A THESIS

FOR THE DEGREE OF DOCTOR OF SCIENCE

GENE IDENTIFICATION, CLONING, EXPRESSION, PURIFICATION AND INDUSTRIAL APPLICATION OF MARINE BACTERIA PRODUCING AGARASES

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CONTENTS

요약문 i

List of Figures v

List of Tables viii

I. Introduction 1

II. Materials and methods 6

1. Isolation of agarase producing bacteria 6

2. Identification of bacteria using 16s rRNA sequence 8

2. 1. Genomic DNA isolation 8

2. 2. 16s rRNA sequence amplification 8

3. PCR amplification of agarase genes 9

3. 1. PCR amplification for *Agarivorans* sp. strains 9

3. 2. PCR amplification for *Cytophaga* sp. strains 10

3. 3. PCR amplification for *Psudoalteromonas* sp. Strains 10

4. Long and Accurate Polymerase Chain Reaction (LA PCR) for detection of full length sequence 12

5. Sequence characterization of agarase genes 15

6. Cloning and expression of agarase genes 15

6. 1. 1. Cloning of AG4 agarase coding sequence into the pMAL-c2x expression vector 15

6. 1. 2. Over-expression and purification of recombinant AG4 agarase enzyme 18

6. 1. 3. Purification of recombinant AG4 using pMAL^{TM} protein fusion and purification system 18

6. 2. 1. Cloning of AG17 agarase coding sequence into the pET16b expression vector 19
6. 2. 2. Over-expression and purification of recombinant AG17 agarase enzyme 21
6. 2. 3. Purification of recombinant AG17 using His Bind purification Kit 21
6. 3. 1. Cloning of AG52 agarase coding sequence into the pET11a expression vector 21
6. 3. 2. Over-expression of recombinant AG52 agarase enzyme 24
6. 4. 1. Cloning of AG52 agarase coding sequence into the pET16b expression vector 24
6. 4. 2. Over-expression of recombinant AG52 agarase enzyme 27

7. Biochemical characterization of recombinant agarase 29
7. 1. Agarase enzyme assay 29
7. 2. Optimum temperature 29
7. 3. Optimum pH 29
7. 4. Thermostability 29
7. 5. Effect of metal ion salts and chelators 30
7. 6. Identification of reaction products after hydrolysis of agar substrates 30

8. Industrial application of neoagaro-oligosaccaride 31
8. 1. Production of neoagaro-oligosaccaride 31
8. 2. Functional activity of neoagaro-oligosaccaride 31
8. 2. 1. Antioxidant activity 31
8. 2. 2. Assay of melanin production 31
8. 2. 3. Cytotoxicity assay 32

III. Results

1. Screening and identification of agarase producing bacteria strains 33

2. Sequence characterization of agarase genes 37
2. 1. AG4 37
2. 2. AG17 39
2. 3. AG31 42

2. 4. AG52 ..... **44**
2. 5. Sequence comparison and classification of AG4, AG17, AG31 and AG52 ..... **47**
2. 6. Phylogenetic analysis of AG4, AG17, AG31 and AG52 genes **50**

3. Expression and purification of recombinant agarase 53
3. 1. Purification of recombinant *Pseudoalteromonas* sp. AG4 agarase **53**
3. 2. Purification of recombinant *Agarivorans* sp. AG17 agarase **55**
3. 3. Purification of recombinant *Pseudoalteromonas* sp. AG52 agarase ... **57**

4. Biochemical characterization of recombinant purified agarases 59
4. 1. Enzyme characterization of purified recombinant AG4 ..... **59**
4. 1. 1. Effect of temperature on the activity of the recombinant AG4 ... **59**
4. 1. 2. Effect of pH on the activity of the recombinant AG4 ..... **59**
4. 1. 3. Effect of temperature on the thermostability of the recombinant AG4 ..... **59**
4. 1. 4. Effect of $CaCl_2$ on thermostability of recombinant AG4 ..... **59**
4. 1. 5. Effect of metal ions salts and chelators on activity of recombinant AG4 ..... **60**
4. 1. 6. Identification of hydrolysis products of the recombinant AG4 on TLC **60**
4. 2. Enzyme characterization of recombinant AG17 ..... **67**
4. 2. 1. Effect of temperature on the activity of the recombinant AG17 . **67**
4. 2. 2. Effect of pH on the activity of the recombinant AG17 ..... **67**
4. 2. 3. The effect of thermostability on purified recombinant AG17 ..... **67**
4. 2. 4. Effect of metal ions salts and chelators on activity of recombinant AG17 ..... **68**
4. 2. 5. Identification of hydrolysis products of the recombinant AG17 on TLC **68**
4. 3. Enzyme characterization of recombinant AG52 ..... **74**
4. 3. 1. Effect of temperature on the activity of the recombinant AG52 . **74**
4. 3. 2. Effect of pH on the activity of the recombinant AG52 ..... **74**

4. 3. 3. Effect of thermal stability on the activity of the recombinant AG52 74
4. 3. 4. Effect of $CaCl_2$ on thermostability of recombinant AG52 74
4. 3. 5. Effect of metal ion salts and chelators on activity of recombinant AG52 75
4. 3. 6. Identification of hydrolysis products of the recombinant AG52 on TLC 75
4. 4. Specific activity of agarases based on to substrate specificity 82

5. Functional activity of neoagaro-oligosaccarides 83
5. 1. Antioxidant assay 83
5. 2. Effect of whitening 83
5. 3. Effect of Neoagaro-oligosaccharide on cell viability 83

IV. Discussion 86

V. References 92

감사의 글 101

요 약 문

한천 (agar)은 홍조류의 세포벽에 존재하는 다당류로 agarose 70%와 agaropcetin 30%로 구성되어진다. agarase는 agar 또는 agarose를 분해하는 효소로써 agarose의 galactose 중합체 중 α-1,3 결합을 절단하여 agarooligosaccharide를 생성하는 α-agarase와 β-1,4 결합을 절단하여 neoagarooligosaccharide를 생성하는 β-agarase로 크게 구분되어진다. α-agarase에 생성된 agarooligosaccharide는 apoptosis 유도 활성, 항암활성, 항바이러스 활성, 항산화 활성, 면역 조절 활성, 항알레르기 활성, 항염증 활성 등이 보고되어져 있고, β-agarase에 의해 생성되는 neoagarooligosaccharide는 세균성장 억제, 항산화 활성, 전분노화 방지, 보습효과, 미백효과 등이 보고되어져 있다.

본 연구에서는 다양한 선택배지를 제작하여 제주 해양 소스로부터 29개의 agarase 생산 균주를 분리하였으며 16s rRNA 유전자 서열을 토대로 유전학적 동정을 실시하였다. 그 결과 *Alteromonas* sp., *Agarivorans* sp., *Aquimarina* sp., *Cellulophaga* sp., *Cytophaga* sp., *Flammeovirga* sp., *Glaciecola* sp., *Microbulbifer* sp., *Pseudoalteromonas* sp., *Rhodobacteraceae* sp., *Ruegeria* sp., *Reinekia* sp., *Shewanella* sp. 등의 다양한 균주들이 확인 되어졌다.

기존 유전자 데이터베이스에 등록된 agarase 유전자 서열 및 아미노산 서열들을 토대로 하여 다양한 primer들을 제작하였고 각 종들을 대상으로 PCR을 수행하였다. 그 결과 4개의 균주 (*Pseudoalteromonas* sp. AG4, *Agarivorans* sp. AG17, *Flammeovirga* sp. AG31, *Pseudoalteromonas* sp. AG52) 로부터 2개의 agarase 유전자 코딩 서열 및 2개의 부분 서열을 확인하였으며, LA PCR을 수행하여 부분서열로부터 전체 코딩서열을 확인하였다. 각각의 agarase에 대한 유전자 분석 결과 *Pseudoalteromonas* sp. AG4는 63 bp의 신호서열을 포함하는 870 bp의 ORF로 되어있고, GH-16 β-agarase domain (D^{22}~K^{287} aa), active sites (Y^{69}, N^{71}, W^{72}, W^{139}, S^{145}, D^{150}, E^{153}, F^{176}, R^{178}, E^{257}, E^{259}) 및 calcium binding modules (E^{47}, F^{48}, N^{49}, G^{91}, A^{92}, D^{82}, W^{83})을 포함하고 있었으며 33 kDa의 분자량이 진단되어졌다. *Agarivorans* sp. AG17 agarase는 60 bp의 신호 서열을 포함하는 2988 bp의 ORF로 되어있고 C-terminal 부분에 transmembrane site (G^{968}~L^{990} aa)가 존재하는 것으로

확인 되었으며 *Agarivorans* sp. JAMB-A11의 agarase와 높은 상동성 (98.6%)을 나타내는 것으로 확인되었다. 진단된 분자량은 107 kDa 이었다. *Flammeovirga* sp. AG31 agarase는 1446 bp의 ORF로 되어있고 다른 종들의 agarase 유전자 서열들과 비교 했을 때 상동성이 매우 낮았다. ORF에는 GH16- β-agarase domain (R^{2}~T^{36}, N^{88}~K^{161} aa), bacterial Ig-like domain 2 (A^{172}~I^{250} aa) 및 carbohydrate binding domain (E^{267}~K^{392}, Q^{413}~I^{478} aa)을 포함하고 있었다. *Pseudoalteromonas* sp. AG52 agarase는 63 bp의 신호서열을 포함하는 870 bp의 ORF가 확인 되었고 GH16-β-agarase domain (D^{22}~K^{287} aa), active sites (Y^{69}, N^{71}, W^{72}, W^{139}, S^{145}, D^{150}, E^{153}, F^{176}, R^{178}, E^{257}, E^{259}) 및 calcium binding domains (Q^{47}, F^{48}, N^{49}, G^{91}, A^{92}, D^{82}, W^{83}).이 확인 되었고, 또한 lipo protein을 형성할 수 있는 신호가 포함되어져 있었다.. *Aeromonas* sp. agarase와 96.8 %의 유전적 상동성을 나타냈으며 분자량은 32 kDa으로 진단되었다.

AG4 agarase mature sequence는 pMal-c2x에 클로닝 하여 *E. coli* BL21 (DE3)를 숙주로 형질전환이 이루어졌으며 IPTG를 이용해 maltose binding protein이 fusion된 agarase의 발현을 유도하였다. pMAL™ protein fusion and purification system을 이용하여 단백질을 순수 정제하고 SDS-PAGE를 통해 72.5 kDa의 fusion된 agarase 단백질을 확인할 수 있었다. AG17 agarase mature sequence는 pET16b vector에 클로닝 하여 *E. coli* BL21 (DE3)를 숙주로 형질전환이 이루어졌으며 IPTG를 이용해 His-tag이 fusion된 agarase의 발현을 유도하고 108 kDa의 fusion agarase를 정제하였다. AG52 agarase는 신호서열이 포함된 것과 포함되지 않은 서열을 각각 pET11a 와 pET16b vector에 클로닝하고 발현을 유도하였다. 신호서열이 포함된 agarase는 발현 양이 미미하여 SDS-PAGE 상에서 확인할 수 없었으나 배양 상등액으로부터 활성을 나타내는 것으로 lipoprotein이 형성되어 세포외로 분비되었음을 확인할 수 있었다. 신호서열이 포함되지 않은 AG52 agarase는 his-tag가 fusion 되어 발현이 유도 되었고 정제 후 33 kDa의 fusion 단백질을 확인 할 수 있었다. 각각(AG4, AG17, AG52 agarase)의 최적 조건 및 특성을 분석하기 위해 정제된 agarase를 가지고 최적 온도 및 pH, 열안정성, 금속이온 효과, 기질 분해 특성을 분석하였다. recombinant AG4 agarase는 55 ℃, pH 5.5에서 최적 활성을 나타냈고 40, 45 ℃에서 2시간동안 열 안정성을 나타냈으며 50, 55 ℃에서도 2시간동안

50% 이상의 활성을 유지하였다. AG4 agarase 서열에 cacium binding module이 포함되어 있어 Ca이 열 안정성에 영향을 미칠 것으로 사료되어 CaCl2을 첨가하여 열 안정성 test를 수행하였지만 40, 45, 50 ℃에서는 Ca을 첨가하지 않을 때와 비슷한 열 안정성을 나타냈고, 55 ℃에서는 오히려 감소하는 경향을 나타냈다. 금속이온 효과 분석에서는 Fe에 의해 활성이 약 120% 증가하였고, Cu, EDTA, Zn에 대해서는 뚜렷한 저해를 나타냈다. TLC 분석에서는 agar를 분해하여 최소 4당을 생산하였고 beta agarase type 이였으며 NA4 및 NA6는 분해하지 못했다.

recombinant AG17 agarase는 65 ℃, pH 5.5에서 최적 활성을 나타냈고, 40, 45 ℃에서는 2시간동안 열에 안정하였으며 50 ℃에서도 2시간동안 80% 활성을 유지하였다. 55, 60, 65 ℃ 에서는 2시간 이후 40% 미만의 활성을 유지하였다. 금속이온 test 결과 Mg 이온에 의해 활성이 140% 이상 증가하였고 Cu, Mn, Zn에 의해 저해 활성이 나타났다. TLC 분석 결과 agar를 분해하여 최소 2당 및 4당을 생성하였고 NA4와 NA6 모두 분해하는 것으로 확인되었다.

recombinant AG52 agarase는 55 ℃, pH 5.5에서 최적 활성을 나타냈고, 열 안정성은 40 ℃에서 2시간 후 40%로 감소했다. 45, 50, 55 ℃에서는 30분 후 40% 미만의 활성 만을 나타내며 빠른 감소를 보였다. Ca을 첨가한 후에도 40 ℃에서만 활성이 조금 증가하였을 뿐 나머지 온도에서는 차이를 나타내지 않았다. 금속이온 효과에서는 Fe 이온 및 K 에 의해 활성이 증가하였고, Cu, EDTA, Zn에 의해 강한 저해 활성을 나타내었다. TLC 분석에서는 agar를 분해 후 최소 4당을 생성하였으며 NA4 및 NA6는 분해하지 못했다.

AG 4, 17, 52 agarase의 기질 특이성을 분석한 결과 AG4, AG17 agarase는 agar와 agarose를 비슷한 수준으로 분해하였고 AG52는 agar를 기질로 했을 때 agarose를 기질로 했을때보다 좋은 활성을 나타냈다. 하지만 각각의 agarase로 carrageenan은 분해하지 못했다. agar에 대한 특이 활성은 AG4 가 204.4 unit/mg, AG17 158.8 unit/mg, AG52 105.1 unit/mg으로 나타났다.

AG4와 AG17 agarase를 이용하여 올리고당을 생산 하였으며 최종 생성물은 NA4와 NA2+NA4로 확인 하였고 이를 이용해 항산화, 멜라닌 저해 활성, 세포 독성 등을 확인 하였다. NA4와 NA2+NA4 둘 다 항산화 활성은 나타냈으나 positive control인 비타민 E 에 비해 미미한 활성을 나타냈다. 하지만 melanin 저해 활성

에서 NA4가 미백 원료로 많이 사용되어지고 있는 albutin 과 비슷한 활성을 나타내었고 세포 독성이 없어 화장품의 미백 원료로 사용이 가능할 것이라 사료 된다.

List of Figures

Figure 1. Structure of the agarose and the two product types produced from α-agarase and β-agarase cleavage.

Figure 2. Sampling area

Figure 3. AG4 agarase coding sequence without including signal sequence cloned into pMal-c2x

Figure 4. AG17 agarase coding sequence without including signal sequence cloned into pET-16b

Figure 5. AG52 agarase coding sequence including signal sequence cloned into pET-11a

Figure 6. AG52 agarase coding sequence without including signal sequence cloned into pET-16b

Figure 7. Identification of agarase producing bacteria by clearing zone screening on agar plates.

Figure 8. The nucleotide and deduced amino acid sequences of the *Pseudoalteromonas* sp. AG4.

Figure 9. The nucleotide and deduced amino acid sequences of the *Agarivorans* sp. AG17.

Figure 10. The nucleotide and deduced amino acid sequences of the *Flammeovirga* sp. AG31.

Figure 11. The nucleotide and deduced amino acid sequences of the *Pseudoalteromonas* sp. AG52.

Figure 12. Lipoprotein signal peptide (signal peptidase II) of *Pseudoalteromonas* sp. AG52.

Figure 13. Schematic representation of the characteristic domains of AG4, AG17, AG31 and AG52 amino acid sequences.

Figure 14. ClustalW paiwise sequence analysis of AG4 and AG52 sequences.

Figure 15. Phylogenetic analysis of AG4, AG17, AG31 and AG52 with selected

agarase genes.

Figure 16. SDS-PAGE analysis of recombinant *Pseudoalteromonas* sp. AG4 purification.

Figure 17. SDS-PAGE analysis of recombinant *Agarivorans* sp. AG17 purification.

Figure 18. SDS-PAGE analysis of the recombinant AG52 agarase.

Figure 19. The effect of temperature on the activity of purified recombinant AG4.

Figure 20. The effect of pH on the activity of the purified recombinant AG4.

Figure 21. The effect of thermostability on purified recombinant AG4 at different temperatures for different time points.

Figure 22. The effect of $CaCl_2$ on thermostability of purified recombinant AG4

Figure 23. Effect of metal ion salts and chelators on purified recombinant AG4

Figure 24. Thin layer chromatography of hydrolysis products of the purified AG4 enzyme on food grade agar and neagaroligosaccharides.

Figure 25. The effect of temperature on the activity of purified recombinant AG17.

Figure 26. The effect of pH on the activity of the purified recombinant AG17.

Figure 27. The effect of thermostability on purified recombinant AG17 at different temperatures for different time points.

Figure 28. Effect of metal ion salts and chelators on purified recombinant AG17.

Figure 29. Thin layer chromatography of hydrolysis products of the purified AG17 enzyme on food grade agar and neagaroligosaccharides.

Figure 30. The effect of temperature on the activity of purified recombinant AG52.

Figure 31. The effect of pH on the activity of the purified recombinant AG52.

Figure 32. The effect of thermostability on purified recombinant AG52 at different temperatures for different time points.

Figure 33. The effect of $CaCl_2$ on thermostability of purified recombinant AG52.

Figure 34. Effect of metal ion salts on enzymatic activity of AG52.

Figure 35. Thin layer chromatography of hydrolysis products of the purified AG52 enzyme on food grade agar and neagaroligosaccharides.

Figure 36. Effect of neoagarooligosaccharides (NA+NA4 and NA4) on the DPPH

scavenging activity.

Figure 37. Whitening effects of neoagarotetrose, neoagarotetrose and neoagarobiose and arbutin at various concentrations in B16F10 cells.

List of Tables

Table 1. Composition of selection plates

Table 2. Primers for amplification of agarase genes

Table 3. Primers for detection of full length sequence from AG31 and AG52

Table 4. Primers for over-expression

Table 5. Identification of agarase producing bacteria by 16s rRNA sequence analysis.

Table 6. Specific activity of agarases accoding to substrates

I. Introduction

Agar

Agar is an important gelatinous substance present in the cell wall of some marine red algae (Rhodophyceaea). It is mainly composed of agarose and agaropectin. Agarose is a hydrophilic hetero polysaccharide, which consists of a linear chain of alternately arranged 3-O-linked β-D-galacto-pyronose and 4-O-linked 3,6-anhydro-α -L-galacto pyronose residues (Duckworth and Yaphe 1971). Agaropectins have the same basic disaccharide-repeating units as agarose although some hydroxyl groups of 3,6-anhydro-α-L-galactose residues are replaced by sulfoxy or methoxy and pyruvate residues (Araki 1966). Agarose and its derivatives are widely used in food, cosmetics, and pharmaceutical industries due to its gelling ability, stabilizing properties, and high viscosity.

Agarase

Agarases are the natural enzymes of certain agarolytic organisms found mostly in marine habitats that hydrolyze the agarose (Araki and Arai 1967; Potin et al. 1993). Also, agarase is the first enzyme in the agar catabolic pathway. Availability of agarases basically in the marine environment, which is consistent with the fact that agar, being a product of marine algae is available to utilize some marine organisms as a convenient carbon and energy source (Hodgson and Chater 1981; Parro and Mellado 1994; Ohta et al. 2005a). Agarases are classified into two groups named as α-agarase and β-agarase based on their cleavage of α-β linkages of agarose, breaking them into oligosaccharides. α-Agarase hydrolyzes α-(1→3) linkages of agarose, producing agaro-oligosaccharises such as agarobiose, while β-agarase hydrolyses β-(1 →4) linkages, resulting neoagaro-oligosaccharises such as neoagarobiose, neoagarotetrose, or neoagarohexaose as the main products. Agaro-oligosaccharides has 3,6- anhydro-α-L-galactose residues at their reducing ends and in contrast, neoagaro-oligosaccharides has D-galactose residues at their reducing, ends (Araki 1959; Hassairi

et al. 2001).

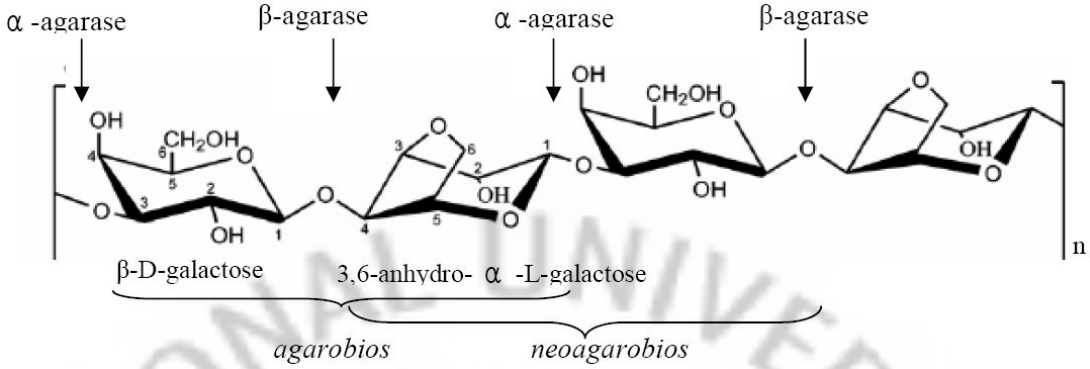

Figure 1. Structure of the agarose and the two product types produced from α -agarase and β-agarase cleavage. The respective cleavage sites are indicated by arrows. The marked agarobiose represent the basic unit of the agaro-oligosaccharide (AOS) cleaved by α-agarase action. The marked neo-agarobiose represents the basic unit of the neo-agarooligosaccharide (NAOS) cleaved by β-agarase. The agarose structure was adapted from Lahaye et al. (Lahaye et al. 1989).

