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Screening of Marine Actinomycetes from Segara Anakan for Natural Pigment and Hydrolytic Activities

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Abstract. Marine actinomycetes have become sources of great interest to natural product chemistry due to their new chemical entities and bioactive metabolites. Since April 2010, we have screened actinobacteria from five sites that represent different ecosystems of Segara Anakan lagoon. In this present study we focus on specific isolates, K-2C which covers 1) actinomycetes identification based on morphology observation and 16S rRNA gene; 2) fermentation and isolation of pigment; 3) structure determination of pigment; and 4) hydrolytic enzymes characterization; Methodologies relevant to the studies were implemented accordingly. The results indicated that K-2C was likely *Streptomyces fradiae* strain RSU15, and the best fermentation medium should contain starch and casein with 21 days of incubation. The isolate has extracellular as well as intracellular pigments. Isolated pigments gave purple color with λmax of 529.00 nm. The pigment was structurally characterized. Interestingly, *Streptomyces* K-2C was able to produce potential hydrolytic enzymes such as amylase, cellulase, protease, lipase, urease, and nitrate reductase.

1. Introduction

Actinomycetes are known as a group of microorganisms that are most responsible for the production of crucial bioactive compounds, particularly antibiotics. The potency of actinomycetes as antibiotic producing bacteria has made these microbial groups become very important. However, the bioactive compounds produced by actinomycetes are not limited against bacteria, but also effective as anti-fungal agents, anti-cancer, and some of them are as anti-virus [1]. Until now, the search of actinomycetes producing new types of bioactive compounds continues.

The research exploration of marine actinomycetes is aimed to explore the indigenous species which are able to produce potentially bioactive compounds, such as pigments, enzymes, anti-bacterial and bioremediation agents. Samples of sediment/soil and water are obtained from the mangrove region as an area of mangrove forest is a source of organic matter of weathering and decomposition of litter that is very beneficial for heterotrophic microorganism such as actinomycetes. Asnani & Ryandini have reported 26 isolates of actinomycetes halotolerant of mangrove areas Segara Anakan that have potency as sources of anti-bacterial compounds. Based on the observation of the colony and cell morphology, actinomycetes isolates generally obtained have morphological properties of actinomycetes genera *Streptomyces* [2].

Certain actinomycetes show the various color of pigments in the hyphae, and various pigment diffused into the fermentation medium such as beige, white, red, green, and brown pigment. Gunasekaran & Poorniammal have reported that *Penicillium* sp capable of producing extracellular red pigment [3]. The red pigment with the molecular formula of anthraquinone produced by *Penicillium oxalicum* has now mass produced as a natural pigment. More recently, Mohanasrinivasan et al., [4] reported that *Streptomyces coelicolor* MSIS1 able to produce the extracellular blue pigment that turns red in acidic conditions. Following these findings, we continue exploring one specific isolate, namely K-2 because of its potency to produce color pigments as well as hydrolytic enzymes. Hence, this study was aimed to identify actinomycetes producing pigment, the isolation, and structure identification of pigment, and to evaluate the hydrolytic capability of isolates.

2. Materials and Methods

2.1. Sites of Sampling

The research was exploratory. Five different sites that represent diverse ecosystems of Segara Anakan Cilacap were selected for the present study. Sediment and water samples were taken in the area of mangrove vegetation with specifications Rhizophora area sp., grassy land, and open land areas. Each location was taken three sampling points. Soil and sludge samples were taken at a depth of soil about 5-8 cm and then put into sterile bottles.

