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Journal of Natural Fibers

ISSN: 1544-0478 (Print) 1544-046X (Online) Journal homepage: www.tandfonline.com/journals/wjnf20

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To cite this article: Shengwen Duan, Lifeng Cheng, Zhengchu Liu, Xiangyuan Feng, Ke Zheng & Yuande Peng (2018) Diversity and Characteristics of Kenaf Bast Degumming Microbial Resources, Journal of Natural Fibers, 15:6, 799-807, DOI: 10.1080/15440478.2017.1369206

To link to this article: https://doi.org/10.1080/15440478.2017.1369206

JOURNAL OF NATURAL FIBERS
2018, VOL. 15, NO. 6, 799–807
https://doi.org/10.1080/15440478.2017.1369206

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Diversity and Characteristics of Kenaf Bast Degumming Microbial Resources

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ABSTRACT

Kenaf is one of the most important natural fiber crops. Traditional degumming with water retting causes serious environmental pollution and reduces the quality of fiber products. The development of kenaf industry is hindered by high production cost. Microbial degumming is suitable for kenaf bast degumming because of its high efficiency, energy conservation, low pollution, and high quality, among others. Through enrichment and screening from water sample, soil sample, and humus sample, we concentrated and identified 92 bacterial strains that could degrade kenaf bast colloid. The strains belonged to 11 genera and 16 species. Five of these strains did not produce cellulase and the weight loss rate of the kenaf bast fiber raw material was more than 20%. These strains belonged to *Bacillus subtilis, Paenibacillus polymyxa, Clostridium acetobutylicum, Bacillus alcalophilus*, and *Erwinia chrysanthemi* and were assigned with serial numbers from K1–K5, respectively. This study is the first to report the function of *Bacillus pumilus, B. alcalophilus, Clostridium tertium, Brevibacillus brevis, Pectobacterium carotovora, E. chrysanthemi*, and *Tyromyces subcaesius* in kenaf bast degumming. Pectinase and mannanase were the key enzymes in the degumming of kenaf bast.

摘要

红麻是一种重要天然纤维作物。传统的水沤法脱胶严重污染环境，损伤纤维产品质量，生产成本高，制约了产业发展。红麻韧皮生物脱胶因具有高效、节能、低污染、高品质等特点，是红麻韧皮脱胶的发展方向。从水样、土壤样以及腐殖质三个样品中富集、筛选出 92 株具有降解红麻韧皮胶质的菌株，隶属于 11 属 16 种。其中 5 株不产纤维素酶，红麻韧皮原料失重率在 20% 以上，编号为 K1 to K5，分别属于 *Bacillus subtilis, Paenibacillus polymyxa, Clostridium acetobutylicum, Bacillus alcalophilus, Erwinia chrysanthemi respectively. Bacillus pumilus, Bacillus alcalophilus, Clostridium tertium, Brevibacillus brevis, Pectobacterium carotovora, Erwinia chrysanthemi, Tyromyces subcaesius* 首次报道具有红麻韧皮脱胶功能。果胶酶及甘露聚糖酶是红麻韧皮脱胶过程中的关键酶。

KEYWORDS

bio-degumming; fiber; kenaf; bast; microbial resources; diversity

关键词

多样性; 生物脱胶; 纤维; 红麻; 韧皮; 微生物资源

Introduction

Kenaf (*Hibiscus cannabinus*) is an annual bast fiber crop that contains 53–66% cellulose, 8–16% lignin, 23–35% pectin, and hemicellulose (Iii et al. 1999; Salleh et al. 2014; Yu and Yu 2007). Kenaf fiber pulp is extensively used in pulp production, papermaking industry (Ohtani, Mazumder, and

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Sameshima 2001; Papadopoulou et al. 2014), textile industry (Ramaswamy, Ruff, and Boyd 1994), and composite fabrication (Davoodi et al. 2010; Salleh et al. 2014). This material is also used to process ethanol, auto parts, decorative plates, potting soil, and adsorbents (Iii et al. 1999).

