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Applying Both Chemical Liquefaction and Enzymatic Catalysis Can Increase Production of Agaro-Oligosaccharides from Agarose

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Abstract Red algae represents an important marine bioresource. One high-value utilization of red algae is the production of agaro-oligosaccharides which have many positive biological effects. However, the lack of an efficient production route seriously limits the application of agaro-oligosaccharides. In this study, we established a green route that combines chemical liquefaction and enzymatic catalysis for the efficient production of agaro-oligosaccharides, and we used the production of neoagarotetraose (NA4) as an example. Agarose (150 g L^{-1}) liquefaction by citric acid was controlled with respect to two targets: a 100% liquefaction rate and a high average degree of polymerization (> 4) of the liquesced agaro-oligosaccharides, which were then catalyzed by β-agarase into an oligosaccharides mixture with a high concentration of NA4 (30.8 g L^{-1}) in a 1-L reaction volume. After purification, we obtained 25.5 g of NA4 with a purity of 92%. This work establishes an easy route for the efficient production of pure agaro-oligosaccharides from agarose.

Key words neoagarotetraose; agarose; agarase; marine polysaccharide; expression

1 Introduction

Red algae is an important marine bioresource for the production of functional sugars and biofuels (Kim *et al*., 2013; Wei *et al*., 2013; Yun *et al*., 2016; Wu *et al*., 2017). The dominant component of red algae is the polysaccharide agarose, which consists of the units D-galactose (D-Gal) and 3,6-anhydro-L-galactose (L-AHG) with alternate α-1,3- and β-1,4-linkages (Chen *et al*., 2015). Agarose can be degraded by an acid or enzyme into agaro-oligosaccharides that contain agaro-oligosaccharides (AOSs) with D-Gal as the non-reducing end and neoagarooligosaccharides (NAOSs) with L-AHG as the non-reducing end (Yun *et al*., 2015). Both NAOSs and AOSs are valuable functional oligosaccharides that feature many positive biological effects, such as anti-aging (Ma *et al*., 2019a), protection against alcoholic liver injury (Jin *et al*., 2017) and the prevention of gut dysbiosis (Higashimura *et al*., 2016). Therefore, the production of agaro-oligosaccharides constitutes a high-value utilization of red algae. Moreover, a NAOS or AOS with a specific polymerization degree (PD) will exhibit specific biological effect. For example, neoagarotetraose (NA4) can protect mice against damage from intense exercise-induced fatigue (Zhang *et al*., 2017b), agaropentaose can protect SH-SY5Y cells against 6-hydroxydopamine-induced neurotoxicity (Ye *et al*., 2019), and NAOSs and AOSs exhibit different skin-whitening effects (Kim *et al*., 2017b). NA4 is a well-known NAOS with many biological functions, including anti-fatigue, the modulation of intestinal microbiota, antiinflammatory properties, and anti-obesity and anti-diabetic effects (Chen *et al*., 2016; Hong *et al*., 2017; Wang *et al*., 2017; Zhang *et al*., 2017a, 2017b). For further clinical application, it is necessary to produce an agaro-oligosaccharide with specific PDs rather than a mixture of NAOSs and AOSs with different PDs.

Specific NAOSs or AOSs with certain PDs can be produced from agarose by agarolytic enzymes such as agarotetraose-producing α-agarase (Liu *et al*., 2019) and NA4-producing β-agarase (Liang *et al*., 2017). Both heterologously expressed agarases (Li *et al*., 2019; Ma *et al*., 2019b) and engineered microorganism cells containing agarases (Gao *et al*., 2019) can be used as catalysts for the production of agaro-oligosaccharides. However, because of the

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gelling properties of agarose, once its concentration is higher than 1%, *i.e.*, the typical concentration used in agarose gel electrophoresis, the agarose solution will transform into a gel or become very sticky, which limits the enzymatic production of NAOS or AOS to a low level. In contrast, the agarose concentration can become very high (307 $g\,L^{-1}$) during acid hydrolysis (Kim *et al.*, 2018), but the degradation products of agarose by acid hydrolysis comprise a complex mixture with consecutively distributed PDs (Lee *et al.*, 2012; Xu *et al.*, 2018). Therefore, an efficient method is needed to produce large amounts of pure agaro-oligosaccharides.

