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Diversity and characterization of ramie-degumming strains

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ABSTRACT: Ramie (*Boehmeria nivea* and *Boehmeria tenacissima*) is a widely used fiber crop. Traditional water retting or chemical boiling method performed in order to extract ramie fiber seriously pollute the environment and severely damage the fiber, so biological method is the general trend of the fiber-extracting industry. Some strains (687), involving 26 genera and 43 species, were collected from the three samples, which produce hydrolyzed circles in the selective culture medium in order to detect the degumming effect and to compare the enzyme activity. Among these strains, 13 of them did not produce cellulase and had a ramie decreasing weight rate above 25 %, which were regarded as efficient ramie-degumming strains named from R1 to R13. R1 to R13 belonged to *Amycolata autotrobutylicun, Bacillus subtilis, Clostridium acetobutylicum, Bacillus subtilis, Rhizobium leguminosarum, Bacteroides finegoldii, Streptomyces lividans, Bacillus amyloliquefaciens, Clostridium acetobutylicum, Pseudomonas brassicacearum, Bacillus pumilus, Bacillus licheniformis, Pectobacterium wasabiae* respectively. *Bacteroides* sp., *Rhizobium* sp. and *Pseudomonas* sp. were firstly reported to be used in ramie-degumming. At the same time, the pectinase was the key enzyme in the ramie-degumming process.

Keywords: fiber, bio-extracting, microorganism, resource

Introduction

Ramie, including *Boehmeria nivea* and *Boehmeria tenacissima*, belongs to Urticaceae, *Boehmeria* genus, which is regarded as one of the oldest textile fiber yield plants (Ghosh and Ghosh, 1995). It grows from 25° S to 39° N, mainly in China, Brazil, India, the Philippines, and Vetnam (Xiong, 2008). Ramie has furrow shaped air containing space, slender dissepiment, tenacity, and porosity. It is a good raw material textile for air permeability, moisture absorption and heat elimination, which is one of the strongest vegetable fiber known in the world today. It possesses highest strength and length, good durability and absorbency with excellent lustre. These remarkable characters make it rather more suitable for use in the manufacture of wide variety of textiles and cordage products (Dempsey, 1975; Mussig et al., 1998). Food and Agriculture Organization of the United Nations reported that 280,000 t ramie was harvested in 410,000 ha planting areas in 2007 in the world (Xiong, 2008).

Ramie contains 20 % ~ 40 % gum mainly composed of pectin and hemicellulose, but only the purely degummed ramie can be used for spinning and weaving. Traditional degumming methods are water retting and chemical boiling, which seriously pollute the environment and severely damage the fiber (Bhattacharyya and Paul, 1976; Gupta et al., 1976). Since 1954, scientists have done researches in biological ramie-degumming and enzymic degumming method (Paul and Bhattacharya, 1979). For bio-procession can override the defects caused by traditional procession, bio-degumming is the general trend of the ramie process (Liu et al., 2001; Zhou, 1978). However, the non-cellulose components are complicated, and the abundance and quantity of enzymes secreted by single microorganism are hard to match specifically with the components of non-cellulose (Puls et al., 1997), so few strains have been chosen successfully for large-scale industrial applications (Basu et al., 2009).

The diversity and character of ramie-degumming strains is reported for the first time, which will promote the development of ramie bio-degumming technology.

Materials and Methods

The water sample was from a large ramie retting pool from 2 to 200 cm in depth with temperature of 24~27 °C, the soil sample was from a farmland sown with ramie from 5 to 20 cm in depth, and the humus sample was from rotten ramie. The three samples were collected from Yuanjiang, China (28°50′ N, 112°22′ E), Fuyang, China (32°55′ N, 115°48′ E) and Xiaoshang, China (30°16′ N, 120° 25′ E), respectively.

The strains T11-01 without degumming function was selected and deposited by the authors, which was chosen as the control bacterium for the ramie degumming strains.

