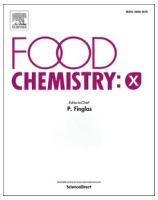
Food Chemistry: X 24 (2024) 101796

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x



Efficient preparation of β-1,3-glucooligosaccharides by microwave-assisted hydrothermal degradation of curdlan and preliminary exploration of its antibacterial activity

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ARTICLE INFO

Keywords:
Hydrothermal hydrolysis
β-1,3-glucooligosaccharides
Separation
Characterization
Antibacterial activity

ABSTRACT

The study focuses on the efficient production of curdlan β-1,3-glucooligosaccharides (CDOS) through microwave-assisted hydrothermal hydrolysis of curdlan. The optimal condition was identified as 170 °C and 30 min, resulting in a curdlan liquefaction yield of over 96%, mainly contains CDOS along with minor amounts of glucose and 5-hydroxymethylfurfural (5-HMF). Oligosaccharides with a low degree of polymerization (DP), specifically DP2 to DP6, were isolated. The yields for DP2 to DP6 were 15.87%, 8.33%, 6.00%, 3.13%, and 1.83%, respectively, with purities over 70%. X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and scanning electron microscopy (SEM) analysis demonstrated that the dense triple helix structure of the crystalline structure was disrupted during degradation. Additionally, CDOS at a concentration of 2.5 mg/mL partially inhibited the growth of *Escherichia coli*. This study concludes that the combination of microwave-assisted hydrothermal hydrolysis with gel filtration chromatography provides an efficient method for producing and purifying CDOS.

1. Introduction

Curdlan is a water-insoluble, linear extracellular homopolysaccharide composed of D-glucose units linked by β-1,3-glycosidic bonds (Chen & Wang, 2020). Its distinctive triple-helix structure in water endows it with advantageous gel properties, making it a prevalent food additive in the industry (Ikeda & Shishido, 2005). Curdlan β-1,3-glucooligosaccharides (CDOS), functional oligosaccharides characterized by their low caloric value, stability, safety, and non-toxicity, have exhibited biological activities in immune stimulation (Tang et al., 2019), as prebiotics (Shi et al., 2018; Xu et al., 2021), and in triggering plant defense responses (Fu et al., 2011; Li et al., 2014). Additional research is necessary to uncover further biological activities of CDOS. Notably, the biological activities of oligosaccharides vary significantly with different degree of polymerization (DP) (Fu et al., 2011).

Various methods are used to degrade curdlan, including chemical, physical, and enzymatic approaches. Chemical hydrolysis involves acid hydrolysis (Li et al., 2013) and oxidative degradation with agents such as H_2O_2 (Wu et al., 2012) or 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) (Tang et al., 2018). Enzymatic degradation, primarily using β-1,3-glucanase (Fu et al., 2015; Gao et al., 2021; Li et al., 2018), and to a lesser extent α-amylase (Qian et al., 2012), has been extensively studied. Research suggests that enzymatic methods are more effective than acid hydrolysis for producing CDOS with a DP of less than 6 (Grandpierre et al., 2008).

Microwave-assisted hydrothermal technology has found wide applications in various fields, including the synthesis of nanomaterials (Li et al., 2021), hydro char production from waste materials (Wang et al., 2020; Wang et al., 2022), extraction of bioactive compounds (Fan et al., 2013; Hu et al., 2022), and depolymerization of polysaccharides (Fan et al., 2013). This technology has successfully produced CDOS from β-D-glucan derived from *Ganoderma lucidum* (Qin et al., 2022) and curdlan (Wang et al., 2017), proving that is an economical, environmentally friendly, and efficient method for CDOS production.

After production, the separation and purification of CDOS are crucial for exploring its biological activities. Common methods for the separation and purification of prebiotic oligosaccharides include membrane separation, adsorption chromatography, and yeast fermentation (Zeng

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https://doi.org/10.1016/j.fochx.2024.101796
Received 22 May 2024; Received in revised form 23 August 2024; Accepted 28 August 2024
Available online 30 August 2024

et al., 2023). Semi-preparative chromatography columns are frequently used for the precise analysis and separation of CDOS (Cheong et al., 2022; Li et al., 2013), while ethanol precipitation, which separates polysaccharides by reducing the dielectric constant of aqueous solutions, provides only coarse fractionation (Qin et al., 2022). Bio-Gel P-2, a refined polyacrylamide bead used in gel filtration chromatography, has proven effective in purifying laminarin oligosaccharides, which are structurally similar to CDOS (Cheong et al., 2022). Consequently, gel filtration chromatography is considered an advantageous method for purifying CDOS.

