Antibacterial and antifungal screening of *Pseudomonas aeruginosa* ISP1RL6 associated with *Eucheuma cottonii*

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Abstract. *Eucheuma cottonii* is a seaweed species that has been cultivated largely in many aquaculture farms across Indonesia mainly for pharmaceutical and food industry purposes. Like any other marine organisms, *E. cottonii* build a close relationship with various endophytic bacteria and these associated bacteria have been assigned to play a prominent role to produce secondary metabolite to protect their hosts from environmental threats such as infection and predation. However, the secondary metabolites potential from endophytic bacteria found in *E. cottonii* has not been much explored. In this present study, we described a bacterial isolate encoded as ISPRL6 which was previously isolated from *E. cottonii* of the Buleleng coastal area. The isolate was identified by amplifying 16S rNA gene fragment, followed by Sanger sequencing. The bacterial isolate was grown in 100 mL ISP-2 liquid media in a shaker for 2 weeks. Subsequently, supernatant was separated from cell mass by filtration and the filtrate was extracted twice using ethyl acetate (1:1, v/v). The ethyl acetate extract was screened against four bacterial tests namely *Staphylococcus aureus, Streptococcus mutans*, *Escherichia coli* and *Klebsiella pneumoniae* using disc diffusion assays. In addition, extracts were also tested against two fungal test namely *Candida albicans* and *Aspergillus flavus*. Molecular identification indicated that the isolate ISP1RL6 was phylogenetically related to *Pseudomonas aeruginosa*. Antibacterial screening showed that *P.aeruginosa* ISP1RL6 inhibited the four bacterial tests at diameter of 9-12 mm. Antifungal tests showed the extract inhibited *C. albicans* and *A. flavus* at diameter of 10.2 ± 0.3 mm and 11.5±1.5 mm, respectively. Overall, these findings served as preliminary important data of seaweeds endophytic bacteria for potential antibacterial and antifungal production.

Keywords: Bioprospecting; natural products; antibiotics; antifungal

1. Introduction

Eucheuma cottonii is one of the most commonly cultivated seaweed species in Indonesia. This seaweed species is rich of carrageenan polysaccharides which are use food industries as stabilizer and gelatinized proteins and as an inactive excipient in tablet for pharmaceutical industry [1].

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Although metabolites of *E. cottonii* have been well studied, exploration of endophytic bacteria associated with this seaweed is still limited. Like other marine bacteria, *E. cottonii* has been reported to build close relationship with group of microorganisms especially bacteria for mutual benefits [2-4]. The host provide shelter and nutrient and as an exchange endophytic bacteria provided secondary metabolites to defend its host for environmental pressures such as by bacterial infections or predations [5]. Many of these bioactive compounds from seaweeds endophytic bacteria are of pharmaceutically important [3, 4, 6-8], which open possibility to obtain valuable novel compounds. Therefore, isolation and characterization of endophytic bacteria from *E. cottonii* should receive a particular attention.

A previous study has successfully isolated 23 endophytic bacteria from *E. cottonii* collected at Buleleng coastal area in Bali [9]. Of these 23 bacterial isolates, an isolate encoded as ISP1RL6 in particular displayed a promising antibacterial activity against a panel of Gram positive and negative bacteria based on agar block method. However, a further characterization is required to verify antibacterial potential produced by these two isolates mainly by performing chemical extraction and screening. Furthermore, bioactive evaluation should also be expanded to screen for antifungal activities which also one of the emerging infection diseases in the society.

This study was designed to characterize isolate ISP1RL6 focusing on molecular identification, morphological observation, antibacterial and antifungal screening. It is expected that the obtained result will provide insight on antibacterial and antifungal properties from endophytic bacteria associated with seaweeds *E. cottonii.*

2. Method

2.1 Molecular identification and phylogenetic analysis

Genomic DNA of ISP1Rl6 were extracted and subsequently amplified by polymerase chain reaction aiming 16S rNA gene following the previously described protocol [10]. The PCR product was sent to Genetika Science (https://ptgenetika.com/) and sequenced bi-directionally.

2.1.1 Scanning microscope electron observation

Cell colonies of ISP1Rl6 were sent to the Mero Foundation (https://merofoundation.org/) following protocols for SEM sample preparation.

2.1.2 Bacterial fermentation and extraction

The isolate ISP1Rl6 was fermented in 100 mL ISP-2 broth at room temperature for 2 weeks at 150 rpm. The filtrate was obtained by separating supernatant from cell mass by filtration and subsequently. The maceration was done using ethyl acetate with equal volume and repeated two times. Subsequently the mixture was separated using a separatory funnel. Finally, the crude extract of each isolate was obtained after evaporation at 40^{o}C in a vacuum evaporator.

