

Analysis of Carotenoids and Identification of Mangrove Sediment Bacteria of Segara Anakan, Cilacap

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Abstract

The excessive consumption of artificial dyes can lead to negative effects on human body. Thus, the invention of natural dyes, such as carotenoids, is needed in order to reduce the negative impacts. Carotenoids are yellow, orange, and reddish yellow pigments produced by plants, animals, algae, and microorganisms. This study was aimed to analyze the carotenoid pigments of mangrove sediment bacteria in Segara Anakan, Cilacap, and to identify species of bacteria that can produce carotenoids. Carotenoids were analysed by using Thin Layer Chromatography (TLC) and UV-Vis spectrophotometer. Meanwhile molecular identification of bacteria was carried by 16S rDNA PCR and DNA sequence was analysed through a program called Basic Local Alignment Search Tool (BLAST). The kinship of bacteria was shown in Phylogenetic tree by using Bioedit and MEGA 5 software. Qualitative analysis by using TLC produced several pigments like: β -carotene, β -isorenieraten, lycopene, flavonoids, chlorophyll a, chlorophyll b and a feofitin with Rf value of 0.36-0.95. Quantitative analysis showed that bacteria KH (greenish yellow), KT (dark yellow) and KM (light yellow) produced carotenoids at $95.30 \mu\text{g.g}^{-1}$, $110.34 \mu\text{g.g}^{-1}$ and $25.349 \mu\text{g.g}^{-1}$. KH, KT and KM were suspected to be bacteria known as *Streptomyces chartreusis*, *Bacillus megaterium* and *Streptomyces chromofuscus* with similarity of 99%. Mangrove sediment bacteria had the potential of producing carotenoids as an alternative of eco-friendly natural dyes.

Keywords: pigment, *Streptomyces*, *Bacillus*, natural dyes

Introduction

In the market, dyes are very often added to food. The use of dyes that is excessive, inappropriate and harmful needs to be avoided because it can have negative impacts on health. Natural dyes can be used as an alternative solution in dealing with the harmful effects of the use of artificial dyes. Natural dyes can be obtained from plants, animals, micro-algae (cyanobacteria), heterotrophic and phototrophic bacteria, and fungi (Britton, 1998; Kurniawan *et al.*, 2010; Gupta *et al.*, 2011). Natural dyes that have been mass produced are β -caroten from *Dunaliella*, astaxanthin from *Haematococcus*, and lutein from *Chlorophyceae* (Campo *et al.*, 2007). Bacteria that are known to be the producers of carotenoid are *Streptomyces* sp., *Brevibacterium maris*, *Paracoccus alcaliphilus*, *Rhodotorula rubra*, *Bradyrhizobium* sp. (Hannibal *et al.*, 2000; Simova *et al.*, 2004; Baskar *et al.*, 2010; Arlita *et al.*, 2013).

Bacteria are potential organisms utilized in the health and industry sector. Natural color pigment or substance that can be produced by bacteria has positive impacts on human health. Palanichamy *et al.* (2011) said *Streptomyces* sp. isolated from sediments on the coast of Chennai, India, is potential as a pigment producer. The new acyclic carotenoids and monocyclic C₄₀ carotenoids were isolated from the novel Gram-negative bacterium *Rubritalea squalenifaciens* and novel species of Gram-negative bacteria belonging to the family Flavobacteriaceae, phylum Bacteroidetes respectively (Shindo and Misawa, 2014).

The utilization of bacteria as the producers of carotenoid pigment can be an alternative to gain dyes that are eco-friendly and safe to be consumed by human. This research is aimed to analyse carotenoid pigment produced by mangrove sediment bacteria of Segara Anakan, Cilacap and to identify bacteria that can produce carotenoid pigment.

Material and Methods

The content of carotenoid was measured by spectrophotometry method (Dharmaraj *et al.*, 2009). Carotenoid was extracted in accordance with Dharmaraj *et al.* (2009) with modifications. Bacterial isolates were inoculated in a starch casein medium with pH 7 of 100 ml, and incubated in a shaker with a rotation speed of 180 rpm and temperature of 28°C for 120 hours. Furthermore, the solution was centrifuged at 6000 rpm for 15 minutes. Bacterial cells were taken and extracted by using maceration method with acetone and methanol (7:3). Then, the extract is centrifuged and dried by using nitrogen gas.

