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Isolation and identification of an agar-liquefying marine bacterium and some properties of its extracellular agarases

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ABSTRACT

Faturrahman, Meryandini A, Junior MZ, Rusmana I (2011) Isolation and identification of an agar-liquefying marine bacterium and some properties of its extracellular agarases. Biodiversitas 12: 192-197. A new agar-liquefying bacterium, designated Alg3.1, was isolated from *Gracilaria* samples collected from the Kuta Coast at Central Lombok in West Nusa Tenggara and was identified as *Aeromonas* sp. on the basis of morphology, biochemical-physiological character and 16S rDNA gene sequencing. The bacterium appeared capable of liquefying agar in nutrient agar-plate within 48 hours of incubation and the agar was completely liquefied after l5 days at 29°C. When the isolate was grown in basal salts solution medium B supplemented with peptone and yeast extract, produced extracellular agarases within a short period of time (4-16 h) and the maximum agarase activity was 0.489 nkat/mL at 36h after incubation.

Key words: *Gracilaria*, agarase, agar-liquefying, *Aeromonas*.

INTRODUCTION

Indonesia is rich country with various kinds of algae. The results of Sibolga expedition shows that there are 782 species of algae in Indonesia which consist of 179 green algae, 134 brown algae and 452 species of red algae (Nontji 2007). One group of red alga, agarophyte produce agar-agar, a complex polysaccharide present in the cell walls, up to 47.34% (Soegiarto and Sulistijo 1985).

Agar-agar can be degraded by several bacterial strains from marine environments and other sources. Agarolytic bacteria are ubiquitous in coastal and estuarine regions; however, they are not exclusively autochthonous in the marine environment, since some reports have shown that they also occur in freshwater, sewage and soil (von Hoffsten and Malmqvist 1974; van der Meulen et al. 1976; Agbo and Moss 1979). Some of bacteria isolates have been identified and classified in to *Actinomyces, Agarivorans, Alterococcus, Alteromonas*, *Microbulbifer*, *Cellulophaga*, *Cytophaga*, *Streptomyces*, *Vibrio*, *Pseudomonas*, *Saccharophagus*, *Pseudoalteromonas, Zobellia*, and *Bacillus* (Macian et al. 2001; Yoon et al. 1996; Jean et al. 2006; Khambhaty 2008). It is possible to utilize of bacteria which can produced agarase enzymes, which can degrade agar into amount of oligosaccharides and D-galactose.

D-galactoses can be catabolytic into piruvic acid via Tagatosa or Leloir pathway by yeast or other bacteria, furthermore the fermented of piruvic acid produce large amounts of alcohol, acetic and formic acids. Beside that, agarase can be used to degrade the cell walls of marine algae for extraction of labile substances with biological activities and for the preparation of protoplasts, as well as isolation of monoclonal hybrids. The polysaccharide fractions can be applied for functional foods. Agarase have applications in food, cosmetics, and medical industries by degrading agar. The polysaccharides produced by hydrolysis of agar can promote immunity in mice by abdominal injection or feeding. Some researches have shown that adding 5% agaropectin to diet suppressed significantly the increasing in cholesterol level in plasma of rats. Anti-hypercholesterolemic effect of rats also was observed (Sie et al. 2009)

In our laboratory, we have isolated a few agar-softening and agar-liquefying bacteria strains from the Kuta Coast of Central Lombok to characterize their extracellular agarases. We describe here the identification of a new agarolytic bacteria strain, *Aeromonas* sp. strain Alg3.1, and identification of hydrolysis product from agarase, and to asses their possibility to produce bioethanol.

MATERIALS AND METHODS

Sampling. Seaweed samples were collected from the Kuta Coast at Central Lombok, West Nusa Tenggara, Indonesia.

