Process Biochemistry 43 (2008) 1195–1201

Contents lists available at ScienceDirect

Process Biochemistry

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

Seawater-retting treatment of hemp and characterization of bacterial strains involved in the retting process

L.L. Zhang [a], R.Y. Zhu [a], J.Y. Chen [b], J.M. Chen [a,*], X.X. Feng [b,**]

[a] School of Biological and Environmental Engineering, Zhejiang University of Technology, No. 6 District, Zhaohui, Hangzhou 310032, China
[b] The Key Laboratory of Advanced Textile Material of Education Ministry, College of Materials and Textiles, Zhejiang Sci-Tech University, Hangzhou 310018, China

ARTICLE INFO

Article history:
Received 2 October 2007
Received in revised form 23 June 2008
Accepted 24 June 2008

Keywords:
Seawater retting
Strains
Characterization
Identification
BIOLOG system
ERIC-PCR

ABSTRACT

Pectin and hemicellulose contents were proportionally lower in the seawater-retted fibers compared with the raw hemp fiber according to the Fourier-transform infrared spectroscopy (FTIR) and chemical analyses. Also, the scanning electron microscopy (SEM) observation revealed the non-cellulosic gummy materials in hemp fibers could be removed to a considerable extent by the seawater-retting treatment. Cultivable bacteria involved in this retting process were isolated, and three of purified strains (*SW-1*, *SW-2*, and *S-SW1*) produced relatively high levels of pectinase activities and had good retting ability. The ERIC-PCR fingerprints of stains *SW-1*, *SW-2*, and *S-SW1* were different, and should be further identified for each isolate. Strains *SW-1* and *SW-2* were identified as *Stenotrophomnas maltophilia*, while strain *S-SW1* was assigned to *Ochrobactrum anthropi* by BIOLOG system. These two species represented rhizosphere bacterial genera, and were possibly introduced by the hemp plants. These organisms seemed potentially capable of producing pectinase, and thus effectively degrading pectin substances in this retting process.

1. Introduction

Hemp is also called *Cannabis sativa*. It is an annual herbaceous plant native to Asia and widely cultivated in many parts of the world [1]. Hemp has traditionally been grown for their valuable and versatile high-quality bast fibers, which have been widely used in the production of cords and clothing, and have potential for reinforcement in polymer–matrix composites [2]. Hemp fibers are formed in the cortical regions of stems. To extract fibers for industrial uses, stems are retted, which is usually a microbial process that separate fiber from non-fiber stem issues. In this process, bast fiber bundles are separated from the core, epidermis, and cuticle and are also separated into smaller bundles and individual fibers. This separation is accomplished by removal of pectin and hemicelluloses from parenchyma cells and the middle lamellae.

Traditionally, two retting methods (dew retting and water retting) have been used, both carried out by pectic enzymes secreted by indigenous microflora. In dew retting, straw is spread on the ground, and pectins are attacked by pectinolytic microorganisms, mainly aerobic fungi [3]. In tank retting, straw is submerged in large freshwater tanks, where a pectinolytic bacterial community is developed [4]. Clostridia are considered to be the major group of bacteria responsible for water retting [5]. Water retting was the method of choice for many years because of high-quality fiber. In the mid-1990s water-retting was mostly discontinued in western countries because of the contamination and consumption of the freshwater [6]. Currently, dew-retting is the primary process used for the industrial production of bast fibers although water retting is still carried out in some places [7]. However, disadvantages also exist for this method that include: low and inconsistent quality, restriction to certain climatic regions, occupation of land for several weeks during retting, and a product contaminated with soil. As a result of these problems, a better retting method has been sought for some time. Enzyme-retting has been evaluated as a replacement for current retting methods. However, cost of the enzymes, and perhaps other less obvious reasons, prevented development of a commercial enzyme-retting process [6]. In fact, water retting still remains the practice most widely used in China to obtain fibers commercially for industrial use, despite continuing research on other methods. With freshwater resource becoming increasingly scarce, an alternative or improvement in water retting will have to play a greater role in dealing with water scarcity and reducing the pollution.

* Corresponding author. Tel.: +86 571 8832 0386; fax: +86 571 8832 0386.
** Corresponding author. Tel.: +86 571 8684 3622; fax: +86 571 8684 3622.
E-mail addresses: jchen@zjut.edu.cn (J.M. Chen), fengxx@zstu.edu.cn (X.X. Feng).