Measurement of carotenoid's quality

Carotenoid's quality of bacterial isolates was measured by employing TLC (Dharmaraj *et al.*, 2009; Baskar *et al.*, 2010). Adsorbent or plate used in TLC was silica gel 60 F₂₅₄. The qualification of control of the TLC used mobile phase variations, that were: methanol: chloroform (1:7,v:v) methanol: chloroform (1:5,v:v), methanol:chloroform (1:9,v:v), hexane: methanol (5:5,v:v) hexane:methanol (3:7,v:v) hexane:methanol (1:9,v:v). The results were visualized on a 254 nm UV light. The analysis of carotenoid's quality with R_f (Retardation factor) value:

$$R_f = \frac{\text{migration distance of extract}}{\text{migration distance of solvent}}$$

Measurement of carotenoid's quantity

Measurement of carotenoid's quantity (Sahara *et al.*, 2013), was done by using *UV-Vis Scan Spectrophotometer* with a wavelength of 400-600 nm. Total carotenoid was counted by using sample comparison with the standard of β -carotene curve (Musfiroh *et al.*, 2008).

Molecular identification of mangrove sediment bacteria that produce carotenoids

DNA was extracted in accordance with Margavey *et al.* (2004) with modification. A total of 1 ml of liquid culture of bacteria was put into a 1.5-mL tube, and then it was centrifuged for 5 min. Next, pellet was homogenized with 400 μ l TE and, again, centrifuged for 5 min. 400 μ l of SET solution and 50 μ l of Lysozim solution were added and incubated at a temperature of 37°C for 1 h. 50 μ l of SDS solution were added and then incubated at a temperature of 65°C for 1 h. Furthermore, 167 μ l of NaCl solution were added and incubated at the the temperature of 65°C for 1 h. 400 μ l of cool chloroform solution were added and then incubated at room temperature for 30 min. After 30 min, the mixture was centrifuged

for 10 minutes and supernatant obtained was moved to a new tube and then a solution of isopropanol in the ratio of 1:1 was added and incubated at -20°C for 24h. The samples then were centrifuged for 5 min. The pellet was homogenized with cool ethanol 70%. The ethanol was evaporated and the pellet formed was dried by wind. The pellet then was resuspended with 50 μ l of TE solution.

The amplification of 16S rDNA PCR fragment was done toward isolates that potentially had carotenoids by using primer R1492 (5'-TACGGCTACCTTGTTACGACTT-3') and F27 (5'-AGAGTTTGATCCTGGCTCAG-3'). The composition of PCR reaction consisted of 7.5 μ l ddH₂O, 2.5 μ l of genomic DNA (50 ng, μ l⁻¹), 1.25 μ l of primer F27 (25 pmol), 1.25 μ l of primer R1492 (25 pmol), and 12.5 μ l of KAPPA MIX PCR (Margavey *et al.*, 2004). The composition was modified by by Riyanti *et al.* (2009).

DNA was amplified by employing an initial denaturation program at a temperature of 80°C for 5 minutes; 30 cycles that consisted of: denaturation (94°C for 30 sec), annealing (55°C for 30 seconds), polymerisation (72°C for 60 sec), and last polymerisation at a temperature of 72°C for 7 min. The result of amplification was analysed by employing electrophoreses, that was 2% agarose and a dye *ethidium bromide* (Margavey *et al.*, 2004).

The results of DNA sequences in bacteria were compared to DNA sequences in the DNA data center. The search was performed with the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information, National Institute for Health, USA (www.ncbi.nlm.nih.gov) (Atschul *et al.*, 1997). Then, a *phylogenetic tree* was made from DNA sequent by employing Bioedit and MEGA 5 software (Zhang *et al.*, 2008).

Result and Discussion

Analysis of carotenoid pigments

Observations based on the color samples of the bacterium used three test samples. Bacteria that contained carotenoid pigments had several color (Figure 1.).

