicrobial and Antibiofilm Activities of Bacterial Strain P-6B from Segara Anakan Against MRSA 2983

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ABSTRACT. Methicillin-resistant *Staphylococcus aureus* (MRSA) infection has become a worldwide concern due to the increasing MRSA resistance to antibiotics. This condition encourages the continuous exploration of new anti-MRSA compounds. Thus, this study explored the effect of carbon (starch, glucose, sucrose, lactose) and nitrogen (casein, yeast extract, urea, peptone) sources for the fermentation medium to produce antibacterial and antibiofilm compounds from bacterial strain P-6B against MRSA 2983. The results showed that six-day incubation produced bioactive extracts with the highest antimicrobial activity (11.361 ± 0.860 mm) and the highest activity to inhibit biofilm formation (89.159 ± 0.293% %). In comparison, three-day incubation produced a bioactive extract with the highest activity to degrade biofilm (86.450 ± 3.260%). Glucose was the best carbon source to produce an extract with antibacterial and biofilm inhibition activities. However, starch was the best carbon source to produce an extract with biofilm degradation activity. Urea was the best nitrogen source to produce extracts with antimicrobial and antibiofilm activities. Species identification based on the 16S rRNA gene revealed a 99.78% similarity with *Ochrobactrum intermedium* strain NBRC 15820 (NR_113812.1). These findings suggested the potency of Ochrobactrum P-6B from Segara Anakan Cilacap as indigenous sources of bioactive compounds with anti-MRSA activities.

Keywords: antibacterial, antibiofilm, MRSA, Ochrobactrum

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a specific *S. aureus* bacteria resistant to methicillin antibiotics (Kemung et al., 2018). Nosocomial infection by MRSA occurs by forming a biofilm layer as a pathogenetic mechanism to infect the human body (Rewatkar & Wadher, 2013). The MRSA biofilms on the surface of tissues are aggregated by an extracellular matrix consisting of protein, DNA, and polysaccharides in the form of polysaccharide intercellular adhesin (PIA) (Boles, Thoendel, Roth, & Horswill, 2010). The colonization of MRSA in the formation of biofilms in the blood circulation can cause bacteremia. Prolonged local bacteremia by MRSA infection eventually causes sepsis (Tissot et al., 2014).

Bacteria that form biofilms are expected to be more resistant to antimicrobial exposure. The resistance occurs because antimicrobial substances' ability to penetrate is reduced due to the formation of aggregates cells, a slower metabolic state of bacteria, and horizontal exchange of resistance genes that are easier to occur between biofilm-forming cells (Khameneh, Diab, Ghazvini, & Fazly Bazaz, 2016). Indeed, WHO (2017) has ranked MRSA as a high priority in the pathogen list for research and development of new antibiotics. The grouping of WHO pathogens list is according to the species and the type of resistance. Thus, exploration for new compounds with antibiofilm activities, particularly against MRSA, becomes necessary.

One strategy that can be used to overcome biofilm formation is to screen bacteria that can produce metabolites as anti-MRSA agents. A thorough review by Kemung et al. (2018) highlights *Streptomyces'* potency to produce various anti-MRSA compounds. Recently, indigenous microbes isolated from mangrove sediment Segara Anakan have been reported to have antibacterial activity against MRSA (Asnani, Amaliyah, & Yuniaty, 2020; Dinda, Asnani, & Anjarwati, 2021). We observed that strain P-6B suggested a potency to produce bioactive extract against MRSA based on qualitative screening. Species identification of P-6B indicated the genus Ochrobactrum. It was predicted to have one gene cluster in region 1.4 involved in the biosynthesis of ambactin from the known non-ribosomal peptide (NRP) cluster (Asnani et al., 2020). Genus Ochrobactrum is found under a wide variety of environmental conditions and is primarily studied for

its potential to degrade heavy metal pollutants (Sharma & Shukla, 2021). Ochrobactrum strain MZV101, a strong oil removal bacteria, exhibits antimicrobial activity against various bacterial pathogens (Zarinviarsagh, Ebrahimipour, & Sadeghi, 2017).