2.2. Actinomycetes Isolation

1 g of sediment or 1 mL of water was mixed with 9 mL of sterile distilled water, shaken until homogeneous and become suspension with a dilution rate of 10^{-1}. From 10^{-1} dilution, the dilution was carried out with sterile seawater to obtain a suspension with a dilution rate of 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. The suspension with 10^{-5} dilution was pipetted into 1 mL and cultured in casein nitrate medium in a sterile petri dish. Media for cultivation was Starch Casein Nitrate (SCN) agar (10 g soluble starch; 1 g Casein; 1 g KNO_3; 0.5 g K_2HPO_4; 0.5 g $MgSO_4.7H_2O$; 0.5 g NaCl; 0.01 g $FeSO_4.7H_2O$; 20 g agar; pH 6.8-7.0) supplemented with nystatin [2]. Incubation was carried out at 28 °C for 7-9 days, or until the actinomycetes colonies grew. The characteristics observed in actinomycetes colonies grow rounded colonies, slow growth, colony surface (could) had hyphae, hard as dusty or velvety. Usually, there was a central point in the middle of the colony and typical colored of the colony.

2.3. Colony Identification

2.3.1. Morphology Identification. The colony morphology and cell identification were done by preparing Henrici's Slide Culture (HSC). The observations of the colony were intended to the form, surface, consistency, edges, color, growth radial and concentric growth. The observation of the presence or absence of cells was directed against the substrate mycelium and aerial mycelium, the appearance of the substrate mycelium, aerial mycelium appearance, presence of spores and structure, the color of spores, as well as the pigment that diffuses into the medium. The observations of the hyphae characteristics were then compared with Bergey's Manual of Determinative Bacteriology.

2.3.2. Species Identification. Identification of actinomycetes species was based on *16S rRNA*. DNA isolation *used* Presto™ Mini gDNA Bacteria Kit Geneaid Instruction Manual. DNA obtained was amplified with 27F and 1492R primers using KAPA Taq™ Extra Host start ReadyMix with dye. DNA electrophoresis used 1% of agarose gel submerged in 1x TAE buffer. Species determination used BLAST (Basic Local Alignment Search Tool).

2.4. Pigment Production.

The pigment production consisted of four stages, namely a) inoculum preparation, b) fermentation medium preparation, c) the pigment production, d) pigment isolation, and e) pigment extraction.

Actinomycetes isolated was a streak on SCN medium and incubated for 8 days at a temperature of 30 °C; then it served as the parent culture. Pigment production was done by inoculating five plugs sized of 0.5 mm from parent culture to 100 ml of liquid medium. The fermentation process was done for 14 t0 21 days at room temperature. Media for fermentation was SCN broth (10 g soluble starch; 1 g Casein; 1 g KNO_3; 0.5 g K_2HPO_4; 0.5 g $MgSO_4.7H_2O$; 0.5 g NaCl; 0.01 g $FeSO_4.7H_2O$; pH 6.8-7.0) supplemented with nystatin. After incubation, the culture producing pigment was centrifuged at 3000 rpm for 30 minutes. The filtrate obtained was an extracellular pigment; while the mycelium obtained was extracted with ethanol to obtain the intracellular pigment [5].

2.5. Structure Determination of Pigment

Pigment extract obtained was purified with silica gel 60 GF254 followed by column chromatography eluted with chloroform: methanol (9: 1). The pure pigment was characterized by UV-Vis spectrophotometry (Shimadzu UV-1800), FTIR spectroscopy (Perkin Elmer Spectrum 100), and NMR Spectrometer (NMR Jeolin 500 MHz).

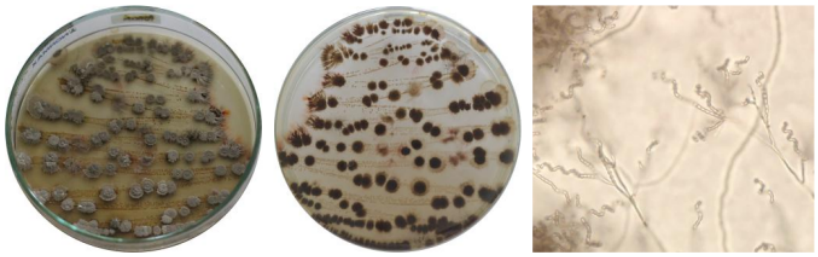
2.6. Hydrolytic Enzymes Characterization

The physiology of the isolates was observed by a series of biochemical tests following the known procedure [6]. Biochemical tests included hydrolytic, enzymatic tests such as starch hydrolysis, CMC hydrolysis, casein hydrolysis, lipid hydrolysis, urea hydrolysis, and nitrate reduction.