Enrichment and isolation of agarolytic bacteria. Erlenmeyer flasks (250 mL) containing sterile river water (100 mL), to which 0.1% (w/v) Oxoid agar had been added, were inoculated with samples and incubated at 29°C on a rotary shaker for 4 d (Agbo and Moss 1979). Samples (0.1 mL) of the cultures were then plated on nutrient agar (with sea water) and a basal salt solution medium B (Hofsten and

Malmqvist 1975) containing (%): $NaNO_3$ (0.2); K_2HP0_4 (0.05); $MgS0_4.7H_20$ (0.02); $MnS0_4.7H_20$ (0.002); $FeS0_4.7H_20$ (0.002); $CaCl_2.2H_2O$ (0.002); Oxoid no. 3 agar (15); adjusted to pH 7.2 before autoclaving at 121°C for 15 min. Plates were incubated at 28°C and examined daily for agarolytic activity, assessed by liquefaction or shallow depressions appearing around the colonies. After 7 d, plates were flooded with Iodine and the appearance of pale-yellow zones around colonies against a brown-violet background was considered indicative of some agar-degrading activity in the absence of the visible signs already referred to. All colonies showing liquefaction or depressions in the agar were picked off and purified by streaking out on mineral or nutrient agar (Agbo and Moss 1979).

Representative agar-degrading strains were maintained in Dubos' solution containing (g L^{-1}): $NaNO_3$ (0.5); K_2HPO_4 (0.1); $MgS0_4.7H_2O$ (0.5)**;** $FeSO_4.7H_2O$ (0.01); adjusted to pH 7.2, dispensed into bijou bottles and sterilized at 121 °C for 15 min.

Colonial and cell morphology. Colonial characteristics and pigmentation were studied on plates of nutrient agar, on marine agar, medium B agar and medium TCBS. Motility (hanging drop), Gram-staining isolates were done on bacteria grown in peptone **(**1.5%**,** w/v) water for 48 h.

Physiological biochemical tests. Strain Alg3.1 was characterized and indentified using standard physiological and biochemical plate and test tube, API 20E kits (ATB system, Biomerieux SA, Marcy-I'Etoile, France). The ability of isolate hydrolyze Starch and cellulose (CMC) was examined by incubating plates of yeast extract agar (Difco) containing 0.2% (w/v) soluble starch for 48h and then flooding with Lugol's iodine or congo red. Antibiotic sensitivity was tested to ampicillin (10 and 30 µg), tetracycline (10µg), vancomycin (30µg), erythromycin (15µg) and rifamicin (5µg) by using paper disk method by Johnson and Case (2007).

Phylogenetic analysis of isolates based on 16S rDNA sequencing. For DNA extraction, bacteria were grown for 2 days at 29°C on MA medium. A single colony of isolate was took with a sterile toothpick, resuspended in 20 mL of sterile distilled water, and heated at 95°C for 10 min to lyses the cells. The lysate was then cooled on ice, briefly centrifuged with a microcentrifuge, and used for PCR amplification.

The DNA coding for the 16S rRNA of isolate was amplified with 63f primer (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387r primer (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi et al. 1998). Amplification was done as follows: each mixture consisted of 2.0 µL of 10x *Taq* reaction buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl, 20 mM $MgCl_2$), 1.2mM of each primer, 0.2mM of each deoxynucleoside triphosphate (Sigma, St. Louis, MO, USA) and 2.5 units of *Taq* DNA polymerase (Takara, Cina), and 1 µg of DNA template in a total reaction volume of 50µL. The reaction mixtures were incubated in a thermocycler GeneAmp PCR System 2400 Perkin Elmer, (New Jersey) at 95°C for 5 min and then put through 30 cycles of 92°C for 30s, 55°C for 30s, and 72°C for 1 min. Successful amplification were confirmed by electrophoresis of 5 µL PCR products on a 1% agarose agar. Finally, the amplified 16S rDNA was purified by using a Qiaquick PCR Purification Kit (Qiagen, Inc., Calif., USA) according to the manual instructions. The amplified 16S rDNA were sequenced directly with an *automatic DNA sequencer* (ABI PRISM 3700 DNA analyzer, Applied Biosystem, Foster City, CA, USA) by using the same primers. The 16S rRNA gene sequence was compared with sequences in GenBank databases (http://www.ncbi.nlm.nih.gov./BLAST) to obtain closely matched species. The phylogenetic tree of the strain Alg3.1 was constructed using biological software MEGA4.