Furthermore, Fan et al. (2020) reported that *Ochrobactrum intermedium* D-2 exhibited potency as quorum quenching bacteria against the pathogen. Quorum quenching has been proposed as a promising strategy to prevent and control quorum sensing-mediated bacterial infections. Accessory gene regulator (*agr*) quorum sensing systems are critical components of MRSA cellular processes. It is a target for inhibiting virulence factor production to treat MRSA infections (Lade & Kim, 2021). Following that report, our research aimed to further analyze the potency of P-6B isolate as antibacterial and antibiofilm towards MRSA 2983.

Production of specific bacterial metabolites occurs under particular fermentation conditions. In this case, determining the optimal incubation time is crucial because microbes will synthesize different metabolites in different growth phases. Chodkowski and Shade (2020) have reviewed that some secondary metabolites, such as antibiotics, increase production at the stationary phases. Additionally, carbon and nitrogen in the medium are essential nutrients for microbial growth and the production of primary and secondary metabolites (Singh et al., 2017). Therefore, optimization of medium is necessary to maximize secondary metabolite production. Our research focused on determining the optimum incubation time for the production of anti-MRSA extracts from the bacterial strain P-6B and exploring the fermentation medium with various carbon and nitrogen sources.

EXPERIMENTAL SECTION

Materials

Bacterial strain P-6B was isolated from mangrove sediments in Segara Anakan Cilacap. The isolate was maintained in starch casein nitrate (SCN) medium consisting of starch (Merck), casein (Merck), KNO_3 (Merck), KH_2PO_4 (Merck), $MgSO_4.7H_2O$ (Merck), NaCl (Merck), $FeSO_4.7H_2O$ (Merck), bacto agar (Oxoid) supplemented with 1% nystatin® (kandistatin). The clinical methicillin-resistance *Staphylococcus aureus* 2983 (MRSA 2983) was the collection of Faculty of Medicine, UNSOED, Purwokerto. The pathogen, MRSA 2983, was cultured in mannitol salt agar (Himedia). The antibiofilm tests used Nutrient Broth (Merck), Mueller-Hinton Agar (Himedia), and Brain Heart Infusion broth (Merck). Other chemicals used were glucose (Merck), sucrose (Merck), lactose (Merck), yeast extract (Merck), urea (Merck), and peptone (Oxoid). DNA isolation used Quick-DNA Fungal/Bacterial Micropep Kit (Zymo Research). PCR was performed using MyTaq™ HS Red Mix (Bioline) with primer 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTTACGACTT).

The Cultivation of Microbes

Cultivation of P-6B isolate followed the procedure described by (Asnani et al., 2020). The isolate was inoculated with a continuous streak on Starch casein nitrate agar supplemented with 1% nystatin®. The culture was incubated for seven days at room temperature before being used. The preparation of MRSA 2983 followed the procedure described by Dinda et al. (2021). MRSA 2983 was inoculated in Nutrient Broth and incubated for 18 to 24 hours. The culture suspension was adjusted to 0.5 units of standard McFarland with a 0.9% NaCl solution.

Determination of the Optimum Incubation Time

Bacterial strain P-6B was grown in a liquid SCN medium and incubated at 90 rpm for seven days at room temperature for inoculum. Next, 10% of the inoculum was inoculated into the new SCN medium and set at 90 rpm for 15 days, with interval sampling of three days (0, 3, 6, 9, 12, and 15 days). After each incubation time, the cultures were centrifuged at 4000 rpm for 10 minutes at 4°C. The mycelium obtained was dried and weighed as the dry weight of the mycelium (g). The supernatant obtained was analyzed for antibacterial activity, inhibition of biofilm formation, and biofilm degradation. The incubation times with the optimum activities for all analyzed parameters were then used for subsequent procedures.

