International Journal of Biological Macromolecules 89 (2016) 25–34

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Production and optimization of curdlan produced by *Pseudomonas* sp. QL212

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ARTICLE INFO

Article history:
Received 2 December 2015
Received in revised form 30 March 2016
Accepted 12 April 2016
Available online 13 April 2016

Keywords:
Curdlan
Pseudomonas sp
Optimization

ABSTRACT

Curdlan is a polysaccharide that consists of β-1,3-linked glucose residues. A polysaccharide-producing bacterium isolated from soil samples was identified as *Pseudomonas* sp. QL212. The polysaccharide was purified to homogeneity via sequential ethanol precipitation, deproteinization, CM ion-exchange, and gel chromatography sequentially. Analysis of the purified polysaccharide revealed that it consisted of many glucosyl residues, and its molecular weight was only 6.18×10^5 Da. This low molecular weight endowed it with excellent solubility. Infrared and nuclear magnetic resonance spectral analysis confirmed that the polysaccharide was curdlan. Single-factor and Response surface methodology experiments were used to optimize the culture medium and conditions. The optimal culture conditions consisted of seed culture age of 12 h, and an incubation temperature of 30 °C, with 10% inoculum and a total fluid volume of 75 mL in a 250-mL Erlenmeyer flask. The maximum curdlan yield of about 5.92 g L^{-1} was achieved with an optimal medium consisting of 30.11 g L^{-1} of sucrose, 5.94 g L^{-1} of yeast extract, and an initial pH of 8.03. To our best knowledge, this is the highest reported yield of curdlan produced by *Pseudomonas* sp., and the curdlan production medium components were much simpler than those in previous reports.

1. Introduction

Curdlan is a neutral essentially linear polysaccharide that consists of glucosyl residues linked with β-1,3 glycosidic bonds. Curdlan aqueous suspensions can be thermally induced to produce gels that do not return to the liquid state, demonstrating unique rheological and thermal gelling properties [1]. Because of this characteristic, curdlan can be used as a food additive to improve the viscoelasticity, stability and creaminess of food, and also plays an important role in moisturizing increase incense agents of cosmetics. Because no enzyme has been detected to digest curdlan in humans, curdlan has been successfully used in diet foods [2–10]. Recently, growing interest has been generated in the biomedical applications of curdlan as a medicine-delivery polymers for sustained drug and drug diffusion, and water-soluble curdlan derivatives have been used as immune recognition sites of dectin-1, and even anti-AIDS agents to inhibit HIV infection [11–20]. Moreover, curdlan can also be used in chemosensor preparation.

β-1,3-glucans exist in cell walls and membranes of fungi, yeasts, algae, bacteria, and plants. Only bacteria can secrete curdlan from the cell, and their production period is shorter than other species, so bacteria are the most important production source of curdlan. Curdlan can be synthesized by a variety of bacteria, such as *Agrobacterium* sp. (previously taxonomically classified as *Alcaligenes faecalis* var. *myxogenes*), *Pseudomonas* sp. and *Bacillus* sp. [21–23]. Most of the current research on bacterially produced curdlan is focused on *Agrobacterium* sp. However, curdlan from different sources exhibited different physical properties [23]. To meet the demand for curdlan with different properties, it is necessary to isolate curdlan-producing bacteria from different sources [24,25]. Aniline blue is a dye that can specifically bind curdlan, so it can be used to isolate curdlan-producing bacteria [1,23].

In this study, a curdlan producing *Pseudomonas* sp. was isolated from soil samples, and curdlan yield reached up to 5.92 g L^{-1} in a simple culture medium. To our best knowledge, this is the highest reported yield about curdlan produced from *Pseudomonas* sp.

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http://dx.doi.org/10.1016/j.ijbiomac.2016.04.027
0141-8130/

Moreover, the molecular weight of this curdlan was 6.18×10^5 Da, lower than the average molecular weight of curdlan. This low molecular weight endowed it with excellent solubility. This curdlan could be promising for application in many areas related to human life.

2. Materials and methods

2.1. Isolation and identification of the curdlan-producing bacterium

Soil samples were obtained from the wet ground near Jiazi Lake at the University of Jinan, and stored at 4 °C in a refrigerator. Primers were synthesized in BGI, and *Taq* enzyme was purchased from Fermentas. Other chemicals were all of analytical grade and purchased from Sigma-Aldrich.

About 10 g of soil samples were added to an Erlenmeyer flask with 100 mL of distilled water and incubated at 30 °C with shaking at 120 rpm for 2 h using a rotary shaker (DHZ-032R, Shanghai, China). One milliliter of the supernatant was added to 9 mL of sterile saline and serial dilutions (10^{-1}–10^{-6}) were successively prepared. Each dilution (0.2 mL) was added and distributed on agar media that contained 10 g L^{-1} of sucrose, 5 g L^{-1} of yeast extract, 0.05 g L^{-1} of aniline blue, and 15 g L^{-1} of agar, and were incubated at 30 °C for 48 h (DNP-9162, Shanghai, China). The blue-stained were isolated and preserved for further experiments.