Our study aimed to optimize microwave treatment conditions to achieve lower temperatures and shorter processing times in the preparation of CDOS, followed by purification using a Bio-Gel P-2 column to isolate CDOS with a specific DP. Additionally, we investigated the antibacterial properties of CDOS, to develop a cost-effective production method for CDOS from curdlan while exploring its potential health benefits.

2. Materials and methods

2.1. Materials and chemicals

Curdlan was sourced from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *Escherichia coli* was obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Bio-Gel P-2 was purchased from Bio-Rad Laboratories (Richmond, CA, USA). A chromatography column, measuring 26 mm × 100 cm, was acquired from Beijing Ruida Henghui Science & Technology Development Co., Ltd. (Beijing, China). All other reagents were supplied by Sigma-Aldrich (St. Louis, MO, USA).

2.2. Microwave-assisted hydrothermal hydrolysis of curdlan

To determine the optimum substrate concentration, curdlan was dispersed in water to achieve concentrations of 1.0%, 2.0%, and 3.0% (w/v). After hydrothermal treatment at 170 °C or 180 °C for 30 min using a microwave digestion system (Preekem WX-7000HP, Shanghai, China), the yield of saccharification, liquefaction, and the formation of 5-hydroxymethylfurfural (5-HMF) were evaluated (refer to Sections 2.4 and 2.6). Subsequently, curdlan was dispersed in water to create a 2.0% (w/v) suspension. The suspensions were hydrolyzed at varying temperatures (160, 170, 180 °C) and for different durations (30, 40, 50, 60 min) to optimize the operation conditions. After hydrolysis, the mixture was centrifuged at 10,000 ×g for 40 min to separate the supernatant.

2.3. Separation and purification of curdlan hydrolysates

The supernatant was freeze-dried for 48 h using a vacuum freeze-dryer (Scientz, Ningbo, China). The concentrated hydrolysate was then separated on a Bio-Gel P-2 column (2.6 mm × 100 cm) using ultrapure water as the mobile phase at a flow rate of 0.3 mL/min. Samples were collected at 10-min intervals by an automatic fraction collector (Huxi, Shanghai, China). The oligosaccharide fractions were initially screened using the 3,5-dinitrosalicylic acid (DNS) method (Wang et al., 2017) and subsequently analyzed by thin layer chromatography (TLC) (refer to Sections 2.4 and 2.5 for details). CDOS fractions with identical DP were pooled and freeze-dried, and the yield was determined by weighing. The yield was expressed as:

$$\text{Yield} = \text{wt. of the separated oligosaccharides}/\text{wt. of the initial curdlan} \times 100\%$$

2.4. Analysis of the liquefaction and saccharification yield of hydrothermal treated curdlan

The unliquefied portion, i.e., the precipitate after centrifugal separation, was weighed after freeze-drying at −70 °C for 48 h. The liquefaction yield of curdlan was calculated as follows:

$$\text{Liquefaction yield} = (\text{wt. of the initial curdlan} - \text{wt. of the unliquefied portion})/\text{wt. of the initial curdlan} \times 100\%$$

The saccharification yield of curdlan was measured at OD_{540} using the DNS method with D-glucose as standard. The saccharification yield was calculated according to the following formula:

$$\text{Saccharification yield} = \text{wt. of reducing sugar}/(\text{wt. of the initial curdlan} \times 1.1) \times 100\%$$

where 1.1 represents the hydrolysis conversion factor for each β-1,3 glycosidic linkage with the addition of a water molecule.

