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Characteristics of a red-pigmented thermotolerant bacteria (GSB-001) isolated from Gedong Songo hot spring, Bandungan, Semarang – Indonesia

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ABSTRACT

INTRODUCTION

Carotenoids are pigment consisting of C_{40} in living organisms, they are synthesized by all photosynthetic organisms and few non photosynthetic organisms such as bacteria and fungi. Carotenoids are various molecules containing carbon and hydrogen such as β-carotene, γ-carotene, lycopene and xanthophylls such as astaxanthin, cantaxanthin, astacene. Among natural resources of pigments, microbial resources have an immense potential to produce good alternative ones to synthetic pigments. Microbial pigments are more promising producers than other natural resources of pigments because they are considered as natural, having no seasonal production problems, growing quikly in cheap culture media and showing high productivity. They grow faster compared to plants and animals and are relatively easy to be manipulated and their growth conditions are easy to produce high pigment content. Thus microbial pigments production are nowadays one of the emerging fields of research to demonstrate their potential for various industrial applications (Kumar *et al.*, 2015). Microorganisms produce many natural pigments such as β-carotene, astaxanthin, canthanxanthin, prodigiosin (Elkenawy *et al.*, 2017; Bathgare *et al.*, 2018). Considering the important use of pigments in various industries, especially food, cosmetics and health, the search for natural pigments from extreme environments such as hot springs is encouraged (Manimala and Murugesan, 2017; Mukherjee *et al.*, 2017). Pigment

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compounds contains bioactive compounds functioned as antioxidant. This is due to the pigment structure can prevent oxidation or neutralizing oxidized compound by adding hydrogen or electron.

Microorganisms from different environment have been isolated to substitute synthetic pigments for industries, like fungal *Monascus* for its monascorubramine red-pigment (Srianta *et al*., 2016) and the yeast *Phaffia rhodozyma* for its astaxantin used as natural feed supplement in aquaculture (Cheng *et al*., 2018). The others microorganisms pigment producer are the yeasts *Rhodotorula* sp., *Rhodosporidium* sp. for synthesis of torulen, torulahordin and β-carotene (Tkačova, 2015; Bonadio *et al*., 2018;). Many bacteria producing pigment are: *Rhodococcus* sp., *Serratia* sp., *Rhodotorula* sp. (Hernández-Almanza *et al*., 2014; Zang *et al*., 2014; Chen *et al*., 2018). Microalgae of *Dunaliella* sp., *Haematococcus pluvialis*, *Chlorella* sp. are also commercial pigment producing (Kleinegris *et al*., 2011; Shah *et al*., 2016).

Our laboratory has isolated red-pigmented thermotolerant bacteria from sediment of hot springs at Gedong Songo in Bandungan – Semarang. Bandungan is one of subdistricts in Semarang Regency, where it is a highland tourist destination with 1200 m above sea level. It has hot springs and geothermal steam vent aerosol, which makes Bandungan a unique environment. Hence, it has microorganisms which are different from other places. Therefore, identification of the isolate found in this region is needed to determine the species.

The purposes of this research were to isolate and identify pigmented thermotolerant bacteria based on 16S rRNA, biochemical and morphological characteristics.

MATERIALS AND METHODS

The red pigmented bacteria was cultivated on Nutrient Agar (NA) at pH 6 and temperature at 28 °C. This culture was conserved at temperature 4 °C.

Morphological and biochemical identifications

The isolate was cultivated on Nutrient Agar media, incubated at ambiant temperature, for 24 h. The colony was identified for its colony characters, cell shape, and Gram staining. Biochemical tests included indole, methyl red, catalase, urease test and carbohydrate fermentation using carbon sources of glucose, lactose, sucrose and maltose (Talaeikhozani *et al*., 2015).

Molecular Identification

DNA extraction

DNA extraction was done by Chelex method (Walsh *et al*., 2013). Briefly, 3 loops overnight culture of endospore-forming bacteria were put into a tube with contains 100 µL ddH_2O and it was added 1 mL saponin 0.5% for overnight at 4 °C. The solution was centrifuged for 10 min with 12,000 rpm and then the supernatant was removed. The pellet was added with 100 µL ddH_2O and 50 µL chelex 20%. The mixture was boiled for 10 min and vortex every 5 min and centrifuged. The supernatant was the DNA and kept at 4 °C.