The degumming methods for kenaf bast mainly include water-retting degumming, dew retting, chemical degumming, biological degumming, oxidation degumming, steam explosion degumming, and so on (Yan et al. 2011; Song et al. 2017; Zheng 2007). The presence of caustic soda, high temperature, and high pressure during chemical degumming process causes the serious environmental pollution and damages the fiber (Ramaswamy, Ruff, and Boyd 1994). As the most widely practiced retting method for kenaf, traditional water retting is performed by submerging the bundles of kenaf in water. The non-cellulose content of kenaf is degradated through natural microbial fermentation, which causes serious environmental pollution and produces unstable products (Banik et al. 2007). Steam explosion degumming is environmentally friendly but produces uneven and incomplete fibers; thus, this method is only regarded as a degumming pretreatment (Gao et al. 2015). Biological degumming uses dedicated microbial strains or enzyme and exhibits various advantages, such as producing low pollution and good-quality products, requires low labor cost, and is suitable for natural fiber processing (Biswas et al. 2013).

This study reports the diversity and characteristics of using microbial bacterial resources for kenaf bast degumming. This study may serve as reference to further screen kenaf bast degumming bacterial strains for kenaf bast degumming and explore the functional genes that promote the application of kenaf bast degumming technologies.

Materials and methods

Enrichment and screening of degumming microorganism

Water sample (100 mL) was extracted in a location 50 cm away from the water surface in the retting pond. Soil sample (50 g) was collected at a spot 10 cm away from the soil surface in the kenaf continuous cropping soil. Afterward, 100 mL of sterile water was added to the soil. The sample was shaken, dispersed, and filtered with gauze. The mixture was then filtered. Humus sample (50 g) was extracted from the decay. About 100 mL of sterile water was added to the humus. The mixture was shaken, dispersed, and filtered with gauze. The mixture was filtered to yield the filtrate. All samples were collected from Xiaoshan, China (30°16′N, 120°25′E), and the sampling temperature was 28°C.

Kenaf bast (30 g) was cut into small pieces (approximately 3 cm) and placed into three bottles with sterile water (100 mL each). Subsequently, 5 mL of water, soil, and humus samples were placed individually in the bottles and on a shaking table with a revolving speed of 180 rpm at 28°C for cultivation and enrichment. About 5 mL of fermentation liquid was extracted after kenaf bast dispersed and evident degumming occurred. The liquid was added to the fresh kenaf bast. This process was repeated for three times. Afterward, 1 mL of the enriched liquid was placed on the plate of the selective medium. The liquid was coated evenly and cultivated at 30°C for 10 h to obtain a single colony. Each liter of selective culture medium included 0.5 g of K_2HPO_4, 0.5 g of NaH_2PO_4, 2.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4{\cdot}7H_2O$, 0.1 g of $CaCl_2$, and 1 g of kenaf bast powder.

Identification of strains

We classified and identified the screened strains based on their 16S rRNA and 18S rRNA gene sequences and on the morphological, physiological, and biochemical characteristics using the Bergey's Manual of Determinative Bacteriology (ninth edition) on screened strains.

The genomic DNA of the strains was extracted with UNIQ-10 Column Strains Genomic DNA Extraction Kit (Shanghai Sangon, China), whereas the genomic DNA of the fungus was extracted with Biospin Fungus Genomic DNA Extraction Kit (Biospin). Polymerase chain reaction (PCR) conditions of rRNA gene in 50 μL of reaction mixture included the forward and reverse primers of 0.5 μM each (16S rDNA 27F: AGAGTTTGATCMTGGCTCAG, 1492R: TACGGYTACCTTGTTACGACTT; 18S rDNA

NS1: GTAGTCATATGCTTGTCTC, NS8: TCCGCAGGTTCACCTACGGA), 25 μL of Taq PCR mix (Shanghai SANGON, China), 50 ng of DNA, and 20 μL of H_2O. Reaction parameters were as follows: predegeneration for 5 min at 95°C, then for 30 cycles, degeneration for 0.5 min at 94°C annealing for 1 min at 52°C, and extension for 1 min at 72°C, and a final extension of 10 min at 72°C. Amplified samples were purified by TaKaRa Co. Dalian, Ltd. and sequenced with dideoxy chain termination method with XL 3730 DNA (Biosystems Applied, USA).