2.5. Qualitatively analysis of curdlan hydrolysates by TLC and MALDI-TOF-MS

The hydrolysate of curdlan was qualitatively analyzed by TLC on a silica gel 60 plate (Merck, Darmstadt, Germany) using a mobile phase of butanol-ethanol-water (3:1:1, v/v/v) and visualized with a solution containing 10% (w/v) sulfuric acid and 0.2% (w/v) 1,3-dihydroxynaphthalene in ethanol (Yun et al., 2013). The plate was then dried at 150 °C for 5 min to develop the color. Further characterization of the hydrolysate was performed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). After treatment, a sample of curdlan (10 μL) was mixed with 10 mM NaCl (6 μL) and 2,5-dihydroxybenzoic acid (DHB) (10 mg/mL, 10 μL) in 50% (v/v) acetonitrile. Subsequently, 1 μL of this mixture was applied to a stainless-steel plate and allowed to dry naturally for uniform crystallization. The MALDI-7090 instrument (Shimadzu, Kyoto, Japan) was used for analysis, set to an acceleration voltage of 20,000 V, a delay time of 200 ns, and operated in reflection mode (Wang et al., 2018).

2.6. Quantitative analysis of curdlan hydrolysates by HPLC

The method was modified from Huang et al. (Huang et al., 2020). High-performance liquid chromatography (HPLC) (Shimadzu LC-20, Kyoto, Japan) equipped with an Asahipak NH2P-50 4E column (Shodex, Japan) was applied to quantify the oligosaccharides in the curdlan hydrolysates. The mobile phase was acetonitrile-water (65:35, v/v) at a flow rate of 0.8 mL/min. The column temperature was maintained at 40 °C, and samples were detected using a refractive index (RI) detector with an injection volume of 10 μL. Additionally, the purity of the separated oligosaccharides was determined using the peak area normalization method (Li et al., 2023).

The 5-hydroxymethylfurfural (5-HMF) was analyzed using a WondaSil C18 column (4.6 mm × 250 mm ID, 5 μm) (Shimadzu, Kyoto, Japan) and an ultraviolet (UV) detector at 284 nm. The column temperature was maintained at 35 °C, and the mobile phase was methanol/water (3/7, v/v) with a flow rate of 0.7 mL/min.

Table 1
The yield of saccharification, liquefaction and 5-HMF of curdlan with different substrate concentrations (1%, 2%, 3%, w/v) at 170 °C 30 min and 180 °C 30 min. Liquefaction and saccharification effect of microwave hydrothermal hydrolysis of curdlan under the condition of substrate concentration of 2.0% at different temperatures (160, 170, 180 °C) and times (30, 40, 50, 60 min). n = 3.

Processing temperature and time		170 °C 30 min		180 °C 30 min
Substrate concentration (%)	1.0%	2.0%	3.0%	3.0%
Saccharification yield (%)	88.15% ± 2.8	86.30% ± 3.3	23.00% ± 4.2	35.04% ± 3.4
Liquefaction yield (%)	96.67% ± 0.1	96.67% ± 0.1	61.67% ± 0.2	80.00% ± 0.2
5-HMF yield (%)	0.35% ± 6.4	0.41% ± 7.6	0.16% ± 8.2	0.47% ± 7.2

Under the condition of substrate concentration of 2.0%						
	Saccharification yield (%)			**Liquefaction yield (%)**		
Processing temperature and time	160 °C	170 °C	180 °C	160 °C	170 °C	180 °C
30 min	5.58% ± 2.8	86.30% ± 3.3	5.32% ± 2.8	74.43% ± 0.2	96.67% ± 0.1	97.86% ± 0.1
40 min	5.06% ± 3.1	93.24% ± 4.1	5.14% ± 2.6	75.89% ± 0.2	96.90% ± 0.1	99.10% ± 0.1
50 min	5.72% ± 2.7	31.23% ± 3.4	5.69% ± 2.5	77.01% ± 0.2	97.17% ± 0.1	99.40% ± 0.1
60 min	8.41% ± 2.7	23.32% ± 2.7	10.37% ± 2.6	76.73% ± 0.2	96.20% ± 0.1	99.90% ± 0.1

2.7. Morphological analysis of curdlan hydrolysates

The surface morphology of curdlan and CDOS (unpurified) was visualized by a scanning electron microscope (SEM, Phenom World, Phenom Pro10102), the samples were dried with an auto critical-point dryer and spread on copper grids coated with carbon support film, followed by coating with gold at 10 kV.

2.8. Structural analysis of curdlan hydrolysates

The Fourier transform infrared spectroscopy (FTIR) analysis of CDOS (unpurified) was determined by a Spectrum Two spectrometer (PerkinElmer, MA, USA) in the wavenumbers range from 4000 to 600 cm^{-1}. X-ray diffraction (XRD) pattern of CDOS was obtained by an X-ray diffractometer (TD-3500, Danton Tongda Science & Technology Co., Ltd., Liaoning, China) in a 2θ range from 5° to 35° with a step width of 0.02°.