Each sample was added to a conical flask containing 200 mL sterile water. After mixing, 15 g ramie was put into the conical flask, then cultured at 30 °C for 20 h. Ten mL the last cultured liquor was taken to another enrichment culture using ramie for 20 h, and then repeated for three times. One mL such enrichment culture liquor was put to dilute and isolate in selective culture medium at 30 °C for 10 h. Finally the single colony was got from the selective culture medium. Each liter of the selective culture medium contained 0.5 g K_2HPO_4, 0.5 g NaH_2PO_4, 2.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, and 0.1 g $CaCl_2$, 1 g ramie powder. The strains were purified and routinely cultured at 30 °C on nutrient broth agar medium (g L^{-1}): 10.0 peptone, 3.0 beef extract, 5.0 NaCl, and 20 agar, and on Martin agar medium (g L^{-1}): 10.0

glucose, 5.0 peptone, 1.0 K_2HPO_4, 0.5 $(NH_4)_2SO_4$, and 20 agar respectively, then maintained as glycerol suspension (20 %, w/v) at -70 °C.

To exclude the strains producing cellulase, the strain suspensions were cultured in the selective medium at 30 °C for 36 ~ 48 h. If hydrolyzed circles appeared around the strains colonies, it was as positive, otherwise, it was as negative (Dong and Cai, 2001). One liter of the cellulose decomposing culture medium contained NH_4NO_3 1.0 g, $CaCl_2$ 0.1 g, K_2HPO_4 0.5 g, $FeCl_3$ 0.02 g, KH_2PO_4 0.5 g, yeast powder 0.05 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, NaCl 1.0 g, fiber powder 8 g and agar 15 g.

Four mL fermentation fluid of strain was put into conical flask containing 200 mL sterile water and dried ramie (M_0). After static cultivating at 30 °C for 1 ~ 6 days, rinsing was performed, after it, drying to a constant weight (M_1), and then the weight decreasing rate was calculated. The weight decreasing rate $V = (M_0 - M_1)/M_0 \times 100$ %. If the weight decreasing rate was above 25 %, it was regarded as the effective degumming strain (Zeng and Xiang, 2007).

The dried ramie was boiled for 2 h in the fixed condenser-Allihn type containing 200 mL of 20 g L^{-1} NaOH. The sample was picked out, washed, and finally dried to a constant weight. We recorded the weight, and calculated the residual gum rate, and evaluated the degumming effect by weight decreasing rate and residual gum rate (Jiang and Shao, 2005).

Morphology, physiology and biochemistry properties of the selected strains were analyzed according to Bergey's Manual of Determinative Bacteriology (9th edition) and identified them by the 16S rRNA or 18S rRNA gene sequence.

The bacteria genomic DNA was extracted using UNIQ-10 Column Strains Genomic DNA Extraction Kit (produced by Shanghai Sangon, China), and the fungus genomic DNA was extracted using Biospin Fungus Genomic DNA Extraction Kit (produced by Biospin).The 50 µL reaction mixture for PCR of rRNA gene contained 0.5 µM each of the forward and reverse primers (16S rDNA 27F: AGAGTTTGATCMTGGCTCAG, 1492R: TACGGYTACCTTGTTACGACTT; 18S rDNA NS1: GTAGTCATATGCTTGTCTC, NS8: TCCGCAGGTTCACCTACGGA), 25 µL Taq PCR Mix (Shanghai Sangon, China), 50 ng of DNA, and 20 µL H_2O. The cycling conditions were as follows: 5 min at 95 °C for pre-denaturation, followed by 0.5 min at 94 °C for denaturation, 1 min at 52 °C for annealing, 1 min at 72 °C for extension for 30 cycles, and, finally, preservation at 72 °C for 10 min. Purification and sequencing were carried out by Dalian TaKaRa Co., Ltd. using the dideoxy chain termination method with 3730 XL DNA sequencer (Applied Biosystems, USA). The 16S rRNA gene sequences were aligned using the Clustal-X program (Thompson et al., 1997). A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) and the maximum-parsimony method (Fitch, 1972) in the MEGA3 Program (Kumar et al., 2004) with bootstrap values based on 1,000 replications (Felsenstein, 1985).