The value of R_f (Table 2.) shows that the extract with KH code allegedly contained β -carotene, flavonoids, feofitin a, chlorophyll a, and chlorophyll b pigment. The one with KT code was suspected to contain β -carotene, flavonoids, and chlorophyll a pigment. While extract with KM code was suspected to contain β -Isorenieraten pigment, chlorophyll a, chlorophyll b and lycopene pigment. Then this

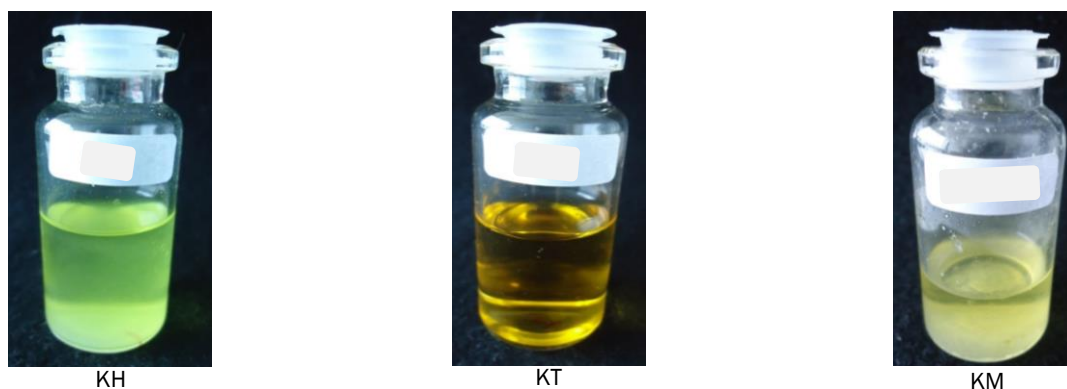


Figure 1. Pigment of the result of sediment bacteria extraction (KH) greenish yellow, (KT) dark yellow, (KM) light yellow.

research would only discuss about carotenoid pigment groups that were β -carotene, β -isorenieraten, and lycopene. The movement of non-polar carotene was faster, marked with the higher Rf than that of other pigments (Rizkina *et al.*, 2013) so that easily identified through visual observation. According to Gross (1991), yellow-orange dye is a sign of carotene pigment group, green-blue is chlorophyll a, while light yellow is xanthophyll pigment group. Carotenoids are divided into two main groups namely polar carotenoid (xanthophyll) and non-polar carotenoid non polar (carotene).

The result of TLC was confirmed by employing UV-Vis spectrophotometer. Based on the analysis of the rough extract of KH, KT dan KM pigment, a spectrum pattern with 3 peaks in the wavelength of 400-600 nm (Figure 2.) could be obtained. They were indicated as a carotenoid pigment (Sahara *et al.*, 2013).

The result of measurement shows that KH extract was indicated for containing carotenoid pigment with three peaks, which were 468.5, 439.5 and 416.0 nm. KT extract shows that maximum wavelengths were 653.5, 474.5 and 364.5. In addition, apparently KM extract contained carotenoid pigment that had wavelengths of 467.5, 439.0 and 416.0. Those three extracts samples are included in carotenoid wavelength category, which is 400-600 nm (Sahara *et al.*, 2013). Besides the comparison between the wavelength of the samples and that of the references, the fathoming of carotenoid content was also held by comparing the curve of spectrophotometer with the standard carotenoid curve of Laughlin *et al.* (2002). Laughlin *et al.* (2002) states that pure β -carotene, when it is measured with the wavelength of 210-700 nm, shows a peak in the wavelength of 452 nm.

It is supported by Barredo (2012) who explain that maximum wavelengths of β -carotene pigment's

are 430, 453 and 447 nm; those of Lycopene are 445, 457 and 504 nm; those of Zeaxanthin are 427, 453 and 447 nm; and those of Lutein are 427, 448 and 472 nm. While Cantaxanthin and Astaxanthin only has one maximum wavelength, that is 470 and 477 nm, respectively. The result of β -carotene degree determining by using ray spectroscopy method appeared on the pigment of sediment bacteria as shown on Figure 3 and Table 2.