2.9. Assays of antibacterial activities

For the assessment of antibacterial activity, *E. coli* was cultured in LB medium at 37 °C and 200 rpm. Subsequently, 20 μL of the activated bacterial culture was added to a microplate containing 200 μL of LB liquid culture medium (Shen et al., 2023). CDOS (unpurified) solutions were then added to achieve final concentrations of 0, 2.5, 5, 10, and 20 mg/mL, respectively (Shen et al., 2023; Chaari, et al., 2016). The absorbance at 600 nm was monitored every 3 h over a period of 24 h.

2.10. Statistical analysis

All experiments were conducted in triplicate (n = 3). Data processing was performed using Excel 2016, and results are presented as mean ± standard deviation (SD). Statistical analyses to determine significant differences were conducted using IBM SPSS Statistics 22, with a P-value of less than 0.05 considered statistically significant. Mass spectrometry data were obtained using Mass++ ver. 2.7.5, and graphs were generated using Origin 2021 software.

3. Results and discussion

3.1. Liquefaction and saccharification yield of hydrothermal treated curdlan

Preliminary experiments showed that curdlan remains in a gel state when heated for up to 30 min at temperatures around 160 °C. Substrate concentration was first optimized based on liquefaction and saccharification yield (Table 1). The results showed that at 170 °C for 30 min with a substrate concentration of 3.0%, curdlan's saccharification and liquefaction rates were the lowest. As the temperature increased, curdlan further decomposed, but the by-product 5-HMF significantly increased. Based on degradation efficiency, the optimal substrate concentration was chosen to be 2.0%.

Subsequently, the hydrothermal treatment temperature and time were optimized (Table 1). The saccharification yield of curdlan was relatively low when treated at 160 °C and 180 °C, achieving maximum yields of 8.41% and 10.37% at 60 min, respectively. At 160 °C, the low yield is likely due to incomplete depolymerization of curdlan, while at 180 °C, excessive degradation might result in the formation of undesirable by-products such as 5-HMF (Wang et al., 2017). According to various studies, glucose can be converted into 5-HMF via either the fructose mechanism or the 3-deoxyglucosone pathway (Zhu et al., 2020). Based on our findings, the optimal treatment condition for curdlan is 170 °C and 30 min.

3.2. Characterization of the hydrolysate

The XRD spectra of curdlan and CDOS are presented in Fig. 1a. Curdlan exhibits strong absorption at 2θ = 19.7°, indicating a semi-crystalline structure (Li et al., 2024). The counts of CDOS at 2θ = 20.4° are significantly lower than those of curdlan, suggesting that the semi-crystalline structure of curdlan has been disrupted. Interestingly, a small diffraction peak is observed at 2θ = 6.56° in CDOS, which relates to the triple helix structure of curdlan (Tao et al., 2021).

Curdlan exhibits characteristic absorption peaks for O—H, C—H, and C—O bonds at 3326 cm^{-1}, 2890 cm^{-1}, and 1031 cm^{-1}, respectively (Fig. 1b). The absorption peak at 890 cm^{-1} indicates the presence of β-glycosidic bonds, while the peak at 1612 cm^{-1} may be associated with the O—H bending vibration of water molecules (Yang et al., 2024). After hydrothermal hydrolysis, the resulting CDOS shows a new characteristic peak for C═O at 1780 cm^{-1} and for COO^- at 1655 cm^{-1}, indicating the formation of new carbonyl and carboxyl groups during degradation (Arli et al., 2011; Li et al., 2024). The original characteristic peaks are still present but with varying intensities, suggesting that while the basic structure is not completely destroyed, significant chemical changes have occurred. These changes indicate that the triple helix structure of curdlan is disrupted during hydrothermal degradation, leading to the disintegration of its semi-crystalline structure and the formation of a loose, porous structure. The newly formed C═O and COO^- groups further confirm the chemical structural changes, which significantly enhance its water solubility and reactivity.

Curdlan has compact aggregates and a regular conformation, while CDOS has a loose and porous structure (Fig. 1c). The structural changes of curdlan significantly affect its water solubility, which may be due to its dense triple helix structure being disrupted during degradation

(Zhang & Edgar, 2014).

