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Enhanced anti-oxidant activity of neoagarooligosaccharides produced by β-agarase derived from *Aquimarina agarilytica* NI125

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Abstract

The neoagarooligosaccharides have received growing attention owing to their physiological activities. The aim of this study was the isolation of agarase-producing bacteria for production of agar hydrolysates with special emphasis on their anti-oxidant potential. An agarolytic strain NI125 was isolated from Nelson's Island, Alexandria, Egypt. Based on 16S rRNA analysis and extensive phenotypic characterization, it was identified as *Aquimarina agarilytica*. Maximum enzyme production was achieved after 24 h incubation at 20°C, and tryptone was recorded to be the best nitrogen source for agarase production. Extracellular agarase was partially purified by ammonium sulfate precipitation. The substrate specificity assay using p-nitrophenyl-α/β-D-galactopyranoside revealed the cleavage of the β-linkage rather than the α-linkage. Neoagarooligosaccharides produced by the partially purified β-agarase expressed promising anti-oxidant properties, with 23% free radical scavenging potential. Notable enhancement of the anti-oxidant potency of the oligosaccharides was achieved (up to 87% scavenging ability) by sulfation of the agar prior to hydrolysis for 12 h with β-agarase. Results obtained suggest the potential application of the produced neoagarooligosaccharides anti-oxidants as promising additives in food and feed products.

Keywords: Agar, β-agarase, Agarolytic activity, Anti-oxidant, Sulfation

1. Introduction

Agar is the main phycocolloid in the cell wall of the red algae. It can be hydrolyzed into oligosaccharides by the action of agarase found in the agarolytic bacteria, and have been isolated mostly from marine habitats (Lee *et al*., 2018; Schultz-Johansen *et al*., 2018). According to their cleavage pattern, agarases are classified into α-agarase (E.C. 3.2.1.158) and β-agarase (E.C.3.2.1.81). Recently, Cheong *et al*., (2018) reported that agar can be hydrolyzed into agaro-oligosaccharides (AOs) or neoagaro-oligosaccharides (NAOs), by the action of α-agarase and β-agarase, respectively.

In general, NAOs exhibit various physiological activities hence are used as food additives,

pharmaceutical and cosmetics ingredients. In several previous studies, NAOs are reported as anti-tumors (Lee *et al*., 2017), anti-inflammatory (Enoki *et al*., 2010; Wang *et al*., 2017), anti-fatigue (Zhang *et al*., 2017), tyrosinase inhibitors (Lee *et al*., 2008), immune modulators (Kang *et al*., 2017), whitening agents with skin-moisturizing properties (Kim *et al*., 2017), anti-obesity, and anti-diabetic (Hong *et al*., 2017a). In addition, Hong *et al*., (2017b) added that the toxicological evaluations indicated that NAOs did not exert any mutational effects, as oral administration to rats and beagle dog models did not express any adverse effects. Moreover, NAOs can potentially serve as prebiotics to stimulate and promote the growth of the bifidobacteria and the lactobacilli, and improve the composition of the gut microbiota (Han *et al*., 2019a).

Furthermore, the anti-oxidant potential is one of the paramount features of NAOs. Zhu *et al*., (2016); Xiao *et al*., (2019b); Zhang *et al*., (2019), have documented their scavenging hydroxyl free radical, scavenging superoxide anion radical and inhibition of lipid peroxidation activities. Previously, Ji *et al*., (2010) thought that the antioxidant merits of NAOs may be exerted by direct eliminating of free radicals, inhibiting their generation, or resisting the activation of the oxidation system. Recently, Liang *et al*., (2018); Wang *et al*., (2018); Chen and Huang, (2019); Song *et al*., (2019) that chemical sulfation of polysaccharides could enhance their water solubility and change their conformation, resulting in the alteration of their biological activities with possible therapeutic uses.

In the current study, agar-degrading *Aquimarina agarilytica* strain NI125 isolated from marine sediments was employed to produce β-agarase for the hydrolysis of native and sulfated agar, and the anti-oxidant potential of the produced agar-hydrolysates was evaluated.

2. Material and methods

2.1. Sample collection, isolation, and screening of agarolytic bacteria

Samples of sediments of the Mediterranean Sea were collected from the vicinity of Nelson's Island, (31° 21' 30.996"N 30° 6' 28.858"E), Alexandria, Egypt. Aliquots of appropriate dilutions were spread onto artificial seawater salts (ASW) agar plates composed of; 6.1 g Tris base (pH 7.2), 12.3 g $MgSO_4$, 0.74 g KCl, 0.13 g $(NH_4)2HPO_4$, 17.5 g NaCl, 0.14 g $CaCl_2$, 0.2 g yeast extract, and 15 g agar\ l (Kim and Hong, 2012). After incubation at 25°C for 2 to 4 d, colonies that formed pits or crater-like depressions were picked and re-streaked on the same medium. Further confirmation of the agarolytic activity was conducted by means of spot inoculation of the purified cultures on the ASW agar plates. Following incubation at 25°C for 24 h, the plates were flooded with Lugol's iodine solution. The appearance of clear zone around the colony indicates its agar-degrading potency (Furusawa *et al*., 2017). The agarolytic bacteria showing obvious agarase activity were selected for further investigations.

2.2. Preparation of the crude agarase enzyme

For agarase production, the selected bacterial isolates were inoculated into ASW broth (24.6 g NaCl, 1.36 g $CaCl_2.2H_2O$, 0.67 g KCl, 6.29 g $MgSO_4.7H_2O$, 4.66 g $MgCl_2.9H_2O$ and 0.18 g $NaHCO_3$), supplemented with 0.2% agar as the sole source of carbon. Cultures were then incubated aerobically at 25 °C with shaking at 180 rpm for 24 h. After incubation, the bacterial cells were removed by centrifugation at 16000 rpm for 15 min., and then the cell-free supernatant was used as the crude enzyme preparation (Gupta *et al*., 2013).

2.3. Quantitative screening for the agarase production

The agarase assay was carried out by estimating the liberated reducing sugars using 3,5-dinitrosalisylic acid (DNS) method in reference to Miller, (1959). Approximately 100 µl of the crude enzyme were added to 3.9 ml of 0.2% agarose solution in Tris-HCl buffer (20 mM, pH 8.0), and then incubated at 40°C for 30 min. After that, about 1 ml of the reaction solution

was mixed with an equal volume of DNS reagent, and then heated in a boiling water bath for 15 min. After cooling to room temperature, the released reducing sugars were estimated by measuring the absorbance at 546 nm against the standard curve of D-galactose. One unit of an enzymatic activity was defined as the amount of enzyme that released 1 µmol of reducing sugar (as D-galactose) from the agar per minute. The protein concentration was measured according to Bradford, (1976), where the bovine serum albumin (BSA) was used for preparing the standard curve.