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doi:10.1016/j.procbio.2008.06.019

Seawater is the only nature resource considered to be inexhaustible, and it is abundant in many countries and regions in the world. The ocean has a high environmental capacity and self-purification ability, and could receive a large amount of wastes without risking the contamination of the marine environment since the wastes are diluted by a vast quantity of seawater in the ocean [8]. According to the above background, if the seawater in some countries, especially in China, where seawater is accessible and abundant, could be exploited for a substitute of freshwater in the retting process, the freshwater consumption would be greatly reduced.

To the best of our knowledge, no study has so far utilized seawater for retting bast fibers. Also, the microbiology of seawater retting of hemp has not been studied. Therefore, this paper attempts to remove non-cellulosic gummy materials from hemp using seawater-retting treatment, and focuses on the characterization of bacterial strains involved in the retting process to search for superior retting organisms.

2. Materials and methods

2.1. Hemp and substrates

The hemp ribbon was supplied by Yunnan Hemp Industry Co. Ltd (Yunnan province, China), which was cut to about 8 cm. The seawater used for the retting was from Beilun Seaport of Ningbo in China. All chemicals used for the experiment were of analytical grade. The pectin, dinitrosalicylic acid (DNS), and D-galacturonic acid were obtained from Sigma (Sigma–Aldrich Chemical Co., USA).

2.2. Seawater-retting experiment

The freeze-dried hemp in 8-cm length (30 g) was dipped in 750 mL seawater in 2-L plastic tanks, and incubated at 28° for 2 weeks. The retted hemp was harvested and used for testing the retting efficiency. The raw hemp and fresh seawater was in tank 1, the sterilized seawater but raw hemp was in tank 2, the sterilized hemp and fresh seawater was in tank 3, and both hemp and seawater in tank 4 were sterilized. The raw hemp was dipped in the freshwater for controls. All the harvested samples were freeze-dried, weighted and stored at −20°.

2.3. Morphological and chemical properties

Scanning electron microscopy (SEM, JSM-5610LV) was used to observe the microstructure and surface morphology of treated and untreated hemp fibers. Samples were prepared for SEM by method described by Henriksson et al. [3]. Fourier-transform infrared spectroscopy (FTIR) spectra were performed using a PerkinElmer 2000 spectrometer. For each sample, a total of 32 scans were accumulated with a resolution of 4 cm^{-1}. The proportions of major fiber components, such as cellulose, hemicellulose, lignin, wax + fat, and pectin fractions present in the retted and unretted samples were determined by method described by Sharma et al. [9]. Each sample was analyzed in triplicate.

2.4. Pectinase activity

Pectinase assay was carried out using the method of Silva et al. [10]. Briefly, 2 mL of filtrate was added to 2 mL of 0.4% pectin in 0.1 M acetate buffer (pH 5.0). After incubated at 45 °C for 30 min, 1.5 mL of DNS reagent was added and boiled for 15 min. The reaction mixture was diluted to 25 mL with de-ionized water. Absorbance at 520 nm was measured to determine the amount of reducing sugars using galacturonic acid as a reference. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of galacturonic acid per minute under the given conditions. All measurements were in triplicate.

2.5. Isolation of pectinolytic bacteria

The liquor in seawater-retting tanks 1 and 2 was diluted serially with medium (10^1–10^7-fold dilution), and 150 μL of each dilution was spread onto agar plates. The plates were incubated at 25° for 2–7 days. The colonies representing different morphologies were picked at random and purified by re-streaking on the same medium plates. The pectin-degrading bacteria were selected by subculturing in MSM medium [11] with pectin as the carbon source and comparing pectinase activity, in which the strains with high pectinase activity were obtained and used for the further studied. The medium (per L) consisted of 16.266 g of K_2HPO_4, 0.899 g of KH_2PO_4, 1.2 g of $(NH_4)_2SO_4{\cdot}7H_2O$, 0.075 g of $CaCl_2$, 2.0 g of agar, and 4.0 g of pectin [11], supplemented with trace elements and vitamins [3].