In this study, we established an easy method for efficiently producing pure agaro-oligosaccharide with a high yield. Using the production of NA4 as an example, we employed a chemical-biological route to produce high volumes of pure NA4. This route has potential for use in the large-scale production of either high-purity NAOSs or AOSs in practical applications.

2 Materials and Methods

2.1 Materials

For the NA4 preparation, we purchased agarose from Sigma (Vetec™ Reagent Grade, USA), and used *Escherichia coli* BL21(DE3) for the expression of AgWH50B with plasmid pET21a (+)-*agWH50B*. The yeast extract and tryptone were purchased from Oxoid (Basingstoke, England). The citric acid monohydrate was purchased from Chinese Medicine Ltd. (China).

2.2 Chemical Liquefaction

Agarose was dissolved in 2.5%, 5%, or 7.5% (w/v) citric acid solutions with a final concentration of 150 $g\,L^{-1}$. The citric acid hydrolysis was performed for 4 h at 90℃ using a sterilizer. After cooling to room temperature, the insoluble material of the liquefied agar was collected by centrifugation at 12000 $r\,min^{-1}$ for 30 min at 4℃. We determined the dry weight of the insoluble material after heating at 105℃ for 12 h in a drying oven. Then, we calculated the liquefaction rate using formula below:

$$\text{Liquefaction rate (\%)} = \frac{\text{Initial agarose (g)} - \text{Insoluble material (g)}}{\text{Initial agarose (g)}} \times 100 .$$

Next, after 0.75 h, 1 h, 2 h, 3 h, and 4 h, the dissolved samples were neutralized to a pH of 7 with 20% (w/v) sodium hydroxide. The concentrations of sugar were determined using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). During the analysis, 300 μL of DNS was mixed with 200 μL sample, which was then boiled immediately for 5 min followed by cooling with cold water. The samples were subsequently diluted with 1.5 mL of water, and the absorbance peak was determined to be 540 nm. We used 200 μL buffer as a control and all measurements were performed in triplicate.

2.3 Preparation of β-agarase and the Enzymatic Catalysis

The engineered strain *E. coli* BL21(DE3)-pET21a (+)-*agWH50B* was cultured for activation using 5 mL of Luria-Bertani medium (1% peptone, 0.5% yeast extract, and 1% NaCl) at 37℃, and was shaken overnight at 220 $r\,min^{-1}$. Then, to produce the target enzyme, 10 mL of the above seed solution was inoculated in 1 L of ZYP-5052 medium (1% tryptone, 0.5% yeast extract, 0.2% $MgSO_4$, 1.25% glycerin, 0.125% glucose, and 10% α-lactose as inducer), and was shaken with 220 $r\,min^{-1}$ at 20℃ for 48 h. The precipitates were collected after centrifugation at 8500 × *g* for 15 min at 4℃, resuspended in binding buffer (20 mmol L^{-1} PBS buffer, pH 7.0), and subsequently disrupted by sonication. Crude extracts were obtained by centrifugation at 8500 × *g* for 20 min at 4℃, then freeze-dried to obtain crude enzyme powder. Using the DNS method (Miller, 1959), the crude enzyme activity level of AgWH50B was dedected to be 0.195 U mg^{-1}. Next, 200 μL of 0.3% (w/v) agarose was digested with 1 mg of crude enzyme at 40℃ for 15 min, and then was boiled for 2 min to terminate the reaction. Then, 300 μL DNS was added to the reaction system and boiled for 5 min to color, and then cooled it to room temperature, after which the absorbance peak was immediately determined to be 540 nm. In this study, one unit of enzymatic activity (U) is defined as the amount of enzyme that produces 1 μmol of reducing sugar per min by hydrolyzing agarose under the assay conditions. Before the last reaction in the NA4 preparation, we optimized the amount of AgWH50B crude enzyme, using 25, 50, or 75 $g\,L^{-1}$ of crude enzyme powder for the optimization with a 10-mL reaction volume at 40℃ for 12 h. We then boiled it for 10 min and obtain the supernatant by centrifugation at 10000 × *g* for 15 min at 4℃. To determine the concentration of NA4, we used high-performance liquid chromatography (HPLC) with a Sugar-Pak I column (Waters, U.S.A.) under the following conditions: a mobile phase of EDTA calcium disodium (50 mg L^{-1}), column temperature of 75℃, flow velocity of 0.5 mL min^{-1}, and detection by a refractive index detector (Liang *et al.*, 2017).