2.4. Phylogenetic analysis of the agarolytic bacterium

The agarolytic strain designated NI125 exhibiting the maximum agarase potential was identified through sequencing of its 16S rRNA gene. In brief, GeneJET™ Genomic DNA Purification Kit (Thermo Scientific, USA) was used for DNA extraction according to the supplier's instructions. The 16S rRNA gene was amplified by the Polymerase Chain Reaction (PCR), using 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') primers. PCR was conducted in a total volume of 50 µl containing 2.5 µl 10X DreamTaq buffer, 50 ng genomic DNA template, 0.2 mM of each dNTP, 0.4 µM of each primer, one unit of Dream Taq DNA polymerase (Thermo Scientific, USA), and nuclease-free water up to 50 µl. The reaction conditions were set as follows; initial denaturation at 95°C for 3 min., denaturation at 95°C for 40 s, annealing at 55°C for 40 s, extension at 72°C for 1.5 min. for 32 cycles, and finally an extension step at 72°C for 8 min.

The amplicon was purified using QIAquick PCR purification kit (Qiagen, Germany), while the purified PCR product was sequenced in both directions using the same primers at Macrogen (Seoul, Korea). The forward and reverse DNA sequence reads were assembled using DNA Baser Sequence Assembler software (v.3.5.3). The produced consensus of 16S rRNA sequence was analyzed using BLAST in NCBI and EzTaxon server database according to Yoon *et al*., (2017). The phylogenetic tree of the strain NI125 was constructed using the neighbor-joining (NJ) method of the MEGA X software, and the bootstrap analysis was based on 1000 replicates.

2.5. Phenotypic and biochemical characterization of the agrolytic bacterium

The strain NI125 was subjected to phenotypic characterization according to the standard methods described by Smibert and Krieg, (1994). This strain was incubated on marine agar 2216 (MA, Difco) plates at 25°C for 36 h, unless otherwise stated. The bacterial cell morphology and type of flagellation were observed using the Transmission electron microscopy (TEM) (JEM-1010; JEOL), after negative staining with 1% (w/v) phosphotungstic acid. The temperature range for the bacterial growth was determined on the MA plates incubated at 4-45°C for 7 d.

The salt tolerance was assayed in synthetic ZoBell medium (Difco) containing different concentrations of NaCl ranging from 0 to 15% (w/v), at intervals of 0.5%. The biochemical assays were conducted following the standard protocols of Tindall *et al*., (2007). The utilization of carbohydrates and metabolism were determined using API 20E strips (bioMérieux, France) according to the manufacturer's instructions, with the exception of the bacterial suspension which was prepared in 2 % (w/v) NaCl.

2.6. Optimization of the conditions for agarase production

The optimal media components and incubation conditions for agarase production were screened using the one-factor-at-a-time (OFAT) method (Jung *et al*., 2012), keeping the other factors constant.

2.6.1. Effect of incubation period

To study the optimal incubation period for maximum agarase production, ASW broth supplemented with 0.2% agar was inoculated with 2% starter culture of the strain NI125, and then incubated

at 25°C. Samples were withdrawn periodically every 6-h intervals over 60 h and assayed for agarase activity.

2.6.2. Effect of incubation temperature

The effect of temperature on optimal agarase production was studied by cultivating the strain NI125 in ASW broth supplemented with 0.2% agar for 24 h, at different temperatures ranging from 12ºC-30ºC, as independent treatments.

2.6.3. Effect of initial pH of the medium

The ASW broth medium supplemented with 0.2% agar was adjusted before sterilization to various levels of pH ranging from 5-10. After 24 h incubation at 20ºC, the cells were harvested by centrifugation and then the cell-free supernatant was analyzed for agarase activity.

2.6.4. Effect of various nitrogen sources

The impact of various nitrogen sources on agarase production was performed using ASW broth containing 0.2% agar and supplemented individually with 1% (w/v) of; casein, tryptone, peptone, beef extract, urea, yeast extract, and $NaNO_3$ as nitrogen sources. The strain NI125 was inoculated and the cultures were incubated at 20ºC for 24 h, after that the agarase potential was examined.

2.7. Partial purification of the agarase enzyme

The strain NI125 was cultivated under optimum conditions for agarase production; consequently, the produced agarase was partially purified using ammonium sulfate fractionation assay as described by Kaur *et al*., (2017). In brief, the culture was centrifuged at 16,000 xg for 10 min., the supernatant was brought to 20% (w/v) saturation by slow addition of powdered ammonium sulfate, and then left at 4°C overnight. After centrifugation at 21,000 xg for 30 min. at 4°C, the supernatant was brought to 80% (w/v) saturation by slow addition of powdered ammonium sulfate, and then left at 4°C overnight again. Then, the formed precipitate was collected by centrifugation at 21,000 xg for 30 min., re-suspended in 20 mM Tris-HCl, and then dialyzed against the same buffer at 4°C for 24 h. The dialysate (partially purified enzyme) was assayed for agarase potency.

2.8. Determination of the substrate specificity

To determine the substrate specificity and cleavage pattern of the partially purified agarase, the enzyme assay was conducted using p-nitrophenyl-α-D-galactopyranoside or p–nitrophenyl-β-D-galactopyranoside as artificial chromogenic substrates (Chi *et al*., 2015). The reaction was carried out at 40°C for 2 h and then terminated by the addition of 1 M Na_2CO_3. The release of the yellow-colored p-nitrophenol was measured by recording the absorbance at 420 nm (A_{420}).

2.9. Preparation of the sulfated agar

Sulfation of agar was performed following the chlorosulfonic acid-pyridine (CSA-Pyr) method according to Xie *et al*., (2016). The sulfation reagent was prepared by dropwise addition of CSA to pyridine at a ratio of 1:4 (v/v) in an ice bath. Subsequently, the mixture was stirred for 30 min. at room temperature. Approximately one gram of agar-agar was suspended in 100 ml of N, N-dimethylformamide (DMF) at room temperature under continuous agitation. After 15 min., the sulfation reagent was added to the agar suspension drop by drop, and then heated in a water bath at 60°C for 4 h with continuous stirring. Afterwards, the mixture was cooled to room temperature and neutralized to pH 7.0 using 4 M NaOH. After that, 95% (v/v) ethanol was added and the mixture was allowed to precipitate at 4°C. After 18 h, the precipitate was re-suspended in dist. water and dialyzed against dist. water; finally the sulfated agar was freeze-dried. The sulfur content of the sulfated polysaccharides was estimated using the benzidine method (Antonopoulos *et al*., 1962). The degree of substitution (DS) designating the average number of sulfo-groups on each sugar residue was calculated on the basis of the sulfur content in reference to Zhang *et al*., (2003).