A number of agar degrading enzymes have been identified and purified from various microorganisms. Except in a few cases, most of these agarolytic organisms are bacteria. To date, agarase producing bacteria of many different genera have been found including species of *Agarivorans* (Ohta et al. 2005a; Lee et al. 2006; Fu et al. 2008b), *Alteromonas* (Leon et al. 1992; Potin et al. 1993; Kirimura et al. 1999; Hassairi et al. 2001; Wang et al. 2006), *Bacillus* (Suzuki et al. 2003), *Cytophage* (Duckworth and Turvey 1968; Van der Meulen and Harder 1975), *Microbulbifer* (Ohta et al. 2004b; Ohta et al. 2004c), *Pseudomonas* (Groleau and Yaphe 1977; Morrice et al. 1983; Lee et al. 2000; Ryu et al. 2001), *Pseudoalteromonas* (Vera et al. 1998; Schroeder et al. 2003; Ma et al. 2007), *Saccharophagus* (Ekborg et al. 2006), *Streptomyces* (Kendall and Cullum 1984), *Thalassomonas* (Ohta et al. 2004a), *Vibrio* (Aoki et al. 1990; Sugano et al. 1993; Araki et al. 1998a; Zhang and Sun

2007; Fu et al. 2008a), *Zobellia* (Jam et al. 2005). However, almost all of the known agarases belong to the β-agarase group. In contrast, α-agarases are poorly known and very few biochemical studies have been reported. For examples, α-agarase purified from *Alteromonas agarlyticus* GJ1B, which hydrolyzed agarose to yield a series of AOS and showed β-galactosidase activity together with agarase activity (Potin et al. 1993). Additionally, novel α-agarase was purified from *Thalassomonas bacterium*, strain JAMB-A33 (Ohta et al. 2005b). Furthermore, α-agarase obtained from marine bacteria has molecular weight of 180 kDa (Potin et al. 1993).

In 1991, a classification of glycoside hydrolase was proposed on the basis of similarities in sequence (Henrissat 1991). A sequence based family contains glycoside hydrolases folding in the same pattern and functioning with the identical mechanism. Furthermore, the evolutionarily, structurally, and mechanistically related families were grouped together in higher hierarchical level termed "clans". Currently, more than 100 families and 14 clans have been reported with details available at CAZy website (http://www.cazy.org/). Therefore, on the basis of primary sequence, β-agarases are classified into three families of glycoside hydrolase (GH) named as GH-16, GH-50 and GH-86. Among the three families of β-agarases, GH-16 is the largest, most heterogeneous and abundant group, with members differing from one another in substrate specificity. Furthermore, GH-16 has served as the basis for the re-division of those in to several sub families (Henrissat and Bairoch 1993). Members of families GH50 and 86 share the same structure of $(\beta/\alpha)_8$ barrel in which the acid/base and the nucleophilic residues are located at the COOH-terminal strands β4 and β7, respectively (Henrissat and Davies 1997; Zverlov et al. 1998). Members of family GH-16 display the general β-jelly roll topology with two predominantly parallel or antiparallel β-sheets forming a long open substrate binding cleft, and two catalytic residues are located in the cleft (Allouch et al. 2003). However, despite the discovery of a large and increasing number of agarases, few of these enzymes have been successfully applied in industry or scientific research. Therefore, agarases have potential applications in the field of biochemistry, food, cosmetic, medical and

aquaculture industries for the production of oligosaccharides from agar or agarose (Kobayashi et al. 1997). Moreover, agarase can be used to degrade the cell walls of marine algae for the preparation of protoplasts and for the extraction of labile substance with biological activities (Araki et al. 1998b).

AOS can be prepared conveniently through acid hydrolysis of agar. However, the hydrolysis product is not homogenous and the hydrolyzing reaction is difficult since it is not easily controllable. Also, acid hydrolysis product is not appropriate for large scale preparation of oligosaccharides with high purity. Therefore, enzymatic degradation of AOS could be an alternative to acid hydrolysis.

Neoagaro-oligosaccharides

In recent years, studies have demonstrated that the oligomer forms derive from agar or agarose exhibits variety physiological and biological activities. The extent of these activities is also correlated with the degree of polymerization and the galactosyl groups on the AOS and NAOS. Neoagarobiose (N2) was reported to process moisturizing effect on skin and and whitening effects on melanoma cells (Kobayashi et al. 1997). Neoagarotetrose (N4), derived from prophyran, was reported to be utilize in vitro by intestinal bacteria, which stimulated the growth of Bacteroides, as well as *Eubacterium* and *Lactobacillus*. The AOS, especially agarohexose (A6) could scavenge reactive oxygen species (ROS) generated by electron leakage and protect cells against apoptosis induced by ROS in a human liver cell L-02 system (Chen et al. 2005). In a previous study, Wang et al., (2004) revealed that agarase digested agar oligosaccharide has antioxidant activities in inhibiting lipid peroxidation, scavenging superoxide anion and hydroxyl free radical. Furthermore, neoagaro-oligosaccharides inhibit the growth of bacteria and slow down the degradation of starch. As additives, they can reduce the caloric value of food.

Overall efforts should be undertaken toward finding more agarases with a high activity from the environment in order to develop new AOS and NAOS products in large scale industrial applications.

In this study, we describe the isolation and identification of agar-degrading marine bacteria from Jeju Island coastal environment. Then, coding sequences of four different agarases were cloned from *Agarivorans* sp., *Flammeovirga* sp, *Pseudoalteromonas* sp. bacteria and purified their recombinant agarases named as AG4, AG17, AG31 and AG52, respectively. Also, purified recombinant agarase enzymes were analyzed for biochemical properties such as specific activities and optimum reaction conditions. Finally, bioactivity studies of NAOS forms derived from agar hydrolyzes by purified agarase have been conducted for developing pharmaceutical and cosmetic products.

II. Materials and methods

1. Isolation of agarase producing bacteria

Agarase producing bacterial strains were isolated from seaweeds, sea animals (shell fish) and sea water of coastal sea areas of Jeju Island, Rep. of Korea (figure 2). The seaweeds and shellfish intestines were macerated and diluted with autoclaved sea water. Those diluted samples were plated on several selection plates as table 1 to screen agarase producing bacteria. First screening was carried out by spreading 10-100 μl of samples on SW, SWY and SWT agar plates. Moreover, different seaweeds were kept on each plate. The plates were incubated at 30 ℃ for 3-5 days. Positive colonies having pit or clear zones were picked out and streaked to SWT, SWY and marine agar plates. Then, the plates were incubated for 2-4 days at 30 ℃. The pure colonies were inoculated in SWT, SWY and marine broth including 0.2% agar and incubated at 30 ℃ in shaking incubator (200 rpm). From the grown bacteria, the stock was prepared by using 20% glycerol and samples were kept at -70 ℃.

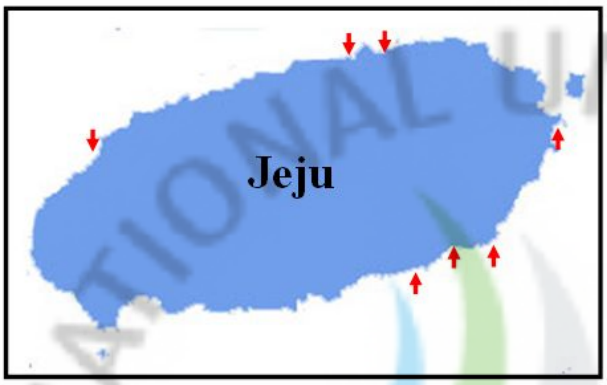

Figure 2. Sampling area

Table 1. Composition of selection plates

Selection plates	Composition
SW agar	only 1.5% agar in seawater
SWY agar	0.3% yeast extract and 1.5% agar in seawater
SWT agar	0.3% Tryptone and 1.5% agar in seawater
Marine agar	commercial marine agar (Difco)

2. Identification of bacteria using 16s rRNA sequence

2. 1. Genomic DNA isolation

The cultured bacteria cells were collected from respective broth after centrifugation at 15000 rpm for 5 min for genomic DNA isolation. The collected cells were re-suspended in 200 ㎕ appropriate solution and incubated at 37 ℃ for 30 min. Proteinase K was used in final concentration of 100 ug/ml and 30㎕ of 10% SDS was added to the samples. They were incubated at 56 ℃ for 30 min. Phenol/chloroform/isoamylachol (25:24:1) solution was mixed with same volume of sample and centrifuged at 12000 rpm for 2 min. Upper layer was moved to new tube and 3M Na-acetate solution was added (1/10 volume). Thereafter, 100% ethanol was added with 2 times volume and kept at -70 ℃ for 2 hours after inverting them for several times. The samples were centrifuged (15000 rpm, 4 ℃, 30 min) and removed the supernatant. To the pellets, 70% ethanol was added and centrifuged (15000 rpm, 4 ℃, 10 min). After removed the supernatant, the samples were dried at room temperature. The dried genomic DNA was dissolved in 100 ㎕ third distilled water.

2. 2. 16s rRNA sequence amplification

Polymerase Chain Reaction (PCR) was carried out for 16s rRNA sequence amplification and isolated genomic DNA were used as templates. Primers used for 16s rRNA sequence amplification were 16s-27F (5'-AGAGTTTGATCMTGGCTCAG-3') as forward primer and 16s-1492R (5'-TACGGYTACCTTGTTACGACTT-3') as reverse primer. The PCR mixture used was 5 ㎕ 10X Ex Taq polymerase, 4 ㎕ of 2.5 mM dNTP, 20 pmole of each primers, 400-600 ng of genomic DNA and 3 units of Ex Taq DNA polymerase (Takara Korea Biomedical Inc, Korea) in 50 ㎕ reaction volume. The initial denaturation step was carried out at 94 ℃ for 5 min. Amplification was carried out in 30 cycles as follows: 94 ℃ for 30 sec, 50 ℃ for 30 sec and 72 ℃ for 90 sec.

Finally, extension step was carried out at 72 ℃for 5 min. The amplified PCR products were checked using 1% agarose gel. The products were purified by PCR purification kit (Bioneer co., Korea) and sequenced at Solgent co. (Korea). The sequences were analyzed using NCBI Blast N program and DNassist program.

3. PCR amplification of agarase genes

For the amplification of partial and coding sequences of agarase genes from each strain, several forward and reverse primers were designed by compare with other agarase genes from NCBI database (Table 2). Firstly, mix primers were used to amplify all the strains. The PCR mixture included 5 ㎕ 10X Ex Taq polymerase buffer, 4 ㎕ of 2.5 mM dNTP, 100 pmole of each primers, 400-600 ng of genomic DNA as template and 3 units of Ex Taq DNA polymerase in 50 ㎕ reaction volume. The initial denaturation step was at 94 ℃ for 5 min. Amplification was carried out in 30 cycles as follows: 94 ℃ for 45 sec, 45 ℃ for 45 sec and 72 ℃ for 45 sec. Final, extension step was carried out at 72 ℃ for 5 min.

3. 1. PCR amplification for *Agarivorans* sp. strains

For amplification of agarase partial gene from *Agarivorans* sp. strains, primers were designed AgF2, AgF3 as forward primer and AgR2, AgR3 as reverse primer. The initial denaturation step the PCR reaction was conducted at 94 ℃ for 5 min. Amplification was carried out in 30 cycles as follows: 94 ℃ for 45 sec, 45 ℃ for 45 sec and 72 ℃ for 90 sec. Finally, extension step was carried out at 72 ℃ for 5 min. For amplification of agarase coding gene from Agarivorans sp. strains, primers were designed as AgF1 and AgR1. In the PCR reaction the initial denaturation step was carried out at 94 ℃ for 5 min and amplification was carried out in 30 cycles as follows: 94 ℃ for 45 sec, 45 ℃ for 45 sec and 72 ℃ for 3 min. The extension step was carried out at 72 ℃ for 5 min.

3. 2. PCR amplification for *Cytophaga* sp. strains

For amplification of agarase partial gene from *Cytophaga* sp. strain, primers were designed as CyF3 and CyR3. For the PCR reaction, the initial denaturation step was carried out at 94 ℃ for 5 min. Amplification was carried out in 30 cycles as follows: 94 ℃ for 45 sec, 45 ℃ for 45 sec and 72 ℃ for 1 min. Finally, extension step was carried out at 72 ℃ for 5 min. For amplification of agarase coding gene from *Cytophaga* sp. AG9, forward primers were designed as CyF1 and CyF2 and reverse primers were designed as CyR1 and CyR2. The initial denaturation step was carried out at 94 ℃ for 5 min. Amplification was carried out in 30 cycles as follows: 94 ℃ for 45 sec, 45 ℃ for 45 sec and 72 ℃ for 1 min. Finally, extension step was carried out at 72 ℃ for 5 min.

3. 3. PCR amplification for *Psudoalteromonas* sp. Strains

For amplification of agarase coding gene from *Psudoalteromonas* sp. strains, primers were designed as PsF1 and PsR1. The initial denaturation step was carried out at 94 ℃ for 5 min and amplification was carried out in 30 cycles as follows: 94 ℃ for 45 sec, 45 ℃ for 45 sec and 72 ℃ for 1 min. Finally, extension step was carried out at 72 ℃ for 5 min.

Table 2. Primers for amplification of agarase genes

Primers (5'→3')		
Mix primers	Forward	AGAF1 : CWTCKTATATWAATGCTTGGC
	Reverse	AGAR1 : TGGYTGRTAATCTTGAAATGG
	Forward	CyF3 : YTNGARTAYTAYATHGAYGG
	Reverse	CyR3 : TTRTANACNCKDATCCARTC
	Forward	SaF1 : TCNATHCAYYTNTAYGAYTTYCC
	Reverse	SaR1 : CCAYTCNGCYTTNACNGG
Primers for *Agarivorans* sp.	Forward	AgF1 : ATGAAGATTAAATTTTTATCTGCAGC (coding)
	Reverse	AgR1 : TTACACTTTACGACGTCTTAG (coding)
	Forward	AgF2 : CAACCGGTGTYGAYAACATTCG (patial)
	Reverse	AgR2 : CTTACGTRGCAAAYGGTTGG (patial)
	Forward	AgF3 : CCGAAGTGGAACTCACCAATAAT (patial)
	Reverse	AgR3 : GTTTTCRCCATCCCAWGCAC (patial)
Primers for *Cytophaga* sp.	Forward	CyF1 : ATGAAAAAAAATTATCTTTTACTGTATTT (coding)
	Reverse	CyR1 : TTATTGGACAATAAGTTTTGTAC (coding)
	Forward	CyF2 : ATGTATTTAATATATCTTAGGTTGGTC (coding)
	Reverse	CyR2 : TTATTTCTCTACAGGTTTATAGATCC (coding)
	Forward	CyF3 : YTNGARTAYTAYATHGAYGG (patial)
	Reverse	CyR3 : TTRTANACNCKDATCCARTC (patial)
Primers for *Pseudoaltero monas* sp.	Forward	PsF1 : ATGAAACAGCTAAAGCTACTAATAGG (coding)
	Reverse	PsR1 : TTACTGGGCTTTATAAACTCGTA (coding)

4. Long and Accurate Polymerase Chain Reaction (LA PCR) for detection of full length sequence

In order to amplify the unknown agarase sequence from agarase partial sequence of strain AG31 and AG52, LA PCR was carried out by LA PCR in vitro cloning kit (Takara Korea Biomedical Inc, Korea). Genomic DNA was extracted using QIAamp DNA mini kit (Qiagen). Then, each genomic DNA was restriction digested separately by *BamH* I, *EcoR* I, *Hind* III and *Xho* I in four separate e-tubes. In brief, 5 ug of sample, 50 units of restriction enzyme, 5 µl of 10x buffer for restriction enzyme were added together and sterilized distilled water was added upto 50 ㎕ (total 50 ㎕ in a tube). Then the samples were incubated at 37 ℃ for 3-5 hrs. For that 5 µl of 3M sodium acetate (pH 5.2) and 137.5. µl (2.5 volumes) of cold ethanol was added. The sample was kept at -20℃ for 1hr and collected the DNA by centrifugation (15000 rpm, 4 ℃, 30 min). The precipitate was rinsed with cold 70% ethanol and dried under reduced pressure. Then it was dissolved in 10 ㎕ of sterilized distilled water.

Each digested product was ligated with *BamH* I, *EcoR* I, *Hind* III and *Xho* I cassette which used as a template for LA PCR. In the ligation reaction 5 ㎕ of digested DNA sample, 2.5 ㎕ of cassette, 15 ㎕ of ligation solution I, 7.5 ㎕ of ligation solution II were combined in the reaction mixture tube and incubated at 16 ℃ for 30 minutes. Then 3 µl of 3M sodium acetate (pH 5.2) and .82.5 µl (2.5 volumes) of cold ethanol was added and kept at -20℃ for 1hr. The DNA was precipitated, rinsed with 70% ethanol and dried under reduced pressure. After that the precipitate was dissolved in 5 ㎕ of sterilized distilled water.

To identify the reverse sequence from known partial sequence, LA31-F1 and LA31-F2 primers were designed as forward primer for AG31 and LA52-F1 and LA52-F2 primers were designed for AG52 (Table 3). The first PCR reaction was performed with forward primer LA31-F1 and reverse primer C1 (from cassette nucleotide sequence) for AG31 and LA52-F1 and C1 for AG52. The PCR mixture

included 5 ㎕ of 10X LA Taq DNA polymerase buffer, 8 ㎕ of 2.5 mM dNTP mixture, 5 ㎕ of $MgCl_2$, 10 pmole of each primer, 1 ㎕ of template and 5 units of LA Taq DNA polymerase in 50 ㎕ reaction volume. The initial denaturation step was at 94 ℃ for 10 min and amplification was carried out in 30 cycles as follows: 94 ℃ for 30 sec, 55 ℃ for 30 sec and 72 ℃ for 4 min. Final extension step was carried out at 72 ℃ for 5 min. This product was used as the template for second PCR reaction.

The second PCR reaction was performed with forward primer LA31-F2 and reverse primer C2 (from cassette nucleotide sequence) for AG31 and LA52-F2 and C2 for AG52. The PCR condition was same as for the first PCR reaction and PCR products were identified by using 1% agarose gel electrophoresis.

To identify the forward sequence from known partial sequence, LA31-R1 and LA31-R2 primers were designed for AG31 as forward primer and LA52-R1 and LA52-R2 primers were designed for AG52. (Table 3). The PCR reaction conditions were similar as above.

Table 3. Primers for detection of full length sequence from AG31 and AG52

Primers (5'→3')		
LA PCR primers for AG31	Forward 1	31LA-F1 : TCCAGATCCGTTGTTAGTAGGAAGATTATATCC
	Forward 2	31LA-F2 : CCAAGTTCCGTTGTAATTAGTGGCAATTGC
	Reverse 1	31LA-R1 : ATGGAATCACAAACGTGGTTAACAGCTTCTAC
	Reverse 2	31LA-R2 : CCATCACAAAGTGATTTAGAAAACCCAGCG
LA PCR primers for AG52	Forward 1	52LA-F1 : TCGTCGCTACGGTGTTCATTGGAA
	Forward 2	52LA-F2 : TAGTTCGCAGCGTTTCAGGTCCTA
	Reverse 1	52LA-R1 : ACGTGCATACGTTGGTCAAACCAC
	Reverse 2	52LA-R2 : TGCCTCCATCGCATCAATTTCCTG
Cassette primers	C1	GTACATATTGTCGTTAGAACGCGTAATACGACTCA
	C2	CGTTAGAACGCGTAATACGACTCACTATAGGGAGA

5. Sequence characterization of agarase genes

The sequenced genes were analyzed by nucleotide BLAST and Protein BLAST of National Center for Biotechnology Information (NCBI) database. The signal peptide amino acid sequence of agarase genes were predicted through a SignalP program (http://www.cbs.dtu.dk/services/SignalP/) and motif prediction was carried out using motif scan prediction program (http://myhits.isb-sib.ch/cgi-bin/motif_scan). For gram negative bacteria lipoprotein site search, LipoP 1.0 Server (http://www.cbs.dtu.dk/services/LipoP/) was used, and for transmembrane site search TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) was used. DNassist version 2.2 program was used for comparison of other sequences and Pairwise and multiple alignment of the agarase genes were performed using the ClustalW multiple alignment 1.8 program (Thompson et al. 1994). MEGA 3.1 (Kumar et al. 2004) was used to produce the phylogenetic tree using the neighbour-joining (NJ) method.

6. Cloning and expression of agarase genes

6. 1. 1. Cloning of AG4 agarase coding sequence into the pMAl-c2x expression vector

Having checked the restriction enzyme sites of the AG4 agarase gene coding sequence, a pair of primers were designed to cloning the coding sequence without including signal sequence into pMal-c2x (New England Biolabs, USA). The sense amplification primer was designed as 5'-gagagagaattcGCTGATTGGAGCCCTTTTAGTATTC -3' having a *EcoR* I site and an antisense primer 5'- gagagaaagcttTTACTGGGCTTTATAAACTCGTAACC -3' containing *Hind* III site. In a total of 50 ㎕ of PCR reaction, 5 ㎕ of Ex Taq DNA polymerase buffer, 4 ㎕ of 2.5 mM dNTP, 500 ng of AG4 gemomic DNA, 20 pmole of each primers and 5 units of Ex Taq DNA polymerase were used. After initial incubation at 94 ℃ for 5 min, 30 cycles were carried out with 30 sec

denaturation at 94 ℃, 30 sec of annealing at 52 ℃, and 1 min of elongation at 72 ℃, followed by a final extension at 72 ℃ for 5 min. The PCR product was analysed using 1% agarose gel and ethidium bromide staining. Thereafter it was purified by the Accuprep™ gel purification kit (Bioneer Co., Korea) and digested with *EcoR* I and *Hind* III restriction enzymes. The expression vector, pMal-c2x, was also digested with the same restriction enzymes as the PCR product and dephosphorylated with calf intestine phosphatase (New England Biolabs, USA) according to the vendor's protocol. Thereafter the vector and PCR product was purified by a 1% agarose gel using Accuprep™ gel purification kit. Ligation was carried out at room temperature for 3 hours with 100 ng of pMal-c2x vector, 70 ng of PCR product and 7.5 ㎕ of ligation mixture (Takara Korea Biomedical Inc, Korea). The ligated product (pMal-c2x;AG4) was transformed into *E. coli* DH5α for subcloning. Positive clones were inoculated in LB ampicillin plates and plasmids were extracted using plasmid extraction kit (Bioneer Co., Korea). The pMal-c2x;AG4 was transformed into *E. coli* expression host cell BL21(DE3).