The best medium for growth and agarase production. To maximize producing of extracelluler agarase, growth and production medium was chosen and the culture conditions were adjusted. The growth and production medium were used marine broth (MB, Merck: Yeast extract 0.1%, Casamino acid 0.5%, NaCl 3.0%, $MgCl_2.6H_2O$ 0.23%, and KCl 0.03%), sea water medium (SWM : bacto-peptone 1.0%, filtered sea water 750 mL, aquadest 250 mL, pH 7.2-7.3), basal salt solution medium B (BSM) and BSM which supplemented with bacto peptone (0.5%, w/v) and yeast extract (0.1%, w/v). Each of medium supplemented with 0.2%, (w/v) agar (Oxoid) and incubated at 29°C for 1 to 5 d on a rotary shaker **(**120 rev. min^{-1}). Cultures were then centrifuged to remove bacteria, the supernatant was used for qualitative activity test. Qualitative agarase activity was measured based on clearance zone of free cell-supernatant of the strain Alg3.1 incubated at 4h on agar plate after that flooding with iodine.

Assay of agarase activity. Selected isolates were cultured in 250 mL Erlenmeyer flasks containing 150 mL basal salt medium B supplemented with 0.2%, (w/v) agar (Oxoid) and incubated at 29°C for 1 to 5 d on a rotary shaker (120 rev. min^{-1}). Cultures were then centrifuged to remove bacteria, the supernatant was treated with 0.2 vol. 2.5% (w/v) Cetrimide and the resulting precipitate was removed by centrifuging. The supernatant was treated with 2 vol. acetone at 4°C for 30 min. The precipitate was collected by centrifuging and redissolved in 5mM KH_2PO_4/Na_2HPO_4 buffer (pH 7.4).

The activity of this crude extracellular agarase was assayed with a solution of agarose (Sigma; 0.2% w/v) in 5mM KH_2PO_4/Na_2HPO_4 buffer (pH 7.4). The agarase preparation (0.5 mL) was mixed with 0.5 mL agarose solution and 2 mL 10mM KH_2PO_4/Na_2HPO_4 buffer (pH 7.4) and incubated at 29°C for 1 h. The reaction was stopped by adding 1 mL copper reagent and the reducing sugars were measured colorimetrically by the method of Dygert et al. (1965) except that any precipitate of undegraded agarose was removed by centrifuging (2600*g*) for 15 min. Blanks of substrate with no enzyme and enzyme with no substrate were treated in the same way. One unit of agarase activity was defined as the release of reducing groups equivalent to 1µmol D-galactose in 1 h at 29°C, pH 7.4.

Thin-Layer Chromatography (TLC). Reactions with purified agarase and agarose were performed in 50 µL reactions containing 40 µL of partial purified agarase and 5 µL of 1% agarose. Galactooligosaccharide, fructooligo-saccharide, and D-galactose (5 µg) were used as standards. The reactions were incubated at 29°C for two hours. The reaction mixture was performed on a Silica Gel 60 glass

plate (F254 Merck, Damstadt, Germany). The plates were developed with 2:1:1 n-butanol:acetic acid:water solution. Degradation products were visualized by using Handy UV Lamp AS ONE 254nm (Japan).

RESULTS AND DISCUSSION

Identification of *Gracilaria*-associated agarolytic bacterium Alg3.1

A bacterial strain that could produce extracellular agarase was isolated from the marine seaweed, *Gracilaria* sp., of the Kuta Coast in Central Lombok, West Nusa Tenggara, Indonesia, based on its capability in liquefying of agar or exerting clearance zone on nutrient agar plate containing agar 1.5%. in the bacteriology laboratory. The strain Ag3.1 was Gram-negative rods, non-fermentative and highly motile, encapsulated, pleomorphic, highly motile, which grows singly or in short chains. colonies, The colony of agarolytic strain Alg3.1 was yellow in TCBS (Figure 1A), flat and large, grayish white in nutrients agar medium (data not shown), appear to like *Vibrio* species. But strain Alg3.1 can utilize lactose so that can not be grouped into *Vibrio*. Cell-free supernatant of strain Alg3.1 produced 42 mm halos of clearing in diameter after 6 h of incubation at 29^{o}C (Figure 1B). This strain rapidly produced a crater of digested agar on nutrient agar plates, in the manner illustrated by v. Hofsten and Malmqvist (1975) (Figure 1C). Moist nutrient agar plates was completely liquefied in about l5 day at 29^{o}C (Figure 1D). Agarolytic bacteria produce visible changes on agar because of the cleavage of polysaccharide chains, ranging from softening of gel to agar pitting and extensive liquefaction (Agbo and Moss 1979). Colonies did not produce diffusible pigment and growth occurred over a wide temperature range (4 to 40^{o}C) with the optimum at 29^{o}C.