2.10. Preparation of the neoagarooligosaccharides (NAOs)

Partially purified β-agarase (100 U) was added to 100 ml of 50 mM Tris–HCl buffer (pH 8.0) containing 1% (w/v) agar (or sulfated agar), incubated at 40°C for 12 h, and then the reaction was stopped by heating the solution in boiling water for 10 min. About two-volumes of absolute ethanol was added to the reaction mixture to remove the high-molecular-massed polysaccharides. After centrifugation, the soluble fraction was collected and lyophilized. The amount of total sugars was determined using the method of phenol-sulfuric acid according to Dubois *et al*., (1956). The optical density was measured at 490 nm, and the values for the total sugars were expressed as D-galactose equivalents.

2.11. Estimation of the anti-oxidant activity of the NAOs

To investigate the anti-oxidant activity of the produced NAOs, DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay was performed (Yang *et al*., 2006). The prepared hydrolysate powder containing NAOs was dissolved in dist. water and then subjected to anti-oxidant activity assays. In brief, 1.0 ml of the hydrolysate solution (1mg/ ml) was mixed with 2 ml of 0.2 mM DPPH dissolved in ethanol. After the mixture was shaken and incubated at room temperature for 30 min. in the dark, the absorbance of the resulting solution was measured at 517 nm. The DPPH radical scavenging effect of the sample was calculated as follows:

Scavenging ability (%) = (1 – absorption of sample/absorption of control) × 100

2.12. Statistical analysis

The measured data were subjected to the analysis of variance (ANOVA) appropriate to the design. The significant differences between treatments were compared with the critical difference at 5% level of probability by the Duncan's test using PASW 17.0 statistics software (SPSS Inc).

3. Results

3.1. Isolation and screening of the agarolytic bacteria

After incubation for 3 d, colonies that formed pits or holes on the plates were selected, and transferred onto new plates until the colony morphology is unchanged. The plates are stained using Lugol's solution to check for the agarolytic potential. About twelve agarolytic colonies forming deep holes in agar are selected and subjected to quantitative screening. Based on the secondary screening, a promising strain designated NI125 exhibiting superior agarase activity is selected for further investigations.

3.2. Phylogenetic analysis

The 16S rRNA gene sequence (1,427 bp) of strain NI125 is deposited in GenBank (Accession No. MK880485). The phylogenetic analysis performed with partial and almost complete sequences of closely related species indicated that strain NI125 is affiliated within the Family *Flavobacteriaceae* belonging to the Phylum *Bacteroidetes*. The BLAST analysis of the NI125 16S rRNA gene revealed that it shares 99.72 % similarity with *Aquimarina agarilytica* ZC1 (Accession No. NR116794.1), 97.32 % with *A. agarivorans* strain HQM9 (Accession No. NR136817.1), and 96.3 % with *A. seongsanensis* strain CBA3208 (Accession No. NR152627.1). The NJ tree showing phylogenetic relationships of strain NI125 and the closest bacteria is presented in Fig. 1.

3.3. The phenotypic characteristics

The agarolytic strain NI125 is a yellow-pigmented, Gram-negative, rod-shaped, and non-motile bacterium. TEM observations by the negative staining with phosphotungstic acid indicated the absence of flagella (Fig. 2). This strain grows at temperature range of 4-30°C, and the growth occurs in the presence of 0.5-4% NaCl. The strain is positive to

oxidase, catalase, alkaline phosphatase, β-galactosidase, and negative to nitrate reduction, indole and H_2S production. The NI125 strain hydrolyzes agar, starch and casein but not cellulose, gelatin and tween 80. It produces acid from glucose, galactose and maltose but not from sucrose and maltose (Table 1). Based on phylogenetic and phenotypic characteristics, the strain NI125 is identified as *Aquimarina agarilytica*.

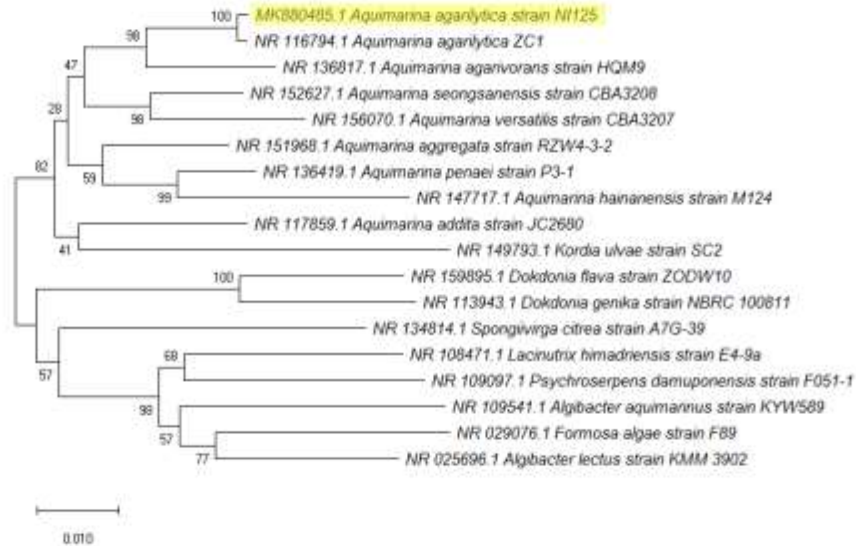

Fig. 1: The phylogenetic tree showing the relationships between *A. agarilytica* strain NI125 and the most closely related species retrieved from GenBank.

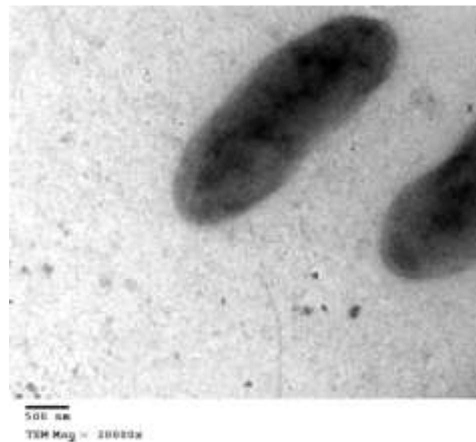

Fig. 2: Transmission electron microscopy (TEM) image of *A. agarilytica* strain NI125 demonstrating the absence of flagella

Table 1: The phenotypic characteristics of *A. agarilytica* strain NI125

Character/Test	Result	Test	Result
Shape	Rod	**Hydrolysis of:**	
Gram stain	-	Agar	+
Motility	-	Starch	+
Growth at 32 °C	-	Cellulose	-
Growth with 5% (w/v) NaCl	-	Casein	+
Oxidase	+	Gelatin	-
Catalase	+	Tween 80	-
Indole production	-	**Acid production from:**	
Nitrate reduction	-	Glucose	+
H_2S production	-	Galactose	+
Alkaline phosphatase	+	Sucrose	-
β-Galactosidase	+	Lactose	-
Urease	-	Maltose	+

Where; +: positive, -: negative

3.4. Optimization of the growth conditions for agarase production

In an attempt to determine the optimum conditions for agarase production, the influence of four factors was investigated; namely incubation period, temperature, initial pH, and different nitrogen sources. The time course of agarase production for 60 h indicated that agarase is produced in low level at the first 6 h of incubation, and then the enzyme production is increased gradually. The maximum production level is observed after 24 to 30 h of incubation. A dramatic decrease in agarase production is observed after prolonged cultivation time (Fig. 3a). The optimum temperature for enzyme production is observed at 20°C, where further increase in the temperature resulted in a suppression of enzyme production (Fig. 3b).