The rRNA sequences were aligned with the Clustal-X program (Thompson et al. 1997). MEGA6 program (Tamura et al. 2013) was used to construct the phylogenetic tree with neighbor-joining (Felsenstein 1985) and maximum-parsimony (Saitou and Nei 1987) methods under parameters of bootstrap values based on 1000 replications (Fitch 1972).

Testing of degumming effect

We established two control groups (with or without strain T11-01) to test the material loss rate (MLR), residual gum rate (RGR), and chemical oxygen demand (COD) during degumming. Strain T11-01 was selected and preserved by previous authors.

Sterilized water (100 mL), a pure culture fermentation liquid (2 mL), and drying constant-weighted kenaf bast (M_0) were all placed into a conical flask. The flask was stored at 30°C for 2-day cultivation. The residues were cleaned and dried to a constant weight (M_1). The formula is MLR $V = (M_0 - M_1)/M_0 \times 100$.

Afterward, 200 mL of water, 20 g/L NaOH, and kenaf bast were added to the fixed condenser-Allihn type that was boiled for 2 h. The residues were cleaned and dried to constant weight, and RGR was calculated.

COD was tested with COD detector ET99718 according to the manufacturer's instructions (Lovibond® Group).

Fiber strength is detected with single-fiber strength tester (Zhang, Jin, and Yu 2013).

Characteristics of enzymes

Cellulase was tested by cultivating the sample at 30°C for 36–48 h. Appearance of hydrolysis circles represents positive results (Dong and Cai 2001). The cellulose degradation medium per liter included 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).

Enzymatic activity refers to the amount of enzyme required to degrade 1 mol of substrate per minute. The enzymatic activity of strains was tested with DNS method (Wang et al. 2009), and the fermentation liquid after 8 h was tested by hydrolysis circle. Oat xylan was used as the substrate, and Congo red was used as the dye in the medium for hydrolysis circle detection (Huang, Wang, and Xiao 2006; Teather and Wood 1982) to test the xylanase activity. Konjac gum was used as the substrate, and trypan blue was used as the dye (Mendoza et al. 1995) to test mannose activity. Finally, orange pectin was used as the substrate, and the brilliant green was used as the dye (Keen et al. 1984) to test the pectinase activity.

Results

Species diversity and distribution of kenaf bast microbial strains

Ninety-two strains that could degrade the colloid of kenaf bast and produce transparent circles in the selective medium were screened from soil (56.52%), water (20.65%), and humus (22.83%) samples (Table 1). We classified and identified these strains according to their morphological, physiological, and biochemical characteristics and 16S rDNA (18S rDNA) gene sequences. Among these 92 strains that belonged to 16 genera and 11 species, 85 (92.39%) were bacteria and 7 (7.61%) were filamentous fungi.

Table 1. Species diversity and distribution of kenaf bast microbial strains.

Genera	Species	Source of strains				
		Soil	Water	Humus	Total	Ratio (%)
Bacillus	*Bacillus subtilis*	12	3	5	20	21.74
	Bacillus cereus	3	3	1	7	7.61
	Bacillus licheniformis	2	2	3	7	7.61
	Bacillus pumilus	3	2	1	6	6.52
	Bacillus alcalophilus	2	3	1	6	6.52
	Others	3	0	0	3	3.26
Paenibacillus	*Paenibacillus polymyxa*	9	2	3	14	15.22
	Paenibacillus macerans	5	1	0	6	6.52
Clostridium	*Clostridium acetobutylicum*	0	0	2	2	2.17
	Clostridium tertium	2	1	2	5	5.43
Bacteroides	*Bacillus vulgatus*	2	1	0	3	3.26
Brevibacillus	*Brevibacillus brevis*	0	0	1	1	1.09
Pectobacterium	*Pectobacterium carotovora*	3	0	0	3	3.26
Erwinia	*Erwinia chrysanthemi*	1	0	1	2	2.17
Aspergillus	*Aspergillus niger*	1	0	1	2	2.17
Penicillium	*Penicillium frequentans*	2	0	0	2	2.17
Mucor	Others	0	1	0	1	1.09
Tyromyces	*Tyromyces subcaesius*	2	0	0	2	2.17
Quantity	Total	52	19	21	92	–
	Ratio (%)	56.52	20.65	22.83	–	100