The enzyme activity was defined as the amount of the enzyme required for 1 µmol substrate to be released per minute. The enzyme activity of the strains was detected in the fermentation liquor at 8 h by DNS method (Wang et al., 2009) and hydrolyzed circles method.

The optimization components in selective plates were as follows: (i) Xylanase activity detection: oats xylan was used as the substrate, and Congo Red as the dyestuff (Teather and Wood, 1982; Huang et al., 2006); (ii) Mannanase activity detection: konjac gum was used as the substrate, and trypan blue as the dye-stuff (Mendoza et al., 1995). (iii) Pectinase activity detection: orange pectin was used as the substrate, and brilliant green as the dye-stuff (Keen et al., 1984).

Results and Discussion

Some (687) strains were collected from the three samples, which produce hydrolyzed circles in the selective culture medium. The strains were identified according to morphological features, physiological-biochemical characteristics and 16S rDNA (18S rDNA) sequences. One hundred seventy two representative sequence data had been submitted to GenBank (sequence numbers: EU982468-EU982547, FJ544315-FJ544406, and GU097444-GU097456). The 687 strains involved 26 genera and 43 species; they belonged to 599 bacteria, 8 actinomycetes, 65 filamentous fungi, and 15 yeasts, accounting for 87.19 %, 1.16 %, 9.46 %, and 2.18 % respectively. The resources were widespread, which were isolated from soil, water, humus, accounting for 42.21 %, 23.44 %, and 34.35 % respectively (Table 1).

Among these 687 strains, 13 of them did not produce cellulase and degrade above 25 % of the non- cellulose, which were regarded as efficient ramie-degumming strains named from R1 to R13. R1, and R7 to R9 were sampled from Fuyang soil, R2 to R6 from Yuanjiang water, and R10 to R13 from Xiaoshan humus. Based on the 16S rRNA gene sequences, the heredity tree of the strains was constructed (Figure 1), and the main phenotypic characteristics (Table 2). R1 to R13 belonged to *Amycolata autotrobutylicun, Bacillus subtilis, Clostridium acetobutylicum, Bacillus subtilis, Rhizobium leguminosarum, Bacteroides finegoldii, Streptomyces lividans, Bacillus amyloliquefaciens, Clostridium acetobutylicum, Pseudomonas brassicacearum, Bacillus pumilus, Bacillus licheniformis, Pectobacterium wasabiae* respectively.

Fifteen kinds of strains have been reported to degrade the non-cellulose of bast fiber crops, including *Clostridium* sp. (He and Feng, 1997; Munshi and Chattoo, 2008), *Micrococcus* sp. (Sun, 1981), *Actinomycete* sp. Brühlmann et al., 1994), *Erwinia* sp. (Peng et al., 1995; Munshi and Chattoo, 2008), *Amycolata* sp., *Streptomyces lividans* Brühlmann et al., 2000), *Paenibacillus polymyxa* (Yang et al., 2001), *Bacillus* sp. (Zheng et al., 2001; Munshi and Chattoo 2008), *Pseudomonas* sp. (Munshi and Chattoo, 2008), *Achromobacter* sp., *Zoogloea* sp., *Cytophaga* sp. (Munshi and Chattoo, 2008), *Aspergillus* sp., *Humi-*

Table 1 – Species diversity and distribution of the ramie-degumming strains.