In the enzymatic catalysis step, we hydrolyzed the neutralized oligosaccharide solution obtained during the liquefaction step using 25 $g\,L^{-1}$ of AgWH50B in a 1-L reaction mixture at 40℃ for 12 h. After the reaction mixture was boiled for 10 min, it was centrifuged at 8500 × *g* for 20 min to obtain a supernatant containing a mixture of NA4 and other oligosaccharides. The supernatant was then freeze-dried it to obtain agaro-oligosaccharide powder for the next step of purification.

2.4 Purification of NA4

The agaro-oligosaccharide powder was then resuspended in hyperpure water, and separated using a Bio-Gel P2 chromatography column, eluting the hyperpure water at a flow rate of 2 mL min^{-1}. Aliquots were sampled every 2.5 min and analyzed by thin-layer chromatography (TLC), and the plates were eluted in a developing solvent com-

posed of n-butanol/acetic acid/water (2:1:1, v:v:v). The agaro-oligosaccharide spots were visualized by soaking the TLC plate in an ethanol solution containing 10% (v/v) H_2SO_4, and then heating it at 100℃ for 5 min. Based on the TLC results, we collected and identified aliquots containing NA4 using the HPLC method described in Section 2.3.

3 Results and Discussion

3.1 Chemical Liquefaction of Agarose

Fig.1 shows an overview of the preparation routes used in this study. First, agarose was liquesced into soluble AOSs by citric acid, which is a food additive. Next, β-agarase AgWH50B (details regarding AgWH50B can be obtained from the study by Liang *et al*. (2017)) was used to hydrolyze the AOSs into NA4 and other agaro-oligosaccharides with low PDs. In Step I, the PDs of the AOSs after liquefaction must be low enough to ensure their complete solubility and easy catalysis by β-agarase in Step II. Meanwhile, AOSs with PDs higher than 4 were hoped to be the products as they are the substrate for β-agarase AgWH50B to produce NA4 in Step II.

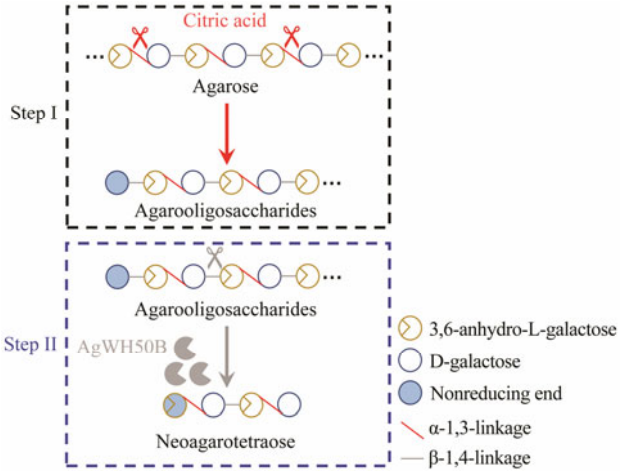

Fig.1 Technical route used in the preparation of neoagarotetraose (NA4) by chemical liquefaction and enzymatic catalysis.