A. agarilytica NI125 produced agarase fairly over a wide pH range of 6.0-8.0, and results showed a severe diminishing of agarase production at pH 8.5 (Fig. 3c). Regarding the effect of various nitrogen sources on agarase production, results demonstrated that all the organic nitrogenous compounds except urea served as good nitrogen sources. The maximum production of agarase is achieved when tryptone or casein are used separately. This is followed by beef extract and yeast extract but with lesser proportion. Significant suppression of agarase production is observed when either urea or $NaNO_3$ are used as nitrogen sources (Fig. 3d).

3.5. Partial purification of the agarase enzyme

The extracellular agarase produced under the optimum conditions was partially purified through ammonium sulfate fractionation of the culture supernatant. The specific enzyme activity is increased from 4.91 U/mg protein to 14.73 U/mg protein after ammonium sulfate precipitation and dialysis. Three-fold purification of the agarase with an overall yield of 52.4% is achieved.

3.6. Determination of the substrate specificity

The partially purified agarase exhibited a strong hydrolytic activity towards p-nitrophenyl-β-D-galactopyranoside (OD_{540}= 0.974), however

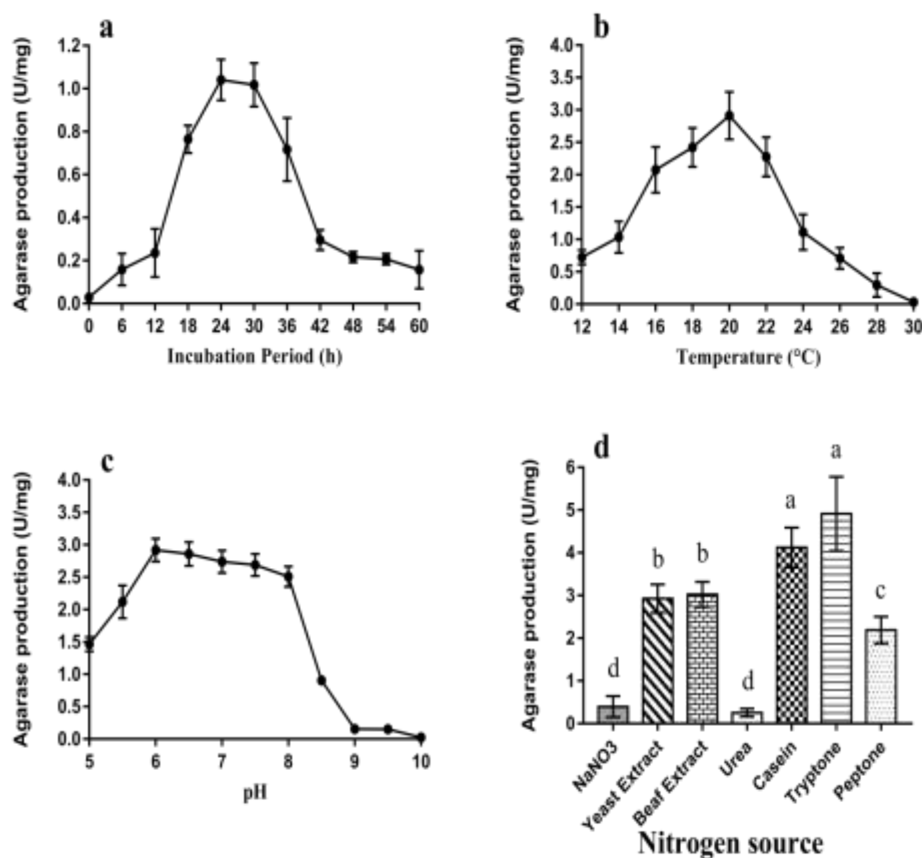

Fig. 3: Impact of incubation period (a), temperature (b), initial pH (c), and various nitrogen sources (d) on β-agarase production by *A. agarilytica* strain NI125. Where; The error bars represent standard deviations (SD). Columns headed by the same letter are not significantly different according to Duncan's multiple range test ($p < 0.05$).

it showed negligible activity towards p-nitrophenyl-α-D-galactopyranoside (OD_{540}=0.004) indicating the specific cleavage of the β-linkage rather than the α-linkage.

3.7. Preparation of the sulfated agar

In the present study, a chemical modification of the agar was performed to increase the sulfur content. Agar was sulfated by the CSA-Pyr method; results revealed that the sulfur content of the sulfated agar derivative is increased by 8.7% (± 0.6), while the DS is increased by 6.09 (± 0.03), compared with the untreated agar.

3.8. Estimation of the anti-oxidant activity of the NAOs

For investigation of the anti-oxidant activity of the NAOs, agar and the sulfated agar were hydrolyzed by the partially purified β-agarase for 12 h, whereas the free radical scavenging activity of the produced

agar-hydrolysates was estimated using the stable free radical DPPH. Results revealed the promising anti-oxidative properties of oligosaccharides obtained by the enzymatic hydrolysis of agar by the β-agarase enzyme derived from strain NI125. Products of the enzymatic hydrolysis showed 23% scavenging ability. On the other hand, the sulfated NAOs exhibited a significant improvement of the radical scavenging activity. More than 87% inhibition of the DPPH is achieved by the NAOs produced by hydrolysis of the sulfated agar through the action of the β-agarase enzyme.

4. Discussion

Marine environments are natural sources of massive products with admirable biological activities. Nowadays, algae-derived oligosaccharides have received eminent attention in numerous applications of the industrial biotechnology. Many algal polysaccharides have commercial interests in various industrial applications. Furthermore, the chemical or enzymatic modification of the marine polysaccharides to produce oligosaccharides with new biophysical and biochemical features is a topic of progressing interest (Jutur *et al*., 2016; Han *et al*., 2019a; Chen *et al*., 2019a; Li *et al*., 2019a). Recent studies of Xu *et al*., (2018); Yu *et al*., (2019) reported the significant bioactivities of the agar oligosaccharides produced by various agarases enzymes derived from agar-degrading bacteria.