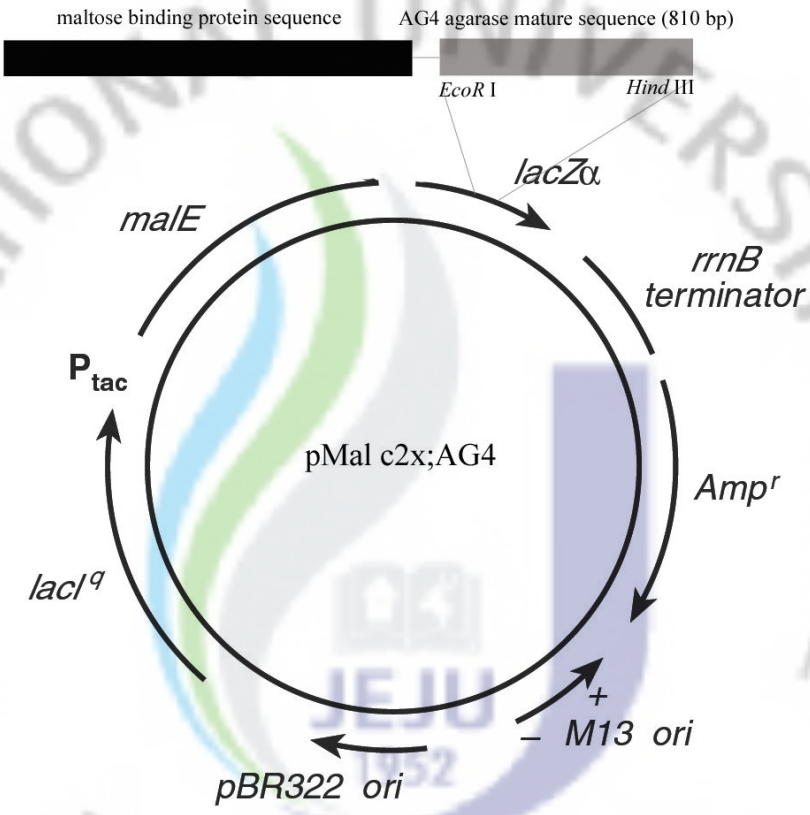

Figure 3. AG4 agarase coding sequence without including signal sequence cloned into pMal-c2x

6. 1. 2. Over-expression and purification of recombinant AG4 agarase enzyme

The recombinant pMal-c2x;AG4 *E. coli* cells were incubated at 37 ℃ with 200 rpm until O.D.600 approached 0.8 in 50 ml LB broth including ampicillin (100 ug/ml) and 10 mM glucose. Isopropyl-ß-thiogalactopyranoside (IPTG) was added for induction at the final concentration of 0.5 mM and incubated for 8 hours at 20 ℃. The cells were harvested by centrifugation at 4000 rpm for 10 min at 4 ℃ and re-suspended with 2.5 ml column buffer (Tris-HCl, pH 7.4, 200 mM NaCl) and frozen at -20 ℃ for over night. After thawing in ice, the bacterial cells were sonicated about 6 times in short pulses for 10 s. The supernatant was taken as crude enzyme after centrifuged at 3500 rpm for 30 min at 4 ℃. The recombinant fusion protein which fused with maltose binding protein (MBP) was purified using pMAL™ protein fusion and purification system.

6. 1. 3. Purification of recombinant AG4 using pMAL™ protein fusion and purification system

In brief, amylose resin was poured into a 1 x 5 cm column and washed with 8 x column volumes of column buffer. The diluted crude extract was loaded at a flow rate of 1 ml/hr. The column was then washed with 12 x column volumes of column buffer and the fusion protein was eluted with elution buffer (column buffer + 10 mM maltose). The elute was collected in 500 µl fractions. SDS-PAGE was performed according to the standard procedure for discontinuous SDS-PAGE. The stacking and separating gels were prepared at 5% and 10% respectively and the gel was stained with Coomassie blue R-250, followed by a standard de-staining procedure. The concentrations of the purified proteins were determined by the method of Bradford using bovine serum albumin (BSA) as the standard (Bradford 1976).

6. 2. 1. Cloning of AG17 agarase coding sequence into the pET16b expression vector

AG17 agarase coding sequence was cloned in to pET16b expression vector (Novagen, USA) without including its signal sequence. Two primers were designed from AG17 agarase coding sequence. The sense amplification primer was designed as 5'-gagagacatatgGCTACCTTAGTCACCTCTTTTG -3' having a *Nde* I site and an antisense primer 5'- gagagaggatccTTACACTTTACGACGTCTTAGTAAAAATAC -3' containing *BamH* I site. In a total of 50 ㎕ of PCR reaction, 5 ㎕ of 10x Herculase DNA polymerase buffer, 4 ㎕ of 2.5 mM dNTP, 500 ng of AG17 genomic DNA, 20 pmole of each primers and 5 units of Herculase DNA polymerase were used. After initial incubation at 94 ℃ for 5 min, 30 cycles were carried out with 30 sec denaturation at 94 ℃, 30 sec of annealing at 50 ℃, and 3 min of elongation at 72 ℃, followed by a final extension at 72 ℃ for 5 min. The PCR product was analysed using 1% agarose gel. Thereafter it was purified by the Accuprep^{TM} gel purification kit. The purified product was digested with *Nde* I and *BamH* I for pET16b cloning. The expression vector, pET16b was also digested with the same restriction enzymes as the PCR product and dephosphorylated with calf intestine phosphatase according to the vendor's protocol. The digested product was ligated transformed into *E. coli* DH5α. After plasmid DNA extraction, the same was transformed into *E. coli* expression host cell BL21 (DE3) for overproduction of AG17 agarase.

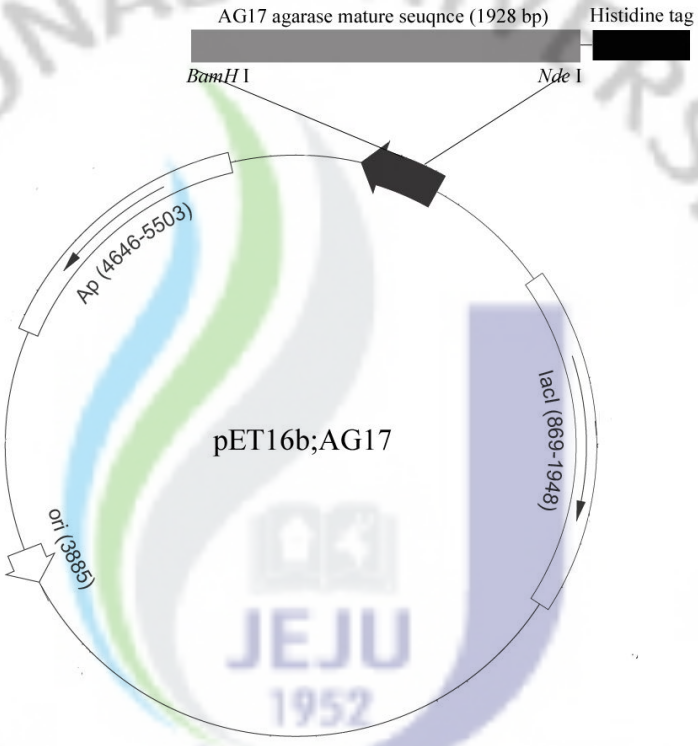

Figure 4. AG17 agarase coding sequence without including signal sequence cloned into pET16b.

6. 2. 2. Over-expression and purification of recombinant AG17 agarase enzyme

The *E. coli* BL21(DE3) cells carrying the agarase gene (pET-16b;AG17) were inoculated in LB ampicillin broth and incubated at 37 ℃ until O.D.600 = 0.6. IPTG was added for induction at the final concentration of 1 mM and incubated at 12 ℃ for 24 h. The crude enzyme was collected after centrifugation at 4000 x g for 20 min at 4 ℃ and re-suspended with 5 ml ice cold 1x binding buffer (8x = 4M NaCl, 160 mM Tris HCl, 40 mM imidazole, pH 7.9) and frozen at -20 ℃ for over night. After thawing in ice, the bacterial cells were sonicated about 6 times in short pulses for 10 s. The supernatant was taken as a crude enzyme after centrifuged at 3500 rpm for 30 min at 4 ℃. The recombinant AG17 fusion protein which fused with His tag was purified using His Bind purification Kit.

6. 2. 3. Purification of recombinant AG17 using His Bind purification Kit

In brief, few milliliters of deionized water was added to 1 x 5 cm polypropylene column to wet the frit and start column flowing. His Bind resin (400 ㎕) was transferred to the column and allowed to resin to pack under gravity flow. Thereafter, the column bed was washed by 3 vol of sterile deionized water, 5 vol of 1x charge buffer and 3 vol of 1x binding buffer to charge and equilibrate the column. The crude extract was loaded at a flow rate of 1 mL/hr. The column was then washed with 10 vol of 1x binding buffer and 6 vol of 1x wash buffer. Finally, the bound fusion protein was eluted with 6 vol of 1x elute buffer. The elutes were collected in 500 µl fractions and respective elutes were run on SDS-PAGE according to the standard procedure for discontinuous SDS-PAGE. The concentrations of the purified proteins were determined by the method of Bradford using bovine serum albumin (BSA) as the standard (Bradford 1976).

6. 3. 1. Cloning of AG52 agarase coding sequence into the pET11a expression vector

To amplify of the AG52 agarase coding region with signal sequence for clone in to

pET11a expression vector (Novagen, USA), two primers were designed from AG52 agarase coding sequence. The sense amplification primer was designed as 5'-gagagacatatgATGAATATATTAAAACTACTATCCTGTTCTAC-3' having a *Nde* I site and an antisense primer 5'-gagagaggatccTTAGTTTGCTTTGTAGACACGT-3' containing *BamH* I site. In a total of 50 ㎕ of PCR reaction, 5 ㎕ of Ex Taq DNA polymerase buffer, 4 ㎕ of 2.5 mM dNTP, 500 ng of AG52 genomic DNA, 20 pmole of each primers and 5 units of Ex Taq DNA polymerase were used. After initial incubation at 94 ℃ for 5 min, 30 cycles were carried out with 30 sec denaturation at 94 ℃, 30 sec of annealing at 50 ℃, and 1 min of elongation at 72 ℃, followed by a final extension at 72 ℃ for 5 min. The PCR product was analysed using 1% agarose gel. Thereafter it was purified by the Accuprep™ gel purification kit. The purified product was digested with *Nde* I and *BamH* I for pET11a cloning. The expression vector, pET-11a was digested with the same restriction enzymes as the PCR product and dephosphorylated with calf intestine phosphatase according to the vendor's protocol. The digested product was ligated into E. coli expression vector, pET11a and ligated pET11a;AG52 was transformed into *E. coli* DH5α. After plasmid DNA extraction, the same was transformed into *E. coli* expression host cell BL21 (DE3) for overproduction of agarase.

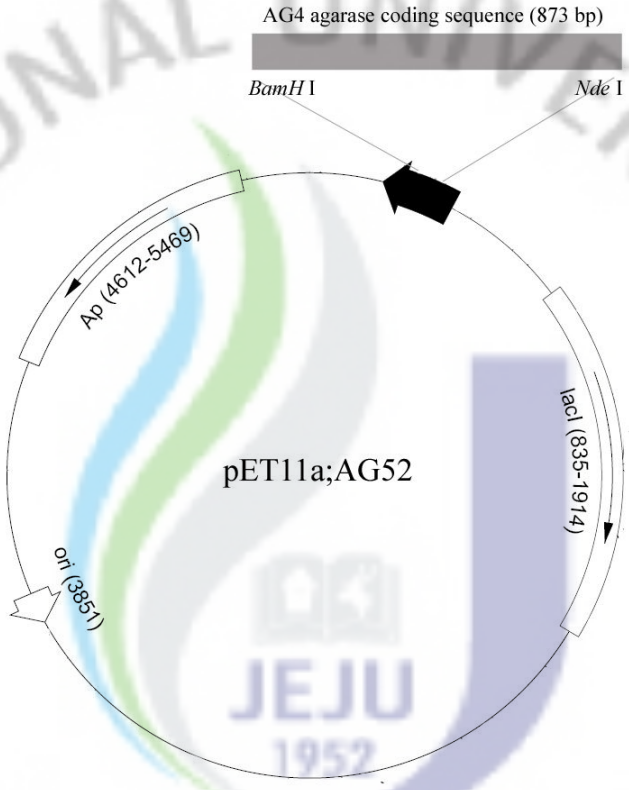

Figure 5. AG52 agarase coding region including signal sequence cloned into pET-11b

6. 3. 2. Over-expression of recombinant AG52 agarase enzyme

The cells were inoculated at 37 ℃ until O.D.600 = 0.8. IPTG was added for induction at the final concentration of 1 mM and incubated for 24 h at 20 ℃. Pellet was collected separately after centrifugation at 15000 rpm for 20 min at 4 ℃. The pellet was resuspended using cold 1x phosphate buffer saline (PBS). After the celll lysis by sonication it was used directly used for the determination of enzyme activity.

6. 4. 1. Cloning of AG52 agarase coding sequence into the pET16b expression vector

To amplify of the AG52 agarase coding region without signal sequence for clone in to pET-16b (Novagen, USA) expression vector, PCR was performed. For clone in to pET-16b, two primers were designed from AG52 agarase coding sequence. The sense amplification primer was designed as 5'-gagagacatatgGCAGATTGGGACGCATATAGTA-3' having a *Nde* I site and an antisense primer 5'-gagagaggatccTTAGTTTGCTTTGTAGACACGTATC-3' containing *BamH* I site. In a total of 50 ㎕ of PCR reaction, 5 ㎕ of Ex Taq DNA polymerase buffer, 4 ㎕ of 2.5 mM dNTP, 500 ng of AG52 gemomic DNA, 20 pmole of each primers and 5 units of Ex Taq DNA polymerase were used. After initial incubation at 94 ℃ for 5 min, 30 cycles were carried out with 30 sec denaturation at 94 ℃, 30 sec of annealing at 52 ℃, and 1 min of elongation at 72 ℃, followed by a final extension at 72 ℃ for 5 min. Thereafter it was purified by the AccuprepTM gel purification kit. The purified product was digested with *Nde* I and *BamH* I for pET-16b cloning. The expression vector, pET16b was digested with the same restriction enzymes as the PCR product and dephosphorylated with calf intestine phosphatase according to the vendor's protocol.

The digested product was ligated into the *E. coli* expression vector, pET-16b and ligated pET-16b;AG52 was transformed into *E. coli* DH5α. After plasmid DNA extraction, the same was transformed into *E. coli* expression host cell BL21 (DE3)

for overproduction of AG52 agarase.

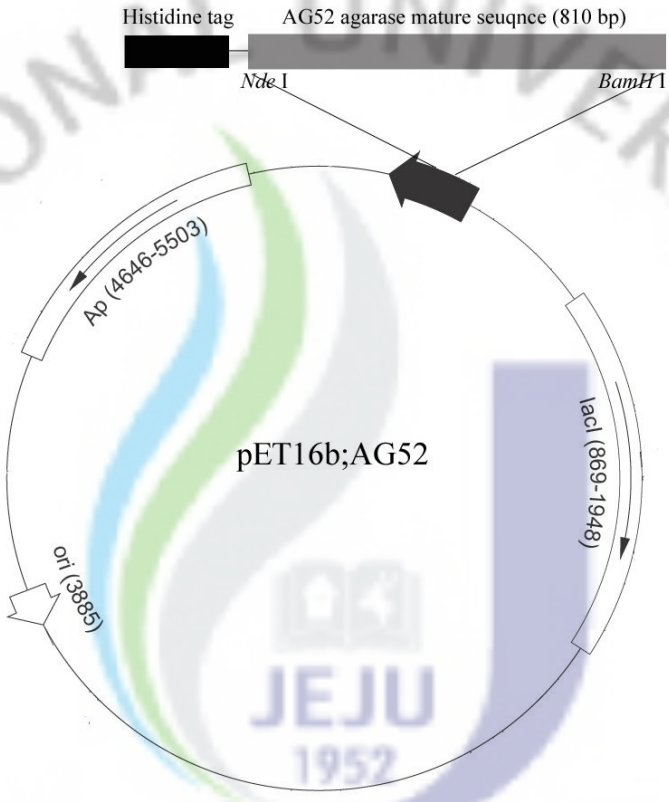

Figure 6. AG52 agarase coding region without including signal sequence cloned into pET-16b

6. 4. 2. Over-expression and purification of recombinant AG52 agarase enzyme

The cells were inoculated at 37 ℃ until O.D.600 = 0.8 and IPTG was added for induction at the final concentration of 0.1 mM and incubated for 24 h at 10 ℃. Cultured cells were collected by centrifugation and resuspended it using cold 1x his binding buffer. The cell lysis was performed by using ultrasonication. Soluble crude protein was collected from the supernatant after centrifugation (15000 rpm for 30 min at 4 ℃). The recombinant crude AG52 fusion protein which fused with His tag was purified using His Bind purification Kit as described above. Matching protein band was identified by 12 % SDS-Polyacrylamide gel electrophoresis (PAGE). Protein content was measured using Bradford method and determined the enzyme activity.

Table 4. Primers for over-expression

Primers (5'→3')		
AG4 agarase amplification	Forward	AG4-F (EcoR I) : gagagagaattcGCTGATTGGAGCCCTTTTAGTATTC
	Reverse	AG4-R (Hind III) : gagagaaagcttTTACTGGGCTTTATAAACTCGTAACC
AG17 agarase amplification	Forward	AG17 (Nde I) : gagagacatatgGCTACCTTAGTCACCTCTTTTG
	Reverse	AG17 (BamH I) : gagagaggatccTTACACTTTACGACGTCTTAGTAAAAA TAC
AG52 agarase amplification	Forward	AG52-F1 (Nde I) : gagagacatatgATGAATATATTAAAACTACTATCCTGTT CTAC
	Forward	AG52-F2 (Nde I) : gagagacatatgGCAGATTGGGACGCATATAGTA
	Reverse	AG52-R (BamH I) : gagagaggatccTTAGTTTGCTTTGTAGACACGT

7. Biochemical characterization of recombinant agarase

7. 1. Agarase enzyme assay

A suitably diluted enzyme solution was incubated in 3rd distill water containing 1% agarose at 43 ℃. Activity was expressed as the initial rate of agar hydrolysis by measuring the release of reducing ends using the 3,5-dinitrosalicylic acid (DNS) procedure (Miller 1959) with D-galactose as the standard. One unit of the enzyme activity was defined as the amount of protein that per minute produced 1 μmol of reducing sugar as D-galactose under condition of the assay.

7. 2. Optimum temperature

The optimum temperatures of each agarases were determined by monitoring the enzymatic activity at temperatures ranging from 40-70 ℃ with 5 ℃ intervals at pH 7.0. Agar (1%) solution and purified agarase were mixed and incubated at each temperature for 30 min. The agarase activity was determined by DNS method as described above.

7. 3. Optimum pH

Optimum pH of each purified agarases were tested at pH 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9. Acetate buffer and phosphate buffer were used for pH 4.5-6 and pH 6.5-9, respectively. Agar (1%) solution and purified agarase were mixed and incubated at 45 ℃ for 30 min and agarase activity was determined by DNS method as described above.

7. 4. Thermostability

The thermostability of purified recombinant agarases were evaluated by measuring the residual activity of the enzyme after incubation at the temperatures between 40 ℃ and their respective optimum temperatures for 30, 60 and 120 min. Each sample was mixed with 1% agar solution and remaining activity was determined by DNS method

as described above.

7. 5. Effect of metal ion salts and chelators

The effect of various metal ions and other chemical reagents on purified recombinant agarase activity were tested by determining the activity in the presence of 2 mM of various ions or chelators ($CaCl_2$, $CuSO_4$, $FeSO_4$, KCl, $MgSO_4$, $MnCl_2$, NaCl and EDTA) in a final concentration. Control was used as without adding metal ion or chelators. Purified agarase and 1% agar were mixed with each reagent and kept at 45 ℃ for 30 min for incubation. The agarase activity was determined by DNS method as described above.

7. 6. Identification of reaction products after hydrolysis of agar substrates

Thin layer chromatography (TLC) was used to identify the hydrolysis products of neoagarooligosaccharides. Neoagarohexanitol (NA6) was purchased from sigma (USA) and neoagarotetrose (NA4) and neoagarobiose (NA2) were prepared by digestion of neoagarohexanitol using commercial beta-agarase (New England Biolab). D-(+)-galactose was purchased from sigma (USA) and all above mentioned were used as standards. Moreover, NA6, NA4+NA2 and food grade agar were used as substrates for the reactions. Reaction of purified agarase and agar were performed in 200 μl reactions containing 20㎕ of purified agarase and 180 μl of 1% agar at 45 ℃ for 30, 60, and 120 min. While NA6 and NA4+NA2 substrates were reacted with 20㎕ of purified agarase separately at 45 ℃ for 120 min. Then the reaction mixtures were applied to a silica gel 60 TLC plate (Merck, Germany). The TLC plates were developed using a solvent system consist of n-butanol: acetic acid: Water (2:1:1, v/v). Resulted oligosaccharides spots after hydrolysis of substrates were visualized by spraying 10% H2SO4 to the plate and heating on hot plate.

8. Industrial application of neoagaro-oligosaccaride

8. 1. Production of neoagaro-oligosaccaride

One gram of agar was dissolved in 100 ml of 3rd distilled water by boiling. The purified AG4 and AG17 agarase were mixed into 1% agar solution, separately and reaction was carried out at 45 ℃ for 24 hours. Degrading pattern of the products (neoagaro-oligosaccaride) was checked using TLC to confirm the hydrolysis of agar. Finally, the samples were dried in freeze dryer and used them to check their respective functional activities.

8. 2. Functional activity of neoagaro-oligosaccaride

8. 2. 1. Antioxidant activity

The level of antioxidant ability was determined by examining the DPPH radical scavenging activity. The volume of 100 ㎕ of neoagarooligosaccharides solution (1 mg/ml NA4 and 1 mg/ml NA2+NA4) was added into 100 ㎕ of DPPH, and incubated at 25 ℃ for 60 min in darkness. The amount of 0.15 mM of DPPH in ethanol was used as the colorimetric reagent. The absorbance was measured at 492 nm using a microplate reader ELISA.