16S rDNA was amplified by PCR using the genomic DNA of the isolated strain as the template. The PCR amplification of the 16S rDNA was performed in a 25-μL reaction mixture [26]. The reaction conditions used were 36 cycles at 94 °C (40 s), 52 °C (40 s) and 72 °C (90 s). The amplified DNA fragment was purified using a gel extraction kit.

2.2. Medium and culture conditions

The curdlan-producing bacterium was inoculated into a 250-mL Erlenmeyer flask containing 50 mL of fermentation medium (pH 7.0) and incubated for 16 h at 30 °C with shaking at 200 rpm, which was used as the initial culture. Then, 10% (v/v) of the initial culture was inoculated in an Erlenmeyer flask (500 mL) containing 100 mL of fermentation medium, and the culture was incubated at 30 °C, on the rotary shaker at 200 rpm for 72 h.

2.3. Purification of the polysaccharide

To obtain the polysaccharide, samples were taken from the fermentation medium and centrifuged for 20 min at 8000 rpm to remove cells and other precipitates (TGL–20 M, Changsha, China). The supernatants were mixed with three volumes of absolute ethanol to precipitate the EPS and let sit for 24 h, after the precipitate was vacuum freeze dried (LGJ-18S, Beijing, China). Impurity proteins were removed using the Sevag method, and the Coomassie Brilliant blue method was used to quantify residual proteins in the polysaccharide [24,26].

To determine the purity of the polysaccharide, it was purified to homogeneity using CM exchange chromatography and Sephacryl S-500 column chromatography, as described by Li et al. [26]. The total sugar content of the polysaccharide was determined with the phenol-sulfuric acid method using glucoses as standard solutions. The methods of anthrone reaction, carbazol-sulfuric acid and Elson–Morgan were used to determine the neutral sugar, uronic acid and amino sugar contents, respectively [26]. The average molecular weight was determined by high-performance size exclusion chromatography-multiangle laser light scattering (Wyatt, DAWN HELEOS) and a refractive index detector (Agilent, G1362A) [24].

2.4. Analysis of the purified polysaccharide

The composition of the purified polysaccharide was determined by analysis of the monosaccharide using high-performance liquid chromatography (HPLC) equiped with a refractive index detector (Thermo U-3000, USA). Approximately 20 mg of purified polysaccharide was completely hydrolyzed at 100 °C for 2 h following the addition of 4 mL of 1 M sulfuric acid, and calcium carbonate was added as a neutralizer until no bubbles were present. The mixture was then centrifuged at 8000 rpm, and the supernatant was filtered and concentrated by vacuum freeze drying. HPLC analysis was carried out on a Shodex Ohpak SB-804 column (8.0 mm × 300 mm, 5 μm) (Agilent). The mobile phase was anhydrous sodium sulfate, and was degassed in an ultrasonic bath prior to use. The flow rate was 0.5 mL min^{-1} and a 5-μL aliquot of the sample solution was injected into the HPLC system. All samples and standards were filtered through 0.45-μm Millipore membrane before use [24].

The acid hydrolyzate of the purified polysaccharide was detected by gas chromatography and mass spectrometry (GCMS-QP 2010, Shimadzu, Japan) following Li et al. [26]. The configuration of the purified curdlan was determined by ^{1}H NMR (nuclear magnetic resonance) and ^{13}C NMR analysis using a Bruker Advance 600 spectrometer (Bruker, Fällanden, Switzerland) [25].

Infrared spectra of the polysaccharide were recorded in a frequency range of 4000–400 cm^{-1} using a Nicolet IR200FT-IR spectrometer (Thermo Fisher Scientific). The KBr tablets were prepared in the following steps: 2 mg of the sample was mixed with 200 mg of analytically pure dry potassium bromide (KBr, purchased from Sigma-Aldrich), and the mixture was ground and compressed into a translucent pellet. Scanning electron microscopy was performed using the following method: the sample was coated with a thin layer of gold by means of ion sputter coating, and then subjected to scanning analysis with an accelerating voltage of 10 kV using a scanning electron microscopy (Quanta 250 FEG, FEI, USA).

2.5. Optimization of the medium and culture conditions of curdlan production

The culture conditions, which included inoculum size, seed age, fluid volume, incubation temperature, agitation rate, and fermentation period, were optimized respectively.

Carbon sources, nitrogen sources and initial pH values of the medium have been considered as three major variables affecting the production of curdlan by *Pseudomonas* sp. [23]. Carbon sources and nitrogen sources were investigated by single-factor experiments, with sucrose, galactose, starch, glucose, maltose, lactose, and dextrin were used as carbon sources; yeast extract, peptone, KNO_3, NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3, and $NaNO_3$ were used as nitrogen sources. Concentrations of the optimal carbon sources and the optimal nitrogen sources, and initial pH levels of culture media on the production of curdlan were further investigated.

The experimental design and statistical analysis were performed according to the response surface methodology for optimization of fermentation parameters. Based on the results of the investigations above, three key factors (carbon source, nitrogen source and the initial pH) significantly affected curdlan production which were selected for further analysis using a Box-Behnken design (BBD). A quadratic model was employed to study the combined effect of three independent variables, which were concentration of the optimal carbon source (25–35 g L^{-1}), concentration of the optimal nitrogen source (5–7 g L^{-1}), and initial pH of the culture medium (7.5–8.5).