Variation of Carbon and Nitrogen Sources

The procedure to determine the optimum carbon source followed Kannan et al. (2014), with modifications. A 10% inoculum was added liquid SCN medium containing 1% of four different carbon sources, which were starch, glucose, sucrose, and lactose. Cultures were incubated at 90 rpm, room temperature. The fermentation was carried out at the optimum incubation time that produces extract with optimum anti-MRSA activity. A similar procedure was applied to determine the optimum nitrogen sources. A 10% inoculum was added liquid SCN medium containing 1% of four different nitrogen sources, which were casein, yeast extract, urea, and peptone. After the optimal incubation time, the supernatant obtained was tested for antibacterial activity, inhibition of biofilm formation, and biofilm degradation.

Antibacterial Activity Test

The extract's ability as an antibacterial compound was tested using the disc paper diffusion method on Mueller-Hinton Agar following the procedure described in CLSI (2019). A total of 30 µl of the extract was added to sterile (6 mm) disc paper, then placed in MHA media that had been inoculated with MRSA. Cultures were incubated at 37°C for 24 hours. The antibacterial activity of each treatment was evaluated by measuring the zone of inhibition around the well with a vernier caliper.

Inhibition of Biofilm Formation

The extract's ability to inhibit biofilm formation was evaluated using a microplate semi-quantitative test following Suzuki et al. (2015) with modification. MRSA was inoculated in Brain Heart Infusion broth supplemented with 1% glucose (BHI-Glu) and then incubated at 37°C for 18 hours. Subsequently, the culture turbidity levels were adjusted to the McFarland standard of 0.5. The biofilm formation inhibitor test was carried out by adding 10 µL of MRSA culture with 100 µL of extract to the microplate and then incubated at 37°C for 24 hours. Biofilm staining used 50 µL of 0.1% crystal violet solution. Measurement of optical density (OD) value was carried out at 595 nm. The OD value obtained was used to calculate the percentage of biofilm formation inhibition using the following formula.

$$\%\ Inhibtion = \frac{\text{OD control} - \text{OD test}}{\text{OD control}}\ x\ 100\%$$

Biofilm Degradation

The ability of the extract to degrade biofilm was also evaluated with a microplate method based on Suzuki et al. (2015) with modifications. A total of 10 µL of MRSA cultures on BHI-Glu medium were cultivated on a microplate for 24 hours at 37°C to form the biofilm. Next, 100 µL of the extract was added, and the incubation was continued for 24 hours at 37°C to expose the extract to the biofilm formed on the walls of microplate. Biofilm staining used 50 µL of 0.1% crystal violet solution. The measurement of the OD value was carried out at 595 nm. The OD value obtained was then used to calculate the percentage of inhibition of biofilm formation using the following formula.

$$\%\ Degradation = \frac{\text{OD control} - \text{OD test}}{\text{OD control}}\ x\ 100\%$$

Species observation and Identification

Macromorphological observations of bacterial strain P-6B focused on colony characters. The observation used a stereo microscope with a magnification of 30x. Micromorphological observations used the Heinrich Slide Culture method using a light microscope with a magnification of 400x. Species identification of P-6B was based on the 16S rRNA gene following the procedure described elsewhere (Asnani et al., 2020). DNA isolation was carried out using the Quick-DNA Fungal/Bacterial Micropep Kit. The DNA obtained was amplified using MyTaq™ HS Red Mix with primers 27F and 1492R. Amplicon DNA was sequenced in two directions with the Sanger sequencing method. The sequences obtained were analyzed with BioEdit 7.2, then aligned to other sequences in GenBank through BLAST nucleotide. The phylogenetic tree construction used MEGA X with construct Maximum Likelihood method and Kimura-2 parameter model with bootstrap 100 (Kumar, Stecher, Li, Knyaz, & Tamura, 2018).

Data Analysis

The research data was in the form of the inhibition index for the antibacterial activity test, the percent inhibition of biofilm formation, and the percent biofilm degradation. Data were analyzed using analysis of variance (ANOVA) at 95% significance levels. The Duncan test followed the results of ANOVA analysis that were significantly different.

RESULTS AND DISCUSSION

MRSA is an infection-causing bacteria that can defend itself by forming a protective layer called a biofilm. MRSA biofilms develop rapidly and form colonies on moist and nutrient-rich surfaces. Biofilm formation is one of the virulence factors of MRSA that contributes to its resistance to antibiotics.