The regression equation for the curve above in the concentration range of 5-50 ppm was $y = 0.0662x - 0.0068$ with $R^2 = 0.99$. The result shows that the level of beta carotene of KT was higher than that of KH and KM. β -carotene concentration in KH and KT were exceed the "limit of quantification" while the concentration of KM could be counted accurately. The problem related to the limited supply of rough extract of bacteria that produced KH and KT pigment curbed us in validating the concentration of β -carotene of KH dan KT extract. KT extract's level of β -carotene is higher than that of other extracts seen from the concentration of the measurement result by using regression equation. Besides, the dark yellow dye owned by KT extract indicated that its carotene content was high. It is supported by Britton *et al.* (1995) that carotene pigment has orange hue. The production of pigment by microorganisms is affected by several factors such as pH, temperature, source of carbon and nitrogen, and time of incubation (Chintapenta *et al.*, 2012).

Carotenoid content produced by KH, KT and KM bacteria was less than that produced by *Micrococcus* sp. Isolated from the sediment, Canada. A research conducted by Ibrahim (2008) shows that *Micrococcus* sp. produces $430 \mu\text{g}\cdot\text{g}^{-1}$ of carotenoid. It means sediment bacteria can produce carotenoid more than carrot and purple carrot, that is between 200 and $300 \mu\text{g}\cdot\text{g}^{-1}$ (Lee *et al.*, 2011). Ibrahim (2008) says that there are some factors affect the production of carotenoid besides light,

that are pH, rate of centrifuge and the temperature of incubation.

Identification of sediment bacteria producing carotenoid pigment

Bacteria used in the process of identification were labelled KM, KH and KT. KT bacteria (Figure 4.) were assumed as a part of *Bacillus megaterium* and the level of similarity was 99%. The result of the research shows that *Bacillus megaterium* could produce dark yellow pigment. Based on the research held by Mitchell et al. (1986) in Khaneja et al. (2009) it is known that *Bacillus megaterium* can produce red pigments. According to Khaneja et al. (2010) there are some *Bacillus* spesies that can produce carotenoid, such as *B. marisflavi*, *B. indicus*, *B. firmus*, *B. altitudinis* and *B. safensis* with yellow, orange and pink detected on the wave length of 455, 467 and 492 nm. It is supported by the research of Duc et al. (2006) that discovers *Bacillus*

indicus produces yellow carotenoid. Nugraheni et al. (2010) in their research about the characteristic of carotenoid of bacterial symbionts in seagrass, *Thalassia hemprichii*, find that *B. licheniformis* produces orange carotenoid. *Bacillus* Genus produce carotenoid pigment with optimum pH, that is 7 (Khaneja et al., 2010).

KM and KH bacteria were *Streptomyces chromofuscus* and *S.chartreusis* that were 99% similar. Some types of *Streptomyces* that have been examined previously are also reported for containing certain kinds of carotenoid for instance the one identified by Dharmaraj et al. (2009), *Streptomyces* sp., isolated from *Callyspongia diffusa* sponge produces lycopene pigment with the maximum wave length is 445 nm and it's Rf value is 0.40. Next, Dharmaraj et al. (2009) found *Streptomyces* strain (AQBMM35) spesies from *Mycale mytilorum* sponge as the producer of phytoene pigment with Rf value was 0.81. Palanichamy et al. (2011) isolated