8. 2. 2. Assay of melanin production

The B16F10 melanoma cells were seeded in 24 well plate with 2 X 105 cells/ml. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 ℃ in 5% CO_2 for 24 hours. Neoagaro-oligosaccarides (NA4 and NA2+NA4) and positive control (Albutin) were treated to the cells in the final concentration of 0.1, 1, 10 and 100 ug/ml and incubated for 72 hours with same condition. The cells were harvested by adding trypsin/EDTA and 10% FBS and moved in to 1.5 ml micro-centrifuge tube. The samples were collected by centrifugation and removed the supernatant. NaOH (1N)

were added to each tubes and incubated at 56 ℃ for 1 hour. The samples were moved in to 96 well plate after mix by vortexing. The melanin content was determined at 405 nm using a ELISA.

8. 2. 3. Cytotoxicity assay

Cells were used with B16F10 melanoma cell and normal human fibroblast for test of cytotoxicity. The cells were cultured in 96 well plate and maintained in DMEM containing 10% FBS. The cells were washed two times with 1 ml PBS, fed fresh media, and treated with α-MSH at 200 nM. The samples were dissolved in PBS and added to the wells by serial dilution (final concentrations : 0.1, 1, 10, 100 ug/ml). After 48 h, the cells were washed twice with PBS and fed 200 μl of fresh media. Twenty μl of MTT (Sigma) solution (5 mg/ml in PBS) was then added to each well and the cells were incubated further for 12 h at 37 ℃. The media were removed and the cells were dissolved in 100 μl DMSO. The conversion of MTT to formazan was quantified from the absorbance at 570 nm using a microplate reader.

III. Results

1. Screening and identification of agarase producing bacteria strains

We isolated 29 agarase producing bacteria strains from the samples (sea water, seaweed, abalone like marine organisms etc.) collected at Jeju coastal environment. Initially, bacteria, which showed agar degrading ability was identified by clearing zone screening on agar plates. The figure 7 showed the representative bacteria grown with clearing zones. In that, agar degradation was varied with the type of bacteria showing different agarase activities. Then, identified colonies were inoculated in specific media to isolate respective genomic DNA. The 16s rRNA sequence amplification was performed using genomic DNA and resulted sequences were analyzed by NCBI Blastn and DNAssist 2.2 programs. The bacteria strains were identified and named based on sequence identity as shown in table 5. The results showed that all identified bacterial strains have over 90% identity to known bacterial strains, which belongs genera of *Alteromonas* sp., *Agarivorans* sp., *Aquimarina* sp., *Cellulophaga* sp., *Cytophaga* sp., *Flammeovirga* sp., *Glaciecola* sp., *Microbulbifer* sp., *Pseudoalteromonas* sp., *Rhodobacteraceae* sp., *Ruegeria* sp., *Reinekia* sp., *Shewanella* sp. etc.

Table 5. Identification of agarase producing bacteria by 16s rRNA sequence analysis. Cloned agarase genes from the identified bacteria are high-lighted with an asterisk (*).

	Strain no.	Matched bacteria strain (based on 16s rRNA sequence)	Identity (%)	Growth Medium
1	AG1	*Rhodobacteraceae bacterium* R11 *Ruegeria* sp. ULA23	98.8 97.1	SW, MA
2	AG2	*Glaciecola* sp. HA02	99.8	SW, MA
3	AG3	*Cellulophaga* lytica MBIC1544	99.6	SW, MA
4	*AG4	*Pseudoalteromonas* sp. BSw20092	99.8	SW, MA
5	AG5	*Alteromonas* sp. MED506	99.8	SW, MA
6	AG6	*Pseudoalteromonas* sp. BSw20092	99.4	SW, MA
7	AG8	*Pseudoalteromonas elyakovii*	99.4	SW, MA
8	AG9	*Persicobacter diffluens* *Cytophaga* sp. I-976	100.0 100.0	SW, MA, SWY
9	AG10	Uncultured bacterium clone 2119 Uncultured bacterium clone DC-OTU6 Marine bacterium GK-2001	99.0 94.0 93.0	SW, SWY
10	AG12	*Agarivorans albus*	99 .0	SW, SWY, MA
11	AG14	Bacterium QM36 *Cellulophaga lytica* strain ACEM21	100.0 100.0	SW, SWY
12	*AG17	*Agarivorans albus* *Agarivorans* sp. JAMB-A11	99.0 99.0	SW, SWY, MA
13	AG18	Sponge bacterium Zo26 *Aquimarina* sp. SW42 clone 147.2 *Flavobacteriaceae* strain SW152	98.96 98.0 99.4	SW, SWY
14	AG19	Gamma proteobacterium YSK-2002 *Agarivorans albus*	99.5 98.7	SW, SWT
15	AG23	*Flavobacteriaceae* strain SW152 Sponge bacterium Zo26 *Aquimarina* sp. HJ055	98.75 98.0 97.0	SW, SWY
16	AG26	Mucus bacterium 63 *Agarivorans albus*	99.87 99.87	SW, SWY
17	AG28	Bacterium s1cb18 Mucus bacterium 63 *Agarivorans albus*	98.5 98.5 98.0	SW, SWY
18	AG29	*Agarivorans albus*	100 .0	SW, SWY
19	*AG31	*Flammeovirga* sp. TJD780	100.0	SW, MA, SWY

20	AG33	Gamma proteobacterium O-011 *Reinekia marinisedimentorum* DSM 15388T *Reinekia blandensis* strain MED 297	96.0 96.0 95.0	SW, SWY
21	AG39	*Agarivorans albus* MKT87	99.0	SW, SWT
22	AG40	*Shewanella pacifica* KMM 3590	100.0	SW, MA, SWY
23	AG42	*Agarivorans* sp. HZ105	100.0	SWY
24	AG43	Bacterium s1cb18 Mucus bacterium 63 *Agarivorans albus* MKT87	99.0 99.0 99.0	SWY
25	AG44	*Aquimarina* sp. HJ055 *Aquimarina muelleri* strain KMM6021	97.0 97.0	SW, MA, SWY
26	AG45	*Shewanella* sp. KMM3299 *Shewanella japonica* isolate KMM 3577 *Shewanella* sp. C111	99.0 99.0 99.0	SW, MA, SWY
27	*AG52	*Pseudoalteromonas citrea* strain CIP 105339 *Pseudoalteromonas* sp. BSw10505	99.0 99.0	SW, MA
28	AG53	*Agarivorans albus* strain:MKT89	99.0	SW, MA
29	AG54	*Microbulbifer* sp. KBB-1	100	SWY, MA

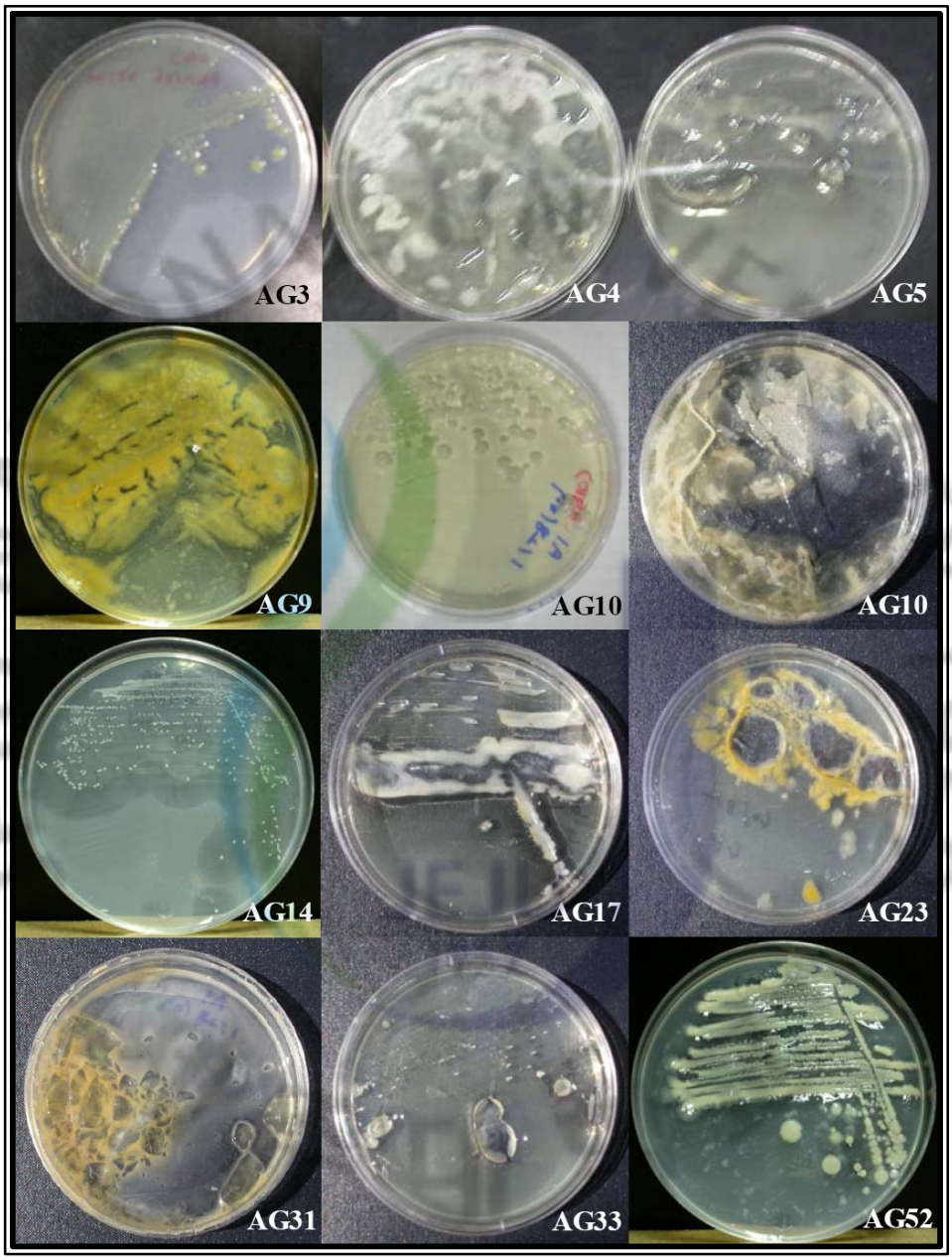

Figure 7. Identification of agarase producing bacteria by clearing zone screening on agar plates.

2. Sequence characterization of agarase genes

From the identified agarase producing bacteria, four agarase-encoding genes were cloned using different primers and LA PCR technique. Four agarase genes were named as AG4, AG17, AG31, AG52 cloned from *Pseudoalteromonas* sp., *Agarivorans* sp., *Flammeovirga* sp., *Pseudoalteromonas* sp., respectively.

2. 1. AG4

Nucleotide and amino acid sequence of the *Pseudoalteromonas* sp. AG4 agarase is shown in figure 8. It has an 870-bp open reading frame (ORF), encoding 290 amino acids. The AG4 has a putative molecular mass of 33 kDa with an isoelectric point (p*I*)of5.9. The signal peptide sequence with 63-bp (21 aa) was identified at the N-terminal sequence with the cleavage site at 21 amino acid position. Also, a characteristic GH-16 β-agarase domain (D^{22}~K^{287} aa), active sites (Y^{69}, N^{71}, W^{72}, W^{139}, S^{145}, D^{150}, E^{153}, F^{176}, R^{178}, E^{257}, E^{259}) and calcium binding modules (E^{47}, F^{48}, N^{49}, G^{91}, A^{92}, D^{82}, W^{83}) were identified from the coding sequence of AG4.

Figure 8. The nucleotide and deduced amino acid sequences of the *Pseudoalteromonas* sp. AG4. The predicted signal peptide sequence is underlined and stop (TAA) codon is high-lighted with an asterisk (*). The GH-16 β-agarase domain is in bold face. Active sites and calcium binding modules are in boxes and dotted boxes, respectively.

2. 2. AG17

Nucleotide and amino acid sequence of the *Agarivorans* sp. AG17 agarase is shown in figure 9. It has a 2988-bp ORF, encoding 995 amino acids. The AG17 has a putative molecular mass of 107 kDa with an isoelectric point (p*I*) of 4.1. The N-terminal 60-bp signal peptide (20 aa) and C-terminal transmembrane (G^{968}~L^{990} aa) sequences were identified in the AG17. Clustal W pairwise identity results reveled that AG17 has the highest nucleotide (98.6%) and amino acid (99.7%) identity to beta-agarase sequence cloned from *Agarivorans* sp. JAMB-A11 (Accession number : AB178483).

ATGAAGATTAAATTTTTATCTGCAGCAATCGCTGCAAGCTTAGCATTGCCATTAAGTGCTGCTACCTTAGTCACCTCTTTTGAGGAAGCC 90
M K I K F L S A A I A A S L A L P L S A A T L V T S F E E A 30
GACTACAGCAGCTCTGAAAACAATACTGAATTCTTGGAAGTGTCTGGAGATGCCACTTCTGAAGTTTCAACTGAACAAGCTACCGATGGT 180
D Y S S S E N N T E F L E V S G D A T S E V S T E Q A T D G 60
AATCAATCCATTAAAGCGTCTTTTGACGCGGCTTTCAAACCAATGGTTGTTTGGAACTGGGGAAGTTGGAACTGGGGCGCTGAAGATGTG 270
N Q S I K A S F D A A F K P M V V W N W G S W N W G A E D V 90
ATGTCAGTAGATGTTGTTAACCCTAACGACACTGACGTCACCTTTGCTATTAAGCTAATTGATAGTGATATTCTTCCTGATTGGGTAGAT 360
M S V D V V N P N D T D V T F A I K L I D S D I L P D W V D 120
GAGTCTCAAACCTCATTGGACTACTTTACGGTTTCAGCTAATACCACGCAGACCTTTAGCTTTAACTTAAATGGCGGCAACGAGTTCCAA 450
E S Q T S L D Y F T V S A N T T Q T F S F N L N G G N E F Q 150
ACTCATGGCGAAAACTTTAGTAAAGATAAAGTTATCGGTGTGCAGTTCATGCTCTCTGAAAACGATCCTCAAGTGTTGTACTTTGACAAC 540
T H G E N F S K D K V I G V Q F M L S E N D P Q V L Y F D N 180
ATTATGGTTGATGGCGAAACAGTCACTCCGCCACCAAGTGATGGTGCAGTGAATACACAAACCGCGCCTGTAGCCACCTTAGCGCAAATC 630
I M V D G E T V T P P P S D G A V N T Q T A P V A T L A Q I 210
GAAGACTTTGAAACCATTCCAGATTACTTACGACCTGATGGTGGGGTAAACGTTTCAACTACTACTGAGATTGTGACTAAAGGCGCTGCA 720
E D F E T I P D Y L R P D G G V N V S T T T E I V T K G A A 240
GCAATGGCTGCCGAGTTTACTGCAGGTTGGAACGGTTTAGTGTTTGCAGGTACTTGGAATTGGGCTGAACTAGGTGAACACACCGCAGTT 810
A M A A E F T A G W N G L V F A G T W N W A E L G E H T A V 270
GCCGTTGACGTTTCAAATACTAGCGATAGCAATATCTGGTTGTACTCACGTATCGAAGATGTAAATAGCCAAGGCGAAACAGCGACTCGC 900
A V D V S N T S D S N I W L Y S R I E D V N S Q G E T A T R 300
GGCGTATTGGTTAAAGCTGGCGAATCGAAAACCATCTACACCAGCTTAAATGACAACCCTTCATTGCTTACTCAAGATGAGCGCGTGTCA 990
G V L V K A G E S K T I Y T S L N D N P S L L T Q D E R V S 330
GCTTTAGGTTTACGTGATATTCCAGCTGACCCAATGAGCGCTCAAAATGGCTGGGGTGATTTTGTTGCTTTAGACAAATCTCAAATTACC 1080
A L G L R D I P A D P M S A Q N G W G D F V A L D K S Q I T 360
GCTATTCGTTACTTCATTGGCGAATTAGCCAGCGGTGAGACTAGCCAAACACTTGTGTTTGATAACATGCGTGTGATTAAAGACCTTAAC 1170
A I R Y F I G E L A S G E T S Q T L V F D N M R V I K D L N 390
CACGAATCAGCCTATGCAGAAATGACTGATGCTATGGGGCAAAACAACTTAGTCACTTATGCAGGTAAAGTTGCCAGCAAAGAAGAGTTA 1260
H E S A Y A E M T D A M G Q N N L V T Y A G K V A S K E E L 420
GCTAAGTTAAGTGATCCGGAAATGGCTGTTTTGGGTGAGTTAACCAATCGCAATATGTACGGTGGTAACCCAGATTCGTCGCCAACTACA 1350
A K L S D P E M A V L G E L T N R N M Y G G N P D S S P T T 450
GACTGTGTGCTCGCTACGCCTGCCTCGTTTAACGCTTGTAAAGACGCTGATGGTAACTGGCAATTGGTAGACCCTGCTGGTAATGCGTTC 1440
D C V L A T P A S F N A C K D A D G N W Q L V D P A G N A F 480
TTCTCAACCGGTGTTGATAACATTCGTTTGCAAGATACTTACACCATGACCGGCGTGTCGAGTGACGCCGAATCTGAGTCTGCACTTCGC 1530
F S T G V D N I R L Q D T Y T M T G V S S D A E S E S A L R 510
CAGTCAATGTTTACAGAAATTCCAAGTGATTATGTAAATGAAAACTATGGCCCTGTGCATAGTGGACCTGTTTCTCAAGGCCAAGCTGTA 1620
Q S M F T E I P S D Y V N E N Y G P V H S G P V S Q G Q A V 540
AGTTTTTACGCTAATAACTTAATTACCCGCCACGCTAGCGAAGACGTATGGCGAGACATTACTGTTAAGCGCATGAAAGACTGGGGCTTT 1710
S F Y A N N L I T R H A S E D V W R D I T V K R M K D W G F 570
AACACCTTAGGTAACTGGACCGATCCAGCGTTGTATGCAAACGGTGACGTTCCTTACGTGGCAAATGGTTGGTCAACCTCTGGTGCCGAT 1800
N T L G N W T D P A L Y A N G D V P Y V A N G W S T S G A D 600
CGTCTTCCCGTTAAACAAATTGGCAGCGGCTACTGGGGACCACTTCCTGATCCGTGGGATGCTAACTTTGCTACCAATGCCGCCACAATG 1890
R L P V K Q I G S G Y W G P L P D P W D A N F A T N A A T M 630
GCTGCAGAGATCAAAGCTCAGGTTGAAGGCAACGAAGAGTACTTAGTGGGTATTTTTGTTGATAACGAAATGAGCTGGGGCAATGTCACT 1980
A A E I K A Q V E G N E E Y L V G I F V D N E M S W G N V T 660
GATGTTGAAGGCTCTCGTTATGCGCAAACGCTAGCAGTGTTCAATACCGACGGCACTGATGCAACAACTAGCCCTGCTAAAAATAGCTTT 2070
D V E G S R Y A Q T L A V F N T D G T D A T T S P A K N S F 690

ATTTGGTTCTTAGAAAACCAGCGTTATACCGGTGGCATTGCTGACCTAAACGCAGCCTGGGGAACCGATTATGCGTCTTGGGATGCGATG 2160
I W F L E N Q R Y T G G I A D L N A A W G T D Y A S W D A M 720
CGCCCAGCGCAAGAGTTAGCTTATGTGGCTGGCATGGAAGCTGATATGCAGTTCCTTGCTTGGCAGTTTGCGTTCCAATACTTCAACACC 2250
R P A Q E L A Y V A G M E A D M Q F L A W Q F A F Q Y F N T 750
GTAAACACGGCATTAAAAGCTGAGTTACCAAACCACTTGTACTTGGGCTCTCGCTTTGCAGATTGGGGACGTACTCCTGATGTAGTAAGT 2340
V N T A L K A E L P N H L Y L G S R F A D W G R T P D V V S 780
GCTGCTGCGGCTGTTGTTGATGTGATGAGTTACAACATCTACAAAGACAGTATTGCAGCTGCCGATTGGGATGCTGATGCCTTAAATCAA 2430
A A A A V V D V M S Y N I Y K D S I A A A D W D A D A L N Q 810
ATTGAAGCCATTGATAAGCCAGTAATTATTGGTGAGTTCCACTTCGGTGCGCTTGATAGCGGTTCGTTTGCAGAAGGTGTAGTAAATGCC 2520
I E A I D K P V I I G E F H F G A L D S G S F A E G V V N A 840
ACTTCGCAACAAGATCGTGCAGACAAAATGGTTAGCTTCTACGAATCAGTAAATGCCCATAAAAACTTTGTAGGTGCGCATTGGTTCCAA 2610
T S Q Q D R A D K M V S F Y E S V N A H K N F V G A H W F Q 870
TACATCGATTCACCATTAACGGGTCGTGCATGGGATGGCGAGAACTACAACGTTGGTTTTGTTAGCAATACTGACACGCCATATACATTG 2700
Y I D S P L T G R A W D G E N Y N V G F V S N T D T P Y T L 900
ATGACAGATGCTGCGCGTGAGTTTAACTGTGGTATGTACGGCACTGACTGCTCTAGCTTAAGCAATGCTACTGAAGCTGCTTCGAGAGCC 2790
M T D A A R E F N C G M Y G T D C S S L S N A T E A A S R A 930
GGTGAGTTGTATACCGGTACCAATATTGGTGTTAGCCACTCTGGCCCAGAAGCGCCAGATCCAGGTGAGCCAGTTGATCCTCCAATTGAT 2880
G E L Y T G T N I G V S H S G P E A P D P G E P V D P P I D 960
CCGCCAACACCACCAACAGGT**GGCGTAACTGGCGGTGGCGGTAGCGCAGGTTGGTTATCGCTACTAGGTTTGGCCGGCGTATTTTTACTA** 2970
P P T P P T G **G V T G G G G S A G W L S L L G L A G V F L L** 990
AGACGTCGTAAAGTGTAA 2988
R R R K V * 995

Figure 9. The nucleotide and deduced amino acid sequences of the *Agarivorans* sp. AG17. The predicted signal peptide sequence is underlined and stop (TAA) codon is high-lighted with an asterisk (*). The C-terminal transmembrane domain (G^{968}~L^{990} aa) is in bold face.