DNA amplification

DNA Amplification was done using the following program: pre denaturation at 95 °C for 3 min, denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extention at 72 °C for 1 min and the last final extention at 72 °C for 7 min. Primers used were 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and primer 1492R (5'-GGYTACCTTGTTACGACTT-3') (Osborne *et al*., 2005). The PCR mixture were 3 µL of DNA template, 1.5 µL of each appropriate primers, 25 µL of KAPA 2GFAST Kits and 19 µL of ddH_2O. Visualization of PCR product was done using gel electrophoresis with agarose concentration at 1% and run with 100V for 30 min. The band of PCR product was seen on gel documentation.

Sequences analysis

Sequenced of DNA product was done at Genetics Science of Indonesia, Inc. Sequence was analyzed used base alignment by Basic Local Alignment Search Tool (BLAST) to establish percentage of base pairs similarity with reference of isolate which be found in gene bank.

Phylogenetic tree

Phylogenetic tree was made using MEGA 5 software. Sequences of bacteria isolate were analyzed and compared with sequence of bacteria which had highest of homology percentage compared to sequence of other bacteria species. Phylogenetic tree was constructed by test of Neighbour-joining tree with Bootstrap method.

Pigment extraction

Serratia marcescens culture at 48, 72 and 96 h were centrifuged, and the pellet was washed with distillated water. Methanol was added and vortexed until pigment extracted. Pigment extract was assayed its antioxidant activity using DPPH methods and the functional groups were also identified by FTIR (Fourier Transform InfraRed Spectroscopy).

Antioxidant activity

Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay was carried out by (Molyneux, 2004) methods and vitamin C commercial used as control. To 0.1 mL pigment extract was added 3.9 mL of DPPH 0.05 mM solution was prepared in methanol, vortexed and followed by incubation at 20 min for 30 °C. DPPH radical scavenging was measured at λ = 517 nm using spectrophotometer. Antioxidant activity represented the percentage of DPPH radical scavenging ability was calculated the following formula as below:

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DPPH radical scavenging (%) = $[(A_0 - A_s/A_0)] \times 100$, where A_0 was the control absorbance, A_s was the sample absorbance.

RESULTS AND DISCUSSION

Thermotolerant red-pigmented bacteria isolate GSB-001

Red-pigmented bacteria isolate GSB-001was isolated from sediment of hot spring Gedong-Songo, Bandungan, Semarang-Indonesia. This isolate has been adapted to grow at 30 °C on Nutrient Agar medium and produced red colony (Figure 1).

Figure 1: Colony of red-pigmented bacteria isolate.

The morphological characteristics of the red-pigmented bacteria isolate had moderate size, irregulare shape, raised elevation and entire margin. This isolate was Gram-negative and rod. Biochemical tests showed that the isolate had negative indole test, positive catalase, negative methyl red and negative urease. This result was the same with the identification carried out by Abdullah *et al.* (2017). Positive catalase on biochemical test showed that this bacteria had catalase enzyme having role in breaking down H_2O_2 to H_2O and O_2.

This isolate was able to ferment glucose, sucrose and maltose, but there was no gas production, so the bacteria isolate could produce acid only. Lactose could not be fermented by this isolate (Table 1). The biochemical test of the isolate was confirmed with the molecular identification to determine the species.

Molecular identification was started with DNA extraction which produced DNA with concentration of 39.8 ng/ul, and purity of 1.42. DNA amplification for molecular identification was done using 27F and 1492R primers which amplified 16S rRNA gene. Low mass ladder marker was used to determine the size of PCR product which had 1500 bp length (Figure 2). Galkiwiecz and Kellog (2008) stated that 27F primer targeted bacteria whereas 1492R was universal primer. Osborne *et al.* (2005), added that primers 27F and 1492R amplified 16S rRNA gene in almost all bacteria known and has been used to study bacteria in many habitats.

The PCR product was then sequenced to find out its sequence and resulted in 1469 bp length. The sequence was analyzed to find its homology sequence from data bank in National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST). The BLAST showed that the isolate had 94% homology with *Serratia marcescens* strain S823.