3. Results and Discussion

Sediment and water samples were collected from Klaces (K) and Ujung Alang (U) areas in Segara Anakan. Sampling time was during the day with the local temperature of 28 ° C and pH waters around 7-8 which corresponded to the growth of actinomycetes such as the temperature of 25-30 ° C and a pH of about neutral pH. Actinomycetes cultivation was carried out on an agar medium Starch-casein nitrate (SCN) supplemented with antifungal nystatin to inhibit mold growth. SCN is a highly sensitive and specific medium for the growth of actinomycetes. Usha et al. managed to isolate 15 isolates of actinomycetes origin by using medium SCN soil [7]. Khan et al. also succeeded in isolating two actinomycetes isolate originating from the sponge using SCN medium supplemented with antifungal and antibacterial [8].

The population of actinomycetes which had colored mycelium was generally obtained from the litter under the mangrove vegetation [2]. This indicates that the litter of mangrove vegetation litter is rich in organic matter because the result of weathering leaf or root exudates. As estuarine areas, Segara Anakan region experiences water movement that causes a typical condition. Only microorganisms with high tolerance to a constantly changing of oxygen, temperature, salinity and humidity are able to survive.



(a) (b) (c)

Figure 1. Colony observation of actinomycetes K-2C: (a) top (b) bottom, (c) mycelium

3.1. Colony Identification

Actinomycetes are a group of bacteria that forms unicellular mycelium that has the appearance of morphology such as mold. Unicellular mycelium is formed consisting of hyphae branched hyphae with a diameter of 0.5-0.8 µm. There are two types of mycelium, the substrate mycelium (vegetative) growth in the medium and aerial mycelium (aerial) growing on the surface of the medium. Aerial hyphae can be white, gray, violet, red, yellow, brown, green or any other color due to the pigmentation. In observation with a light microscope on a glass slide, aerial mycelium was refractive and bright, while the substrate mycelium is transparent and dark [1].

Based on morphology observation, the colony of K-2C has a size of 6 mm, red pigmentation to medium, irregular shape, powdery surface, convex elevation, margin undulate, gray aerial mycelium, and brown substrate mycelium; Morphological properties of K-2C were suggested to be similar with actinomycetes genera *Streptomyces*. Vegetative hyphae form a mycelium was branched. Aerial hyphae forming three or more mature spore chains, and a spiral-shaped sectional existed (figure 1).

Determination of the species of the isolate K-2C was done using 16S rRNA. The isolated DNA was amplified using PCR and sequenced in two-way forward and reverse with 27F and 1492R primers to get the base sequence. Determination of the base sequence of K-2C was carried out by pairwise alignment method in Bioedit program. The results of Bioedit program is as follows:

CATGCAGTCGACGATGAACCACCTTCGGGTGGGGATTAGTGGCGAACGGGTGAGTAACA
CGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATA
CTGACCTGCCAAGGCATCTTGGCGGGTCGAAAGCTCCGGCGGTGCAGGATGAGCCCGCG
GCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGA
GAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCACACTCCTACGGGAGGCAGCAG
TGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACG
GCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGA
AGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGT
CCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGGTTGTGAAAGCCCG
GGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCG
GAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCANGAGGAACACCGGTGGCGAAGGC
GGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGTGGAGCGAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTTG
TCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAC
TCAA

Total nucleotides	= 834 base pairs
Molecular weight	= 253152.00 Daltons, single stranded
Molecular	= 508419.00 Daltons, double stranded
Composition G+C	= 59.83%
Composition A+T	= 40.17%