2.1.3 Antibacterial and antifungal screening

The obtained crude extract from each isolate was assessed for their antibacterial and antifungal activities using triplicate sterile 6 mm paper discs as previously described [11]. Four bacterial tests used in the screening were *Streptococcus mutans* FNCC 0405, *Klebsiella pneumoniae* ATCC 700603, *Staphylococcus aureus* ATCC 25923, and *Escherichia coli* ATCC 25922. While, the two test fungal species were *Candida albicans* and *Aspergillus flavus*. Ethyl acetate was also included as negative control. Antibiotic levofloxacin and antifungal nystatin

were included as positive control for antibacterial and antifungal tests, respectively. While, ethyl acetate was used as negative control. Agar plates containing crude extracts and target bacteria or fungi were placed in a 37^{o}C incubator for 48 hours. Positive results were indicated by the formation of inhibition zone surrounding paper discs which were measured using a digital caliper.

3. Result and Discussion

Morphological observation of ISP1RL6 under SEM showed that have rod structures with diameter of 3µm as shown in Figure 1. Furthermore, nucleotide BLAST of the isolat indicated that it was closely related to *Pseudomonas aeruginosa* (Figure 1 and Table 1). *Pseudomonas aeruginosa* have been regarded to have wide distribution from marine to terrestrial habitats [12]. Thus, it is not surprising that these two isolates were among bacteria found in seaweeds.

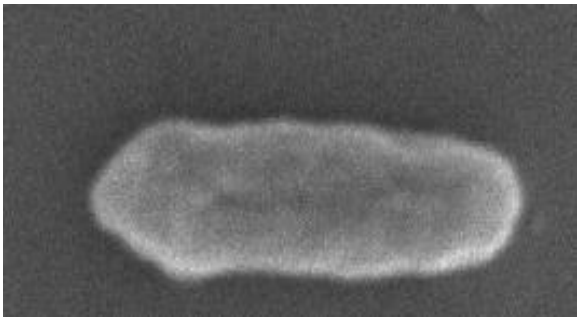
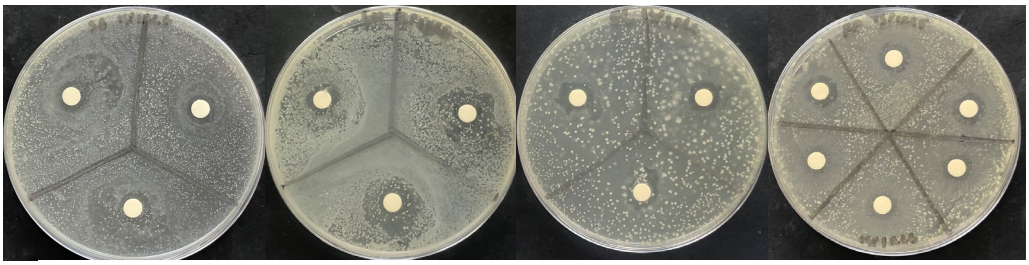


Figure 1. SEM imaging of unicellular of isolate ISP1RL6 under 30.000 magnification

Results of antibacterial screening of ethyl acetate crude extracts of these two isolates were summarized in Table 2 and Figure 2. Ethyl acetate extracts could inhibit *S. mutans* FNCC 0405 at average diameter of 12 mm. While the rest of bacterial tests were inhibited at range of 9–11 mm (Table 2). Comparison with positive control levofloxacin showed that the zone of inhibition made by these two crude extracts were much lower. However, the result is arguable because the antibiotic used is a pure compound which was indeed much stronger compared to ethyl acetate crude extracts. Nevertheless, comparison with negative control has clearly indicated result of antibacterial activities observed in ethyl acetate crude extracts were produced by these two isolates. This finding confirmed antibacterial potential of *P. aeruginosa* ISP1RL6 that was reported from the initial study [9]. Furthermore, antibacterial activities against Gram positive and negative found in this study were in line with previous studies that have reported antibacterial activities from marine *P. aeruginosa* [11, 13, 14].



A B C D

Figure 2. Antibacterial screening of crude extracts in triplicate against *S.aureus* ATCC 25923 (A), *S. mutans* FNCC 0405 (B), *E. coli* ATCC 25922 (C) and *K. pneumoniae* ATCC 700603 at the lower side (D).