The isolated strains were cultivated in shake flasks at shake flasks at 30° and 140 rpm in 1-L Erlenmeyer flasks with 300 mL of MSM medium with pectin as the carbon source. Samples for measurement of pectinase activity were taken periodically during the cultivation. Insoluble material was removed from the culture filtrates by centrifugation at 5000 × g, and pectinase activity of supernatants was determined according to the DNS method mentioned above.

2.6. Retting experiment by pectinolytic isolates

Pectinolytic isolates were incubated in 500-mL MSM medium with pectin as carbon source at 28° for 48 h and the culture was used for retting tests. The freeze-dried hemp (2 g) being autoclaved in 6 mL distilled water was dipped in 40 mL culture and cultivated at 28° for 36 h. After the culture was decanted the retted hemp was washed with distilled water three times and the retting efficiency was assessed by weight loss and residual pectin analysis by the described method [12].

2.7. ERIC-PCR amplification

DNA was isolated using a Qiagen DNeasy Tissue kit. The ERIC-PCR was preformed as described by de Bruijn [13] with a PTC-200 DNA Engine® Thermal Cycler (BIO-RAD, Laboratories Inc.). The primers used were the ERIC1 (5′-ATGTAAGCTCCTGGG GATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′). The reaction mixture in a 25-μL volume contained 2 μL of 2.5 mM dNTP; 2.5 μL of 10× buffer; 2 μL of 25 mM $MgCl_2$; 0.5 U Taq DNA polymerase; 12.5 pmol of ERIC1 and ERIC2 primers. The PCR conditions were: (95 °C/7 min) 1×, (94 °C/1 min, 52 °C/1 min, and 65 °C/8 min) 30×, and (65 °C/10 min) 1×. After the reactions, 8–10 μL of the ERIC-PCR products were separated on 1% agar gels, and stained with ethidium bromide. Images were documented using a GelDoc 2000 and Quantity one software (BIO-RAD).

2.8. Identification of strains by BIOLOG system

Ahead of the BIOLOG analysis, gram reaction was determined with 3% potassium hydroxide [14], and spores formation was determined by phase-contrast microscopy. The isolates were identified on the basis of their carbon substrate oxidation patterns using the standard protocols (BIOLOG, Hayward, CA, USA). BIOLOG GN and GP microplates, each containing 95 individual carbon substrates, plus a negative control, were used to identify isolates. The isolates were prepared according to BIOLOG instructions that include culturing on BIOLOG Universal Growth (BUG) agar, preparation of a standardized liquid suspension based on turbidity and inoculation of one GP2 MicroPlate™ per culture. After incubation (at 37 °C for 24 h), the optical density at 590 nm produced from the reduction of tetrazolium violet in each well was read using a BIOLOG Microplate reader in conjunction with the MicroLog software (Release Version 4.20.04). A correct identification was attained when the similarity index (SIM) and genetic distance (DIS) values were >0.500 and <5.00, respectively [15].

3. Results and discussion

3.1. Chemical and morphological properties of hemp

Infrared spectra of unretted and retted hemp fibers are shown in Fig. 1. Pectin contains both esterified and carboxylic acid groups in the structure. The FTIR spectrum of the raw hemp showed a band at 1733 cm^{-1}. The presence of this band indicated the existence of pectin [16]. In general, the spectrum of seawater-retted hemp is similar to that of the raw hemp. However, the vibration peak at 1733 cm^{-1} attributed to the C=O stretching of methyl ester and carboxylic acid in pectin and hemicellulose disappeared from the seawater-retted hemp. This indicated the removal of pectin and partial hemicelluloses by the seawater-retting treatment. The band at 1372 cm^{-1} was attributed to a C–H vibration in the cellulose, and the band at 897 cm^{-1} corresponds to β-D-glycosides [17]. This indicated cellulose content in hemp fibers did not exhibit significant changes during the seawater retting. The bands at 1626 cm^{-1} and 1514 cm^{-1} were assigned as aromatic vibration [17], indicating the existence of lignin in the unretted and retted hemp fibers.

The chemical composition proportions of hemp fibers are listed in Table 1. The proportions of cellulose, hemicellulose, pectin, and lignin fractions present in the retted and unretted fiber samples were significantly different. Comparisons of pectin and hemicellulose contents of the samples have shown that pectin and