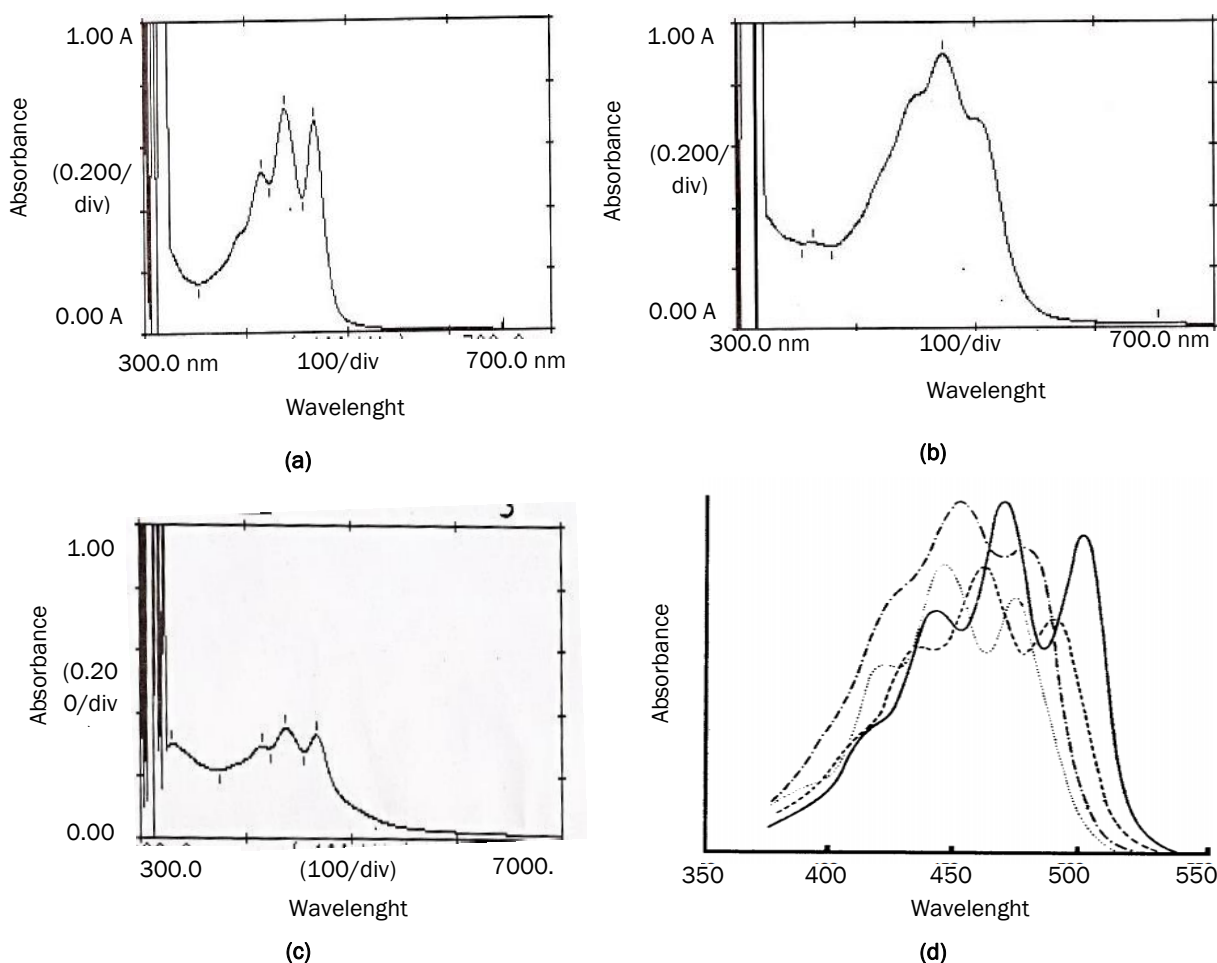


Figure 2. The result of absorbance of UV-Vis spectrophotometer (a) KH extract (b) KT extract (c) KM extract (d) absorbance spectrum of lycopene (—), γ -carotene (----), β -carotene (-.-.-), α -carotene (.....) in petroleum ether (Amaya, 2001)

Table 1. Fathoming of kind of pigment based on Rf value by using TLC

Isolate	Spot	Color	Rf value	fathoming Pigmen Type	Literature	Reference
KH	1	Yellow	0.94	β -carotene	0,94-0,97	Ati et al. (2006)
	2	Yellow	0.86	Flavonoid	0,86-0,89	Ati et al. (2006)
	3	Grey	0.8	pheophytin a	0.74-0.82	Heriyanto and Limantara (2006)
	4	Grey	0.64	chlorophyl a	0,57-0,64	Heriyanto and Limantara (2006)
	5	Bluish green	0.6	chlorophyl a	0,57-0,64	Heriyanto and Limantara (2006)
	6	Yellow	0.48	chlorophyl b	0,48-0,56	Heriyanto and Limantara (2006)
KT	1	Dark yellow	0.94	β -carotene	0,94-0,97	Ati et al. (2006)
	2	Grey	0.64	chlorophyl a	0,57-0,64	Heriyanto and Limantara (2006)
	3	Bluish green	0.6	chlorophyl a	0,57-0,64	Heriyanto and Limantara (2006)
	4	Light yellow	0.36	Flavonoid	0,32-0,40	Ati et al. (2006)
KM	1	Dark yellow	0.95	β -Isorenieratene	-	Baskar et al. (2010)
	2	Bluish green	0.6	chlorophyl a	0,57-0,64	Heriyanto and Limantara, (2006)
	3	Yellow	0.48	chlorophyl b	0,48-0,56	Heriyanto and Limantara, (2006)
	4	Light brown	0.40	Lycopene	-	Dharmaraj et al. (2009)