Determination of Optimum Incubation Time

The fermentation process to produce anti-MRSA extract was carried out to determine the optimum incubation time. The growth curve of P-6B (**Figure 1**) shows that the exponential phase occurred from day 6 and reached maximum on day 12. The antibacterial activity of the P-6B extract increased from day 0 and reached the highest activity on day six with an inhibition zone diameter of 11.361 ± 0.860 mm. After that, the antibacterial activity decreased until the 15th day. The results of the variance analysis (Sig. <0.05) indicated that the incubation time significantly affected the diameter of the inhibition zone. The Duncan test further confirmed that antibacterial activity with an incubation time of six days was the highest value and significantly different. Based on this analysis, the 6-day incubation time was suggested for the fermentation process to produce extract with the highest antibacterial activity.

The percentage inhibition of biofilm formation by P-6B extract increased from day 0 to optimal incubation on day six, with a percent inhibition of 89.159 ± 0.293% (**Figure 2**). After reaching the optimal incubation time, the percentage of inhibition decreased. The analysis of variance (Sig. <0.05) showed that the incubation time was significantly affected the inhibition of biofilm formation. Further, the Duncan test concluded that some interactions showed no significant difference. However, the best treatment was the six-day incubation time, which gave the highest inhibition value of biofilm formation. Based on this analysis, the 6-day incubation time was proposed for the fermentation process to produce extract with the highest percentage of inhibition of biofilm formation. The percentage of biofilm degradation by the P-6B extract reached its optimum on the third day of incubation with a percentage of degradation of 86.450 ± 3.260% (**Figure 2**). After getting the optimum incubation time, the degradation decreased. The variance analysis (Sig. <0.05) indicated that the incubation time significantly affected biofilm degradation. Further Duncan test suggested that some interactions were not significantly different.

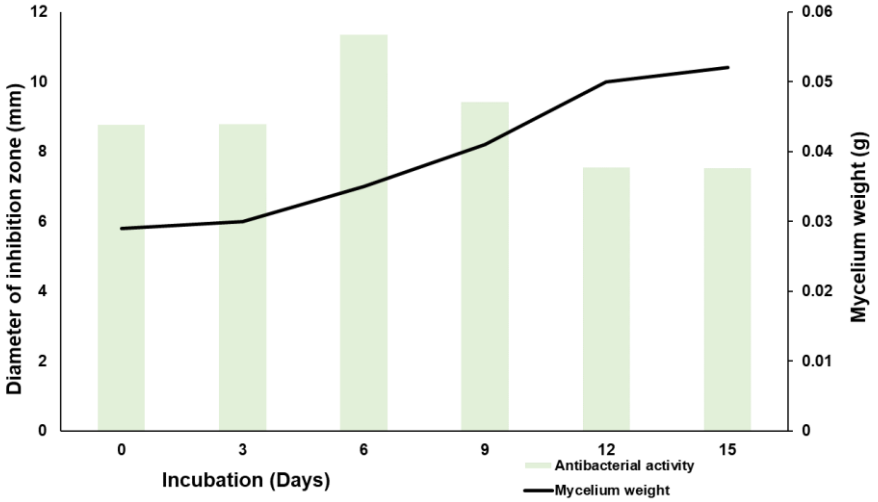


Figure 1. Production of antibacterial extract with incubation times. The dried mycelium weight (g) corresponds with antibacterial activity measured as the diameter of inhibition zone (mm);