Table 1: Morphological and biochemical characteristics of red-pigmented thermotolerant bacteria isolate GSB-001

Tests	Morphological		Biochemical
	Colony	Cells	
Color	Red	-	-
Size	Moderate	Rod	-
Shape	Irregular	-	-
Elevation	Raised	-	-
Margin	Entire	-	-
Gram	-	Negative	-
Indole test	-	-	Negative
Catalase test	-	-	Positive
Methyl red test	-	-	Negative
Urease test	-	-	Negative
Carbohydrate Fermentation			
Glucose	-	-	Positive
Lactose	-	-	Negative
Sucrose	-	-	Positive
Maltose	-	-	Positive

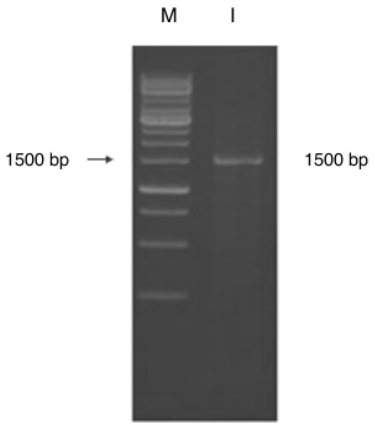

Figure 2: PCR product of 16S rRNA amplification of red-pigmented thermotolerant bacteria isolate GSB-001 (M=marker; I =isolate).

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Phylogenetic tree

Phylogenetic tree was constructed using *neighbor-joining* (NJ) and showed that the high relationship between GSB-001 isolate with pigment-producing bacteria (Figure 3). Phylogenetic tree showed that bacteria isolate GSB-001 had closely relation with *S. marcescens* strain S823.

Serratia marcescens is a Gram-negative bacteria that can be found in many habitats such as soil, water and plant. This bacteria belongs to the opportunistic pathogenic bacteria in the family Enterobacteriaceae. It produces a red pigment that is a secondary metabolite known as prodigiosin. It grows from 5 - 40 °C and pH 5-9.

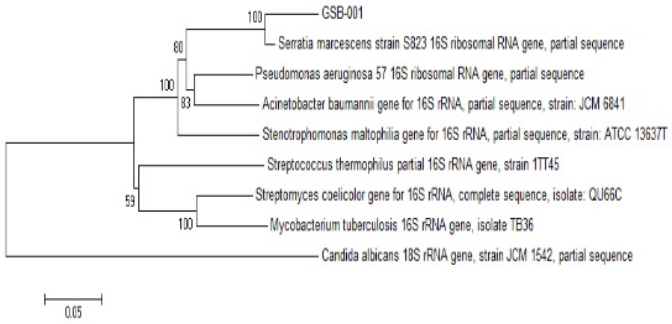

Figure 3: Phylogenetic tree between red-pigmented bacteria isolate GSB-001 and *S. marcescens* strain S82, based on 16S rRNA has 94% similarity and gaps 1%.

Pigment of *S. marcescens*

Red pigment extration was done using methanol solvent 70% (Figure 4). Pigment production increased in accordance with the incubation time. The red pigment produced by *S. marcescens* is prodigiosin (Srimanthi *et al*., 2017) and the bacterial cell has to be broken to get out its intracellulare pigment. Result showed that 48 h of culture produced the lowest pigment concentration compared to 72 and 96 h of culture. On the contrary, Rakh *et al*. (2017) showed that *Serratia rubidaea* JCM 124OT produced the highest prodigiosin at 48 h of incubation. Production of prodigiosin pigment is related with the increasing of biomass cells (Haddix and Shanks, 2018).

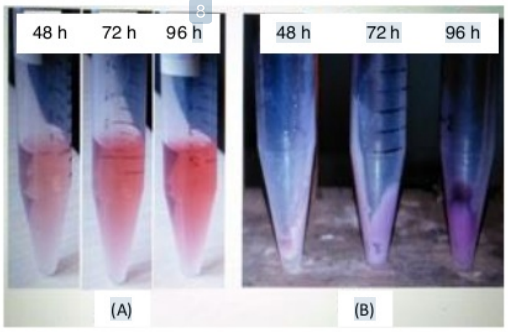

Figure 4: Red pigment of *S. marcescens*: (A) Extract pigment in methanol 70% (B) Pigment after evaporation.