Based on the BLAST analysis, isolate K-2C was identified to have similar sequences of 99% with a *Streptomyces fradiae* strain RSU15. Hangstrom et al., stated that isolates which have similarities sequences of the 16S rRNA gene could represent the same species while the sequence similarities between 93% -97% could represent the same identity at the level of genus, but different at the species level [9]. In other words, the difference between the 16S rRNA gene sequences of isolates stored in the data center if it is less than 3% showed the same species, while if more than 3% indicates a

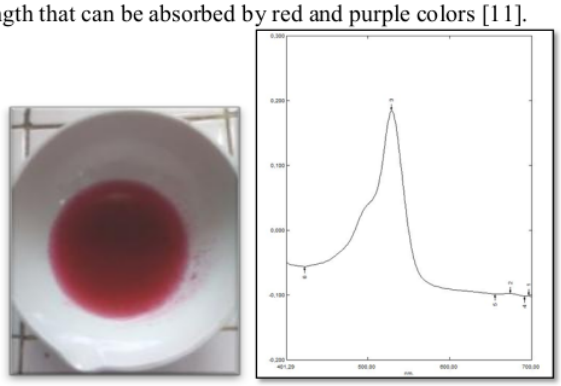
different species from the data center. From BLAST searches, K-2C taxonomy can be seen, as follows:

Domain: Bacteria
Phylum: Actinobacteria
Classic: Actinomycetes
Ordo: Actinomycetales
Familia: Streptomycetaceae
Genus: Streptomyces
Species: *Streptomyces fradiae* strain RSU15

3.2. Pigment of Actinomycetes K-2C

Aside from being able to produce antibiotics, actinomycetes are also able to produce the pigment that includes all rainbow colors. The bioactive pigments produced by microbes halotolerant has been reviewed. Those pigments have specific color and have an activity, such as anti-cancer, antibacterial, anti-inflammatory, antioxidant, as well as antibiotics. Asnani & Ryandini have also been reported marine actinomycetes expressing the color pigment. Observation of the pigment was observed on colony color and pigmentation to the substrate [5].

The microbes pigment can be isolated from three general stages, namely the cultivation of microbes, microbial fermentation to produce pigment, and pigment isolation. Measurement of the pigment concentration can be carried out using a UV spectrophotometer at the optimum wavelength (λ) for a particular color. For example, the yellow pigment can be observed on λ 390 nm, while the red pigment on λ 500 nm [10]. In this study, *Streptomyces* K-2 C produced purple pigment within 21 days of fermentation time, with optimum λ at 529.00 nm (figure 2). The wavelength in the range of 500 – 560 nm is the wavelength that can be absorbed by red and purple colors [11].



(a) (b)

Figure 2. Pigment production from actinomycetes K-2C: (a) purple pigment, (b) optimum λ at 529.00 nm

The highest production of purple pigment was obtained with fermentation with modified casein nitrate medium, and the lowest production was obtained from that of yeast extract-malt extract medium. Microbial fermentation medium is used for microbial growth as well as for the pigment production. Optimization of the fermentation medium can be done by varying sources of carbon and nitrogen sources. Palanichamy et.al., reported that antibiotics actinorhodin red-bluish can be isolated from *Streptomyces* sp in the fermentation medium using glycerol and mannose as a source of C; and Na-casein peptone as a source of N [12]. Optimization of fermentation medium in order for *Penicillium* sp to excrete extracellular red pigment was using soluble starch as a source of C and peptone as a source of N, at a temperature of 30 °C and pH 9.0. The optimum condition of fermentation medium was able to increase the production of red pigment up to sevenfold [3].

FTIR analysis was conducted to determine the functional groups of isolated pigment. Infrared spectra of purple pigment were shown in Figure 3 while the data of wave number and related functional groups was presented in table 1. Interpretation of IR spectra indicated that *Streptomyces* K-2C pigment contained a cyclic core, carbonyl, hydroxyl, alkyl (CH3 and CH2), non-conjugated one and secondary NH.