Generally, most of the described agarolytic bacteria were isolated from marine habitats including; *Gayadomonas joobiniege* (Asghar *et al*., 2018), *Winogradskyella* sp, *Colwellia* sp., *Lacinutrix* sp., *Olleya* sp., *Paraglaciecola* sp. (Sánchez Hinojosa *et al*., 2018), *Agarivorans gilvus* (Yang *et al*., 2018), *Cellulophaga omnivescoria* (Ramos *et al*., 2018), and *Flammeovirga* sp. (Chen *et al*., 2019b). Nevertheless, few bacteria from soil have been reported to produce these agarases such as; *Cohnella* sp. (Li *et al*., 2015), *Streptomyces lavendulae* (Wu *et al*., 2017), and *Microbacterium* sp. (Parashar and Kumar, 2018). In the present study, the most promising agarolytic bacterial strain NI125 isolated from marine sediments is identified phylogenetically and phenotypically as *A. agarilytica*, which belongs to *Flavobacteriaceae*. Few previous studies of Kim *et al*., (2014); Han *et al*., (2019b) have described several agarolytic strains belonging to the same family including *Cellulophaga algicola* and *Flavobacterium faecale*.

The optimization studies revealed the significant impact of incubation period and temperature on the production of the extracellular agarase by *A. agarilytica* NI125. The current results showed that maximum production of agarase is achieved at low temperature (20°C); however, the optimum temperature for production of the same enzyme by the *Pseudoalteromonas* strain JYBCL was recorded to be 25°C (Jung *et al*., 2012). Regarding the nitrogen source, tryptone and casein stimulate maximum production of agarase by *A. agarilytica* NI125. The optimum nitrogen source for production of agarase is varied; organic nitrogen sources are preferred by some bacteria while inorganic nitrogen is favorable for others. Yeast extract, tryptone, and peptone were recorded to be the best nitrogen source for agarase production by *Agarivorans albus* YKW-34, *Pseudoalteromonas* sp. JYBCL 1, and *Rhodococcus* sp. Q5, respectively (Fu *et al*., 2009; Feng *et al*., 2012; Jung *et al*., 2012). It is assumed that the organic nitrogen sources can easily replenish the existing internal pool of amino acids within the microbial cell, thus facilitating the availability of these amino acids for protein synthesis. On the other hand, Lakshmikanth *et al*., (2006); Roseline and Sachindra, (2016) revealed that ammonium nitrate or sodium nitrate induced significant production of agarase by *Pseudomonas aeruginosa* AG LSL-11, and *Acinetobacter junii* PS12B. By employing the OFAT optimization method, more than 4-fold increment in agarase production by *A. agarilytica* NI125 is achieved. However, several studies of Abd El Aty *et al*., (2014); Potla Durthi *et al*., (2019); Khan *et al*., (2019) pointed that further enhancement in enzyme production could be attained by using the central composite design optimization approaches, such as

response surface methodology (RSM) or Taguchi-based design.

In the present investigation, the substrate specificity assessment suggests that partially purified agarase belongs to β-agarase that produces neoagarooligosaccharides from agar. These findings are consistent with the results of several recent studies dealing with agarases from various sources, describing the cleavage of β-bonds in agar and agarose (Han e*t al*., 2019b; Li *et al*., 2019b; Choi *et al*., 2019; Chen *et al*., 2019b). Agarases are characterized as α-agarases and β-agarases according to the cleavage pattern. Li *et al*., (2018); Hafizah *et al*., (2019); Liu *et al*., (2019); Lee *et al*., (2019) revealed that the basic products of the α-agarases and β-agarases are agarobiose and neoagarobiose, respectively. Results of this study revealed the significant anti-oxidant potential of the NAOs produced by hydrolysis of agar using β-agarases derived from *A. agarilytica* NI125. It was reported that agar-derived oligosaccharides have high economic values, due to their physiological and biological activities. In agreement with these findings, Zhu *et al*., (2016) demonstrated that oligosaccharides produced by the enzymatic treatment of agar with agarase enzyme derived from *Stenotrophomonas* sp. NTA had inhibitory effects on hydroxyl, DPPH, and ABTS radicals, with potent anti-oxidative potency. Similarly, oligosaccharides derived from agar by *Vibrio natriegens* β-agarase exhibited excellent anti-oxidative activity (Zhang *et al*., 2019).

It is worth mention that agar sulfation before hydrolysis resulted in the production of NAOs with notable enhanced anti-oxidant potential. Upon hydrolysis of the sulfated agar derivatives with DS value of 6.09 which is higher than the un-sulfated agar, the produced oligosaccharides demonstrated 87% scavenging ability; however, the oligosaccharides produced from the un-sulfated agar expressed 28% scavenging ability only. The previous study of Wang *et al*., (2004) suggested that oligosaccharides with the sulfate group or with higher molecular masses showed stronger anti-oxidative activities than those without the sulfate group or with smaller molecular masses. The current findings agree with the recent investigations suggesting that sulfate modification is an effective method to improve the antioxidant activities of various polysaccharides (Xiao *et al*., 2019a; Olasehinde *et al*., 2019; Huang *et al*., 2019). It was thought that sulfation of the polysaccharides promotes their scavenging ability to free radicals by activation of hydrogen atoms on the anomeric carbons, thus providing stronger hydrogen supply capacity and reduce the aggressiveness of the free radicals. Thus, these sulfated polysaccharides possess a greater capacity to donate hydrogen to the superoxide anion (Chen *et al*., 2015). Beside the anti-oxidative activities, agar-derived oligosaccharides may inhibit the growth of bacteria, slow down the degradation of starch, and used as low-calorie additives to improve food qualities (Giordano *et al*., 2006). Moreover, Li *et al*., (2014); Zhang *et al*., (2019) revealed that NAOs obtained from the enzymatic hydrolysis of agarose stimulated the growth of *Bifidobacteria* sp., *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Sterptococcus thermophilus in vitro* and *in vivo* without side effects. In this study, *A. agarilytica* strain NI125 is found to produce an extracellular β-agarase enzyme that digests agar producing biologically active oligosaccharides with promising anti-oxidative properties. Furthermore, the present work shed light on the crucial role of agar sulfation prior to hydrolysis with respect to enhancing the anti-oxidant power of the produced oligosaccharides.

Conclusion

The present investigation explores the potential of a newly isolated psychrophilic marine bacterium, *A. agarilytica* strain NI125, for production of β-agarase. More than 4-folds enhancement in the productivity is achieved by cultivation of this strain in ASW broth supplemented with 1% tryptone for 24 h at 20°C. The partially purified enzyme exhibited a specific activity of 14.73 U/mg protein, with apparent potential towards the beta linkage, so is classified as β-agarase. The anti-oxidant potential of agar hydrolysates is significantly improved by sulfation of agar before the

enzymatic hydrolysis. The produced oligosaccharides possessing up to 87% scavenging ability; thus could be used as promising additives in food and feed products. Nevertheless, further investigations are required to study the β-agarase at the molecular level, and to characterize the sulfated NAOs with special emphasis on their physiological activities.