We treated 150 g L^{-1} of agarose using citric acid with concentrations ranging from 2.5% to 7.5% at 90℃. As shown in Fig.2A, when the citric acid concentration was 2.5% and the treatment time was 0.75 h, the agarose liquefaction rate was 92%, whereas in all other conditions, the liquefaction rate for each sample was 100%. This means that 0.75 h was not long enough to achieve complete liquefaction. As shown in Fig.2B, with increasing the citric acid concentration, the molar concentration of the reducing sugars (MCRS) increased, and a higher MCRS was attained by increasing the treatment time. The initial agarose concentration (150 g L^{-1}) was the same in all the samples, so the higher PD, *i.e*., the higher the relative

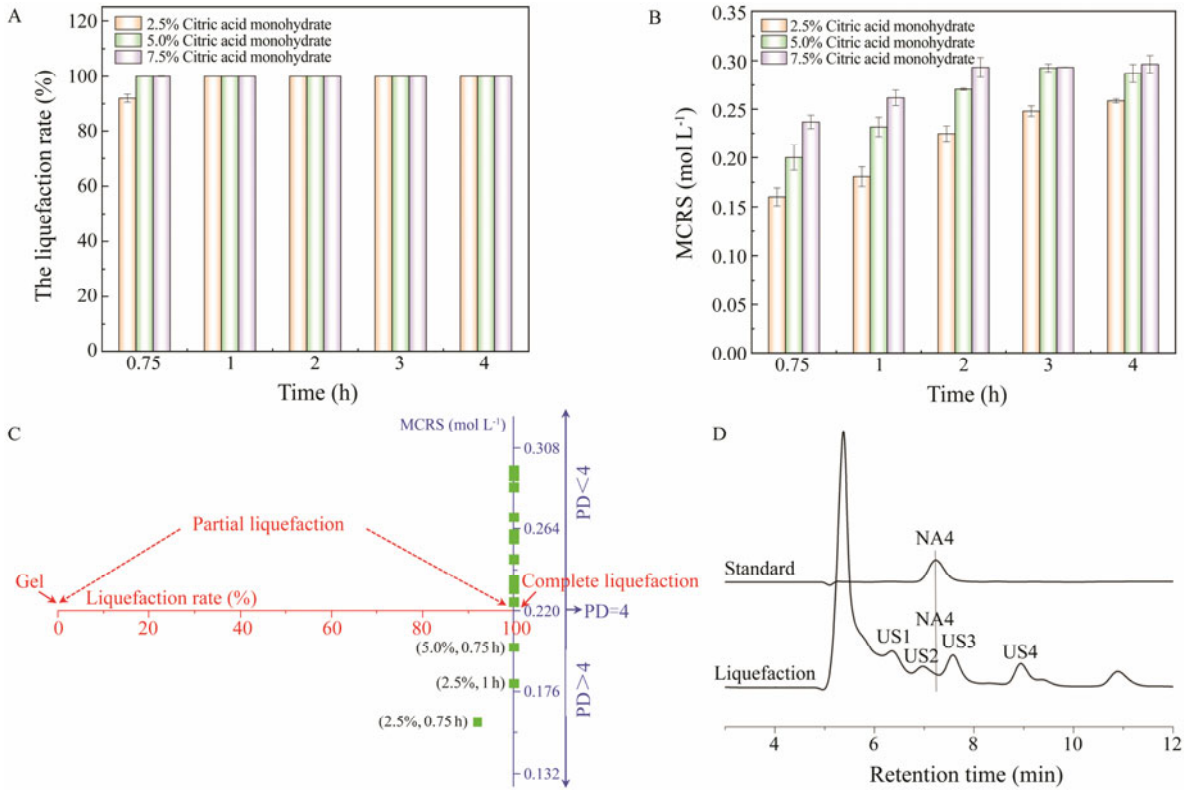

Fig.2 Chemical liquefaction of agarose. (A) Liquefaction rate of agarose after chemical liquefaction. (B) Molar concentration of reduced sugars (MCRS) after chemical liquefaction. (C) Scheme for screening the optimal liquefaction conditions. (D) Oligosaccharides produced during the chemical liquefaction step. US, unknown saccharide; PD, degree of polymerization; MCRS, molar concentration of reducing sugars. All measurements were performed in triplicate. Error bars indicate the standard deviation of measurement.

