Industrial Crops and Products 97 (2017) 518–524

Contents lists available at ScienceDirect

Industrial Crops and Products

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

Research paper

Isolation of *Bacillus cereus* P05 and *Pseudomonas* sp. X12 and their application in the ramie retting

Qian Wang[a,b], Hong-gao Chen[b], Gang Fang[b], An-qing Chen[b], Peng Yuan[b], Jian-she Liu[a,*]

[a] College of Environmental Science and Engineering, Donghua University, Shanghai, 201620, China
[b] Engineering Research Center for Clean Production of Textile Dyeing and Printing, Ministry of Education, Wuhan Textile University, Wuhan, 430200, China

ARTICLE INFO

Article history:
Received 14 July 2016
Received in revised form
14 December 2016
Accepted 27 December 2016

Keywords:
Ramie
Retting
Dominant bacteria
Isolation
Bioaugmentation

ABSTRACT

Two bacterial strains that express high levels of pectinase and xylanase, named P05 and X12, were isolated from the soil of a ramie garden using pectinase and xylanase selective medium. A bioaugmentation experiment for ramie retting using these bacterial strains showed a significant increase in retting rate when equal ratios of P05 and X12 were mixed into the retting solution at 20% of the volume. Using bioaugmentation, the retting process was completed at 60 h, reducing the retting period by 50%, and the gum removal ratio improved by 79.6%. In addition, the breaking strength of the resulting fiber was increased by 52.3%. Analysis of bacterial population structure and enzyme activity levels in the bioaugmented retting system showed that P05 and X12 quickly became the dominant bacteria within the retting solution microflora, and improved the microflora's ability to produce key retting enzymes. The maximum pectinase and xylanase activities in the bioaugmented retting system were 80.84 U/mL and 50.51 U/mL, respectively, which were 68.98% and 64.21% higher respectively when compared to the control. Thus, addition of P05 and X12 into the retting solution resulted in accelerated gum degradation and fiber separation.

1. Introduction

Ramie (*Boehmeria nivea*) produces one of the strongest and longest plant fibers with a silky appearance. Products that use ramie fiber include clothing, industrial packaging, twine, cordages, canvases, and car covers (Brühlmann et al., 2000). However, decorticated ramie fiber contains about 30% gum, which consists primarily of pectin and hemicellulose. The conventional retting process consumes high levels of NaOH and energy, which can result in environmental pollution. It is therefore imperative to find substitutive techniques for retting fibers (Zheng et al., 2001).

There are three biological retting methods for ramie: enzymatic, bacterial, and the use of artificial microflora. Enzymatic retting removes gum from ramie using pectinase (Akin et al., 2001) and xylanase (Beg et al., 2001). Purchasing purified pectinase and xylanase can be expensive. Furthermore, these enzymes are unable to completely remove gum from ramie, necessitating the need to identification of more effective retting enzymes. Bacterial cultures from natural environments can be used for retting, and these bacteria are primarily from the genera *bacillus* (Basu et al., 2009), *Pseudomonas* (Kashyap et al., 2001), *Enterobacter* (Rajesh et al., 2013) and *Aspergillus.* Retting using pure bacterial cultures takes longer than retting with purified enzymes, as natural bacteria strains have low enzyme activity. In addition, the procedure is aseptic and technically difficult to prepare, making this method less practical for industrial applications. While the shortcomings of retting with purified enzymes and pure bacterial cultures can be avoided by the use of artificial flora instead, this method also has its drawbacks; specifically, the loss of pro-retting bacterial strains during microflora preparation, and the difficulty of obtaining the bacterial consortium (Banik et al., 2007; Sabra et al., 2010). Modern bio-degumming techniques using enzymatic degumming or pure bacterial cultures mentioned above have not yet been applied at an industrial scale due to high cost, long retting period, and low fiber quality.

The majority of industries in Southeast Asia, including China and India, use the ancient technique of bio-degumming by water retting for flax, ramie, jute and hemp (Kozlowski and Maria, 2009). Water retting has been practiced in China for more than 2000 years, and involves the immersion of ramie stems in a pond or river; relying on the microbial community in the water to decompose gum. Although water retting is simple and economic, it can be time consuming and difficult to control since the retting process depends on natural conditions such as climate, weather,

* Corresponding author.
E-mail address: liujianshe@dhu.edu.cn (J.-s. Liu).

http://dx.doi.org/10.1016/j.indcrop.2016.12.047
0926-6690/

Table 1
Experimental system of bioaugmented ramie retting.