Table 1. The BLASTn results of ISP1RL6 16S rRNA gene based on NCBI database

Description	Accession number	Query cover	Percentage identity	e-value	Max score
Pseudomonas aeruginosa strain 2022CK-00828 chromosome, complete genome	CP117749.1	100%	99.76%	0.0	1500
Pseudomonas aeruginosa strain N83 16S ribosomal RNA gene, partial sequence	CP117527.1	100%	99.76%	0.0	1500
Pseudomonas aeruginosa strain M63 16S ribosomal RNA gene, partial sequence	OQ405862.1	100%	99.76%	0.0	1500
Pseudomonas aeruginosa strain L42 16S ribosomal RNA gene, partial sequence	OQ405771.1	100%	99.76%	0.0	1500
Pseudomonas aeruginosa strain L40 16S ribosomal RNA gene, partial sequence	OQ405769.1	100%	99.76%	0.0	1500
Pseudomonas aeruginosa strain L36 16S ribosomal RNA gene, partial sequence	OQ405765.1	100%	99.76%	0.0	1500
Pseudomonas aeruginosa strain L34 16S ribosomal RNA gene, partial sequence	OQ405763.1	100%	99.76%	0.0	1500
Pseudomonas aeruginosa strain C45 16S ribosomal RNA gene, partial sequence	OQ405514.1	100%	99.76%	0.0	1500
Pseudomonas aeruginosa strain C35 16S ribosomal RNA gene, partial sequence	OQ405504.1	100%	99.76%	0.0	1500
Pseudomonas aeruginosa strain C32 16S ribosomal RNA gene, partial sequence	OQ405501.1	100%	99.76%	0.0	1500

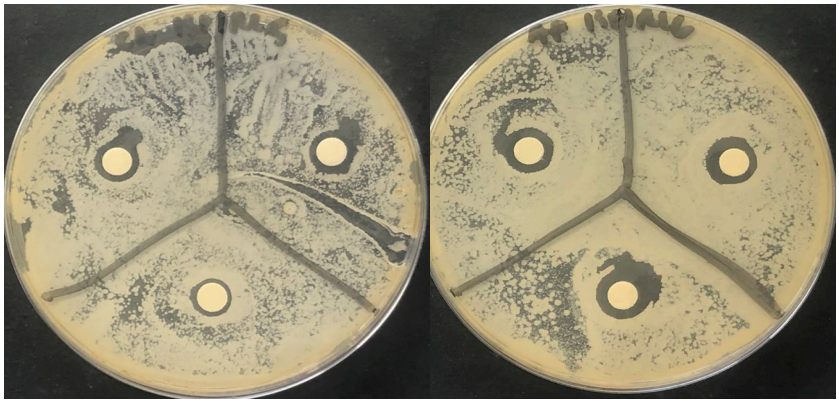
Table 2. Antibacterial screening of ethyl acetate crude extracts *P. aeruginosa* ISP1RL6. Diameter zone of inhibition was shown in mm

Sample	*S. mutans* FNCC 0405	*K. pneumoniae* ATCC 700603	*S. aureus* ATCC 25923	*E. coli* ATCC 25922
Ethyl acetate extracts of *P. aeruginosa* ISP1RL6	12.1±1.6	9.8±0.4	9.8±0.3	11.1±0.3
Ethyl acetate only (negative control)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Levofloxacin	29.0±2.2	22.6±0,7	20.5±0.3	17.4±0.4

Antifungal screening of the two crude extracts was summarized in Table 3 and Figure 3. The crude extracts of ISP1RL6 strongly inhibited both fungal tests at zone of diameter > 10 mm [15] (Table 3). The observed antifungal activities were much lowed compared to antifungal nystatin; however, this finding has confirmed antifungal that could be synthesized the *P. aeruginosa* ISP1RL6 isolate. Despite the fact that the exact compound produced by the isolate was not identified, this finding has confirmed potential of marine *P. aeruginosa* as antifungal producer. A previous study highlights that *P. aeruginosa* from marine sediment could synthetize antifungal rhamnolipids which actively inhibited *Fusarium oxysporum* [16]. Furthermore, *P. aeruginosa* has been assigned to produce antifungal phenazine which was evidently active against *C. albicans* in biofilms [17]. The capability of *P. aeruginosa* to produce antifungal strains was supported by genomic analysis which described the presence of secondary metabolite gene mainly associated with phenazine [18].

Table 3. Antifungal screening of ethyl acetate crude extracts *P. aeruginosa* ISP1RL6. Diameter zone of inhibition was measured in mm

Sample	***C. albicans***	***A. flavus***
Ethyl acetate extracts of *P. aeruginosa* ISP1RL6	10.2±0.3	11.5±1.5
Ethyl acetate only (negative control)	0.0±0.0	0.0±0.0
Nystatin	14.9±0.8	18.6±5.9



A B

Figure 3. Antifungal screening of IPS1Rl6 crude extracts against (A), *C. albicans* (B), *A. flavus*

4. Conclusion

As a conclusion, the current study gave a preliminary important finding of the antibacterial and antifungal potential produced by marine *Pseudomonas aeruginosa*. A follow up research will be focused to explore antibacterial and antifungal of crude extract *P. aeruginosa* ISP1RL6 against multidrug resistance strains. Furthermore, some culture conditions such as volume of media, type of nutrient, temperature and duration of fermentation could still be optimized to obtain a better production of active compounds. Moreover, analysis of chemical constituents of ethyl acetate crude extracts should be done by applying quantitative techniques such as HPLC, LC-MS and GC-MS to identify a more precise antibacterial and antifungal compounds.

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