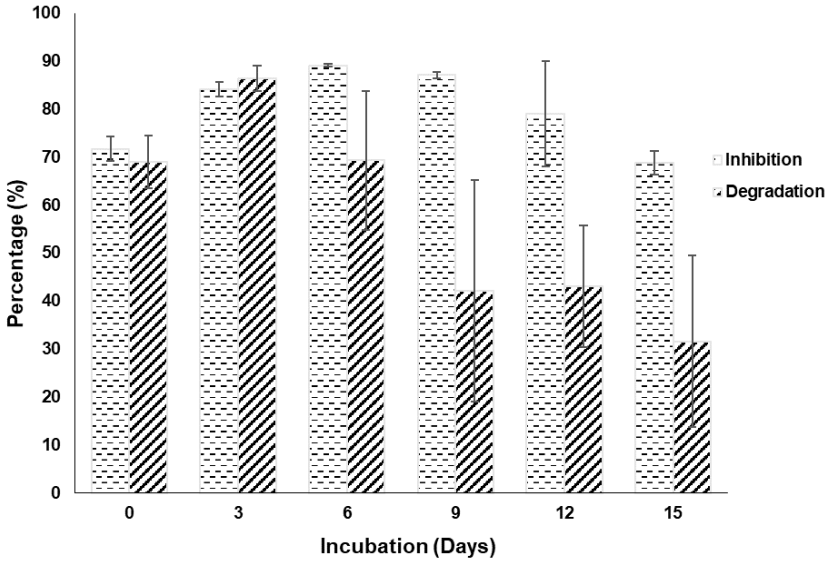


Figure 2. Production of antibiofilm extract with incubation times. The inhibition of biofilm formation and biofilm degradation activities of the textracts was measured as a percentage (%).

However, the best treatment was the incubation time of three days that yielded the highest biofilm degradation. Based on this analysis, the 3-day incubation time was recommended for the fermentation process to produce extract with the highest percentage of biofilm degradation.

The mangrove ecosystem in Segara Anakan Cilacap is rich in microbial diversity. The present study results suggested the potency of P-6B to produce antimicrobial and antibiofilm extract against MRSA 2983. The ability of the P-6B extract as antibacterial also suggested its ability to inhibit biofilm formation. Memariani et al. (2019) stated that compounds with antibacterial activity against microbes that form biofilms could also be used to inhibit the formation of those microbial biofilms. Indeed, the mechanisms of inhibition of biofilm formation are mostly the same as an antibacterial mechanism. On the contrary, biofilm degradation has a different mechanism. It leads to an enzymatic activity that can break down the biofilm matrix, weakening microbial aggregation on biofilms (Lee et al., 2016).

The difference in incubation time is in accordance with the bioactive compounds produced. In this study, the extracts for antibacterial and biofilm inhibition were produced by incubation for six days, while the extract for biofilm degradation was produced only for three days incubation. These results were consistent with those of Xie et al. (2019). They stated that the production of antibacterial compounds by actinomycetes takes longer than the production of compounds that can degrade the extracellular layer of MRSA. Oja et al. (2015) reported that *Streptomyces violaceoruber* (DSM-40701) produced antibiofilm compounds against *S. aureus* with an incubation time of four days. Suzuki et al. (2015) reported that the

Streptomyces sp. strain MC11024 produced antibiofilm compounds against *S. aureus* and MRSA N315 with three incubation days. In other reports, Streptomyces sp. SBT343 has antibiofilm compounds against staphylococcal bacteria with an incubation time of ten days (Balasubramanian et al., 2017).

Determination of Optimum Carbon and Nitrogen sources

The best carbon and nitrogen sources for the fermentation medium were determined based on the highest antibacterial activity, inhibition of biofilm formation, and biofilm degradation. The production of P-6B extract with antibacterial activity was carried out with an incubation time of six days. The results of the antibacterial activity test showed that glucose was the best carbon source (10.617 ± 0.646 mm) and urea was the best nitrogen source (12.426 ± 2.152 mm) in producing antibacterial extracts (**Figure 3**). The variance analysis results (Sig. <0.05) showed that carbon and nitrogen sources significantly affected the inhibition zone's diameter. Further, the Duncan test concluded that antibacterial activities were significant differences between treatments. The highest inhibition zone was produced by fermentation medium using glucose as a carbon source and urea as a nitrogen source.