Degumming function of the selected strains

Among these 92 strains, 5 strains did not produce cellulase and could degrade more than 25% of the noncellulosic fibers. These strains were assigned with serial numbers from K1 to K5. The 16S rRNA sequences of these five strains were submitted to GenBank (sequence numbers: KT897713–KT897717). K1, K2, and K5 were obtained from the soil, K3 was extracted from water body, and K4 was obtained from humus. The main physiological and biochemical characteristics of the five strains are shown in Table 2. Strains K1–K5 belonged to *Bacillus subtilis, Paenibacillus polymyxa, Clostridium acetobutylicum, Bacillus alcalophilus*, and *Erwinia chrysanthemi*, respectively (Figure 1).

Strains K3 and K4 had slow growth. After inoculation, their fermentation liquids were clear within 24 h, and then they started to become turbid. Kenaf bast softened after 60 h of fermentation, and the fiber was dispersed after 72 h. The fermentation liquids of the other strains remained clear within 4 h, and then they started to become turbid after 10 h. The kenaf bast softened after 24 h of fermentation, and the fiber was dispersed after 40 h.

Strain K5 exhibited the best degumming effect with MLR and RGR of 28.14% and 18.58%, respectively, followed by strains K1 and K4. Strains K2 and K3 had the worst degumming effect with MLRs of 23.29% and 21.59%, respectively, and RGRs of 19.54% and 21.25%, respectively. The

Table 2. Physiological and biochemical characteristics of K1–K5.

Characteristic	Strains				
	K1	K2	K3	K4	K5
Casein	+	+	+	+	+
Gelatin	+	+	+	+	+
Starch	+	+	+	+	–
Catalase	+	+	–	+	+
Urease	+	–	–	–	–
D-Glucose	+	+	+	+	+
L-Arabinose	+	+	–	+	+
D-Xylose	+	+	–	+	+
D-Mannitol	+	+	+	+	+
Indole	–	–	–	–	+
Nitrate reduction	+	+	–	–	+

"+" Represents the positive, and "–" the negative.

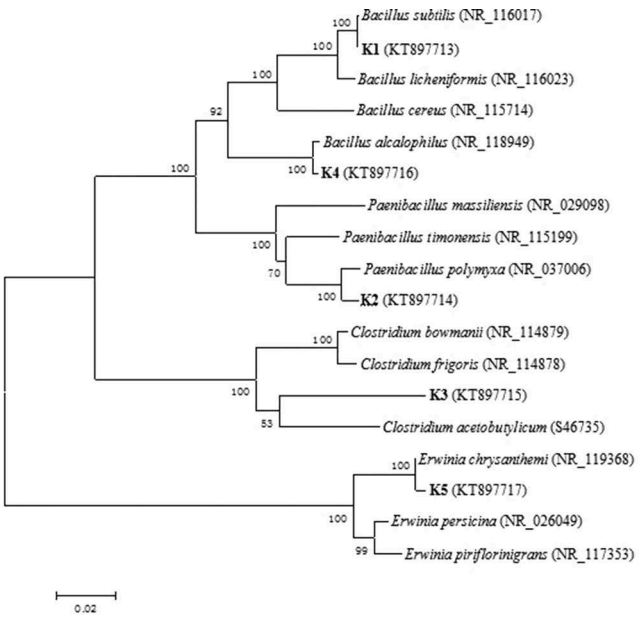

Figure 1. Phylogenetic tree diagram constructed using the neighbor-joining method based on the 16S rRNA gene sequences of stains K1–K5. Numbers at the nodes represent bootstrap percentages based on 1000 samplings. The scale bars represent 0.05 substitutions per site. We also constructed the phylogenetic tree diagram with the maximum-parsimony method. The results in these two diagrams were essentially consistent (date not shown).

Table 3. Degumming effects of different strains.