2. 3. AG31

Nucleotide and amino acid sequence of the *Flammeovirga* sp. AG31 agarase is shown in figure 10. Based on the 446-bp partial sequence, full-length (1597-bp) AG31 agarase sequence was cloned by LA PCR. It has 1446-bp ORF encoding 482 amino acids. The blast results indicated that the similarity of the deduced amino acid sequence of AG31 gene with the existed proteins were very low. The AG31 has a putative molecular mass of 52 kDa with an isoelectric point of 3.8. Motif scan results showed that AG31 amino acid sequence was consisted with characteristic GH16- β-agarase domain (R^{2}~T^{36}, N^{88}~K^{161} aa), bacterial Ig-like domain 2 (A^{172}~I^{250} aa) and carbohydrate binding domain (E^{267}~K^{392}, Q^{413}~I^{478} aa). Members of this family are found in bacterial and phage surface proteins such as intimins. Bacterial Ig-like domain of AG31 agarase was 54.4% similar to a Ig domain protein from *Flavobacterium johnsoniae* UW 101, 46.8% similar to a non-catalytic region of agarase from *Microscilla* sp. PRE1 beta-agarase, 35.4% similar to a Ig domain protein from *Anaerocellum thermophilum* DSM6725.

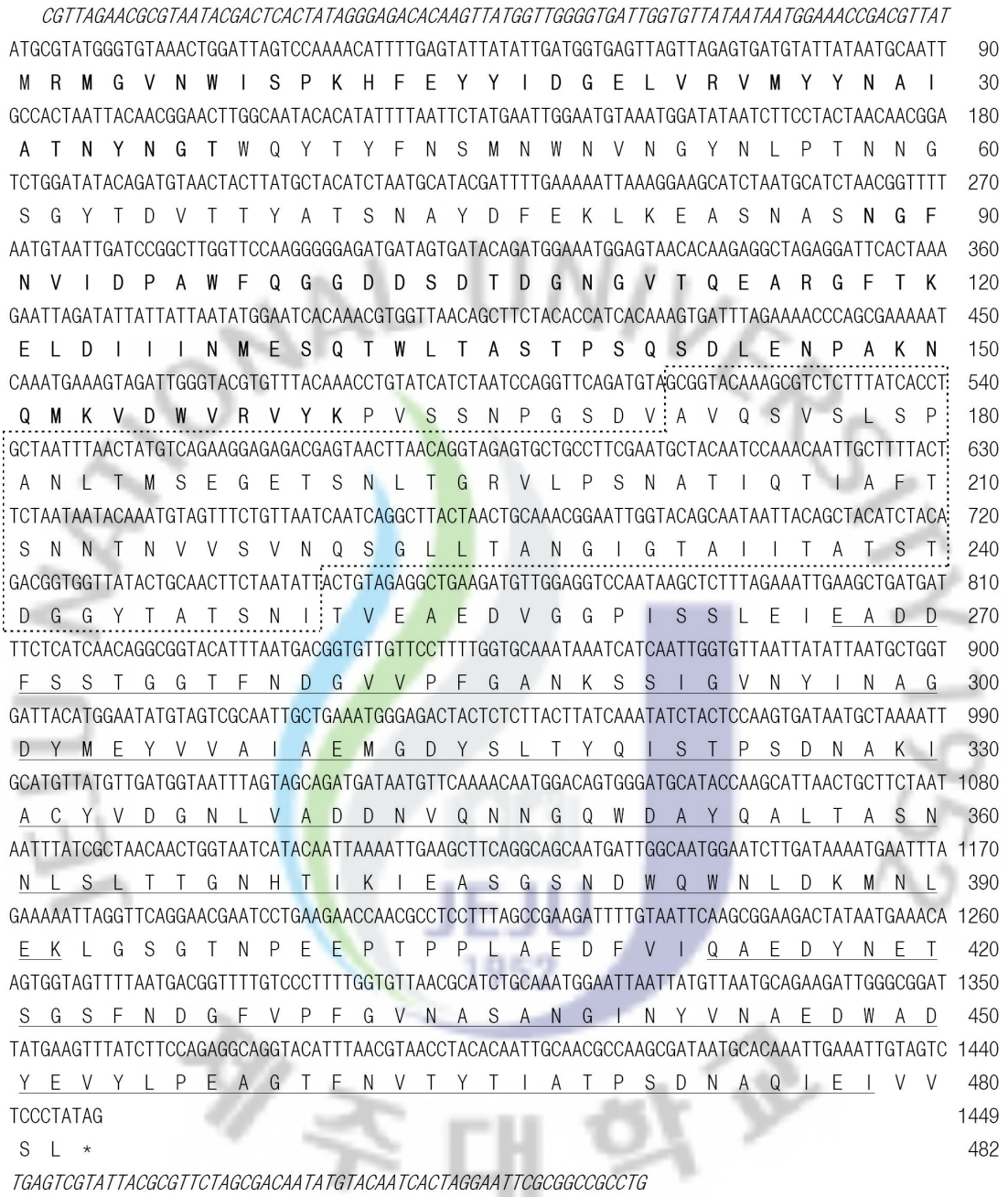

Figure 10. The nucleotide and deduced amino acid sequences of the Flammeovirga sp. AG31. The predicted GH-16 β-agarase domain is in bold and stop (TAG) codon is high-lighted with an asterisk (*). The Bacterial Ig-like domain group 2 is in dotted box and carbohydrate binding module family 6 are indicated by a underline.

2. 4. AG52

Initially, partial AG52 agarase sequence (651-bp) was amplified from *Pseudoalteromonas sp*. Then, primers were designed based on the partial sequence and full-length sequence was cloned by performing LA PCR. The complete nucleotide and deduced amino acid sequences of *Pseudoalteromonas sp.* AG52 are shown in figure 11. The full-length of AG52 was consisted 1234-bp with a 870-bp coding sequence encoding 290 amino acids. The AG52 has a putative molecular mass of 32 kDa with an isoelectric point of 5.8. The signal peptide (1 ~ 21 aa) and lipoprotein signal peptide (signal peptidase II) were identified at the N-terminal sequence of AG52 (figure 12). Additionally, AG52 was consisted with characteristic GH16-β-agarase domain (D^{22}~K^{287} aa), active sites (Y^{69}, N^{71}, W^{72}, W^{139}, S^{145}, D^{150}, E^{153}, F^{176}, R^{178}, E^{257}, E^{259}), and calcium binding domains (Q^{47}, F^{48}, N^{49}, G^{91}, A^{92}, D^{82}, W^{83}). The clustal W pairwise comparison results showed that AG52 nucleotide sequence has 96.8% nucleotide identity to β-agarase sequence from *Aeromonas* sp. (Accession number : U61972).

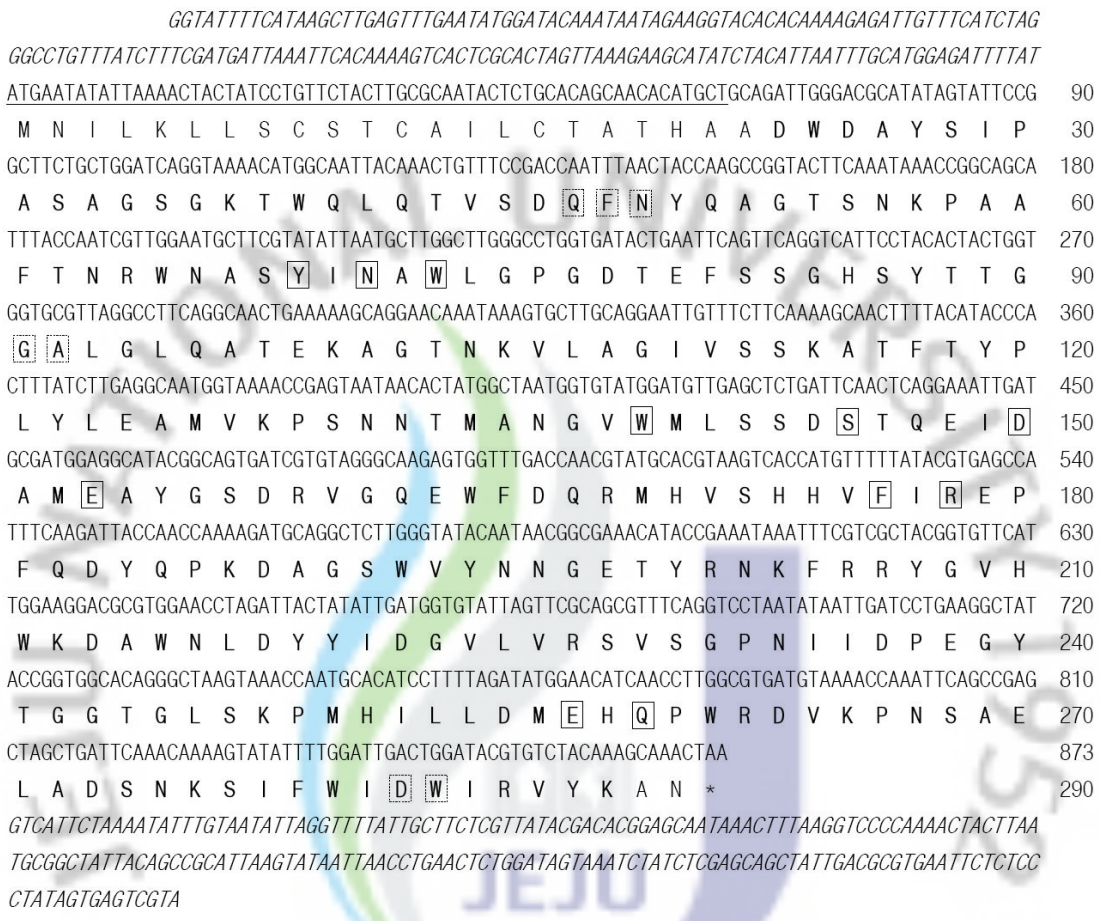

Figure 11. The nucleotide and deduced amino acid sequences of the *Pseudoalteromonas* sp. AG52. The predicted signal peptide sequence is underlined and stop (TAA) codon is high-lighted with an asterisk (*). The GH-16 β-agarase domain is in bold face. Active sites and calcium binding modules are in boxes and dotted boxes, respectively.

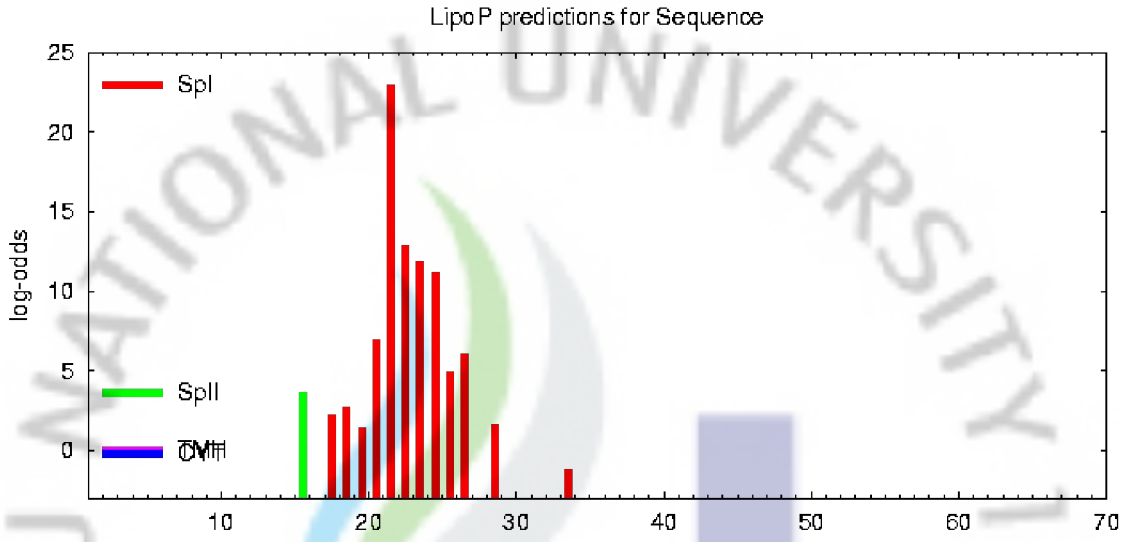

Figure 12. Lipoprotein signal peptide (signal peptidase II) of *Pseudoalteromonas* sp. AG52. The deduced AG52 amino acid sequence was analyzed for the prediction of lipoproteins and signal peptides in Gram negative bacteria by LipoP 1.0 (http://www.cbs.dtu.dk/services/LipoP/) program.

2. 5. Sequence comparison and classification of AG4, AG17, AG31 and AG52

Sequence based classification of AG4, AG17, AG31 and AG52 agarase sequences was done based on the molecular size, specific motifs and active sites present in the amino acid sequences. Schematic representation of the characteristic domains of AG4, AG17, AG31 and AG52 amino acid sequences are shown in figure 13. AG17 was the longest amino acid sequence (995 aa) among all agarase genes in this study. Interestingly, AG4 and AG52 sequences were exactly same with 290 amino acids. Signal peptide was identified in AG4, AG17 and AG51 sequences. However, AG31 did not show the signal peptide sequence. GH-16 beta agarase domain was identified in AG4, AG31 and AG52 but not in the AG17 sequence. The comparison results reveled that AG31 has some additional active site domains such as bacterial Ig- like domain and carbohydrate binding module, which were not identified in AG4, AG17 and AG52. In contrast, no consensus signature was found in AG17. Additionally, AG4 and AG52 sequences were analyzed by pairwise clustal W alignment since both showed the same amino acid length and GH-16 beta agarase domain. The pairwise alignment of AG4 and AG52 is shown in figure 14. It was revealed that AG4 and AG52 were 85% identity in amino acid level.

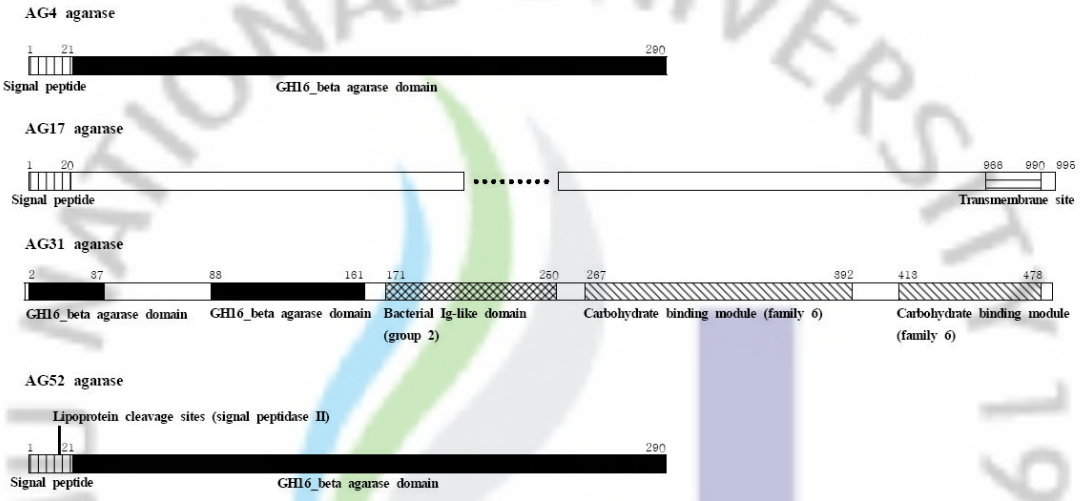

Figure 13. Schematic representation of the characteristic domains of AG4, AG17, AG31 and AG52 amino acid sequences. Important domain and sites were named in each sequence.

Figure 14. ClustalW paiwise sequence analysis of AG4 and AG52 sequences. A residue with shading represents the completely conserved amino acid residues.

2. 6. Phylogenetic analysis of AG4, AG17, AG31 and AG52 genes

To determine the relationship between different agarases cloned this study with the known agarse sequences, a phylogenetic tree was constructed based on their primary amino acid sequences using the Neighbor-joining method in MEGA3.1 (Figure 15). Phylogenetic results showed that AG4 and AG52 were more closely related to each other and those were grouped with family GH-16 of the beta agarase group. AG17 showed closer relationship with another group of beta agarase known as GH-50 family. In contrast, AG31 was positioned separately in this phylogenetic analysis without showing strong relationship to known agarase sequences included into this study.

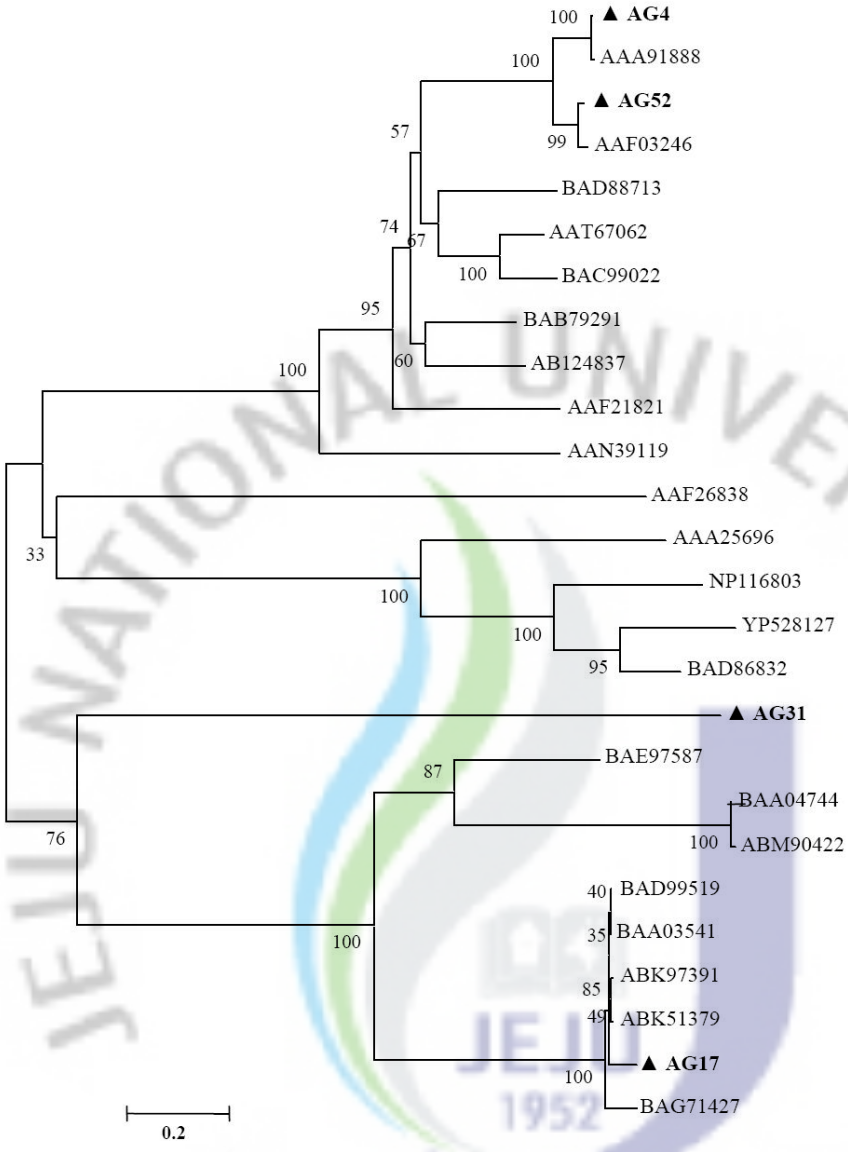

Figure 15. Phylogenetic analysis of AG4, AG17, AG31 and AG52 with selected agarase genes. Phylogenetic analysis was done by NJ method in MEGA3.1 based on sequence alignment using ClustalW (1.81). Numbers indicate the bootstrap confidence values of 1000 replicates. The accession numbers of the selected agarase sequences are as follows: ▲ AG4 beta-agarase (*Pseudoalteromonas* sp.) in this study; AAA91888, beta-agarase I (*Pseudoalteromonas atlantica*); ▲ AG52 beta-agarase (*Pseudoalteromonas* sp.) in this study; AAF03246, beta-agarase (*Aeromonas* sp); BAD88713 beta-agarase (*Pseudomonas* sp. ND137); AAT67062 beta agarase I

(*Saccharophagus degradans*); BAC99022 agarase (*Microbulbifer elongates*);AB79291, agarase (*Pseudomonas* sp. ND137); AB124837, agarase (*Microbulbifer thermotolerans*); AAF21821, beta-agarase B precursor (*Zobellia galactanivorans*); AAN39119, agarase precursor (*Pseudoalteromonas* sp. CY24); AAA25696, beta-agarase precursor (*Pseudoalteromonas atlantica*); NP116803, beta-agarase precauser (*Microscilla* Sp.); YP528127, agarase (*Sacchrophagus degradans*); BAD86832, agarase (*Microbulbifer thermotolerance*); ▲ AG31, agarase (*Flammeovirga* sp.) in this study ; BAE97587, beta-agarase (*Alteromonas* sp.);BAA04744, beta-agarase (*Vibrio* sp.). ABM90422, beta-agrase (*Agarivorans* sp. QM38);BAD99519, agarase (*Agarivorans* sp. JAMB-A11]. BAA03541, beta-agarase (*Vibrio* sp. JT0107); ABK97391, beta agrase (*Agrovorans* sp. JA-1). ABK51379; agarase (*Agarivorans* sp.QM38). ▲AG17, (*Agarivorans* sp.) in this study; BAG71427 agarase (*Vibrio* sp. PO-303).

3. Expression and purification of recombinant agarase

3. 1. Purification of recombinant *Pseudoalteromonas* sp. AG4 agarase

Pseudoalteromonas sp. AG4 agarase mature sequence was cloned into pMal-c2x expression vector that fused with a maltose binding protein (MBP) tag at the N-terminal. The recombinant AG4 was over expressed in *E. coli* BL21 (DE3) cells by IPTG induction. The 12% SDS-PAGE results in different steps in the recombinant AG4 purification is shown in figure 16. The analysis results indicated that approximately 72.5 kDa strong band in the induced cells (lane 2), but not in the same position of un-induced cells (lane 3). Then, the recombinant AG4 fusion protein was purified from the soluble fraction of induced cells. The purified recombinant AG4 was identified as an approximately 72.5 kDa strong specific band (lane 4). Therefore, the resulted protein was in agreement with our predicted molecular weight of 30 kDa (without signal peptide), since the MBP has a 42.5 kDa molecular mass.