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5. References

Abd El Aty, A.A.; Wehaidy, H.R. and Mostafa, F.A. (2014). Optimization of inulinase production from low cost substrates using Plackett-Burman and Taguchi methods. Carbohydrate Polymers. 102(1): 261-268.

Antonopoulos, C.A.; Daniel, A.F.; Havanka, R.; Briggs, T.; Haslewood, G.A.D. and Flood, H. (1962). A Modification for the Determination of Sulphate in Mucopolysaccharides by the Benzidine Method. Acta Chemica Scandinavica. 16: 1521-1522.

Asghar, S.; Lee, C.R.; Park, J.S.; Chi, W.J.; Kang, D.K. and Hong, S.K. (2018). Identification and biochemical characterization of a novel cold-adapted 1,3-α-3,6-anhydro-l-galactosidase, Ahg786, from *Gayadomonas joobiniege* G7. Applied Microbiology and Biotechnology. 102(20): 8855-8866.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 72(1–2): 248-254.

Chen, L. and Huang, G. (2019). Antioxidant activities of sulfated pumpkin polysaccharides. International Journal of Biological Macromolecules. 126: 743-746.

Chen, X.; Li, L.; Chan, Z.; Zeng, R.; Lin, M. and Lin, H. (2019a). One-Step Process for Environment-Friendly Preparation of Agar Oligosaccharides From *Gracilaria lemaneiformis* by the Action of *Flammeovirga* sp. OC4. Frontiers in Microbiology. 10.

Chen, X.; Lin, H.; Jin, M.; Zeng, R. and Lin, M. (2019b). Characterization of a novel alkaline β-agarase and its hydrolysates of agar. Food Chemistry. 295: 311-319.

Chen, Y.; Zhang, H.; Wang, Y.; Nie, S.; Li, C. and Xie, M. (2015). Sulfated modification of the polysaccharides from *Ganoderma atrum* and their antioxidant and immunomodulating activities. Food Chemistry. 186: 231-238.

Cheong, K.L.; Qiu, H.M.; Du, H.; Liu, Y. and Khan, B. (2018). Oligosaccharides Derived from Red Seaweed: Production, Properties, and Potential Health and Cosmetic Applications. Molecules. 23(10): 2451.

Chi, W.J.; Lee, C.R.; Dugerjonjuu, S.; Park, J.S.; Kang, D.K. and Hong, S.K. (2015). Biochemical characterization of a novel iron-dependent GH16 β-agarase, AgaH92, from an agarolytic bacterium *Pseudoalteromonas* sp. H9. FEMS Microbiology Letters. 362(7).

Choi, U.; Jung, S.; Hong, S.-K. and Lee, C.R. (2019). Characterization of a Novel Neoagarobiose-Producing GH42 β-Agarase, AgaJ10, from *Gayadomonas joobiniege* G7. Applied Biochemistry and Biotechnology. 189(1): 1-12.

Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; Smith, F. (1956). Colorimetric Method for Determination of Sugars and Related Substances. Analytical Chemistry. 28(3): 350-356.

Enoki, T.; Okuda, S.; Kudo, Y.; Takashima, F.; Sagawa, H. and Kato, I. (2010). Oligosaccharides from agar inhibit pro-inflammatory mediator release by inducing heme oxygenase 1. Bioscience, Biotechnology and Biochemistry. 74(4): 766-770.

Feng, Z.; Peng, L.; Chen, M. and Li, M. (2012). *Rhodococcus* sp. Q5, a novel agarolytic bacterium

isolated from printing and dyeing wastewater. Folia Microbiologica. 57(5): 379-386.

Fu, X.T.; Lin, H. and Kim, S.M. (2009). Optimization of medium composition and culture conditions for agarase production by *Agarivorans albus* YKW-34. Process Biochemistry. 44(10): 1158-1163.

Furusawa, G.; Lau, N.-S.; Suganthi, A. and Amirul, A.A.A. (2017). Agarolytic bacterium *Persicobacter* sp. CCB-QB2 exhibited a diauxic growth involving galactose utilization pathway. Microbiology Open. 6(1): e00405.

Giordano, A.; Andreotti, G.; Tramice, A. and Trincone, A. (2006). Marine glycosyl hydrolases in the hydrolysis and synthesis of oligosaccharides. Biotechnology Journal. 1(5): 511-530.

Gupta, V.; Trivedi, N.; Kumar, M.; Reddy, C.R.K.; Jha, B. (2013). Purification and characterization of exo-β-agarase from an endophytic marine bacterium and its catalytic potential in bioconversion of red algal cell wall polysaccharides into galactans. Biomass and Bioenergy. 49: 290-298.

Hafizah, N.F.; Teh, A.H. and Furusawa, G. (2019). Biochemical Characterization of Thermostable and Detergent-Tolerant β-Agarase, PdAgaC, from *Persicobacter* sp. CCB-QB2. Applied Biochemistry and Biotechnology. 187(3): 770-781.

Han, Z.L.; Yang, M.; Fu, X.D.; Chen, M.; Su, Q.; Zhao, Y.H. and Mou, H.J. (2019a). Evaluation of Prebiotic Potential of Three Marine Algae Oligosaccharides from Enzymatic Hydrolysis. Marine Drugs. 17(3): 173.

Han; Zhang; Yang. (2019b). Biochemical Characterization of a New β-Agarase from *Cellulophaga Algicola*. International Journal of Molecular Sciences. 20(9): 2143.

Hong, S.J.; Lee, J.H.; Kim, E.J.; Yang, H.J.; Park, J.S. and Hong, S.K. (2017a). Anti-Obesity and Anti-Diabetic Effect of Neoagarooligosaccharides on High-Fat Diet-Induced Obesity in Mice. Marine Drugs. 15(4): 90.

Hong, S.J.; Lee, J.H.; Kim, E.J.; Yang, H.J.; Park, J.S. and Hong, S.K. (2017b). Toxicological evaluation of neoagarooligosaccharides prepared by enzymatic hydrolysis of agar. Regulatory Toxicology and Pharmacology. 90: 9-21.

Huang, L.; Huang, M.; Shen, M.; Wen, P.; Wu, T.; Hong, Y. and Xie, J. (2019). Sulfated modification enhanced the antioxidant activity of *Mesona chinensis* Benth polysaccharide and its protective effect on cellular oxidative stress. International Journal of Biological Macromolecules. 136: 1000-1006.

Ji, J.; Wang, L.; Wu, H. and Luan, H.M. (2010). Bio-function Summary of Marine Oligosaccharides. International Journal of Biology. 3(1).

Jung, C.; Kwon, H.; Park, C. and Lee, J. (2012). Optimization of *Pseudoalteromonas* sp. JYBCL 1 culture conditions, medium composition and extracellular β-Agarase activity. Biotechnology and Bioprocess Engineering. 17(5): 937-945.