Tabel 2. Level of β -carotene pigment of mangrove sediment bacteria

Sample	Absorbance	Level of β -carotene ($\mu\text{g.g}^{-1}$)
KH	0.721	95.30
KT	0.899	110.34
KM	0.354	25.34

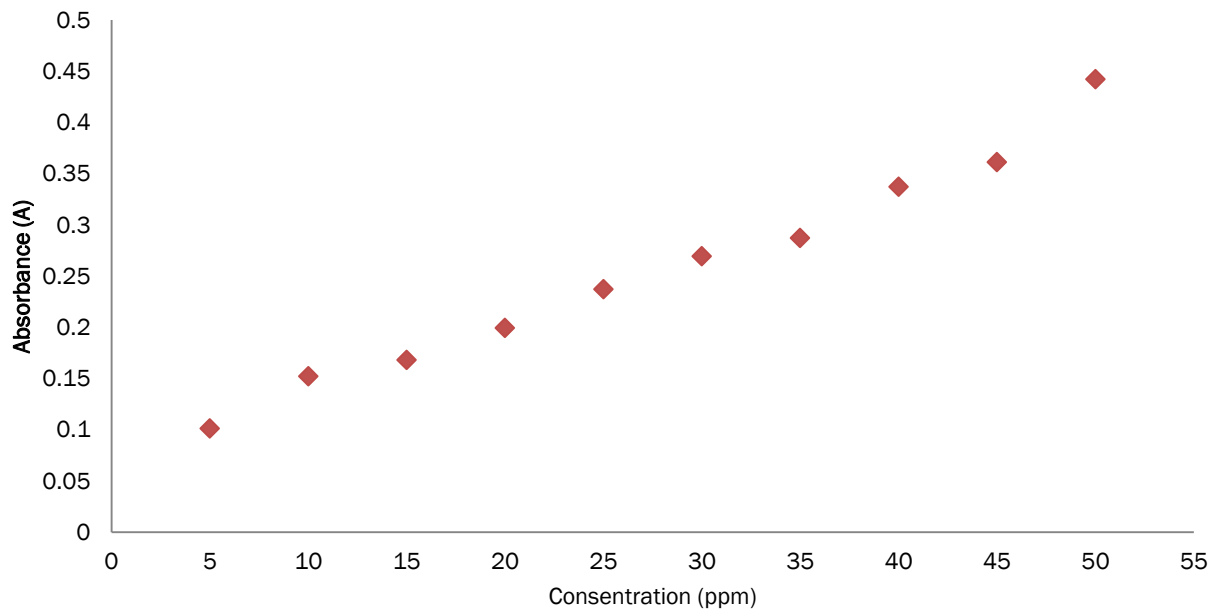


Figure 3. The curve of β -carotene calibration

S.coelicolor and *S. violaceoruber* bacteria from the sediment of sea and soil in India as the producers of actinorhodin pigment that has red-blue dye. Bose et al. (2013) in their research state that *Streptomyces* sp. T1027, lighted by a lamp during the culture, can produce β -carotene pigmen. Baskar et al. (2010)

also discovered that *Streptomyces* sp. isolated from some spesies of fish such as *Tendania anhelan*, *Epinephelus diacanthus*, *Cyprinus carpio* and *Osepherenemous gourami* can produce several tyoes of carotenoid like phytoene, phytofluene, α -carotene, β -carotene and β -isorenieratene.