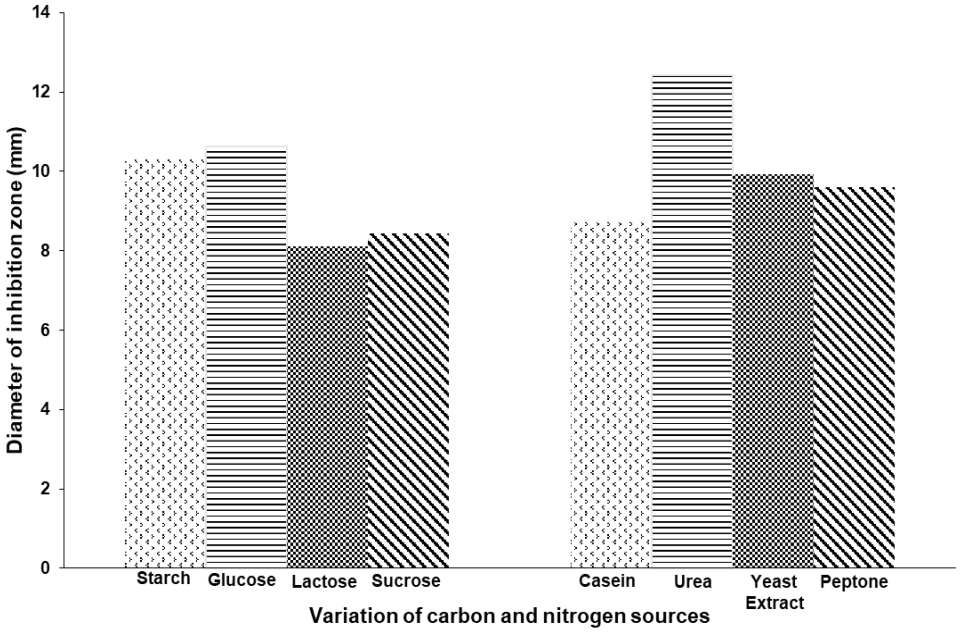


Figure 3. Antibacterial activity of extract P-6B with different sources of carbon and nitrogen.

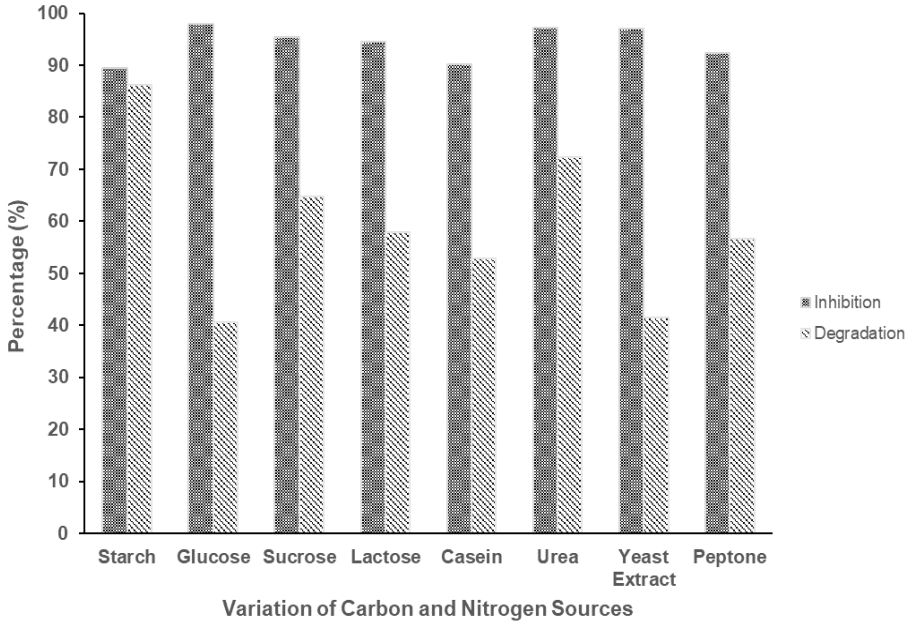


Figure 4. Inhibition of biofilm formation and biofilm degradation from the extract P-6B with different carbon and nitrogen sources.

Production of P-6B extracts with MRSA 2983 potential to inhibit the biofilm formation of MRSA 2983 was carried out with an incubation time of six days. The results showed that the highest inhibition of biofilm formation was produced from extracts using glucose (97.968 ± 0.151%) as a carbon source and urea (97.144 ± 0.516%) as a nitrogen source (**Figure 4**). The results from the variance analysis (Sig. <0.05) showed that carbon and nitrogen sources significantly affected biofilm formation inhibition. Further Duncan's test indicated that some interactions were not significantly different. However, the best treatment with the highest inhibition of biofilm formation was glucose as a carbon source and urea as a nitrogen source.