molecular mass, the lower was the MCRS. If the average PD is 4 after liquefaction, the MCRS would be 0.22 mol L^{-1}, so the actual MCRS must be lower than 0.22 mol L^{-1}. Therefore, the liquefaction conditions must be controlled to achieve two goals: a 100% liquefaction rate and an MCRS lower than 0.22 mol L^{-1}. In other words, in Fig.2C, the desired sample-condition data point should be just on the MCRS coordinate axis (vertical line) and lower than the liquefaction rate coordinate axis (horizontal line). There are only two conditions that qualify: 2.5% citric acid for 1 h and 5% citric acid for 0.75 h. The MCRS value in the condition (2.5%, 1 h) was 0.18 ± 0.01 mol L^{-1}, which is lower than that (0.20 ± 0.01 mol L^{-1}) in the condition (5%, 0.75 h), which indicates that the average PD in the condition (2.5%, 1 h) is higher than that in the condition (5%, 0.75 h). Therefore, the optimal condition in Step I was 2.5% citric acid at 90℃ for 1 h. The HPLC results in Fig.2D showed that no NA4 was produced after chemical liquefaction. However, we observed one unknown oligosaccharide (US1, US2) with a PD higher than NA4, and three other unknown oligosaccharides (US3, US4) with PDs lower than NA4.

In previous studies, acetic acid and HCl were used in the preparation of oligosaccharides from agarose (Kazłowski *et al*., 2008; Kim *et al*., 2012). In this study, for the agarose liquefaction, citric acidused as a food additive was employed (Henry *et al*., 1985). The citric acid treatment seems to be an extra step compared to the one-step enzyme hydrolysis method (Kim *et al*., 2017a). However, without citric acid treatment, prior to hydrolysis by agarase, the agarose must be melted at high temperature and then cooled to the appropriate temperature for enzymatic hydrolysis. The time and workload associated with this pretreatment are comparable to that (90℃, 1 h) of our chemical liquefaction step. Moreover, citric acid can be used to pretreat agarose in high concentrations, which enables large-scale enzymatic preparation.

3.2 NA4 Production by β-agarase

Fig.3 shows that with increases in the enzyme concentration, the titer of NA4 decreased, whereas the titers of US3, US4, and US5 increased. The highest titer of NA4 (30.8 g L^{-1}) was produced using 25 g L^{-1} of β-agarase AgWH50B. Fig.3A shows that US1, whose PD is higher than that of NA4, was completely degraded after hydrolysis by AgWH50B. However, the titer of US3, another by-product whose PD is slightly lower than NA4, significantly increased. Therefore, it is important that US3 is removed in the purification step. Fig.4 shows the time course of NA4 formation in Step II. At 12 h, the NA4 titer reached 30.8 ± 0.52 g L^{-1} with a productivity of 2.57 g L^{-1} h^{-1} in 1 L of reaction broth.

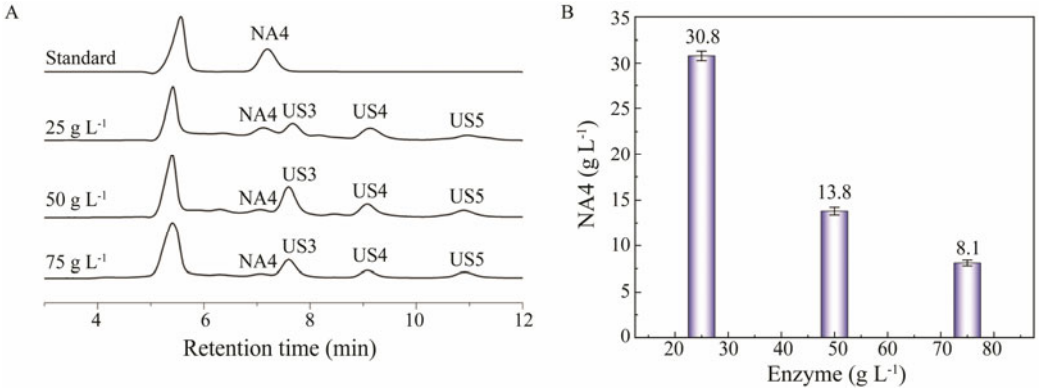

Fig.3 Optimization of enzymatic catalysis. (A) Oligosaccharides produced using different amounts of enzyme. (B) NA4 produced using different amounts of enzyme. US, unknown saccharide. All measurements were performed in triplicate. Error bars indicate the standard deviation of measurement.