Genera	Species	Strains quantity and substrate of isolation				Ratio
		Soil	Water	Humus	Total	
		CFU				%
	subtilis	28	15	19	62	9.02
	cereus	10	5	8	23	3.35
	megaterium	7	8	10	25	3.64
	alvei	6	0	3	9	1.31
Bacillus	*comesii*	6	1	7	14	2.04
	pumilus	2	0	0	2	0.29
	amyloliquefaciens	3	4	1	8	1.16
	licheniformis	0	3	2	5	0.73
	others	46	26	31	103	14.99
	polymyxa	25	18	12	55	8.01
Paenibacillus	*macerans*	13	17	4	34	4.95
	amyloliquefaciens	5	0	4	9	1.31
	others	19	15	26	60	8.73
	felsinews	5	0	0	5	0.73
	pectinovorum	0	2	7	9	1.31
Clostrdium	*tertium*	4	1	2	7	1.02
	acetobutylicum	1	1	0	2	0.29
	others	5	4	12	21	3.06
Brevibacillus	*brevis*	11	2	3	16	2.33
	others	3	2	0	5	0.73
	carotovora	7	11	16	34	4.95
Pectobacterium	*amylovora*	6	0	5	11	1.60
	chrysanthemi	1	0	3	4	0.58
	others	2	1	11	14	2.04
	aeruginosa	16	6	3	25	3.64
	alcaligenes	0	2	0	2	0.29
Pseudomonas	*putida*	0	0	7	7	1.02
	brassicacearum	1	1	0	2	0.29
	others	3	0	1	4	0.58
Amycolate	*autotrophica*	0	0	1	1	0.15
Rhizobium	*leguminosarum*	1	0	0	1	0.15
Pectobacterium	*wasabiae*	1	0	0	1	0.15
Bacterides	*finegoldii*	2	3	1	6	0.87
Micrococcus	/	1	2	3	6	0.87
Natronococcus	/	1	0	0	1	0.15
Alcaligenes	/	0	0	2	2	0.29
Natronobacterium	/	3	1	0	4	0.58
	niger	1	0	1	2	0.29
Aspergillus	*flavus*	1	0	0	1	0.15
	oryzae	1	0	0	1	0.15
	others	0	0	1	1	0.15
Penicillium	*frequentans*	3	0	0	3	0.44
	roquefortii	0	0	2	2	0.29
	others	2	0	1	3	0.44
Cryptococcus	*albidus*	3	1	1	5	0.73
	others	0	3	0	3	0.44
	rulamacerans	0	0	2	2	0.29
Rhodoto	*minuta*	0	1	0	1	0.15
	rubra	2	0	0	2	0.29
	others	1	1	0	2	0.29
Tyromyces	*subcaesius*	2	0	2	4	0.58
	others	2	0	2	4	0.58

Continue...

Table 1 – Continuation.

Fomes	*lignosus*	4	0	2	6	0.87
	others	2	0	2	4	0.58
Phanerochete	*chrysosporium*	4	1	4	9	1.31
	others	1	0	2	3	0.44
Trametes	*sanguinea*	5	0	0	5	0.73
	others	2	1	2	5	0.73
Ceriporiopsis	*subvermispora*	2	0	1	3	0.44
	others	1	0	1	2	0.29
Mucor	/	4	0	1	5	0.73
Fusarium	/	1	0	1	2	0.29
Streptonyces	*tenebrarius*	1	0	4	5	0.73
	lividans	1	2	0	3	0.44
Total		290	161	236	687	/
Percent		42.21	23.44	34.35	/	100