The results of several biochemical and physiological test for strain Alg3.1 are shown in Table 1. The strain was susceptible to ampicillin, tetracycline, vancomycin, erythromycin and rifamicin. The strain Alg3.1 was aerobic, oxidase positive, arginine dihydrolase positive, urease and gelatinase positive, utilized D-glucose, D-galactose, D-manitol, D-fructose, sucrose, lactose, agar, agarose, carrageenan, arabinogalactan, galactomannan, and starch as sole carbon source, and reduced nitrate to nitrite. Strain Alg3.1 can be distinguished from *Aeromonas salmonicida* subsp. *pectinolytica* MEL and *A. salmonicida* subsp. *salmonicida* by its utilization of N-acetyl glucosamine, citrate and indole production.

Phylogenetic analysis of 16S rRNA

Partial 16S rDNA sequence of Alg3.1 could be used for identification taxonomic of isolate. Alignment of nucleotide sequence of the 16S rDNA with sequence in GenBank database showed maximum homology with those of *Aeromonas* species and appeared to be 98% identical to *Aeromonas salmonicida* subsp. *salmonicida* strain VA-K2-V5 (Figure 2). However, we must point out that strain Alg3.1 differs from the type strain of this strain in some properties. Based on Gram staining, morphology, biochemical, physiology and 16S rDNA sequence analysis, the agarolytic strain Alg3.1 was grouped into the genus of *Aeromonas*. There has not been report on agarase production from this genus. Searching agarolytic bacteria in GenBank database showed that there is not discover *Aeromonas* species, and phylogenetic tree of some agarolytic bacterium presented in Figure 3. Consequently, these result indicated that the *Gracilaria*-associated bacteria strain Alg3.1 is a novel agarolytic bacteria producing extracellular agarase enzyme.

Agarolytic activity of strain Alg3.1

Most of the reported agar-degrading enzyme producers are marine microorganisms active in algae cell wall decomposition. Because agarases are the enzymes that hydrolyzes agar, they have been isolated from the surface of rotted red algae in the South China Sea coast in Hainan Island, decomposing algae in Niebla in Chile and in Halifax in Canada, and decomposing *Porphyra* in Japan (Fu and Kim 2010). Curiously, decomposition of agar by microorganisms appears to be performed almost entirely by gram-negative bacteria, although few if any gram-positive bacteria have been identified as producers of alginate degrading enzyme (Khambhaty et al. 2008).

A **B** **C** **D**

Figure 1. Agarolytic bacterial colonies on TCBS medium with 2% agar (A), clearance zone of free cell-supernatant of the strain Alg3.1 incubated at 4h on agar plate after flooding with iodine (B), and agar liquefying by agarolytic bacterium strain Alg3.1 after incubated 3 d (C) and 15 d (D) in solid medium.

Table 1. Phenotypic characteristics of *Aeromonas* strain Alg3.1

Characteristics	*Aeromonas* strain Alg3.1	*A. salmonicida* subsp. *pectinolytica* MEL	Characteristics	*Aeromonas* strain Alg3.1	*A. salmonicida* subsp. *pectinolytica* MEL
Morphology	Rods	Rods	Substrate utilization:		
Gram-reaction	-	-	Glucose	+	Nd
Motility	+	-	Mannitol	+	Nd
Aerobic growth	+	+	Sucrose	+	+
Anaerobic growth	-	-	Maltose	-	Nd
Optimum pH	7.5		Mannose	-	Nd
Optimum temperature	25-29^{o}C		Arabinose	-	Nd
Oxidase	+	Nd	Sorbitol	Nd	+
Urease	+	+	Lactose	+	+
Elastase	Nd	+	NAceG	-	+
PNPG	-	Nd	K-glukonat	+	+
Indole production	-	+	Capric acid	-	Nd
Hydrolysis of			Adipic acid	-	Nd
Gelatin	+	Nd	Malic acid	+	Nd
Starch	+	Nd	Citrate	-	+
Esculine	+	+	Phenilacetat acid	-	Nd
Nitrate	+	Nd			