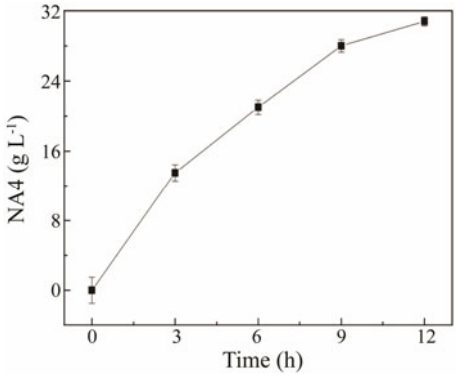

Fig.4 Time course of NA4 formation during enzymatic catalysis by 25 g L^{-1} of β-agarase AgWH50B. US, unknown saccharide. All measurements were performed in triplicate. Error bars indicate the standard deviation of measurement.

In most of the previous studies on agarose degradation, oligosaccharides were produced at laboratory scales to study their function or activity. So a much lower initial agarose concentration was enough, such as 5.5 g L^{-1} (Kazłowski *et al*., 2015) or 2 g L^{-1} (Li *et al*., 2007). An exception was an agaro-oligosaccharides production at the medium scale, in which the researchers used 289 g of agarose as a substrate in 3-L buffer for hydrolysis by agarase, which means the initial agarose concentration was 96.3 g L^{-1} (Pan *et al*., 2010). In this study, combining both chemical liquefaction and enzymatic catalysis in the preparation of NA4 enabled the use of a larger concentration of substrates (up to 150 g L^{-1}) and consequently drastically reduced the amount of water, which facilitates the subsequent purification steps.

3.3 Purification of NA4

In total, we collected 100 aliquots after purification by Bio-Gel P2 chromatography (Li *et al*., 2007; Jang *et al*., 2009; Lin *et al*., 2019). The TLC results shown in Fig.5A reveal that purified NA4 was present in aliquots 30 to 50, the details of which are shown on another TLC plate (Fig.5B). These results indicate that aliquots 38–42 were well purified, but aliquots 30–37 and 43–50 contained a few other oligosaccharides and required further purification. The Bio-Gel P2 column results for the different aliquots after the second purification showed in Fig.5C suggested that the purified NA4 were in aliquots 20–28. Aliquots 38–42 from the first purification and 20–28 from the second purification were examined by HPLC, which revealed that purified NA4 had been successfully acquired. After vacuum distillation and freeze-drying, 25.5± 0.31 g of NA4 powder was isolated, the purity of which, based on the HPLC results, was calculated to be 92%.

β-Agarase has been used previously to produce NAOSs, whereby a mixture of 6.4 g L^{-1} NA4 and 3.8 g L^{-1} neoagarobiose (NA2) were produced from 10 g L^{-1} of agarose (Seo *et al*., 2014). Using 35 g of agar as the substrate, 53 mg of purified NA4 was also obtained after enzyme hydrolysis followed by purification (Jang *et al*., 2009). By producing NAOSs with a PD range of 2–22, 13.18 g of NA4 was prepared from 289 g of agarose with a concentration of 2.46%, which is the highest concentration of purified NA4 that have been reported (Pan *et al*., 2010). Using the engineered *E. coli* BL21(DE3) containing a β-agarase, 0.45 g L^{-1} of NA2 was also produced from 2 g L^{-1} of agarose (Gao *et al*., 2019). As shown in Table 1, the 25.5 g of NA4 obtained in this study is the highest production of any kind of agaro-oligosaccharide ever pro-