The FTIR spectrum of compounds showed wave lenght between 1500-3500 cm^{-1}. Specific peak at 1740 cm^{-1} represented of C=O (carbonyl) stretching group. This result matched with the research of Charttejee *et al.* 2017. Based on theory, the carbonyl group present in 1820-1600 cm^{-1}. The other peak at 3364 cm^{-1} (stretching in N-H) represented amine functional group. The wave number at 3015 cm^{-1} showed C–H (from C=C) (Figure 5).

Antioxidant activity

DPPH is stable radical and frequently used to test the antioxidant activity of pigment. It changes its color from purple at free radical state to yellow at stable compound by antioxidant reaction, where antioxidant gives one electron to DPPH hence the free radical is neutralized. An electron not coupled with DPPH gives strong absorbance, maximal at λ = 517 nm and purple coloured.

The potency of antioxidant from red pigmented bacteria was measured with DPPH and the pigment extracts in methanol were incubated at different time. Result showed that the antioxidant activity of cultures incubated at 48, 72 and 96 h were 49,11%; 46,59% and 37,53%, respectively (Figure 7). The C vitamin used as a control showed at 24,43%. The data proved that the longer incubation time, the lower antioxidant activity was. Pawar *et al.* (2015) stated that pigment of *Serratia* sp. had the potential as antioxidant. Ibrahim *et al.* (2014) showed that *S. marcescens* IBRL USM 84 pigment has the ability as antibacterial against several bacteria.

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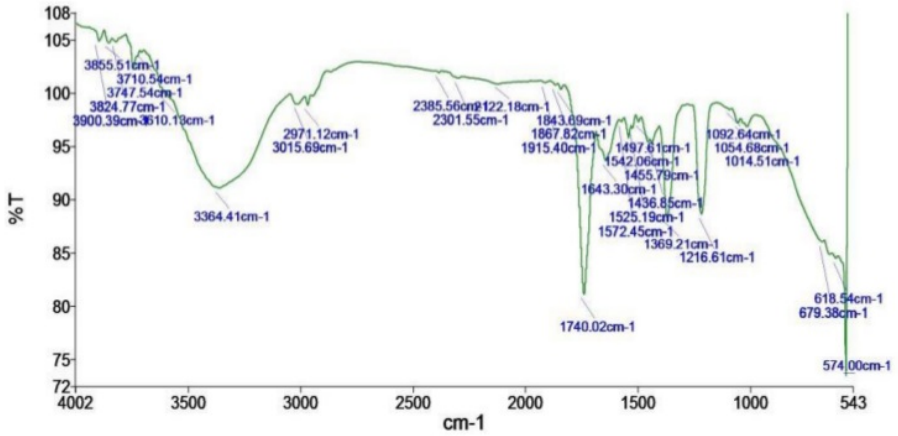

Figure 5: FTIR spectrum for crude pigment of *S. marcescens*.

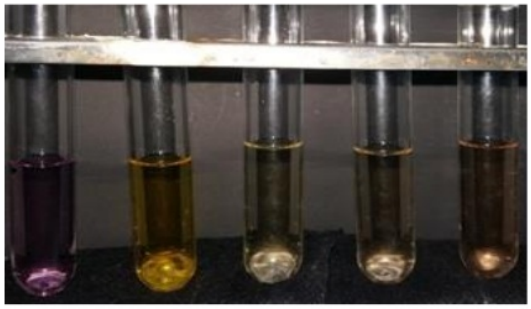

Figure 6: Antioxidant activity of *S. marcescens* with DPPH method.

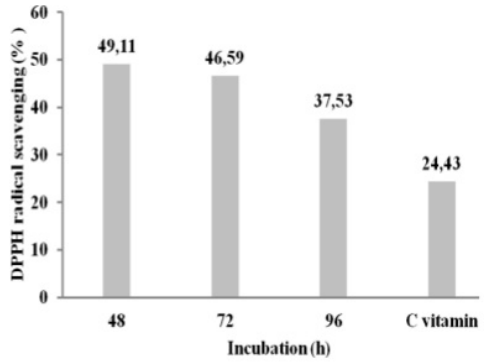

Figure 7: DPPH radical scavenging of *S. marcescens* cultured at 48, 72 and 96 h.

CONCLUSION

Based on molecular, biochemical and morphological identification, the red-pigmented bacteria isolated from Gedong Songo hot spring, Semarang was *S. marcescens.* Based on DPPH radical scavenging test, it showed that *S. marcescens* had potency as antioxidant.

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