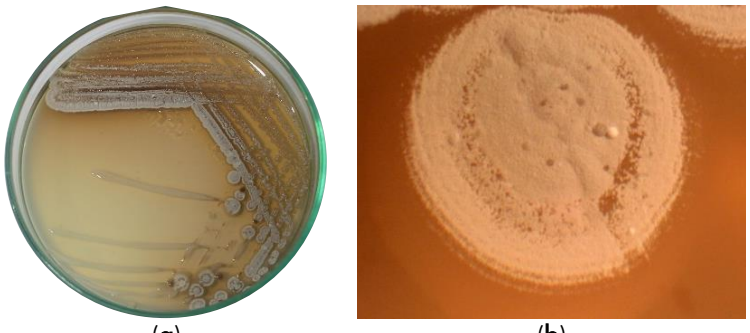
The production of P-6B extract, which could degrade the biofilm of MRSA 2983, was carried out with an incubation time of three days. The results showed that the highest percentage of biofilm degradation was obtained from fermentation medium using starch (86.158 ± 2.395%) as a carbon source and urea (72.349 ± 7.939%) as a nitrogen source (**Figure 4**). The results of the variance analysis (Sig. <0.05) indicated that the sources of carbon and nitrogen significantly affected biofilm degradation. Further, the Duncan test showed significant differences between treatments, with the highest biofilm degradation coming from starch as a carbon source and urea as a nitrogen source.

The composition of the fermentation medium must provide all the required components of the compound. The main components that must be present are carbon and nitrogen for microorganism cell metabolism, cell growth, and to produce specific metabolites. However, the difference in carbon and nitrogen sources in the fermentation medium consequently provides different activities. In this study, glucose was the best carbon source for producing antibacterial extracts and inhibition of biofilm formation, while starch showed the highest biofilm degradation activity. Similarly, Kannan et al. (2014) reported that glucose is the best carbon source to produce antibacterial compounds from the actinomycetes group. Likewise, Nandhagopal et al. (2017) also use starch to produce bioactive compounds for MRSA biofilm degradation.

Nitrogen sources from complex organic compounds function for microbial growth and increase the production of bioactive compounds (González, 2018). Nitrogen is a macronutrient that plays a role in protein biosynthesis to prepare cell structure and cell function. Nitrogen in the fermentation medium can also induce specific enzymes according to the nitrogen source used. Thus, the nitrogen source in the fermentation medium will differ depending on the type of bacteria and bioactive compounds desired. In this study, the treatment of variations in nitrogen sources suggested that urea is the best nitrogen source for the fermentation medium. Urea is known to have the ability to lyse cells and tissues and damage biofilms originating from clinical isolates (Rasmussen et al., 2016). Sanawar et al. (2018) also reported that urea reduced protein and carbohydrate concentration in the EPS biofilm matrix by> 50%. Urea destroys biofilms by breaking hydrogen bonds between amides and carbonyl groups in proteins. Differently, Suzuki et al. (2015) used casein in the fermentation medium Streptomyces sp. strain MC11024 to inhibit the *S. aureus* biofilm Streptorubin B as an MRSA biofilm inhibitor. Balasubramanian et al. (2017) used yeast extract to produce bioactive compounds that inhibit the Staphylococcal biofilm by Streptomyces sp. SBT343. Sharma and Manhas (2019) used casein as a nitrogen source in fermentation medium to produce antibacterial compounds from Streptomyces strain M7 against MRSA and VRE.

Species Observation and Identification

Bacterial strain P-6B was isolated from sediment mangrove in Kalipanas area, Segara Anakan, Cilacap. The bacterium was obtained from sediment samples which were pretreated with 1.5% phenol (Asnani & Oedjijono, 2019). The colonies of the P-6B bacteria strain grew slowly and were tightly attached to the agar medium (**Figure 5**). The colonies were circular with powdery surfaces. The edges of the colonies tended to be flat with raised elevation. The colony surface was gray, the reverse colony was dark brown, and colored pigmentation to the medium was observed. Based on the 16S ribosomal RNA gene, the P-6B isolate has 99.78% similarity to the *Ochrobactrum intermedium* strain NBRC 15820 (NR_113812.1).