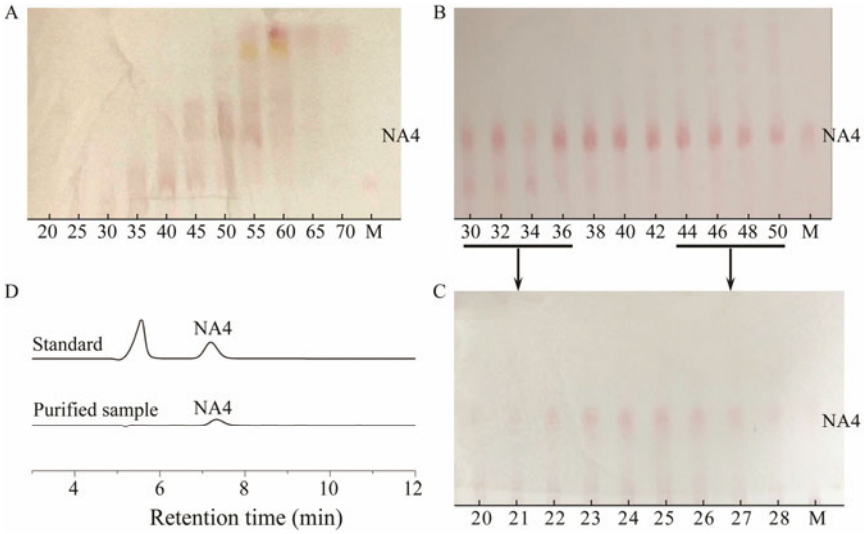

Fig.5 Purification of NA4 by gel chromatography. (A), (B) TLC results for different aliquots from the first purification. (C) TLC results for the different aliquots from the second purification. (D) HPLC results for purified NA4.

Table 1 The production of agaro-oligosaccharides (PD≤8) by chemical and/or biological methods in previous researches

Product	Amount (g)	Purity	Yield (g g^{-1})	Substrate (g)	Method	Reference
NA2	16.76	100%	0.058			
NA4	13.18	100%	0.046	289	β-agarase	Pan *et al*., 2010
NA6	20.88	100%	0.072			
AOSs	0.051	–	0.21	0.5	Cellulase	Kang *et al*., 2014
NA2†	3.8 g L^{-1}	–	–	10 g L^{-1}	β-agarase	Seo *et al*., 2014
NA4	6.4 g L^{-1}	–	–			
NA2	0.029	63.6%	0.058			
NA4	0.065	66.6%	0.13	0.1	β-agarase	
NA6	0.085	71.9%	0.17			
NA8	0.075	64.8%	0.15			Kazłowski *et al*., 2015
A2	0.11	65.6%	0.075			
A4	0.36	81.4%	0.24	1.5	HCl	
A6	0.39	77.7%	0.26			
A8	0.29	76.8%	0.19			
NA4	0.224	100%	0.448	0.5	β-agarase	Xu *et al*., 2018
NA2	0.45 g L^{-1}	–	–	2 g L^{-1}	β-agarase	Gao *et al*., 2019
NA4	18.4 g L^{-1}	–	–	20 g L^{-1}	β-agarase	Ma *et al*., 2019
NA4 and NA6	1.41 μmol mL^{-1}	–	–	0.3 g	β-agarase	Li *et al*., 2019
NA4	25.5	92%	0.17	150	Citric acid + β-agarase	This study

Note: † The purity and yield were not calculated if there was no purification step.

duced by any method to date. Considering the purity and yield of the products, the advantage of our green chemical-biological route is obvious. In the future, we expect that by combining controlled chemical liquefaction with one or more other agarolytic enzymes such as α-agarase (Liu *et al*., 2019), α-neoagarobiose hydrolase (Jiang *et al*., 2020), and/or β-galactosidase (Yang *et al*., 2018), AOSs or NAOSs with different PDs can be efficiently produced. Furthermore, our method is easy to scale up to produce agaro-oligosaccharides at both pilot-project and industrial levels to yield high-purity NAOS or AOS for practical application.

4 Conclusions

In this work, we established a route that combines controlled chemical liquefaction with enzymatic catalysis in the preparation of purified NA4. Using 2.5% citric acid, we liquesced 150 g L^{-1} of agarose dissolved in 1 L of water at 90℃ for 1 h to obtain an agaro-oligosaccharide solution with an MCRS value of 0.18 ± 0.01 mol L^{-1}. Enzymatic catalysis was then performed using β-agarase AgWH50B to produce NA4, followed by the use of gel chromatography to purify NA4. Ultimately, we obtained 25.5 ± 0.31 g NA4 with a purity of 92%, which is the highest production of NA4 to date. Our proposed method is suitable for producing many pure agaro-oligosaccharides and enables their large-scale production.

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