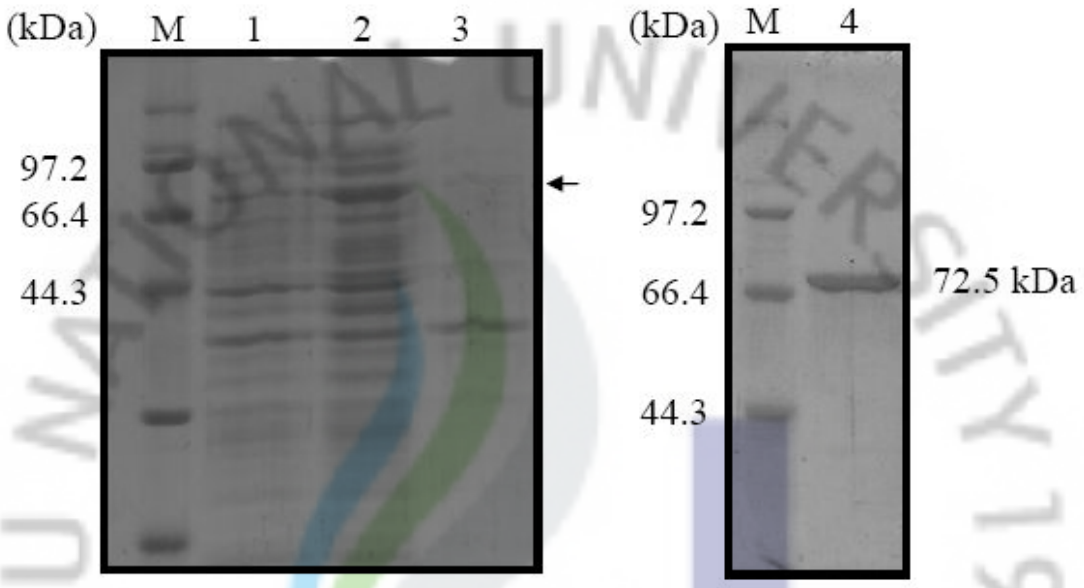

Figure 16. SDS-PAGE analysis of recombinant *Pseudoalteromonas* sp. AG4 purification. Protein samples are separated by 12% SDS-PAGE and stained with Coomassie brilliant blue. M: molecular mass marker (BioRad). Lane 1: total cellular extract from *E. coli* BL21 (DE3) before induction; lane 2, total cellular soluble extract after induction; lane 3: total cellular insoluble extract after induction; lane 4: purified recombinant AG4 agarase.

3. 2. Purification of recombinant *Agarivorans* sp. AG17 agarase

Agarivorans sp. AG17 agarase mature sequence was cloned to pET16b expression vector that fused with a histidine tag at the N-terminal. The recombinant AG17 was over expressed in *E. coli* BL21 (DE3) cells by IPTG induction and purified as a his-tag fusion protein. The 12% SDS-PAGE results in different steps in the recombinant AG17 purification is shown in figure 17. It was very clear that AG17 was highly induced (lane 2) compare to un-induced cells (lane 1). Then, the recombinant AG17 fusion protein was purified from soluble crude protein extract by Ni^{2+} binding column (lane 4). The purified fusion protein size was identified as 108 kDa fusion protein, which is identical to mature protein size together with his-tag fusion protein.

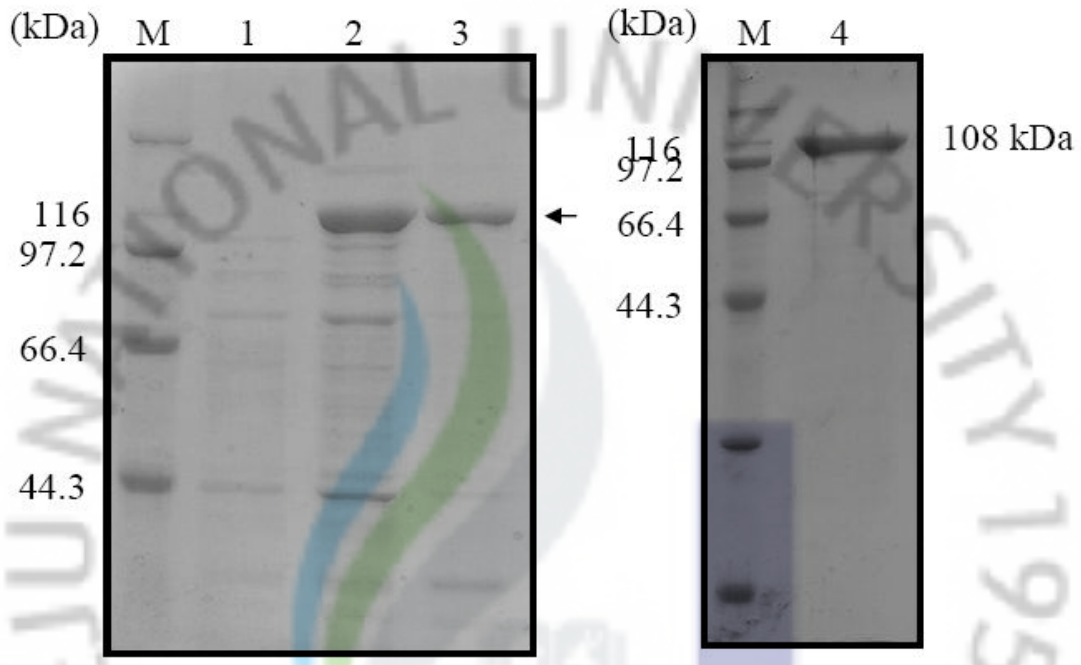

Figure 17. SDS-PAGE analysis of recombinant *Agarivorans* sp. AG17 purification. Protein samples are separated by 12% SDS-PAGE and stained with Coomassie brilliant blue. M: molecular mass marker (BioRad). Lane 1: total cellular extract from *E. coli* BL21 (DE3) before induction; lane 2, total cellular soluble extract after induction; lane 3: total cellular insoluble extract after induction; lane 4: purified recombinant AG4 agarase.

3. 3. Purification of recombinant *Pseudoalteromonas* sp. AG52 agarase

Recombinat purification of AG52 agarase was done using both pET11a and pET16b expression vectors and His-tag protein purification system. The AG52 agarase coding region with signal sequence was in pET11a + *E. coli* BL21(DE3) system without any fusion protein. Using pET11a, the expression of recombinant protein could not identify as the specific band by SDS-PAGE since expression level was very low (data not shown). Howerer, the AG52 agarase induced supernatant showed the agarase activity in an intra-cellular both. While, recombinant AG52 agarase in pET16b was successfully purified using histidine tag at N-terminal in the *E. coli* BL21(DE3) using soluble fraction by Ni^{2+} binding column (Figure 18, lane 4). The purified fusion protein size was around 33 kDa, which was accordance with the predicted molecular mass of AG52.

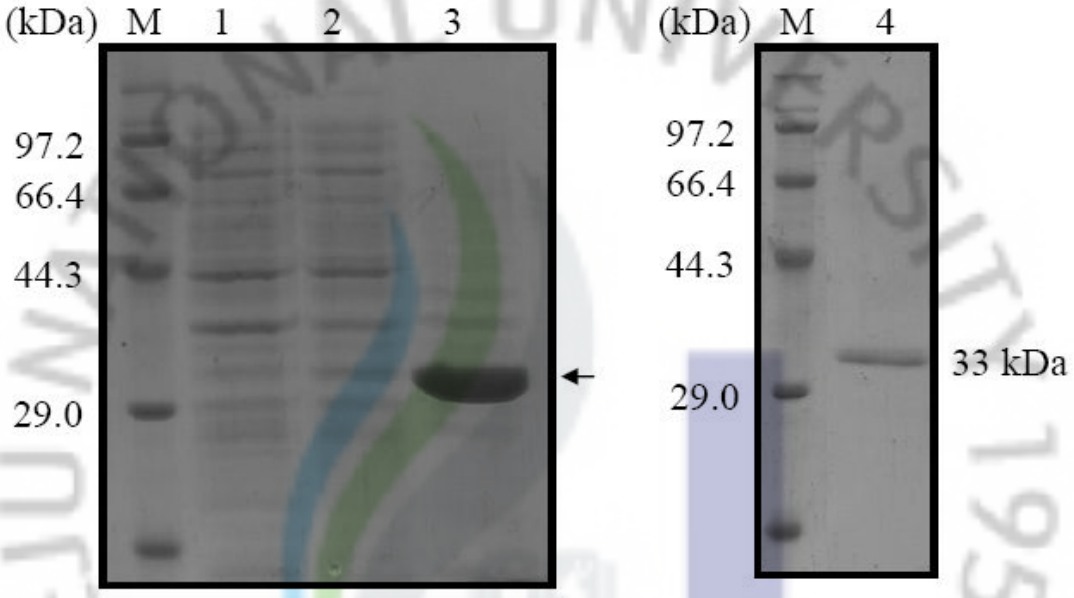

Figure 18. SDS-PAGE analysis of the recombinant AG52 agarase. M, molecular mass marker; lane1, total cellular extract from *E. coli* BL21 (DE3) before induction; lane 2, total cellular soluble extract after induction; lane 3, total cellular insoluble extract after induction; lane 4, purified recombinant AG52 agarase.

4. Biochemical characterization of recombinant purified agarases

4. 1. Enzyme characterization of purified recombinant AG4

4. 1. 1. Effect of temperature on the activity of the recombinant AG4

The optimum temperature of purified recombinant AG4 was determined at different temperatures ranging from 40-65 ℃ in 5 ℃ intervals. Optimal temperature of recombinant AG4 is shown in figure 19 and it was found to be 55 ℃. When the temperature was increased from 55 ℃ the relative activity was started to decrease. However, relative activity was remained 50% at the temperature of 65 ℃.

4. 1. 2. Effect of pH on the activity of the recombinant AG4

The optimum pH of AG4 was 5.5 in acetate buffer and it was stable at pH 5-6.5, retaining more than 80% of relative activity (figure 20). Further, more than 50% of relative activity was remained at pH ranging from 4.5-8.0 in acetate and phosphate buffers.

4. 1. 3. Effect of temperature on the thermostability of the recombinant AG4

The thermal stability of AG4 is shown in figure 21. Four different temperatures (40, 45, 50 and 55 ℃) were used to check thermal stability of AG4 with respect to different incubation time points at 30, 60 and 120 min. The enzyme was retained its thermal stability (almost 100% of relataive activity) at 40 and 45 ℃ upto 120 min. However, the enzyme was fairly stable at 50 and 55 ℃ for 60 min and the thermal stability was retained about 80%. Even though, their thermel stability was decreased at 120 min compared to 60 min, the relative activity was retained around 60%.

4. 1. 4. Effect of $CaCl_2$ on thermostability of recombinant AG4

The effect of $CaCl_2$ on thermostability of purified recombinant AG4 is given in figure 22. By addition of 2 mM $CaCl_2$ in a final concentration of the reaction

mixture did not show much differences when compared with the samples which did not use $CaCl_2$ at different temperatures. Interestingly, it was observed that, with the addition of $CaCl_2$ to the reaction mixture, the thermostability was drastically decreased at 55 ℃ from 70-30% during the period of 30-120 min.

4. 1. 5. Effect of metal ions salts and chelators on activity of recombinant AG4

The effect of metal ion salts and chelators on AG4 enzymatic activity was investigated as inhibitors or activators at optimum conditions. Figure 23 shows that AG4 relative activity was inhibited more than 90% by divalent metal salts such as CuSO4 and ZnSO4 and also by metal chelator EDTA (each at 2 mM). Moreover, divalent metal salt 2mM MnCl2 also inhibited the activity by 40%. In contrast, FeSO4 enhanced the relative activity when compared to control which has no metal ion salts or chelators. However, There was no difference was observed in relative activity of AG4 which included NaCl, MgCl2 and CaCl2 in the reaction mixture when compared with the control samples.

4. 1. 6. Identification of hydrolysis products of the recombinant AG4 on TLC

Hydrolysis patterns of the purified agarase AG4 against food grade agar, Neoagarohexanitol (NA6) and mixture of neoagarotetrose (NA4) and neoagarobiose (NA2) are shown in figure 24. When food grade agar was used as a substrate, with following the time, reaction products were observed on TLC plate clearly. One product was identified as NA6 and other was identified as NA4. The results showed that final product which obtained from agar hydrolysis by AG4 was NA4 and could not able to hydrolyze NA6 and NA4 in this study.

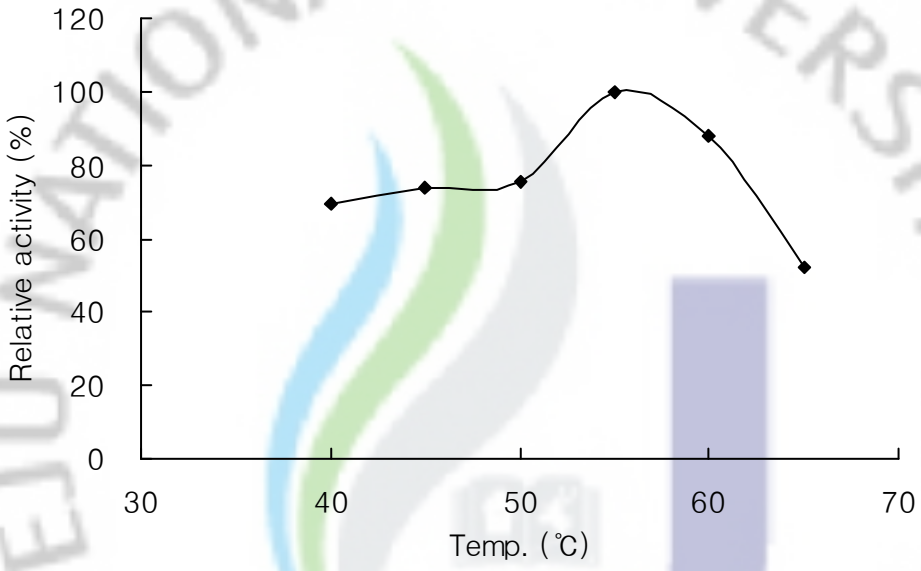

Figure 19. The effect of temperature on the activity of purified recombinant AG4. The effect of temperature on the enzyme activity was determined under standard assay conditions at temperature ranging between 40-65 ℃.

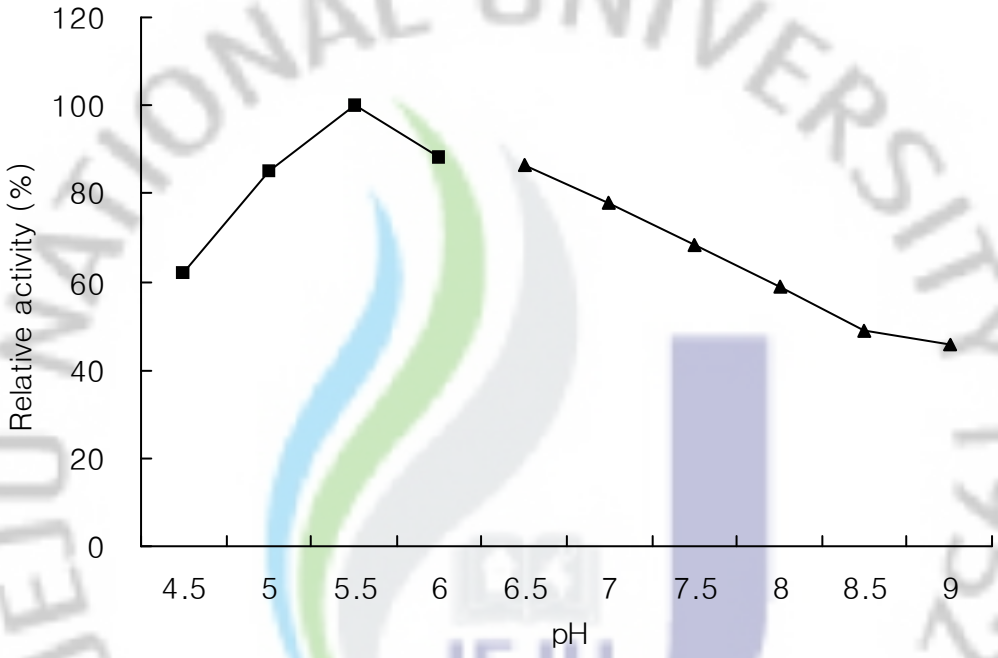

Figure 20. The effect of pH on the activity of the purified recombinant AG4. The effect of pH on the enzyme activity was determined under standard assay conditions using acetate (pH 4.5-6) and phosphate buffer (pH 6.5-9).

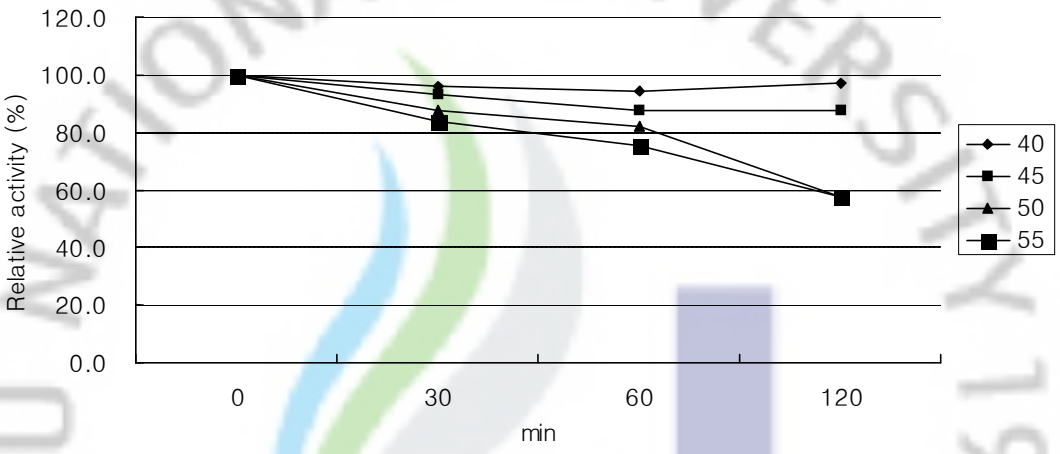

Figure 21. The effect of thermostability on purified recombinant AG4 at different temperatures for different time points. Thermostability of the recombinant AG4 determined by measurement of residual activity under standard assay conditions after incubation at 40, 45, 50 and 55 ℃ for 30, 60 and 120 min.

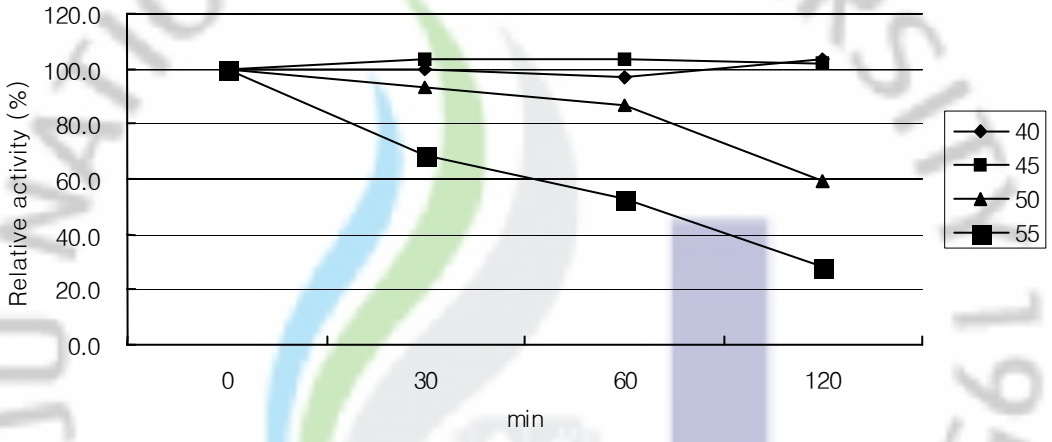

Figure 22. The effect of CaCl2 on thermostability of purified recombinant AG4

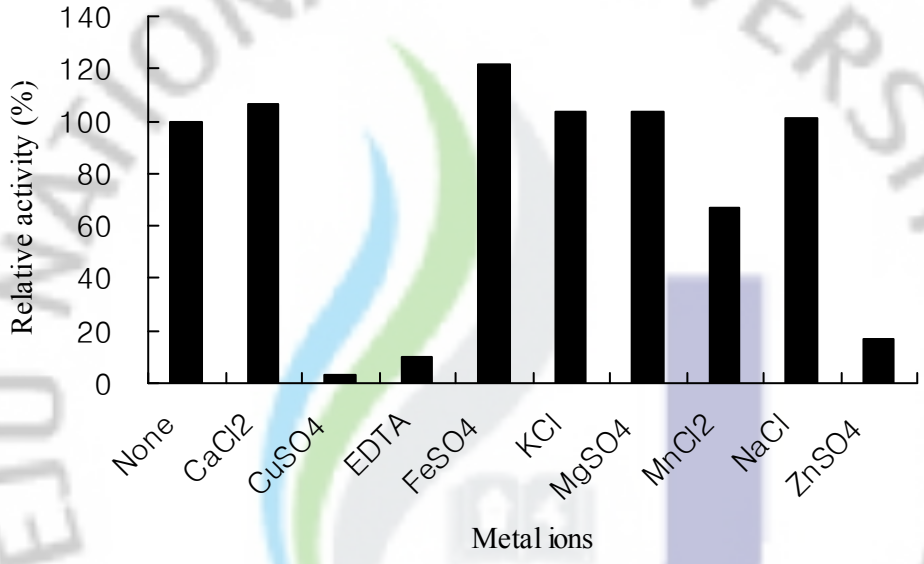

Figure 23. Effect of metal ions and metal salts on purified recombinant AG4

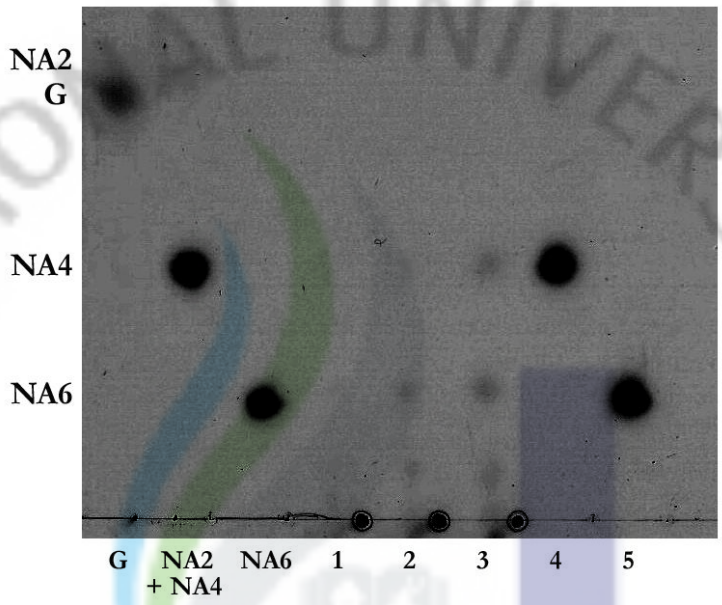

Figure 24. Thin layer chromatography of hydrolysis products of the purified AG4 enzyme on food grade agar and neagaroligosaccharides. Reaction of purified AG4 and agar were performed in 200 µl reactions containing 20ul of purified agarase and 180 µl of 1% agar at 45 oC for 30, 60, and 120 min. NA6 and NA4+NA2 substrates were reacted with 20 ul of purified AG4 separately at 45 oC for 120 min. Neoagarohexaitol (NA6), neoagarotetrose (NA4), neoagarobiose (NA2) and D-(+)-galactose (G) were used as standards.