(**a**) (**b**)

Figure 5. Observation of Ochrobactrum P-6B, (**a**) colony surface, (**b**) single colony with stereo microscope 30x.

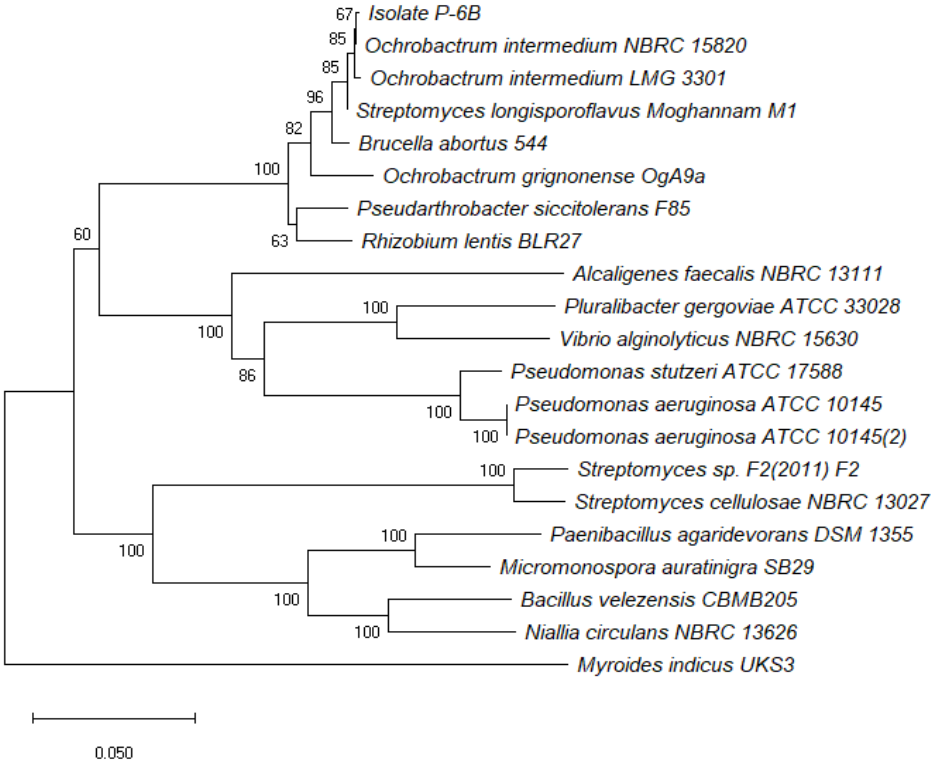


Figure 6. The phylogenetic tree of Ochrobactrum P-6B and related species were obtained from the Maximum Likelihood method and the Kimura-2 parameter model. The numbers on the branch node were bootstrap values expressed as a percentage of 100 replications.

The construction of the phylogenetic tree validated the relationship of Ochrobactrum P-6B with other species. Evolutionary analyzes were conducted in MEGA X (Kumar et al., 2018). The evolutionary relationship was inferred using the neighbor-joining method. The optimal tree with the sum of branch length = 1.13588203 is shown in Figure 6. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. The tree is drawn to a 0.050 scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. This analysis involved 21 nucleotide sequences. There were a total of 1594 positions in the final dataset. All ambiguous positions were removed for each sequence pair (pairwise deletion option).

CONCLUSIONS

Our research highlights the potency of indigenous marine Ochrobactrum P-6B from the sediment mangrove in Segara Anakan Cilacap as antibacterial and antibiofilm sources against MRSA 2983. Indeed, the mangrove forest offers a unique environment with diverse antibiotic-producing microbes. Further research is required to purify and characterize the bioactive compounds with antibiofilm activities from Ochrobactrum P-6B as an alternative therapy for MRSA infection.

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