Group	Inoculums			Pond water (L)	Raw ramie (kg)
	P05 (L)	X12 (L)	Inoculation ratio (%)		
1	2.5	2.5	10	45	5
2	5	5	20	40	5
3	7.5	7.5	30	35	5
4	10	10	40	30	5
5	12.5	12.5	50	25	5

and aboriginal microbial communities (Marek et al., 2008). Attention has now shifted to bioaugmentation retting, where selected strains/mixed cultures of bacteria are added to natural retting systems to enhance the efficiency of gum removal. For example, adding fungal complexes to flax during water retting has a positive effect on the fiber separation and quality of the resulting flax fiber (Repečkienė and Jankauskienė, 2009). Hemp water retting takes a significantly shorter time and generates higher quality fiber when bioaugmented with two selected strains of pectinolytic bacteria (Di Candilo et al., 2010). Similarly, addition of bacteria with polygalacturonase, pectin lyase, and xylanase activity significantly reduces the retting period and increases fiber strength during jute fiber degumming (Das et al., 2012). These studies about bioaugmentation studies suggest that bioaugmented retting is a promising technique to solve problems encountered in traditional water retting of fiber, yet it has rarely been applied in ramie retting.

In the ramie industry, it is imperative to develop an alternative biological retting method to reduce pollution, reduce cost, simplify operations, and enhance efficiency and quality. In the present study, we tested bioaugmentation methods to improve the traditional water retting process. Two dominant retting bacteria, P05 and X12, were isolated from garden soil in which ramie was growing, and inoculated into the retting tank. We then optimized technological parameters to improve retting conditions. Our results suggest that inoculating ramie during water retting greatly increased the separation process speed, and remarkably improved the ramie fiber quality. In addition, we investigated the zymologic and microbiological mechanisms in order to improve the retting process with these microbes.

2. Materials and methods

2.1. Experimental material

Raw ramie, variety Zhongzhu No.1, was provided by Hubei Xinnong Eco-Ramie Co., LTD, China. Ramie bark was manually stripped from the core, dried in the field, and stored for approximately two years.

2.2. Experimental method

2.2.1. Isolation and preservation of retting dominant bacteria

Sampling: The upper 10 cm portion of the soil was collected from a garden that has grown ramie for over 12 years, located in Xianning, China (29°87′N, 114°28′E). Pond water was collected from a retting pond nearby the ramie garden. Five hundred liters of soil leachate and pond water were sampled respectively, and then mixed together.

Isolation and preservation: The mixture of soil leachate and pond water was streak plated on media containing pectin and xylan, cultured for 48 h at 42 °C, and stained using Congo red. Bacterial colonies with hydrolysis circles were selected for isolation until pure colonies were obtained by microscopic examination. The pure bacteria cultures were inoculated onto beef extract peptone media, and were preserved after culturing for 24 h at 42 °C with 150 r/min shaking.

2.2.2. Optimization of the bioaugmentation conditions for retting

Two strains were selected, named P05 and X12, and cultured for 48 h at 42 °C with 150 r/min shaking in a liquid beef extract peptone media, respectively. Culture volume was gradually increased until it was sufficient for the retting bioaugmentation experiment. All bioaugmentation experiments were conducted at 42 °C, and the culture was turned over every 12 h.

The effect of each strain on ramie retting was determined as follows: 10 L P05 enlarged cultures or 10 L X12 enlarged cultures or 10 L mixtures of equal volume of P05 and X12 were added to 40 L pond water with 5 kg ramie at 24 h after retting was initiated. The inoculum was replaced by 10 L pond water in the control. Experiments on the effects of different inoculation scale on the ramie retting were conducted, as shown in Table 1. Pure cultures were added at 24 h after retting was initiated.

Effects of inoculation stage on ramie retting was determined by starting the retting process by combining 40 L of pond water with 5 kg of ramie, then inoculating the mix with 5 L of P05 and X12 inoculum at 0 h, 24 h, 48 h, 72 h and 96 h after retting was initiated. Effects of temperature on the ramie retting were determined similarly by adding 5 L of P05 and X12 inoculum at 24 h after the retting was initiated, then culturing the mixture at 30 °C, 37 °C, 42 °C, 45 °C and 48 °C. Lastly, influences of pH on retting were determined by mixing 5 kg ramie and 40 L pond water, culturing the mixture at 42 °C, and adjusting the initial pH of the retting solution to 5.5, 6.5, 7.5, 8.5 or 9.5. Five liters of P05 and X12 inoculum were then inoculated into the tanks after 24 h of culturing.

Retting was considered completed once three ramie retting workers affirmed that the ramie fiber had separated into cotton-like fiber. The retting period was defined as the time between initiation of the retting process and receipt of fiber quality approval by the workers.

Data was analyzed with SPSS 19.0, using one-way ANOVA test for one-way analysis of variance. Where differences were found to be significant, Duncan's method was used for multiple comparisons.

2.2.3. Evaluation of gum removal rate and bundle fiber breaking strength

Gum removal rate and bundle fiber breaking strength were determined after the ramie fibers were cleaned under running water and dried. The method of analysis of gum removal rate was conducted as previously reported (Das Gupta et al., 1976), and bundle fiber breaking strength was determined according to China Textile Industry Association standards (1986). The surface features of the ramie fibers were examined by scanning electron microscopy (SEM) (Phenom Pro, Philips, Netherlands).

2.2.4. Enzymatic analysis

Optimal retting occurred when 40 L of pond water and 5 kg ramie were mixed at 42 °C, pH 8.5, and 20% of inoculum containing equal ratios of the two bacterial strains at 24 h after retting