3.3. TLC and MALDI-TOF-MS analysis of the hydrolysate

According to the TLC results shown in Fig. 1d, curdlan was not completely degraded at 160 °C. Prolonged treatment for 60 min at this temperature led to increased degradation, as evidenced by darker spots on the TLC plate, indicative of oligosaccharide formation (DP2-DP5). In contrast, treatment at 180 °C resulted in the significant production of monosaccharides and by-products such as 5-HMF. Additionally, a small quantity of low molecular weight CDOS with a DP of less than 4 was observed.

At 170 °C, a diverse range of degradation products was observed, including D-glucose and oligosaccharides ranging from DP2 to DP6. Furthermore, Fig. 2a shows that after hydrolysis at 170 °C for 30 min, CDOS with a continuous DP ranging from 2 to 7 were detected using MALDI-TOF-MS analysis. This finding is consistent with the types of oligosaccharides identified in the TLC analysis, further validating the effectiveness of the selected hydrolysis conditions for generating CDOS from curdlan.

3.4. Separation and purification of the hydrolysate

Gel filtration chromatography was employed to isolate CDOS with a specific single DP. As depicted in Fig. 3a, this method effectively separated oligosaccharides with DPs ranging from 2 to 6. The yields for oligosaccharides with DP2 through DP6 were recorded as follows: 15.87% for DP2, 8.33% for DP3, 6.00% for DP4, 3.13% for DP5, and 1.83% for DP6, as shown in Fig. 3b.

HPLC analysis revealed that CDOS ranging from DP1 to DP6 were the primary products of curdlan hydrolysis under the conditions of 170 °C for 30 min. The isolated CDOS with individual DPs constituted the main components of the fractions, as illustrated in Fig. 3c and Fig. 2b-f. These CDOS fractions exhibited relatively high purity, with the purity levels for DP2 through DP6 measured at 93.27%, 91.36%, 81.37%, 80.12%, and 71.35% (Fig. 3b).

3.5. Antibacterial activity of CDOS

Most research on CDOS has focused on their prebiotic activity, as they are readily fermented by various intestinal probiotics. However, studies on their antibacterial functions are relatively scarce. β-glucooligosaccharides derived from barley β-glucan, which are characterized by 2–3 consecutive β-1,4 linkage bonds interspersed with a single β-1,3 linkage, have been shown to regulate the selective growth and enhance the antimicrobial activity of probiotics without promoting the proliferation of pathogens. This suggests a potential for CDOS and similar β-glucooligosaccharides to not only support beneficial gut flora but also contribute to maintaining intestinal health by inhibiting harmful bacteria. Further exploration into the antibacterial properties of CDOS could reveal additional health benefits and applications, particularly in the development of functional foods designed to enhance