Jutur, P.P.; Nesamma, A.A. and Shaikh, K.M. (2016). Algae-derived marine oligosaccharides and their biological applications. Frontiers in Marine Science. 3: 1-5.

Kang, D.R.; Yoon, G.Y.; Cho, J.; Lee, S.J.; Lee, S.J.; Park, H.J. and Jung, I.D. (2017). Neoagarooligosaccharides prevent septic shock by modulating A20-and cyclooxygenase-2-mediated interleukin-10 secretion in a septic-shock mouse model. Biochemical and Biophysical Research Communications. 486(4): 998-1004.

Kaur, A.; Bhatia, S. and Phutela, U. (2017). Partial Purification and Characterization of Cellulolytic Enzymes Extracted from *Trichoderma reesei* Inoculated Digested Biogas Slurry. Journal of Pure and Applied Microbiology. 11(4): 1953-1963.

Khan, Y.M.; Munir, H. and Anwar, Z. (2019). Optimization of process variables for enhanced

production of urease by indigenous *Aspergillus niger* strains through response surface methodology. Biocatalysis and Agricultural Biotechnology. 20.

Kim, J.H.; Choi, B.H.; Jo, M.; Kim, S.C. and Lee, P.C. (2014). *Flavobacterium faecale* sp. nov., an agarase-producing species isolated from stools of Antarctic penguins. International Journal of Systematic and Evolutionary Microbiology. 64(8): 2884-2890.

Kim, Ji; Yun, E.; Yu, S.; Kim, K. and Kang, N. (2017). Different Levels of Skin Whitening Activity among 3,6-Anhydro-l-galactose, Agarooligosaccharides, and Neoagarooligosaccharides. Marine Drugs. 15(10): 321.

Kim, J. and Hong, S.K. (2012). Isolation and Characterization of an Agarase-Producing Bacterial Strain, *Alteromonas* sp. GNUM-1, from the West Sea, Korea. Journal of Microbiology and Biotechnology. 22(12): 1621-1628.

Lakshmikanth, M.; Manohar, S.; Patnakar, J.; Vaishampayan, P.; Shouche, Y. and Lalitha, J. (2006). Optimization of culture conditions for the production of extracellular agarases from newly isolated *Pseudomonas aeruginosa* AG LSL-11. World Journal of Microbiology and Biotechnology. 22(5): 531-537.

Lee, C.H.; Lee, C.R. and Hong, S.K. (2019). Biochemical characterization of a novel cold-adapted agarotetraose-producing α-agarase, AgaWS5, from *Catenovulum sediminis* WS1-A. Applied Microbiology and Biotechnology. 103(20): 8403-8411.

Lee, D.G.; Jang, M.K.; Lee, O.H.; Kim, N.Y.; Ju, S.A. and Lee, S.H. (2008). Over-production of a glycoside hydrolase family 50 β-agarase from *Agarivorans* sp. JA-1 in *Bacillus subtilis* and the whitening effect of its product. Biotechnology Letters. 30(5): 911-918.

Lee, M.H.; Jang, J.H.; Yoon, G.Y.; Lee, S.J.; Lee, M.G.; Kang, T.H. and Jung, I.D. (2017). Neoagarohexaose-mediated activation of dendritic cells via Toll-like receptor 4 leads to stimulation of natural killer cells and enhancement of antitumor immunity. BMB Reports. 50(5): 263-268.

Lee, Y.; Jo, E.; Lee, Y.J.; Hettiarachchi, S.A.; Park, G.H.; Lee, S.J. and Oh, C. (2018). A novel glycosyl hydrolase family 16 β-agarase from the agar-utilizing marine bacterium *Gilvimarinus agarilyticus* JEA5: The first molecular and biochemical characterization of agarase in genus *Gilvimarinus*. Journal of Microbiology and Biotechnology. 28(5): 776-783.

Li, G.; Sun, M.; Wu, J.; Ye, M.; Ge, X.; Wei, W. and Hu, F. (2015). Identification and biochemical characterization of a novel endo-type β-agarase AgaW from *Cohnella* sp. strain LGH. Applied Microbiology and Biotechnology. 99(23): 10019-10029.

Li, Jiang; Xie, M. and Gao, Y. (2019a). Identification and biochemical characterization of a novel exo-type β-agarase Aga3463 from an Antarctic *Pseudoalteromonas* sp. strain. International Journal of Biological Macromolecules. 129: 162-170.

Li, Jun; Cai, C.; Yang, C.; Li, J.; Sun, T. and Yu, G. (2019b). Recent Advances in Pharmaceutical Potential of Brown Algal Polysaccharides and their Derivatives. Current Pharmaceutical Design. 25(11): 1290-1311.

Li, M.; Li, G.; Zhu, L.; Yin, Y.; Zhao, X.; Xiang, C.; Wang, X. (2014). Isolation and Characterization of an Agaro-Oligosaccharide (AO)-Hydrolyzing Bacterium from the Gut Microflora of Chinese Individuals. PLoS ONE. 9(3): e91106.

Li, R.K.; Chen, Z.; Ying, X.J.; Ng, T.B. and Ye, X.Y. (2018). A novel GH16 beta-agarase isolated from a marine bacterium, *Microbulbifer* sp. BN3 and its characterization and high-level expression in *Pichia pastoris*. International Journal of Biological Macromolecules. 119: 1164-1170.

Liang, L.; Ao, L.; Ma, T.; Ni, Y.; Liao, X.; Hu, X. and Song, Y. (2018). Sulfated modification and anticoagulant activity of pumpkin (*Cucurbita pepo*, *Lady Godiva*) polysaccharide. International Journal of Biological Macromolecules. 106: 447-455.

Liu, J.; Liu, Z.; Jiang, C. and Mao, X. (2019). Biochemical Characterization and Substrate Degradation Mode of a Novel α-Agarase from *Catenovulum agarivorans*. Journal of Agricultural and Food Chemistry. 67(37): 10373-10379.

Miller, G.L. (1959). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Analytical Chemistry. 31(3): 426-428.

Olasehinde, T.A.; Mabinya, L.V.; Olaniran, A.O. and Okoh, A.I. (2019). Chemical characterization, antioxidant properties, cholinesterase inhibitory and anti-amyloidogenic activities of sulfated polysaccharides from some seaweeds. Bioactive Carbohydrates and Dietary Fibre. 18.

Parashar, S. and Kumar, N. (2018). Studies on agarolytic bacterial isolates from agricultural and industrial soil. Iranian Journal of Microbiology. 10(5): 324-333.

Potla Durthi, C.; Pola, M.; Kola, A.K. and Rajulapati, S.B. (2019). Screening, optimization of culture conditions and scale-up for production of the L-Glutaminase by novel isolated *Bacillus* sp. mutant endophyte using response surface methodology. Biocatalysis and Agricultural Biotechnology. 18.