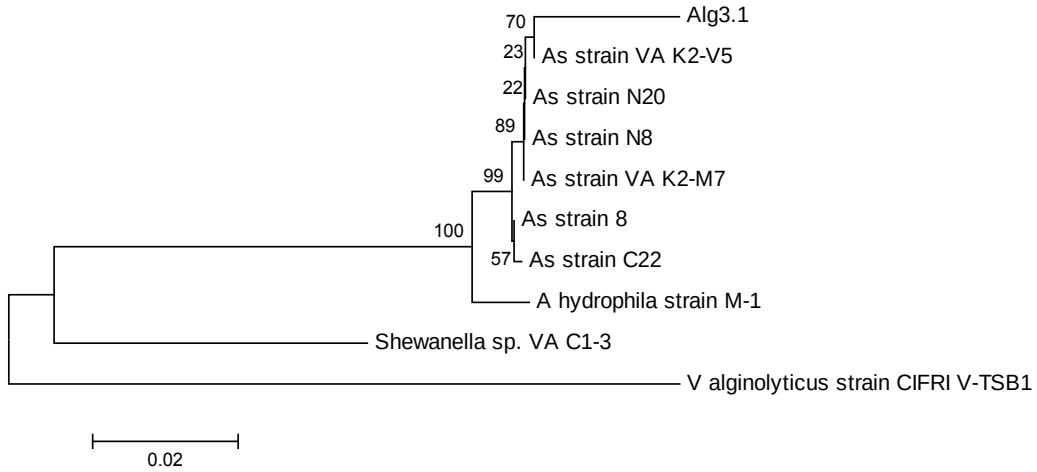

Figure 2. Phylogenetic tree based on 16S rDNA gene sequencing showing the relationships between the agar-degrading strain Alg3.1, *Aeromonas* spp. and related genera. Their GenBank accession numbers for the bacteria in the tree are: *Aeromonas salmonicida* subsp. *salmonicida* strain VA_K2-V5, GQ869652.2; *A salmonicida* strain N20, HM244937.1; *A salmonicida* strain 8, HQ533268.1; *A salmonicida* strain 8, HQ533268.1; *A salmonicida* strain C22, HQ259698.1; *A salmonicida* strain N8, HM244936.1; *A. hydrophila* strain M-1, HQ609947.1; and *Shewanella* sp. VA_C1-3, GQ869648.1 and *V. alginolyticus*, JF784015.1 were used as outgroup.

To clarify the level of agar degradation, the cell growth during the batch fermentation of the strain Alg3.1 to produce agar-degrading enzyme and the change of agar-degrading enzyme activity in the supernatants of the culture broth were investigated. Figure 4 shows the growth curve of *Aeromonas* Alg3.1 and the production of agarase in the presence of agar, where is growth and production agarase walk successive. The strain *Aeromonas* Alg3.1 readily released agarase into the medium yielding monosaccharide or agarooligosaccharide. The biomass reached maximum and the agar-degrading enzyme activity was 0.425 nkat/mL after cultivation for 44 h and the maximum activity was 0.489 nkat/mL at 36h after incubation. During the logarithmic phase of growth the enzyme activity showed a rapid increase. But the activity decreased before cell entering to the stationary and decline phase.

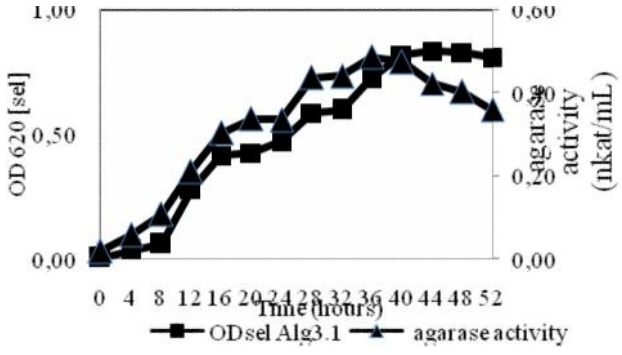

Figure 4. Time courses for cell growth of strain Alg3.1 and agar-degrading enzyme activity change in culture broth. Cells were grown in a BSM supplemented peptone and yeast extract at 120 rpm at 29^{o}C, pH 7.5. Cell growth was estimated by optical density at 620 nm (OD).