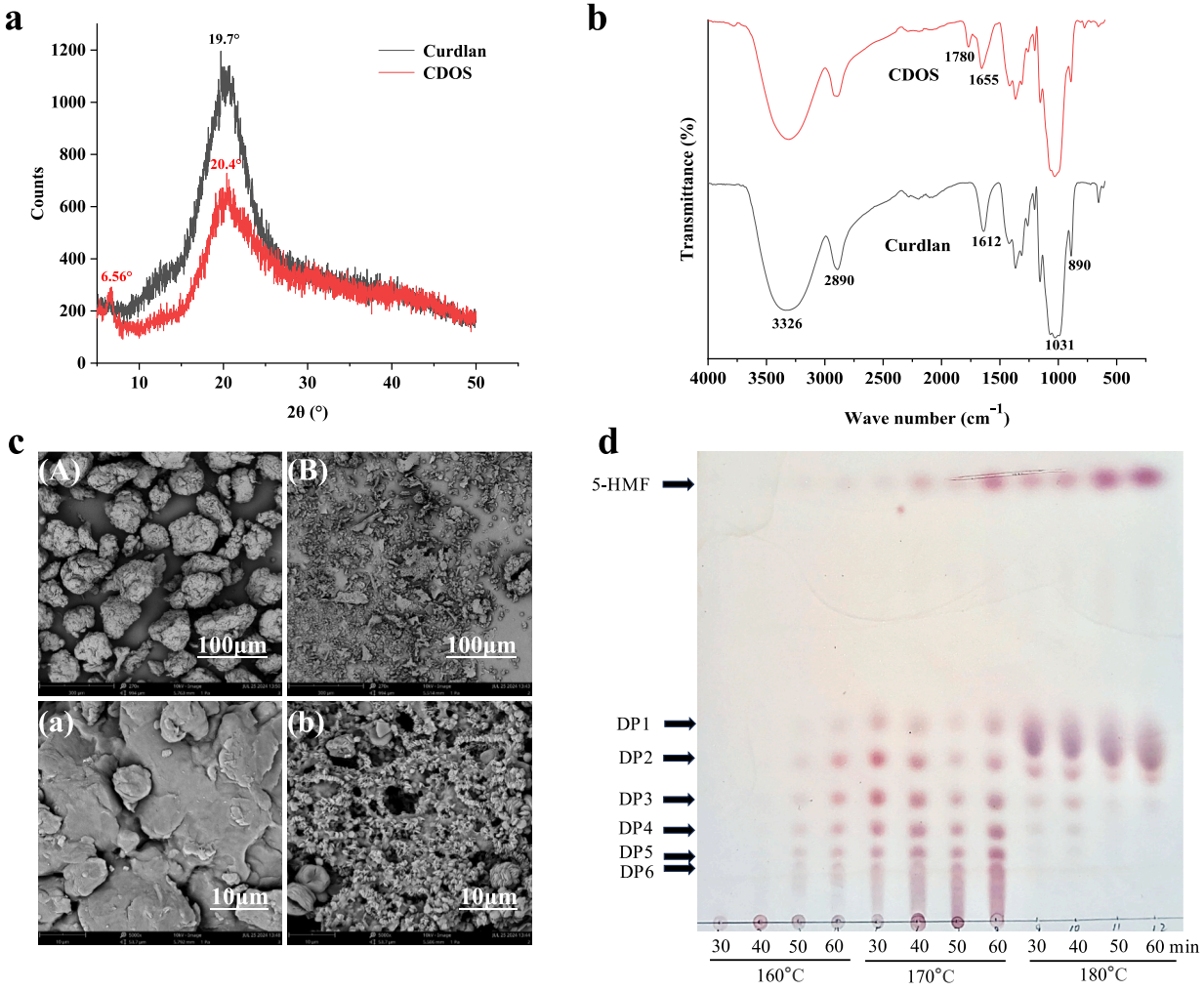

Fig. 1. Characterization of CDOS (unpurified). (a) XRD spectra and (b) FTIR patterns of curdlan and CDOS, respectively. (c) SEM analysis of curdlan (A/a) and CDOS (B/b), the image magnification is ×250 and × 5000, respectively. (d) TLC analysis of hydrothermal hydrolysates of curdlan under different conditions. n = 3.

gastrointestinal health (Lee et al., 2020).

Fig. 3d illustrates the inhibitory effect of various concentrations of CDOS on the growth of *E. coli*. Concentrations below 10 mg/mL exhibited partial inhibitory effects, while those above 10 mg/mL significantly inhibited the growth of *E. coli*. The OD_{600} demonstrated a decrease with increasing CDOS concentration after 24 h, indicating a concentration-dependent increase in the inhibitory effect of CDOS on *E. coli* growth.

Current antibacterial mechanisms of oligosaccharides include inhibition mediated by interactions with cell membranes and cell walls, inhibition of biofilm formation, anti-adhesion properties, induction of binding to harmful factors, and indirect inhibition mechanisms, as outlined by Liu et al. (2023). The indirect inhibition mainly involves the prebiotic functions of oligosaccharides, which can foster beneficial microflora that compete with pathogens, and elicitor functions that can activate plant defense responses. Therefore, CDOS, known for its prebiotic capabilities and its role in activating defense mechanisms in plants, may exert its antibacterial activity through these indirect mechanisms. Further research is essential to explore additional potential antibacterial mechanisms of CDOS and to fully understand how these properties can be harnessed in medical and agricultural applications.