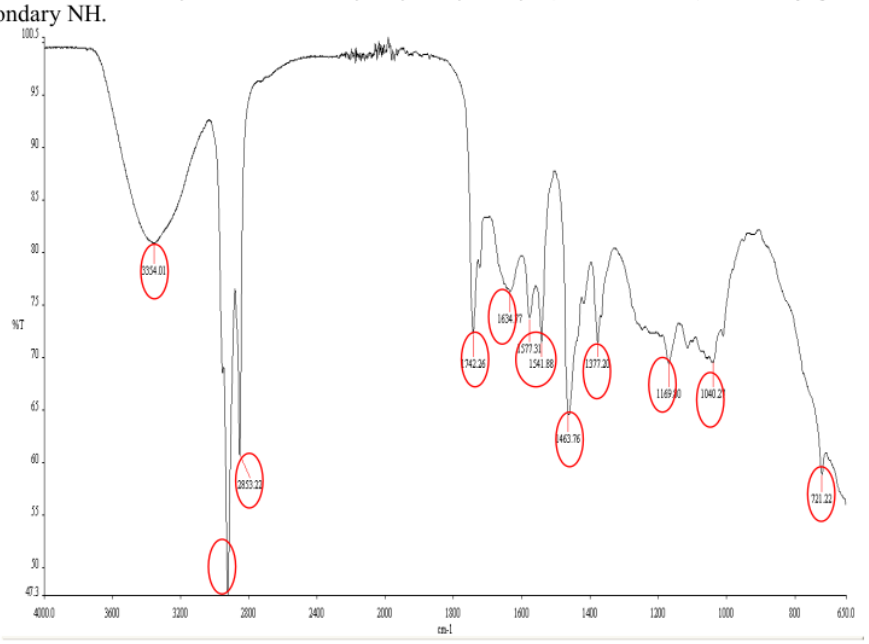


Figure 3. IR spectrum of pigment from *Streptomyces* K-2C

Table 1. FTIR data of purple pigment

Wavenumber (cm^{-1})	Frequency range (cm^{-1})	Correlated functional groups
2853.22	2850-3000	C-H alkane
1742.26	1650-1780	C=O ester
1634.77	1620-1680	C=C non conjugated
1577.31	1550-1640	N-H secondary
1463.76	1450-1470	$-CH_2-$
1377.20	1350-1370	$-CH_3$
1169.80	1000-1300	C-O ester
1040.27	990-1060	C-OH cyclic
721.22	650-1000	=C-H

1H-NMR spectra analysis was done to describe different types of hydrogen atoms in the pigment molecule. The information that can be obtained from the 1H-NMR spectrum is the chemical environment of hydrogen atoms, the number of hydrogen atoms in each environment and the cluster structures adjacent to each hydrogen atom [13]. 1H-NMR spectrum of the pigment Streptomyces K-2C is shown in Figure 4.

1H-NMR spectrum of the pigment Streptomyces K-2C showed a number of signals that appear in the chemical shift (δ) at 0.5 to 5.5 ppm. These data indicated aliphatic compounds. A chemical shift at 0.5 to 2.0 ppm was the signal for the H atoms bonded to C in a sp3 manner. Chemical shift at 0.87

ppm (3H) indicated the H atoms as $–CH_3$; δ = 1.29 ppm (2H) indicated the H atoms as $–CH_2$; and δ = 1.62 ppm (2H) indicated the H atoms as $–CH_2$ as well. Chemical shift at 2.34 ppm (2H) was a signal of H atom $–CH_2$ bonded to C heteroatoms. Chemical shift at 3.72 ppm (1H) indicated the H atoms as a form of –CH bonded to C heteroatoms. Chemical shift at 5.35 ppm (1H) is a signal to H bound to C double bond (-C = H). Based on the interpretation of the 1H-NMR spectrum, the purple pigment has H atoms in the form of methyl and methylene bonded to C Sp3; H form methylene attached to the C-heteroatom; Metin H form attached to the C-heteroatom; and H attached to C double bond.