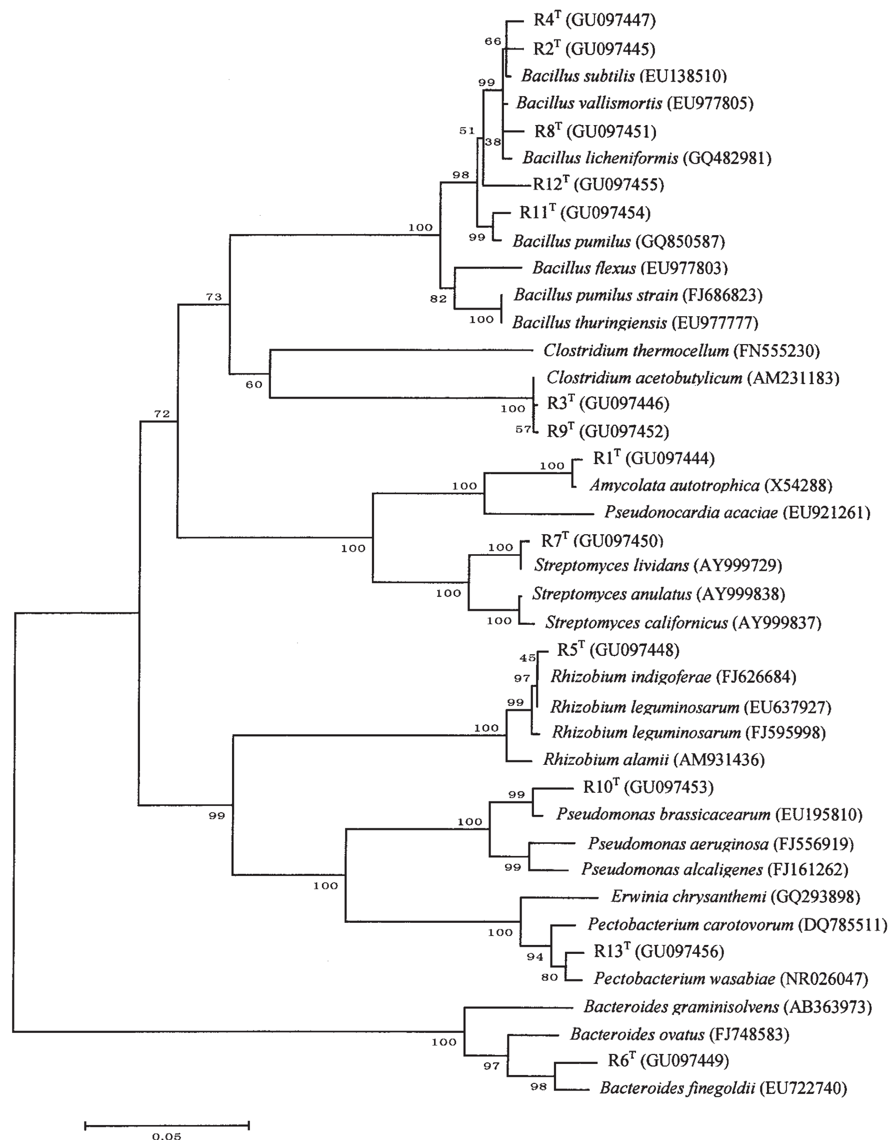

Figure 1 – Phylogenetic tree generated using the neighbour-joining method based on the 16S rRNA gene sequence of selected strains. Numbers at nodes represent bootstrap percentages based on 1000 samplings. The scale bars represent 0.05 substitutions per site. The maximum-parsimony tree showed essentially the same topology (data not shown).

Table 2 – Physiological and biochemical properties of the selected strains.

Characteristic	Strains												
	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13
Casein	-	+	+	+	+	-	+	+	+	+	+	+	+
Gelatin	-	+	+	+	+	-	+	+	+	+	+	+	+
Starch	-	+	+	+	-	-	+	+	+	-	+	+	-
Catalase	+	+	-	+	+	-	+	+	-	+	+	+	+
Urease	+	+	-	+	+	-	-	+	-	-	-	-	-
D-Glucose	+	+	+	+	+	-	+	+	+	+	+	+	+
L-arabinose	+	+	-	+	+	-	+	+	-	-	-	+	+
D-xylose	-	+	-	+	+	-	+	+	-	-	+	+	+
D-Mannitol	+	+	+	+	+	-	+	+	+	-	+	+	+
Indole	-	-	-	-	-	-	-	-	-	-	-	-	+
Nitrate reduction	+	+	-	+	+	-	+	+	-	+	-	+	+

Note: "+", stand for positive; "-", stand for negative.

Table 3 – Material losing rate (MLR) and residue gum rate (RGR) of the raw ramie after degumming.

Strains	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13
	%												
MLR	25.3	31.2	32.4	29.2	30.7	32.2	25.7	30.4	33.5	31.9	30.6	28.5	34.7
RGR	19.4	16.9	15.7	18.3	17.6	15.4	18.7	18.3	15.2	16.6	17.5	18.9	14.6

Table 4 – The hydrolyzed circles and enzyme activity of strains in different substrate plates.