Strains	K1	K2	K3	K4	K5	T11-01	Control group without added strains
MLR (%)	26.31	23.29	21.59	25.07	28.14	5.17	2.72
RGR (%)	19.06	19.54	21.25	19.39	18.58	40.06	42.56
Fiber breaking strength (cN d/tex)	4.26	3.57	4.41	4.02	4.12	3.96	2.57

MLR: Material loss rate; RGR: residual gum rate.

MLR and RGR of the experimental group (includes strain T11-01 with degumming function) were 5.17% and 40.06%, respectively, whereas those of the control group (without added strains) were 2.72% and 42.56%, respectively. Fiber-breaking strengths of strain K1 and K5 were 4.26 and 4.12 cN d/tex, respectively, and the dates of strain K3 and T11-01 were 4.41 and 3.96 cN d/tex, respectively (Table 3).

The COD of the fermentation liquid increased gradually during degumming with prolonged time. The COD of the fermentation liquid of strain K5 increased from 2.5 to 13.7 g/L within 24 h, which was higher than those of other four strains. The COD of strain K3 was the lowest with the range of 1.9–6.5 g/L. The COD of the control group was between 0.12 and 2.31 g/L (Figure 2).

Enzymatic characterizations of the degumming strains

Strain K2 did not produce hydrolysis circles on the plate of the medium using xylanase. By contrast, the other four strains showed high enzymatic activity and formed evident hydrolysis circles on the plate of the medium with three different hydrolysis methods. The experimental group (with strain T11-01 without degumming function) did not produce hydrolysis circles on the plate of the medium using pectinase. The enzymatic activities of mannanase and xylanase were 325 and 257 IU/mL, respectively. The rank of the pectinase activity from the highest to the lowest was K5, K1, K3, K2, K4,

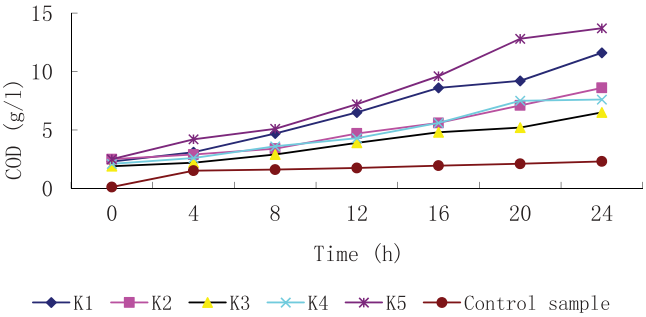

Figure 2. Variation trends of CODs during the degumming of kenaf bast.

Table 4. Hydrolyzed circles and enzymatic activity of strains in the different substrate plates.

Strains	K1	K2	K3	K4	K5	T11-01
Pectinase	+	+	+	+	+	–
	97.3	39.6	40.1	46.2	152.8	0
Mannase	+	+	+	+	+	+
	71.2	60.1	32	82.5	52.7	325
Xylanase	+	–	+	+	+	+
	65.8	0	121.3	53.7	11.2	257

"+" Represents the presence of hydrolysis circles. "–" Represents the absence of or no evident hydrolysis circle formed. The data represent enzymatic activity. The unit for enzymatic activity is international units per milliliter (IU/mL).

and T11-01; that of the mannanase activity was T11-01, K4, K1, K2, K5, and K3; and that of the xylanase activity was T11-01, K3, K1, K4, K5, and K2 (Table 4).

Discussion

Specific kenaf bast degumming strains

Thirteen strains, namely, *Aspergillus niger* (Zheng 2007), *P. polymyxa* (Sun 1981), *Bacillus licheniformis, B. subtilis, C. acetobutylicum, Clostridium felsineum* (Donaghy et al. 1990), *Bacillus vulgatus, Bacillus cereus, Paenibacillus macerans, Penicillium frequentans, Sclerotium rolfsii, Mycelia sterilia*, and *Macrophomina phaseoli* (All et al. 1972), have the ability to degrade noncellulosic fibers from kenaf bast. The diversed community in the samples of kenaf bast degumming mainly composed of *Aeromonadales, Bacillales, Bacteroidales, Burkholderiales, Clostridiales, Enterobacteriales, Lactobacillales, Pseudomonadales, Rhodocyclales, Sphingobacteriales, Selenomonadales, Sphingomonadales*, and *Xanthomonadales* (Visi et al. 2013).