4. Conclusions

In this study, we refined the microwave-assisted hydrothermal conditions to effectively depolymerize curdlan. Through gel filtration chromatography, we successfully isolated CDOS with DP ranging from 2 to 6. The overall yield of CDOS (DP2–6) was 35.2%, characterized by

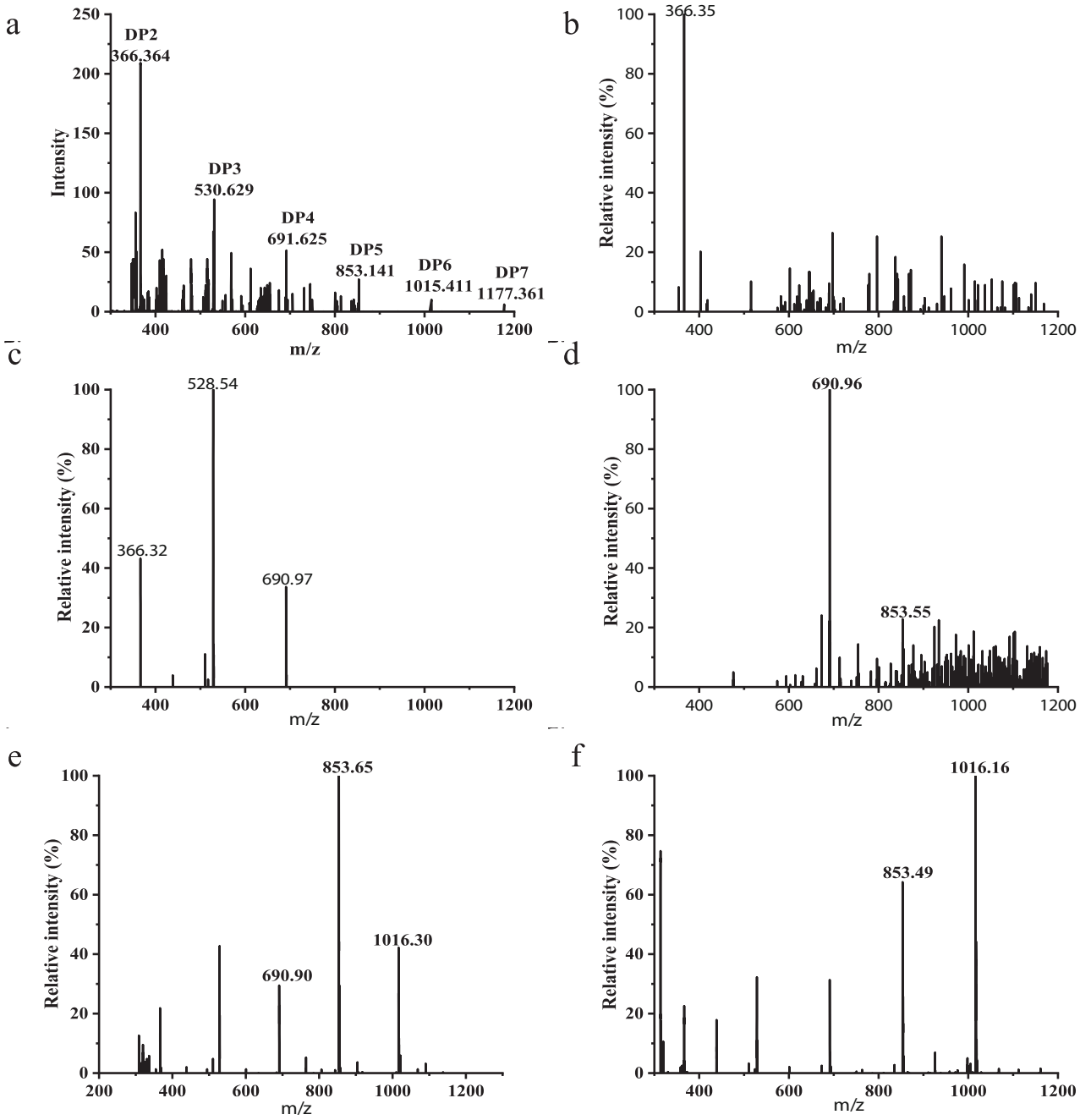

Fig. 2. MALDI-TOF-MS analysis for CDOS mixture (a), and DP2 (b), DP3 (c), DP4 (d), DP5 (e), DP6 (f) after separation and purification. The *m/z* ratio of CDOS from DP2-DP7 in the form of $[M + Na]^+$ are approximately 365, 527, 689, 853, 1015 and 1177, respectively.