4. 2. Enzyme characterization of recombinant AG17

4. 2. 1. Effect of temperature on the activity of the recombinant AG17

The effect of temperature on the activity of AG17 is shown in figure 25. The optimum temperature of purified recombinant AG17 was determined at different temperatures ranging from 40-70 ℃ in 5 ℃ intervals. With in the range of the tested temperatures the activity of the enzyme increased up to 65 ℃ and it was shown as the optimal temperature for the enzyme. Once the temperature rose above 65 ℃, the activity of the enzyme decreased drastically. However, relative activity was retained about 45% at the temperature of 70 ℃.

4. 2. 2. Effect of pH on the activity of the recombinant AG17

The effect of pH on the purified enzyme is shown in figure 26. To determine the effect of pH on the activity of the recombinant enzyme, two different buffering systems were used. Higher activity was obtained in the range of pH from 4.5-8.5 and in all that range, more than 90% of relative activity was retained. However, optimum pH was obtained at pH 5.5 in acetate buffer.

4. 2. 3. The effect of thermostability on recombinant AG17

Figure 27 shows the thermal stability of AG17. Six different temperatures (40, 45, 50, 55, 60 and 65 ℃) were used to determine the thermal stability of AG17 with respect to different incubation time points at 30, 60 and 120 min. The enzyme was retained its thermal stability (almost 100% of relative activity) at 40 and 45 ℃. Moreover, about 80% of relative activity was retained at 50 ℃ up to 120 min. Even though, the thermal stability was decreased at 55, 60 and 65 ℃ at the time of 120 min (up to 40 % relative activity) , their thermal stability was retained between 60-80% at 60 min.

4. 2. 4. Effect of metal ions salts and chelators on activity of recombinant AG17

The effect of metal ion salts and chelators on activity of purified AG17 enzyme is shown in figure 28. Purified recombinant AG17 relative activity was inhibited more than 40% by divalent metal salts such as CuSO4 and ZnSO4 and also by FeSO4 and MnCl2 by 20% (each at 2 mM). Moreover, divalent metal salt 2mM MnCl2 also inhibited the activity by 40%. In contrast, MgSO4, NaCl, KCl enhanced the relative activity when compared to control. However, There was no difference was observed in relative activity of AG17 which had CaCl2, FeSO4 and EDTA in the reaction mixtures when compared with the control samples.

4. 2. 5. Identification of hydrolysis products of the recombinant AG17 on TLC

Hydrolysis patterns of the purified agrase AG17 against food grade agar, Neoagarohexanitol (NA6) and mixture of NA6 and neoagarotetrose (NA4) are shown in figure 29. When the AG17 is reacted with food grade agar, NA4 and NA2 were detected on TLC plate at early stage of the reaction and following the time, the amount of NA2 production was increased, which was detected at higher amount at 60 and 120 min compared to 30 min of hydrolysis. More over, when the AG17 was reacted with NA6 and mixture of NA2+NA4 at 120 min, two reaction products were observed as NA4 and NA2 on TLC. The main final product which obtained from AG17 was NA2 and it could hydrolyze all the substrates which used in this study.

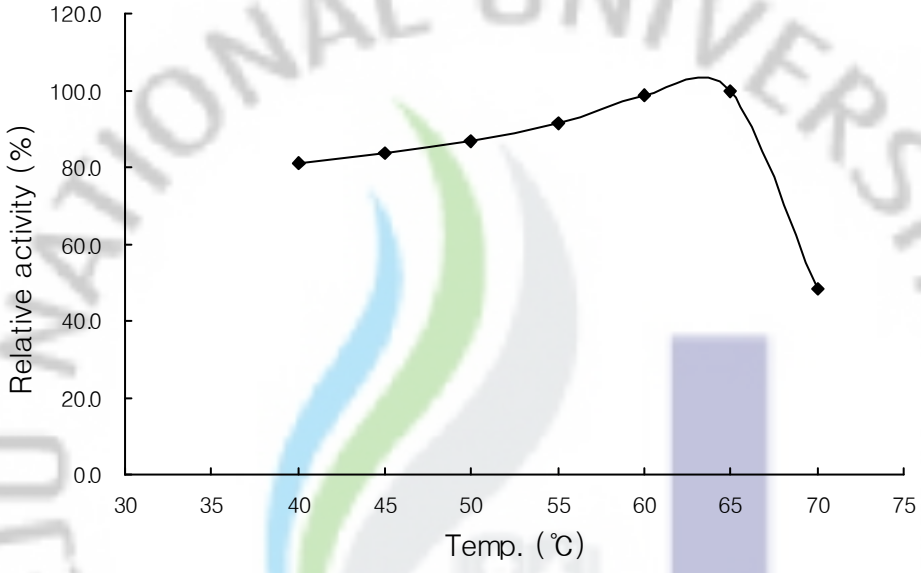

Figure 25. The effect of temperature on the activity of purified recombinant AG17. The effect of temperature on the enzyme activity was determined under standard assay conditions at temperature ranging between 40-70 ℃.

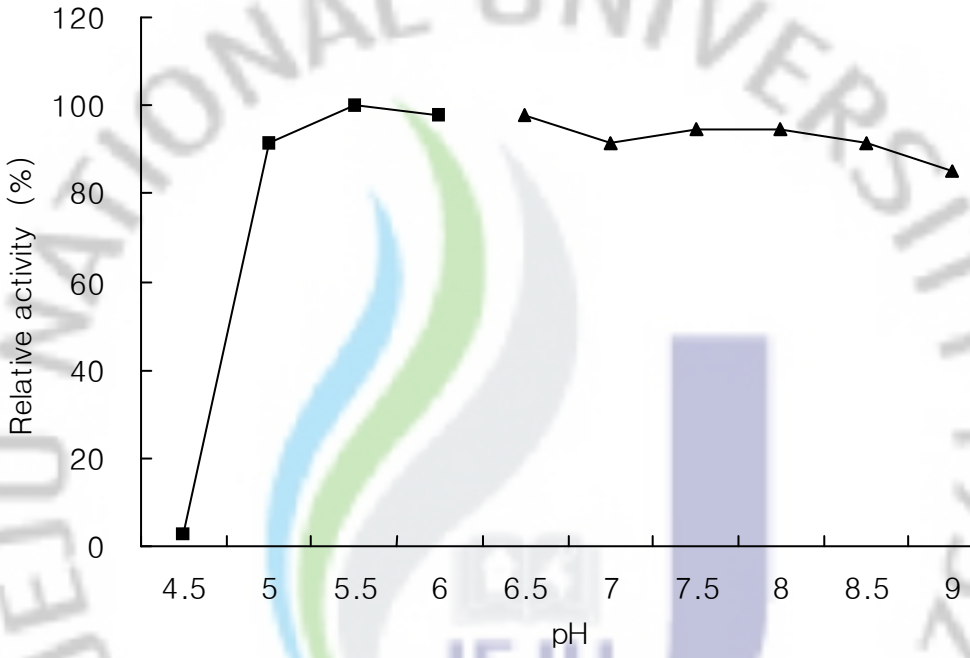

Figure 26. The effect of pH on the activity of the purified recombinant AG17. The effect of pH on the enzyme activity was determined under standard assay conditions using acetate (pH 4.5-6) and phosphate buffer (pH 6.5-9).

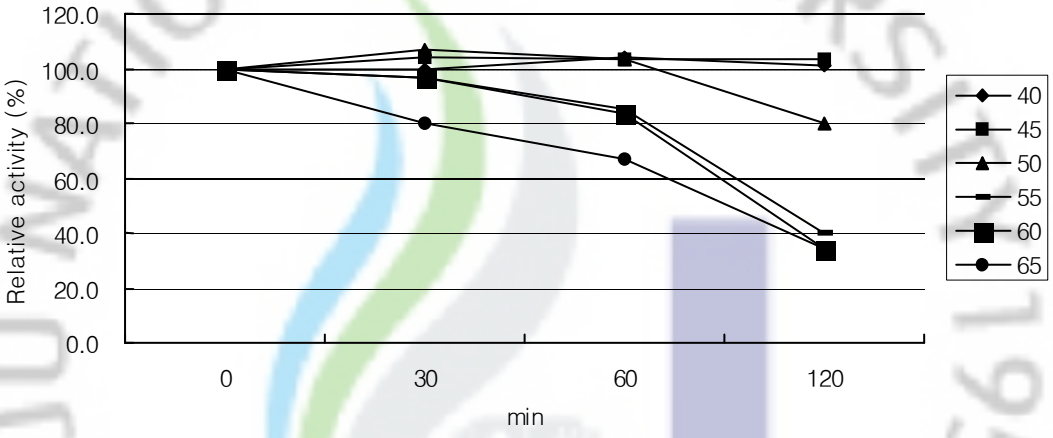

Figure 27. The effect of thermostability on purified recombinant AG17 at different temperatures for different time points.

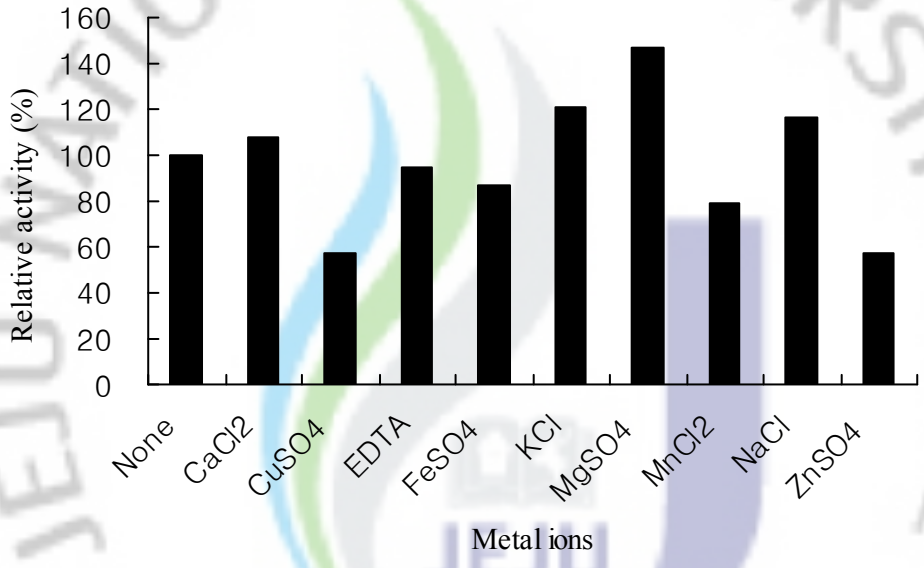

Figure 28. Effect of metal ions and metal salts on purified recombinant AG17.

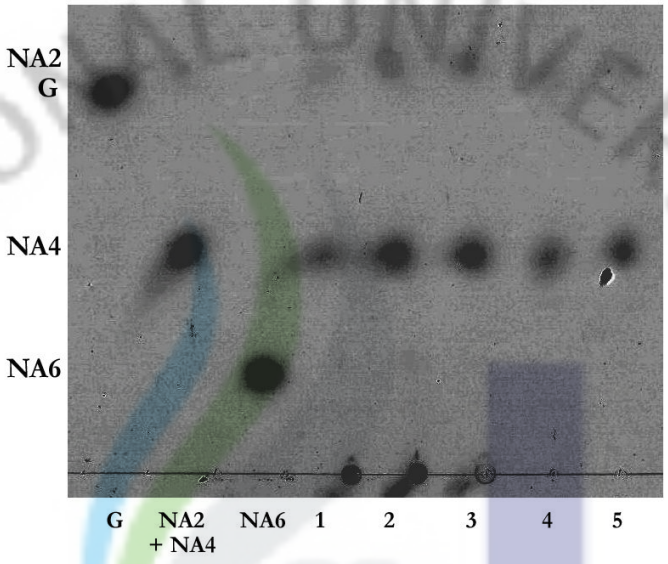

Figure 29. Thin layer chromatography of hydrolysis products of the purified AG17 enzyme on food grade agar and neagaroligosaccharides. Reaction of purified AG17 and agar were performed in 200 µl reactions containing 20 ul of purified agarase and 180 µl of 1% agar at 45 ℃ for 30, 60, and 120 min. NA6 and NA4+NA2 substrates were reacted with 20 ul of purified AG4 separately at 45 ℃ for 120 min. Neoagarohexaitol (NA6), neoagarotetrose (NA4), neoagarobiose (NA2) and D-(+)-galactose (G) were used as standards.

4. 3. Enzyme characterization of recombinant AG52

4. 3. 1. Effect of temperature on the activity of the recombinant AG52

The optimum reaction temperature of the purified recombinant AG52 agarase was 55 ℃ (figure 30). At 40 and 50 ℃ the relative activity was 90 and 92%, respectively in comparison with the maximum. However, more than 50% of relative activity was observed at 40 and 60 ℃.

4. 3. 2. Effect of pH on the activity of the purified recombinant AG52

The effect of pH on the purified enzyme is shown in the figure 31. The effect of pH on the enzyme activity was determined at 45 ℃ in the pH range of 4.5-9 and it showed that the enzyme was stable under the conditions of this assay (more than 60% relative activity) in the range of buffers from pH 4.5-9. However, the enzyme exhibited maximum activity at pH 5.5.

4. 3. 3. Effect of thermal stability on the activity of the recombinant AG52

Figure 32 shows the effect of temperature on the stability of AG52. Four different temperatures (40, 45, 50 and 55 ℃) were used to check thermal stability of AG52 with respect to different incubation time points at 30, 60 and 120 min. The figure shows that enzyme relative activity was decreased when the temperature was increased with respect to time points and the enzyme was inactivated at temperatures of 45, 50 and 55 (relative activity 20-30 %) when it was incubated for 30 min. Moreover, the enzyme was fairly stable up to 40 ℃ for 60 min and relative activity was retained up to 50%.

4. 3. 4. Effect of CaCl2 on thermostability of recombinant AG52

The effect of CaCl2 on thermostability of purified recombinant AG17 is given in figure 33. By addition of 2 mM CaCl2 in a final concentration of the reaction mixture did not show any differences when compared with the samples which did

not use CaCl2 at different temperatures.

4. 3. 5. Effect of metal ion salts and chelators on activity of recombinant AG52

The effect of metal ion salts and chelators on activity of purified AG52 enzyme is shown in figure 34. AG52 relative activity was totally inhibited by divalent metal salts such as CuSO4 and ZnSO4 (each at 2 mM). More over, 2 mM EDTA inhibited the enzyme activity by 60%. In contrast, FeSO4 and KCl enhanced the activity of AG52 and MgSO4, NaCl, CaCl2 and MnCl2 did not show much difference when compared with the control.

4. 3. 6. Identification of hydrolysis products of the recombinant AG52 on TLC

Hydrolysis patterns of the purified agrase AG52 against food grade agar, Neoagarohexanitol (NA6) and mixture of NA6 and neoagarotetrose (NA4) are shown in figure 35. When the AG52 is reacted with food grade agar, two clear spots were observed on TLC plate at 30, 60 and 120 min after the reaction. According to the standards, the hydrolysis products were identified as NA6 and NA4. More over, when AG52 was reacted with NA6, the final product obtained was NA4. However, when AG52 was reacted with mixture of NA2+NA4 at 120 min, no distinct reaction products was observed suggesting that the final product, which obtained from AG52 was NA4.

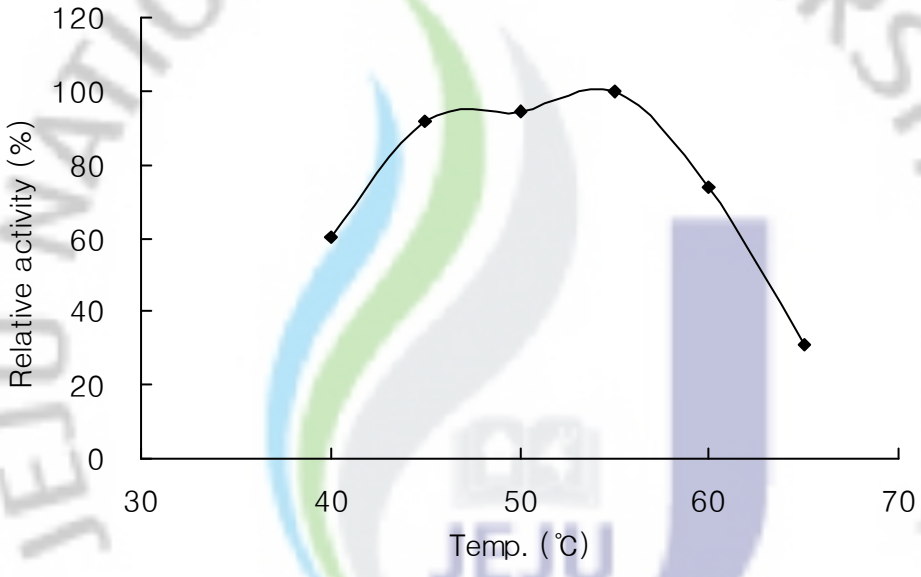

Figure 30. The effect of temperature on the activity of purified recombinant AG52.

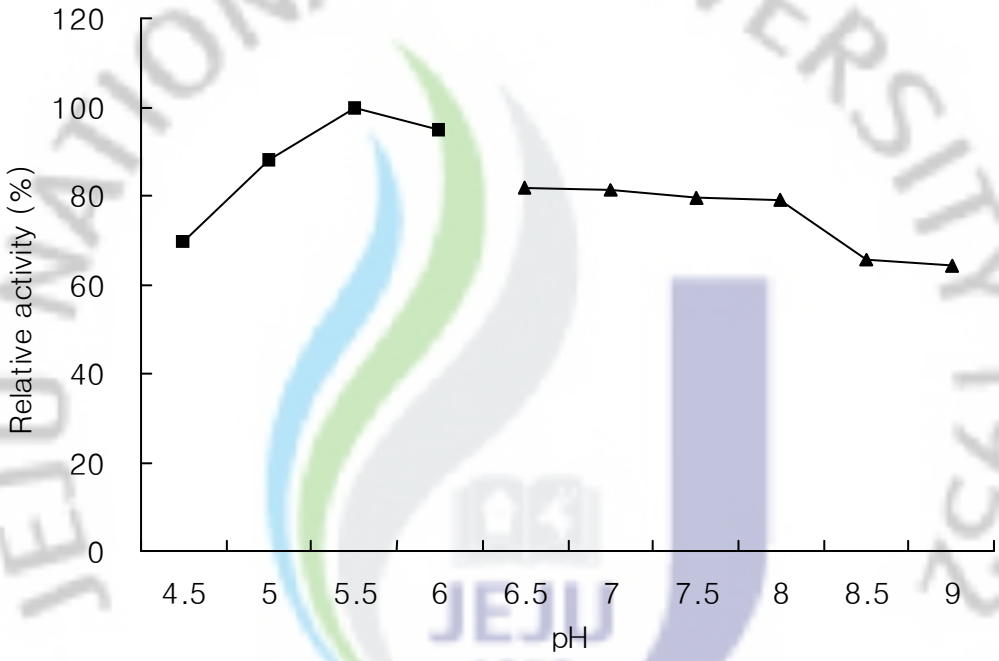

Figure 31. The effect of pH on the activity of the purified recombinant AG52. The effect of pH on the enzyme activity was determined under standard assay conditions using acetate (pH 4.5-6) and phosphate buffer (pH 6.5-9).

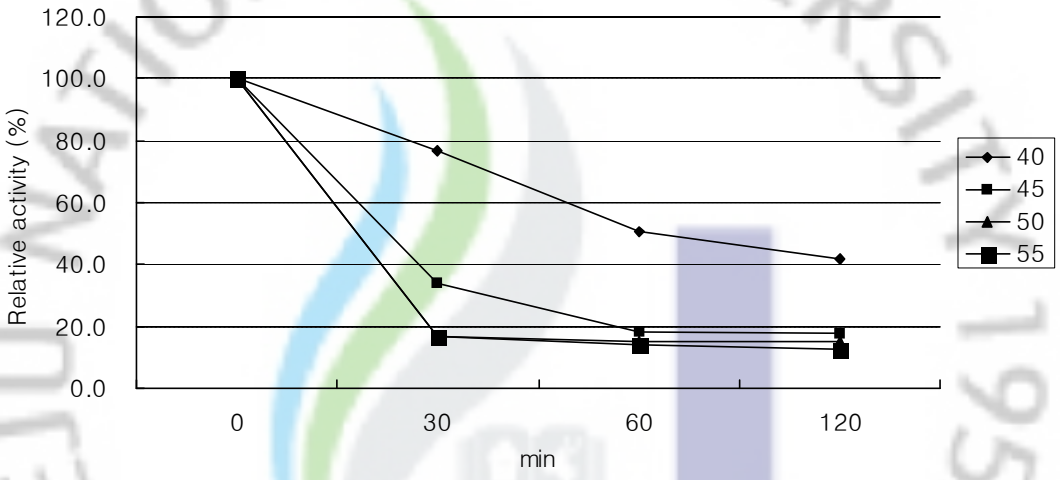

Figure 32. The effect of thermostability on purified recombinant AG52 at different temperatures for different time points. Thermostability of the recombinant AG52 determined by measurement of residual activity under standard assay conditions after incubation at 40, 45, 50 and 55 ℃ for 30, 60 and 120 min.