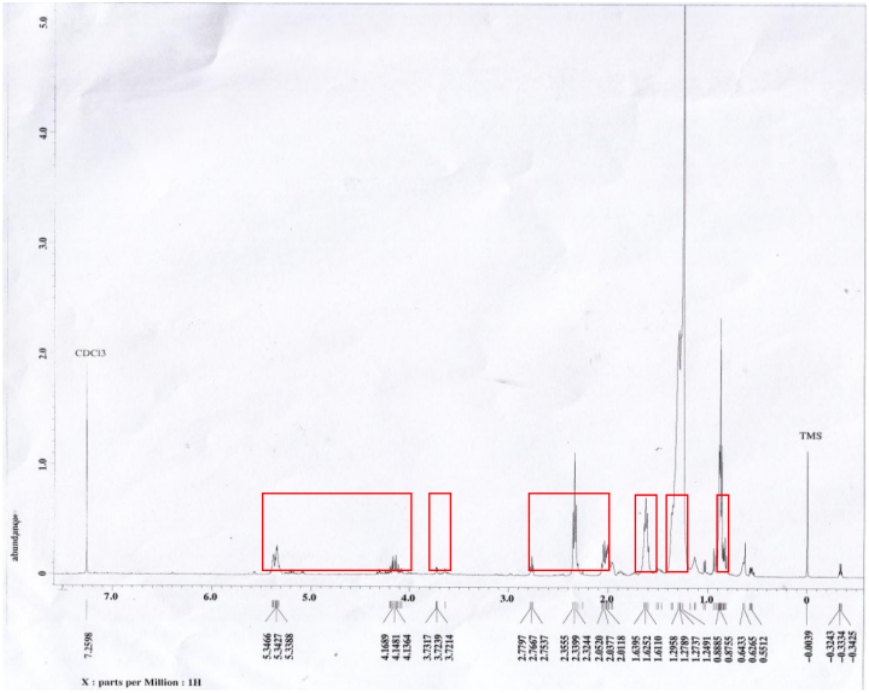


Figure 4. 1H-NMR spectrum of pigment from Streptomyces K-2C

3.3. Hydrolytic Enzyme Characteristics

The other actinomycetes potency is its ability to produce hydrolytic enzymes. The results from biochemical tests revealed the ability of *Streptomyces* K-2C in hydrolyzing starch, casein, lipids, urea, cellulose and reduced nitrate. These positive results indicated that the isolate were able to produce hydrolytic enzymes such as amylase, cellulase, protease, lipase, urease and nitrate reductase (table 2).

The further quantitative study indicated that the cellulolytic ability of K-2C was higher (cellulase index of 20.06 mm) compared to the amylolytic ability (amylase index of 6.26 mm) (figure 5). Indeed, the areas of mangrove forest are sources of organic matter of weathering and decomposition of litter that is very beneficial for heterotrophic microorganism such as actinomycetes. Thus, marine actinomycetes obtained from mangrove areas in Segara Anakan, such as *Streptomyces* K-2C, allegedly able to act as decomposers organic materials. In this regard, the hydrolytic ability of *Streptomyces* K-2C will have a promising prospect for industrial application.

Table 2. Biochemical Tests of *Streptomyces* K-2C

	Results
Enzyme Production:	
- Amylase	+
- Protease	+
- Lipase	+
- Cellulase	+
- Nitrate reductase	+
Carbohydrate Utilization	
- Galactose	-
- Sucrose	+
- Raffinose	+
- Mannitol	+
- Arabinose	+
- Xylose	-

Starch hydrolysis (6,16mm) CMC hydrolysis (20,06mm)

Figure 5. Amylolytic and cellulolytic ability of Streptomyces K-2C

4. Conclusion

Marine actinomycetes K-2C was identified as *Streptomyces fradiae* strain RSU15. The isolate was able to produce purple pigment with λmax of 529.00 nm within 21 days of fermentation. Partial structure of the pigment was identified using FTIR and NMR. However further characterization is needed to have a complete pigment structure. Interestingly, the isolate was also able to produce potential hydrolytic enzymes namely amylase, cellulase, protease, lipase, urease, and nitrate reductase.

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