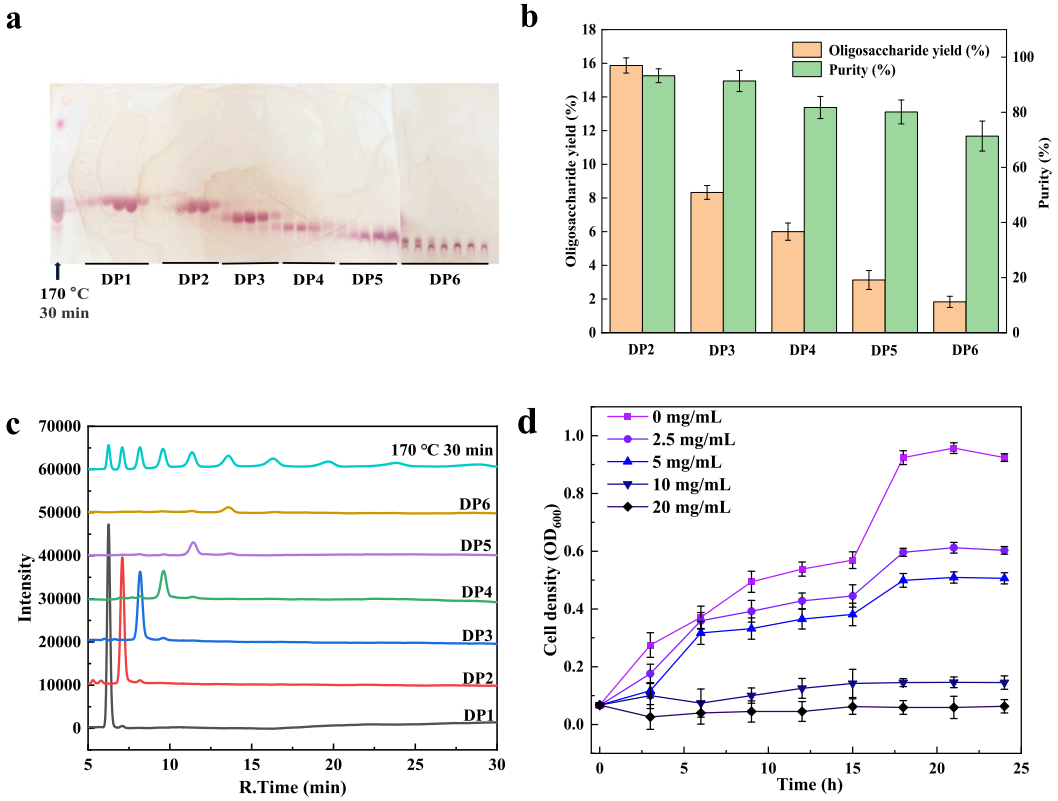

Fig. 3. TLC (a) and HPLC (c) analysis of separated products with different DPs (1–6) and original sample (170 °C/30 min) before separation. The yields and purity (b) analysis of separated β-1,3-glucooligosaccharides with DPs from 2 to 6. (d) The effect of different concentrations of CDOS (0, 2.5, 5, 10, 20 mg/mL) on the growth of *E. coli* for 24 h. n = 3.

high purity levels (over 70%), with DP2 exhibiting a purity of 93.3%. The morphological and structural characteristics indicate the formation of new C═O and COO^- groups during the degradation of curdlan, and the disruption of the triple helix structure of curdlan increases its water solubility. Additionally, CDOS showed a concentration-dependent inhibition of *E. coli,* with complete inhibition observed at 10 mg/mL. These findings not only confirm the effective separation and purification of CDOS but also enhance our understanding of and potential applications for curdlan-derived oligosaccharides. Our results lay a solid foundation for future research and the industrial application of CDOS in various functional contexts, encouraging continued exploration in this promising area.

CRediT authorship contribution statement

Meiling Zhou: Methodology, Visualization, Validation, Writing – original draft. **Meiling Dan:** Methodology, Visualization, Validation, Writing – original draft. **Guohua Zhao:** Supervision. **Damao Wang:** Project administration, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgements

This work was supported by National Natural Science Foundation of China (no. 32172158), Chongqing Municipal College Student Innovation and Entrepreneurship Training Program (S202310635076), the Shuangcheng cooperative agreement research grant of Yibin, China (XNDX2022020012), and Postgraduate mentor team-building program of Southwest University (XYDS201905).

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