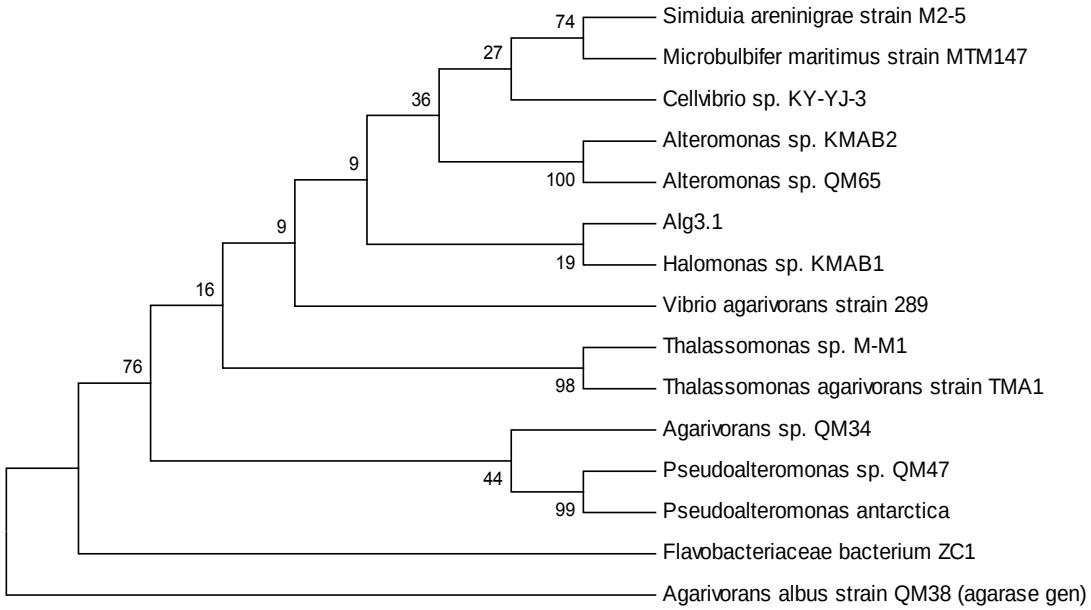

Figure 3. The genetic relationship between Alg3.1 with other genera of agarolytic bacteria

The activity of agarase crude extract of Alg3.1 is 2.9 fold higher than *P. antartica* N-1, 0.1667 nkat/mL (Vera et al. 1998). Numerous reported data indicate that agarases purified from genus of *Vibrio* have lower specific activities, which are 7.54 and 20.8 U/mg from strain PO303 (Araki et al. 1998) and 6.3 U/mg from strain JT0107 (Sugano et al. 1993). Agarases from genus Agarivorans show medium specific activities, which are 57.45 and 76.8 U/mg from strain HZ105 (Hu et al. 2008) and 25.54 U/mg from strain YKW-34 (Fu et al. 2008). Agarases from genus *Alteromonas* and *Pseudoalteromonas* exhibit high specific activities, which are 83.5 U/mg from *Alteromonas* sp. SY37-12 (Wang et al. 2006), 234 U/mg from *Alteromonas* sp. C-1 (Leon et al. 1992).

To maximize producing intracellular agarase, growth and production medium was chosen and the culture conditions were adjusted. Table 2 showed that the best medium for produce intracellular agarase is basal salt solution medium (Medium B) which supplemented with bacto peptone (0.5%, w/v) and yeast extract (0.1%, w/v). Strain Alg3.1 needs growth factor such as bacto peptone and yeast extract to grow optimum, and the production of agarase influenced by salt concentration. In medium B, we can regulate salt concentration as according to need of the bacteria.