Strains	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	T11-01
	U mL^{-1}													
Pectinase	+	+	+	+	+	+	+	+	+	+	+	+	+	-
	12.7	53.5	92.6	32.5	40.4	53.3	18.4	39.2	104	47.2	32.7	21.7	121.2	0
Mannase	-	+	+	+	+	+	+	+	+	-	+	+	+	+
	0	38.1	38.1	11.9	22.3	42.2	15.6	32.8	101	0	27.4	20.6	148.2	427
Xylanase	+	+	+	+	+	+	+	+	+	-	+	-	-	+
	29	102	247	247	29	124	17.6	33.8	183	0	52.7	11.2	117	334

Note: The enzyme activity was defined as the amount of the enzyme required for 1 μmol substrate to be released per minute, U. The content of enzyme activity was shown by U mL^{-1} of fermentation liquor. The substrates used in enzyme activity measurement of xylanase, mannanase, and pectinase were oats xylan, konjac gum, and orange pectin respectively. "+", stand for hydrolyzed circles; "-", stand for no hydrolyzed circles or no significant hydrolyzed circles; the data stand for the enzyme activity of strains.

cola sp., and *Penicillium* sp. (Baracat et al., 1989; Deshpande and Gurucharanam, 1985). Seven kinds of them belonged to ramie-degumming strains, i.e., *Clostridium* sp. (He and Feng, 1997), *Actinomycete* sp. (Brühlmann et al., 1994), *Erwinia carotovorum* (Peng et al., 1995), *Amycolata* sp., *Streptomyces lividans* (Brühlmann et al., 2000), *Bacillus subtilis* (Sun et al., 1979), *Bacillus pumilus* (Basu et al., 2009), some other alkalophilic *Bacillus* (Cao et al., 1992; Zheng et al., 2001), and *Aspergillus* sp. (Deshpande and Gurucharanam, 1985). Therefore, *Bacteroides* sp., *Rhizobium* sp. and *Pseudomonas* sp. were firstly reported to be used in ramie-degumming.

Both R1 and R7 grew slowly. After vaccination, the degummed solution were clear in the early 48 h and it became muddy in 72 h. Ramie became soft in 90 h and the fiber dispersed in 120 h. After vaccination, the degummed solutions of other 11 strains were clear in 5 h, and became muddy in 12 h. Ramie became soft in 24 h and dispersed naturally in 36 h.

By degumming, non- cellulose had been degraded by 30.5 %, and the residual gum rate was 17.16 %. Among them, R13 had the best degumming effect, whose average rate of material loss was 34.7 %, and the residual gum rate was 14.6 %. The second was R3 and R9, but R1 and R7 were the worst, whose average rates of material loss were 25.3 % and 25.7 % respectively, and the residual gum rates were 19.4 % and 18.7 % respectively (Table 3).

Strains R2, R3, R4, R5, R6, R7, R8, R9 and R11 formed obvious hydrolyzed circles and higher enzyme activity in the three different culture medium plates, while R1 could not form hydrolyzed circles in konjac gum plate, R12 could not form hydrolyzed circles in xylan plate, and R10 could not form hydrolyzed circles in both konjac gum plate and xylan plate (Table 4). The control stain T11-01 without degumming function could not form hydrolyzed circles in pectin plate, but could form hydrolyzed circles in both konjac gum plate and xylan plate.

All the 13 strains of degumming strains secreted pectinase. R10, R12 and R13 did not secret xylanase, and R1 and R10 did not secret mannanase either. R10 and R13 had powerful degumming ability. The control

strain T11-01 did not secret pectinase, but secreted xylanase and mannanase. Cao et al. selected four strains designated as NT-2, NT-6, NT-33 and NT-82, which all could produce pectinase (Cao et al, 1992). Pectinolytic activity played an active role in degumming of ramie bast fibers (Brühlmann et al, 1994; Brühlmann et al, 2000; Zheng et al., 2001). Xylanase also played an important role in the degradation of residual gum (Zheng et al., 2001), and, we found strain R10, R12 and R13 did not secret xylanase, however, they had better degomming function. Therefore, pectinase was the key enzyme of ramie degumming, and the xylanase and mannanase were just assistants.

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