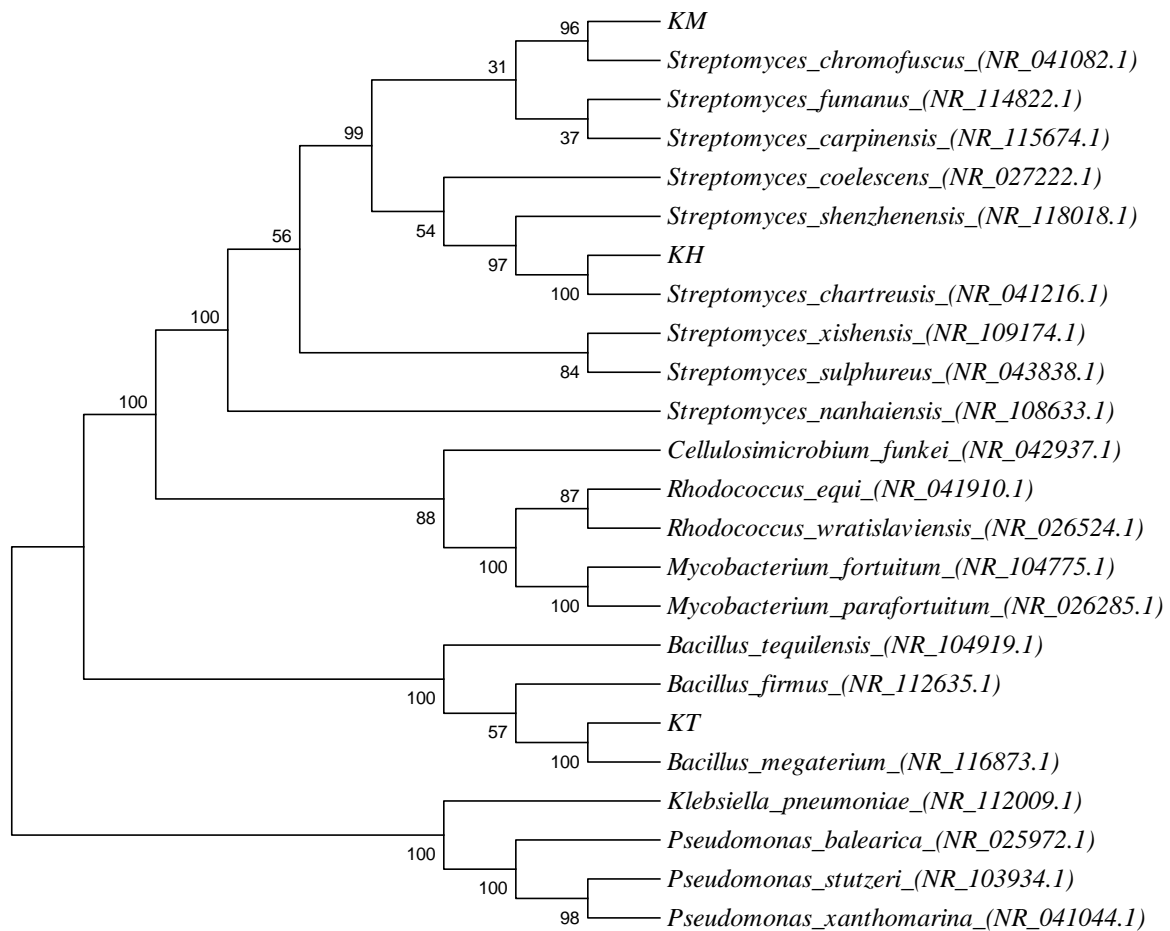


Figure 4. Genetic relation of mangrove sediment bacteria. The formation of dendrogram was based on PCR 16S rDNA. For the sequence alignment ClustalW Multiple Alignment was employed. While for the analysis, the researcher used Neighbor-joining, phylogeny test with Bootstrap method and 1000 repetitions, as well as kimura 2-parameter model.

Conclusion

Mangrove sediment bacteria of Segara Anakan-Cilacap could produce carotenoid pigment of carotene group, which were β -carotene, β -isorenieratene and lycopene. KH, KT and KM bacteria were known can produce carotenoid sebesar $95.30 \mu\text{g}\cdot\text{g}^{-1}$, $110.34 \mu\text{g}\cdot\text{g}^{-1}$ and $25.34 \mu\text{g}\cdot\text{g}^{-1}$. The result of identification shows that KT, KM and KH bacteria had similarity with *Bacillus megaterium*, *Streptomyces chromofuscus* and *Streptomyces chartreusis*.

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