Table 2. Growth and qualitative agarase activity of Alg3.1 strain in various medium for 24h incubation. Qualitative activity was measured based on diameter of clearing zone by using cell-free supernatant which incubated in solid medium for 4 h at 29°C

Growth media	Growth (cfu/mL)	Clearing zone (mm)
Marine broth	6.0×10^7	26.5
Sea water medium	7.4×10^7	32.5
Basal salt solution medium B	4.1×10^7	21
BSM + peptone + yeast extract	1.1×10^8	38

Identification of reaction product

The result of visualization with UV 254 nm shows a large amount of two kind of agarooligosaccharide as major product (Figure 5). These result indicated that this strain have multi extracellular agarase enzymes, which could cleavage agarose into neoagarotetraose by agarase I, then, neoagarotetraose is cleaved at by neoagarotetraose hydrolase-or agarase II-to yield neoagarobiose. Finally, neoagarobiose is degraded by periplasmic α-neoagarobiose hydrolase to the D-galactose and 3,6-anhydro L-galactose, which are metabolized by intracellular enzymes.

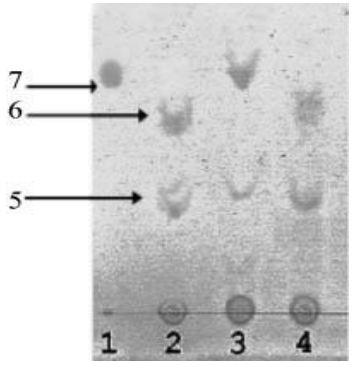

Figure 5. TLC of the products of agarose hydrolyzed by the partial purified agarase enzyme. Standard (1,2), Abn1.2 (3), Alg3.1 (4), neoagarooligosaccharide (5), fructooligosaccharide (6) and D-galactose (7)

Numerous reports show that agarolytic bacteria can produce agarase enzyme that vary. A part of these strains only produce agarase type I like *Pseudomonas* SK38, other has only agarase II like *Agarivorans* JAMB-A11, there also that can to produce agarase I and II like in *P. atlantica*.

Possibility to produce bioethanol from agarophyte

Increase on world's energy demand and the progressive depletion of oil reserves motivate the search for alternative energy resources, especially for those derived from renewable materials such as biomass (Saxena et al. 2009). Global concern about climate change and the consequent need to diminish greenhouse gases emissions have encouraged the use of bioethanol as a gasoline replacement or additive (Balat et al. 2008). Bioethanol may also be used as raw material for the production of different chemicals, thus driving a full renewable chemical industry.

Substitution bioethanol as one of energy source has been selected as an alternative source for the fossil fuel substitution. The marine seaweed, such as Agarophyte, can be used for the production of bioethanol. The main component of agarophyte such as *Gracilaria* consists of a complex biopolymer cellulose, hemicelluloses, agar or carrageenan (Abbot and Dawson 1978). Agar is composed of two fractions, agarose and agaropectin. Agarose, the main constituent, is a neutral polysaccharide that forms a linear chain structure consisting of repeating units of agarobiose, which is an alternating polymer of D-galactose and 3,6-anhydro L-galactose linked by alternating β-(1, 4) and α-(1,3) bonds (Allouch et al. 2003; Flament 2007). The product of agar-degradation consist of neoagarotetraose as the major end product, neoagarobiose, D-galactose and 3,6-anhydro L-galactose (Hosoda et al. 2003; Michel et al. 2006).

This strain can degrade and utilized several complex polysaccharides, such as agar, agarose, starch, and carrageenan. Although Alg3.1 can hydrolyze carboxy methyl cellulose but can not utilize it as carbon source solely (Table 3). These results suggest that agar-liquefying Alg3.1 might be a good candidate as a producer bioethanol from agarophyte, because if we mix this strain with other bacteria or yeast so D-galactoses can be catabolytic into piruvic acid via Tagatosa or Leloir pathway, furthermore the fermented of piruvic acid produce large amounts of alcohol, acetic and formic acids.

Table 3. Polysaccharides degradation and utilization by the strain Alg3.1

Polysaccharides	Degradation	Utilization
Agar	+	+
Agarose	+	+
Soluble Starch	+	+
carrageenan	+	+
CMC	+	-

CONCLUSION

The *Gracilaria*-associated bacteria strain alg3.1 is a new report of agarolytic bacteria from *Aeromonas* genera which can produce extracellular agarase enzyme. This strain have two kind of agarooligosaccharides as major product and can degrade and utilized various of complex polysaccharides, such as agar, agarose, starch, and carrageenan.

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