Strains K1–K5 showed good degumming effect for kenaf bast, with degradation rate to non-cellulose material of more than 25% and produced no cellulosic fiber. The MLR and RGR of K5 reached 28.14% and 18.58%, respectively. *B. subtilis* and *E. chrysanthemi* could be significantly applied to kenaf bast degumming. Strain K3 has the highest fiber breaking strength. However, the fiber-breaking strength of the control group that may contain cellulose degradation enzyme was only 2.57 cN d/tex. This study is the first to report that *Bacillus pumilus, B. alcalophilus, Clostridium tertium, Brevibacillus brevis, Pectobacterium carotovora, E. chrysanthemi*, and *Tyromyces subcaesius* could be used in kenaf bast degumming.

Key enzymes in kenaf bast degumming

All five degumming strains produced pectinase and mannanase. Strain T11-01 in the control group produced mannanase and xylanase, but not pectinase, indicating its lack of a degumming function. Therefore, pectinase was the key enzyme in kenaf bast degumming. The strain K5 had higher pectinase activity but lower mannanase and xylanase activities than K1. Moreover, the degumming effect of K5 was better than that of K1. These characteristics indicate that pectinase was more important than mannanase and xylanase during degumming. Stain K2 had 1.24% lower pectinase activity, 46.76% higher mannanase activity, and 1.7% higher MLR than K3; however, this strain had 0% xylanase. When the pectinase activity of the strains was similar, mannanase exhibited the main effect on degumming, whereas xylanase only played an auxiliary role.

Studies on the degumming mechanism of key enzymes showed that pectinase is a crucial degumming enzyme (Zheng 2007). Pectinase plays an important role in the degumming of kenaf bast, ramie, flax, hemp, and other herbaceous fiber plants (Kozlowski et al. 2006).

Biological degumming as the key for developing the kenaf industry

Traditional water-retting method requires 60–90 days. However, strains K3 and K4 could complete microbial degumming of kenaf bast within 72 h. Strain K1, K2, and K5 could soften the kenaf bast within 24 h and disperse the fiber within 40 h. The biological degumming cycle of kenaf bast was shortened by 97%, compared with traditional methods (Kozlowski et al. 2006).

The CODs of all five strains in the biological degumming of kenaf bast increased with the fermentation time. The COD of the fermentation liquid of K5 was the highest with the range from 2.5 to 13.7 g/L, whereas that of K3 was the lowest with the range from 1.9 to 6.5 g/L. Traditional water-retting method causes serious pollution and reduces the fiber quality (Yu and Yu 2007). For the chemical degumming method, the COD content reached 2000–3800 mg/L (Zawani, Abdullah, and Abdan 2015). The use of NaOH or other strong corrosive chemical reagents in chemical degumming facilitates the degradation of noncellulosic fiber but significantly damages the fiber quality (Parikh, Chen, and Sun 2006; Ramaswamy, Ruff, and Boyd 1994).

Traditional kenaf bast degumming restricts the development of kenaf bast industry. However, biological degumming technology is expected to change the processing in the kenaf industry and promote its sustainable development.

Conclusion

Ninety-two stains with kenaf bast degumming function were isolated and screened from water, soil, and humus samples. These strains belonged to 11 genera and 16 species. This study can help identifying the functional genes of degumming. Strains K1–K5 did not produce cellulase and increased the weight loss rate of the raw material to more than 20%. Strains K1–K5 belonged to *B. subtilis, P. polymyxa, C. acetobutylicum, B. alcalophilus*, and *E. chrysanthemi*, respectively, and can be applied in production. Pectinase and mannanase acted as key enzymes in the degumming of kenaf bast.

Funding

This work was supported by the China Agriculture Research System for Bast and Leaf Fiber Crops (CARS-19-E24), the Chinese Agricultural Science and Technology Innovation Project (ASTIP-IBFC08), the Natural Science Foundation of Hunan Province (2016jj3126), the Fundamental Research and Incremental Budge of Chinese Academy of Agricultural Sciences (Y2016PT36), and the Project of Scientific Elitists in National Agricultural Research.

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