Ramos, K.R.M.; Valdehuesa, K.N.G.; Nisola, G.M.; Lee, W.K. and Chung, W.J. (2018). Identification and characterization of a thermostable endolytic β-agarase Aga2 from a newly isolated marine agarolytic bacteria *Cellulophaga omnivescoria* W5C. New Biotechnology. 40: 261-267.

Roseline, L.T. and Sachindra, N.M. (2016). Characterization of extracellular agarase production by *Acinetobacter junii* PS12B, isolated from marine sediments. Biocatalysis and Agricultural Biotechnology. 6: 219-226.

Sánchez Hinojosa, V.; Asenjo, J. and Leiva, S. (2018). Agarolytic culturable bacteria associated with three antarctic subtidal macroalgae. World Journal of Microbiology and Biotechnology. 34(6): 73.

Schultz-Johansen, M.; Bech, P.K.; Hennessy, R.C.; Glaring, M.A.; Barbeyron, T.; Czjzek, M. and Stougaard, P. (2018). A Novel Enzyme Portfolio for Red Algal Polysaccharide Degradation in the Marine Bacterium *Paraglaciecola hydrolytica* S66T Encoded in a Sizeable Polysaccharide Utilization Locus. Frontiers in Microbiology. 9.

Smibert, R. and Krieg, N. (1994). Phenotypic characterization. In Gerhardt P, Murray RGE, Wood WA, Krieg NR (eds), Methods for General and Molecular Bacteriology. American Society for Microbiology, Washington, DC. pp. 607-654.

Song, J.; Wu, Y.; Jiang, G.; Feng, L.; Wang, Z.; Yuan, G. and Tong, H. (2019). Sulfated polysaccharides from *Rhodiola sachalinensis* reduce D-gal-induced oxidative stress in NIH 3T3 cells. International Journal of Biological Macromolecules. 140: 288-293.

Tindall, B.J.; Sikorski, J.; Smibert, R.A. and Krieg, N.R. (2007). Phenotypic Characterization and the Principles of Comparative Systematics. In Methods for General and Molecular Microbiology, Third Edition.

Wang, J.; Jiang, X.; Mou, H. and Guan, H. (2004). Anti-oxidation of agar oligosaccharides produced by agarase from a marine bacterium. Journal of Applied Phycology. 16(5): 333-340.

Wang, W.; Liu, P.; Hao, C.; Wu, L.; Wan, W. and Mao, X. (2017). Neoagaro-oligosaccharide monomers inhibit inflammation in LPS-stimulated macrophages through suppression of MAPK and NF-κB pathways. Scientific Reports. 7.

Wang, Z.; Xie, J.; Shen, M.; Nie, S. and Xie, M. (2018). Sulfated modification of polysaccharides: Synthesis, characterization and bioactivities. Trends in Food Science and Technology. 74: 147-157.

Wu, H.; Chen, G.; Bian, Y.; Zeng, W.; Sun, B. and Liang, Z. (2017). Identification and characterization of a new agar-degrading strain with the novel properties of saccharides inhibition and nitrogen fixation. Journal of Microbiology. 55(6): 475-482.

Xiao, H.; Fu, X.; Cao, C.; Li, C.; Chen, C. and Huang, Q. (2019a). Sulfated modification, characterization, antioxidant and hypoglycemic activities of polysaccharides from *Sargassum pallidum*. International Journal of Biological Macromolecules. 121: 407-414.

Xiao, Q.; Liu, C.; Ni, H.; Zhu, Y.; Jiang, Z. and Xiao, A. (2019b). β-Agarase immobilized on tannic acid-modified Fe_3O_4 nanoparticles for efficient preparation of bioactive neoagaro-oligosaccharide. Food Chemistry. 272: 586-595.

Xie, J.H.; Wang, Z.J.; Shen, M.Y.; Nie, S.P.; Gong, B.; Li, H.S. and Xie, M.Y. (2016). Sulfated modification, characterization and antioxidant activities of polysaccharide from *Cyclocarya paliurus*. Food Hydrocolloids. 53: 7-15.

Xu, S.Y.; Kan, J.; Hu, Z.; Liu, Y.; Du, H.; Pang, G.C. and Cheong, K.L. (2018). Quantification of Neoagaro-Oligosaccharide Production through Enzymatic Hydrolysis and Its Anti-Oxidant Activities. Molecules. 23(6): 1354.

Yang, B.; Wang, J.; Zhao, M.; Liu, Y.; Wang, W. and Jiang, Y. (2006). Identification of polysaccharides from pericarp tissues of litchi (*Litchi chinensis* Sonn.) fruit in relation to their antioxidant activities. Carbohydrate Research. 341(5): 634-638.

Yang, X.; Liu, Z.; Jiang, C.; Sun, J.; Xue, C. and Mao, X. (2018). A novel agaro-oligosaccharide-lytic β-galactosidase from *Agarivorans gilvus* WH0801. Applied Microbiology and Biotechnology. 102(12): 5165-5172.

Yoon, S.H.; Ha, S.M.; Kwon, S.; Lim, J.; Kim, Y.; Seo, H. and Chun, J. (2017). Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. International Journal of Systematic and Evolutionary Microbiology. 67(5): 1613-1617.

Yu, S.; Yun, E.J.; Kim, D.H.; Park, S.Y. and Kim, K.H. (2019). Anticariogenic Activity of Agarobiose and Agarooligosaccharides Derived from Red Macroalgae. Journal of Agricultural and Food Chemistry. 67(26): 7297-7303.

Zhang, M.; Zhang, L.; Wang, Y. and Cheung, P.C.K. (2003). Chain conformation of sulfated derivatives of β-glucan from sclerotia of *Pleurotus tuber-regium*. Carbohydrate Research. 338(24): 2863-2870.

Zhang, N.; Mao, X.; Li, R. W.; Hou, E.; Wang, Y.; Xue, C. and Tang, Q. (2017). Neoagarotetraose protects mice against intense exercise-induced fatigue damage by modulating gut microbial composition and function. Molecular Nutrition and Food Research. 61(8): 1600585.

Zhang, Y.H.; Song, X.N.; Lin, Y.; Xiao, Q.; Du, X.P.; Chen, Y.H. and Xiao, A.F. (2019). Antioxidant capacity and prebiotic effects of *Gracilaria neoagaro* oligosaccharides prepared by agarase hydrolysis. International Journal of Biological Macromolecules. 137: 177-186.

Zhu, Y.; Zhao, R.; Xiao, A.; Li, L.; Jiang, Z.; Chen, F. and Ni, H. (2016). Characterization of an alkaline β-agarase from *Stenotrophomonas* sp. NTa and the enzymatic hydrolysates. International Journal of Biological Macromolecules. 86: 525-534.