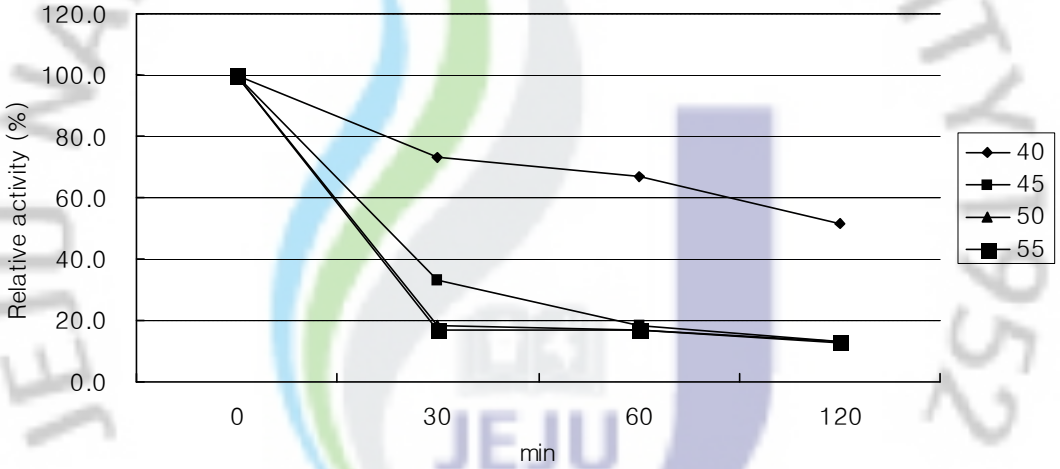

Figure 33. The effect of $CaCl_2$ on thermostability of purified recombinant AG52

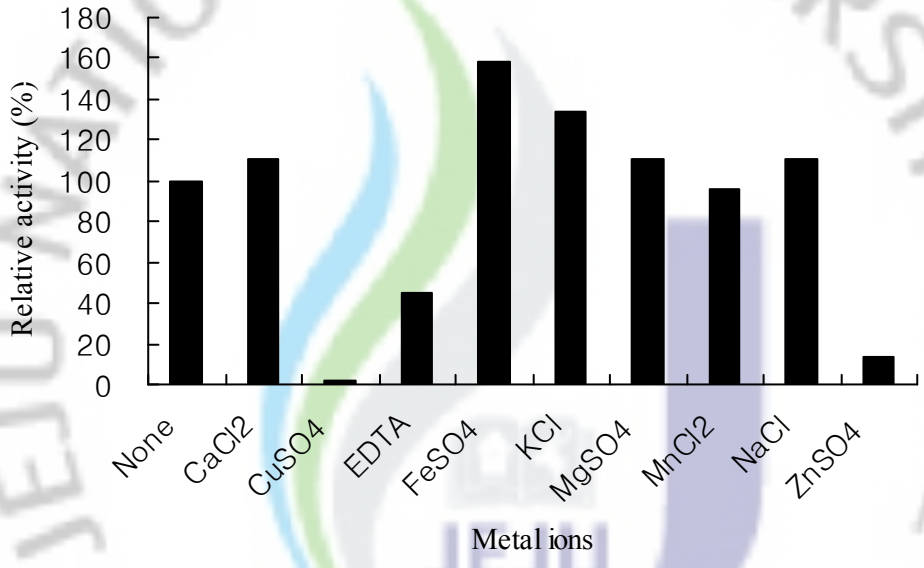

Figure 34. Effect of metal ion salts on enzymatic activity of AG52.

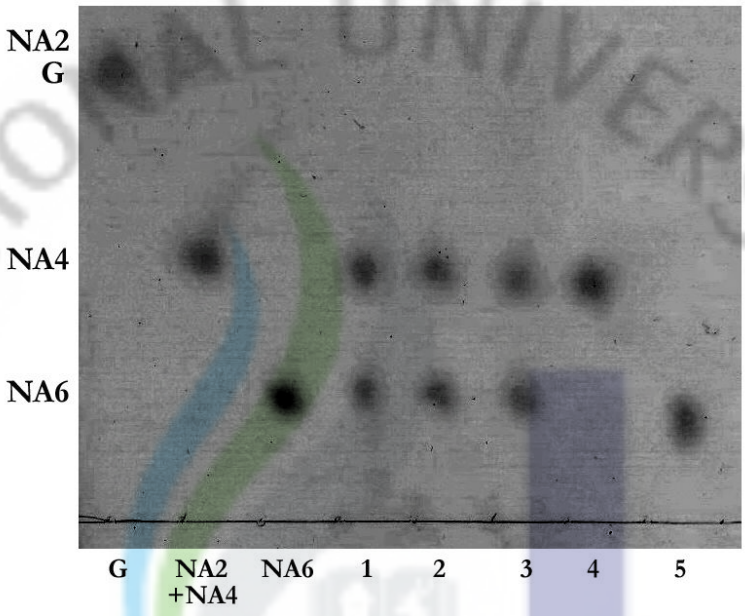

Figure 35. Thin layer chromatography of hydrolysis products of the purified AG52 enzyme on food grade agar and neagaroligosaccharides. Reaction of purified AG52 and agar were performed in 200 µl reactions containing 20ul of purified agarase and 180 µl of 1% agar at 45 oC for 30, 60, and 120 min. NA6 and NA4+NA2 substrates were reacted with 20 ul of purified AG4 separately at 45 oC for 120 min. Neoagarohexaitol (NA6), neoagarotetrose (NA4), neoagarobiose (NA2) and D-(+)-galactose (G) were used as standards.

4. 4. Specific activity of agarases based on substrates specificity

Table 6 summarizes the results of specific activities of purified recombinant agarases toward different substrates. The purified AG4 enzyme showed 204.4 and 207 unit/mg specific activity towards agar and agarose, respectively. The specific activity was performed at 55℃in acetate buffer (pH 5.5) supplemented with 2mM $FeSO_4$. Moreover, recombinant purified AG17 enzyme showed 158.8 and 155.3 unit/mg specific activity towards agar and agarose, respectively. The specific activity was performed at 65℃ in acetate buffer (pH 5.5) supplemented with 2mM $MgSO_4$. Further, the specific activity of AG52 was 105.1 and 79.5 unit/mg towards agar and agarose, respectively. The specific activity was performed at 55℃ in acetate buffer (pH 6.0) supplemented with 2mM FeSO4 in 30 min reaction. However, non of these enzymes showed activity towards the substrate carrageenan.

Table 6. Specific activity of agarases accoding to substrates

	Substrates		
	Agar	Agarose	Carrageenan
AG4	204.4 unit/mg	207.5 unit/mg	0 unit/mg
AG17	158.8 unit/mg	155.3 unit/mg	0 unit/mg
AG52	105.1 unit/mg	79.5 unit/mg	0 unit/mg

5. Functional activity of neoagaro-oligosaccarides

5. 1. Antioxidant assay

The level of antioxidant activity was determined by measuring the DPPH radical scavenging activity (figure 36). The results showed that NA2+NA4 and NA4 showed lower activity than commercial antioxidant vitamin C and E. However, NA2+NA4 and NA4 showed about 50% DPPH scavenging activity while commercial antioxidants showed about 90% activity.

5. 2. Effect of whitening

The whitening effects of neoagarotetrose (NA4), mixture of neoagarobiose and neoagarotetrose (NA4+NA2) and arbutin were evaluated by measuring melanin content in melanocytes (figure 37). In this study, B16F10 melanoma cells were whitened by 10 and 100 ug/mL of NA4 and commercial whitening compound arbutin had similar whitening effect at the same concentration. Highest activity was observed in NA4 treated samples when compared with NA4+NA2.

5. 3. Effect of Neoagaro-oligosaccharide on cell viability

Neoagarotetrose (NA4), mixture of neoagarobiose and neoagarotetrose(NA4+NA2) were not cytotoxic to B16F10 melanoma cell and normal human fibroblast at concentrations up to 0.1 ~ 100 ug/ml.

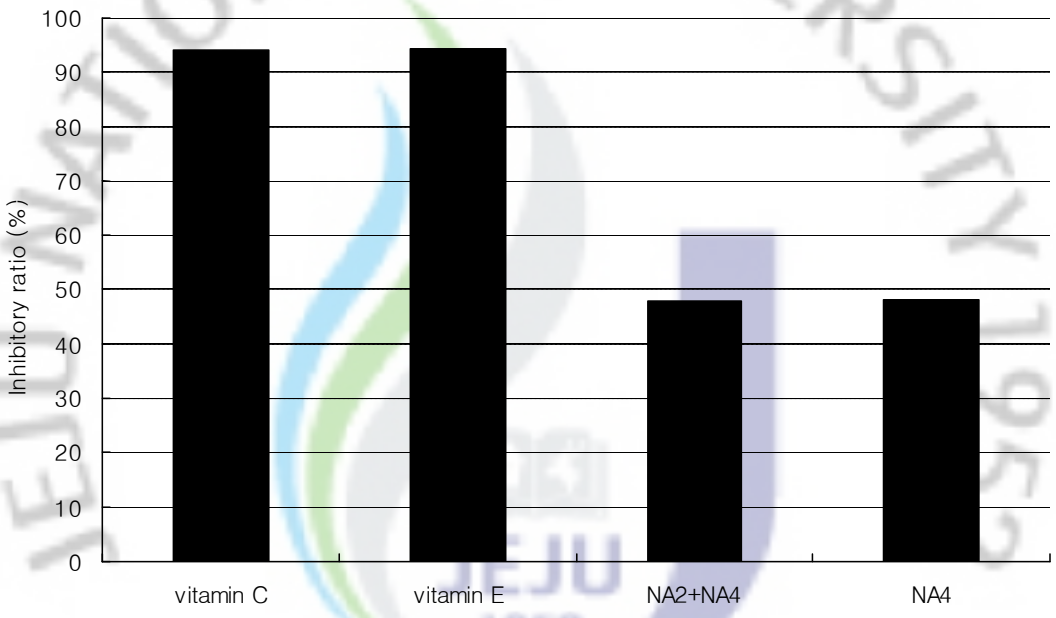

Figure 36. Effect of neoagarooligosaccharides (NA+NA4 and NA4) on the DPPH scavenging activity.

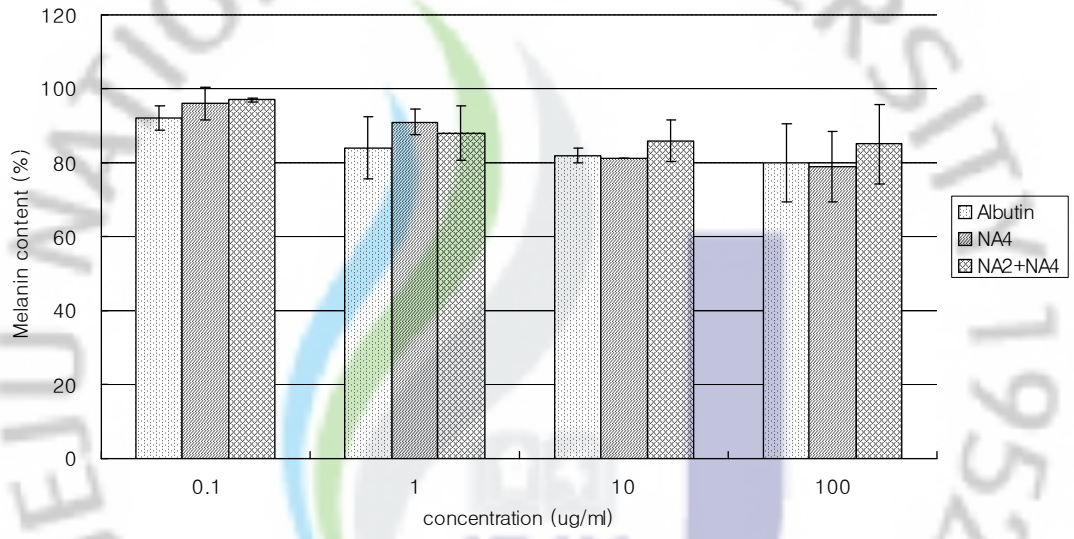

Figure 37. Whitening effects of neoagarotetrose, neoagarotetrose and neoagarobiose and arbutin at various concentrations in B16F10 cells

IV. Discussion

In this study, we have identified different agarase producing bacteria, cloning, recombinant enzyme purification of selected agarases, and functional characterization of these enzymes with respects optimum conditions. Additionally, potential further applications of oligomer forms derived from agar were tested with respect to different bioactivities such as whitening, antioxidant and cytotoxic effects.

First, we screened agarase producing marine bacteria strains isolated from different marine sources at Jeju Island costal environment. Different types of selection plates were used to identify the different agarase producing bacteria. Generally, selection plates were used with rich medium including agar. Alternatively, we used SW agar plate for first selection, which has only agar as carbon source that allows for growing different kind of bacteria. Then, the selected agarase positive colonies from SW plates were streaked on SWT, SWY and marine agar plates for second selection. Finally, we were able to screen 29 agarase producing marine bacteria strains. The strains include *Agarivorans* sp., *Aquimarina* sp., *Cellulophaga* sp., *Cytophaga* sp., *Flammeovirga* sp., *Glaciecola* sp., *Microbulbifer* sp., *Pseudoalteromonas* sp., *Reinekia* sp. and *Shewanella* sp. etc. that identified by 16s rRNA nucleotide sequences. All the identified agarase producing bacteria were Gram negative type in this study. Similarly, almost all the agarase producing bacteria were identified from Gram negative type. There are numerous reports on agarase producing bacteria. In previous study, agarase producing bacteria were identified from different bacterial genera such as *Agarivorans* (Ohta et al. 2005a; Lee et al. 2006; Fu et al. 2008b), *Alteromonas* (Leon et al. 1992; Potin et al. 1993; Kirimura et al. 1999; Hassairi et al. 2001; Wang et al. 2006), *Bacillus* (Suzuki et al. 2003), *Cytophage* (Duckworth and Turvey 1968; Van der Meulen and Harder 1975), *Microbulbifer* (Ohta et al. 2004b; Ohta et al. 2004c), *Pseudomonas* (Groleau and Yaphe 1977; Morrice et al. 1983; Lee et al. 2000; Ryu et al. 2001), *Pseudoalteromonas* (Vera et al. 1998; Schroeder et al. 2003;

Ma et al. 2007), *Saccharophagus* (Ekborg et al. 2006), *Streptomyces* (Kendall and Cullum 1984), *Thalassomonas* (Ohta et al. 2004a), *Vibrio* (Aoki et al. 1990; Sugano et al. 1993; Araki et al. 1998a; Zhang and Sun 2007; Fu et al. 2008a), *Zobellia* (Jam et al. 2005). More importantly, we have identified *Aquimarina* sp., *Cellulophaga* sp., *Flammeovirga* sp., and *Reinekia* sp. as new agarase producing bacteria, which were not reported in previously. However, cloned agarases genes were belonged to *Pseudoalteromonas* sp. (AG4 and AG52) *Agarivorans* sp. (AG17) *Flammeovirga* sp. (AG31), which has been reported, previously.

Beta-agarase is a glycosyl hydrolase family 16 (GH16) member that hydrolyzes the internal beta-1,4-linkage of agarose, producing neoagaro-oligosaccharides. While beta-agarases are also found in two other families derived from the sequence-based classification of glycosyl hydrolases (GH50, and GH86) the GH16 members are most abundant including agarase, carrageenase, glucanases etc. Agarases of AG4, AG31 and AG52 strains contained catalytic domains, GH16. GH16 family group members were known from bacteria as DagA (accession number: AAA91888), *P. atlantica* ATCC19262, AagA (accession number : AAF03246) from *Aeromonas* sp. strain B9, AgaA (accession number: AB124837) from strain JAMB-A94, AgaA7 (accession number: BAC99022) from *Microbulbifer* sp. strain JAMB-A7, AagA (accession number : BAB79291) from *Pseudomonas* sp. strain ND137, AgaB (accession number: AAF12821) from *Zobellia galactanivorans* Dsij, AgaA (accession number : AAF21820) from *Z. galactanivorans* Dsij, AgaA (accession number : AAN39119) from *Pseudoalteromonas* sp. strain CY24, DagA (accession number : CAB61795) from *Streptomyces coelicolor* A3(2). *Pseudoalteromonas* sp. AG4 and AG52 agarase amino acid sequences have 85% similar and both are detected active sites of 11 and calcium binding modules of 7 to same position. However, *Pseudoalteromonas* sp. AG52 agarase have more similarity to *Aromonas* sp. agarase.

Members having bacterial Ig-like domain are found in bacterial and phage surface

proteins called intimins. Intimin is a bacterial cell-adhesion molecule that mediates the intimate bacterial host-cell interaction. It contains three domains; two immunoglobulin-like domains and a C-type lectin-like module implying that carbohydrate recognition may be important in intimin-mediated cell adhesion (Kelly et al. 1999). Bacterial Ig-like domain was first identified from agarase of *Vibrio* sp. strain PO-303 (Dong et al. 2007). Therefore, AG31 in this study is the second report of agarase sequence with bacterial Ig-like domain (group 2). Also, AG31 agarase has carbohydrate binding modules family 6. The insoluble polymers are recalcitrant to microbial degradation, and enzymes that catalyze this process generally contain non-catalytic carbohydrate binding modules (CBMs). It was shown that potential activity is given by increasing substrate binding (Henshaw et al. 2006). AG52 agarase sequence showed the predicted lipoprotein signal at N-terminal. Lipoprotein signal is one of secretion system from gram-negative bacteria (Pugsley 1993). Therefore, AG52 could be supported by lipoprotein signal for the enzyme secretion. We tried to express this with signal sequence by pET11a. However, expression level was very low. We checked the activity from supernatant of inducted cells. The activity showed 1 unit/ml.min and intracellular protein showed 1.4 unit/ml.min. This result suggest that lipoprotein signal sequence can lead target protein to outside from the *E. coli* cells.

Agarase enzymes share a common ancestor and have diverged significantly in their primary sequence (Allouch et al. 2003). Pairwise comparison of the sequences of family GH-16 reveals the existence of several sub-families. Similarly our phylogenetic analysis results showed different sub families GH-16. Also, it is further confirmed that AG4 and AG52 are belongs to GH-16 sub-family of β-agarase since both sequences were grouped with members of GH-16 sub-family. Additionally, it could be confirmed that AG31 also belongs to β-agarase GH-16 sub family even thought it is separated from AG4 and AG52 since AG31 has the GH-16 domain. In contrast, AG17 is deviated from known agarase sequences and positioned separately,

suggesting that it may be a new glycoside hydrolase. Although, the sequence based GH families contain enzymes of similar fold and identical molecular mechanism, they are known to often group together enzymes of varying substrate specificity. Interestingly, family GH-16 is such a "polyspecific" family and contains enzymes hydrolyzing widely different substrates such as keratin-sulfate, β-1,3 (4) glucans, xyloglucan, k-carrageenan, and agrose. Therefore, we have selects different substrates such as agar, agarose and carrageenan (Allouch et al. 2003).

Generally most of the GH16 beta-agarases that have been characterized so far, has temperature optimum around 40 ℃ and a optimum pH around neutral to mild alkaline (Zhang and Sun, 2007). However, in contrast, the recombinant AG4, AG17 and AG52 showed optimum activity at 55, 65 and 55 ℃ and pH 5.5. As found in other beta-agarases, we found that $MgSO_4$, NaCl and KCl acted positively on the activity of the recombinant AG17 and to certain extent, KCl on the activity of AG52 suggesting that source strains of these purified agarases are marine inhabitant. However, in this study, among divalent metal ion salts, $FeSO_4$ acted positively on AG4 and AG52. More over, as reported in previous studies, in this study the $CaCl_2$ did not show significant effect on the activity of the recombinant enzymes at final concentration of 2mM. Interestingly, even though, the calcium binding module is present in the AG4 gene sequence, apparently inhibitory effect at higher temperatures (55 ℃) of AG4 was observed with the $CaCl_2$. In contrast to this study, it has been reported that $CaCl_2$ could stabilize the activity of GH86 beta-agarase AgaO and enhanced the activity of AgaB of the *Pseudoalteromonas* sp. (Ohta et al. 2004; Ma et al. 2007). However, similar results have been reported by Zhang and Sun (2007) with the no measurable effect on the activity of the recombinant beta agarase AgaV enzyme (isolated from V134, a marine isolate of the Vibrio genus) at concentrations of up to 10 mM $CaCl_2$ and exerted an apparently inhibitory effect at higher concentrations. It has been reported that, even though, there are functionally similarities in hydrolysis of beta 1,4-linkages in specific substrates, there is high

degree of heterogeneity in amino acid sequences molecular masses, catalytic properties and substrate specificities.

It has been reported that in the initial stage, the purified agarase hydrolyzed agar or agarose to generate many oligosaccharides with various degrees of polymerization (Lee et al., 2006). Even though AG4 agarase sequence was 2 bp (2 aa) and 5 bp (4 aa) different compare to *Pseudoalteromonas* sp. KJ 2-4 alpha-agarase gene (accession number : AY488029) and *Pseudomonas atlantica* beta-agarase gene (accession number : M73783), after identifing the degrading patten of agar, neoagarohexaitol and neoagarotetrose by TLC analysis, the results showed that AG4 agarase was endo beta-agarase type. Moreover, after 30, 60 and 120 min incubation with AG17, the main products observed were NA6 and NA4 and also to some extent of NA2. These results suggest that the purified recombinant enzymes are beta agarases that catalyzed the hydrolyzis of beta 1,4-linkages in agar.

Also, it has been recognized that agarases have potential wide applications in many areas of industry and scientific research. It has been reported that neoagarobiose has good potential for functional cosmetics due to its moisturizing and whitening effect on skin (Kobayashi et al. 1997). Since generated simple neo-oligosaccharides from complex polysaccharides has whitening and antioxidant activities, the hydrolysis products which identified here, has potential to use in industries such as cosmetics as well as medical. In this study, neooligosaccharides prepared by agarase showed antioxidative activities by scavenging production of free radicals. Several explanations of in vitro antioxidation mechanism of polysaccharide have been reported (Zhou et al. 2002). DPPH scavenging activity is easy-use in vitro assay to assess the activity of various antioxidants through proton donation mechanism. In this study, the reaction mechanism of agar oligosaccharide, need to be studied more in details in future.

In conclusion different bacteria, which produced agarase were identified from Jeju

Island coastal environment. Molecular cloning and sequence characterization of four agarase genes showed that three of them (AG4, AG31 and AG52) are belongs to GH-16 β agarase family while other one AG17 is new member of agarase family. Purified recombinant proteins of respective agarases were functionally active. More importantly, our purified agarases are applied in the production of neoagarohexaose, neoagarotetrose and neoagarobiose from agar. Preliminary studies showed that resulted neoagaro forms have biological activates such as whitening and antioxidant effects suggesting these purified agarase enzymes would be have potential for the industrial applications.

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감사의 글

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