

Isolation and Identification of Symbiotic Bacteria from *Brasenia schreberi* and Analysis of their Polysaccharide Producing Characteristics and Antioxidant Activity

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Abstract

Brasenia schreberi (water shield), a perennial freshwater aquatic plant of the family *Nymphaeaceae*, is rich in polysaccharides with positive biological activity and potential application value. As an indicator plant of the environment in wetlands, *B. schreberi* original and wild water environment faced increasing deterioration and has been listed as a critically endangered species in several countries of East Asia. Seeking an alternative method to produce *B. schreberi* mucilage polysaccharide is of top priority. The morphological structure of the stem and leaf of *B. schreberi* was studied. Well-developed glandular cells and aerenchyma tissue were observed in the stem and leaves of water shield, which were related to its aquatic habitat and polysaccharide secretion and then three endophytic bacteria were successfully isolated and purified from its stem and leaf and identified as *Micromonospora* sp. YG-1, *Xylariaceae* sp. strain UT-X and *Psychrobacter pulmonis* strain T-15. Symbiotic bacteria from *B. schreberi* produced polysaccharides with similar spectral peaks and chemical functional groups to those of *B. chinensis*. Further, analysis of hydroxyl free radicals showed that symbiotic bacteria in water shield had significant antioxidant activity against hydroxyl free radicals. This study provides a theoretical and practical basis for further study using symbiotic bacteria to produce polysaccharides to overcome the environmental limitations of the growth and development of *B. schreberi*.

*The first two authors contributed equally to this work and can be considered co-first authors.

Keywords

Brasenia schreberi, Endosymbiont, HPLC, IR Spectrum, Polysaccharide

1. Introduction

Extracellular polysaccharides are attracting increasing attention in the field of medicine because of their positive biological activity and potential application value [1]. Research revealed that extracellular polysaccharides produced by two marine microorganisms, *Fusarium oxysporum* and *Aspergillus ochraceus*, showed strong anti-lipid peroxidation ability and free-radical (e.g. superoxide anions) scavenging potential [2] and extracellular oligosaccharides of an endophytic bacteria (14-DS-1) found in *Codonopsis pilosula* activate macrophages of the immune system and affect their migration ability and microfilament morphology [3]. Moreover, evidence indicates that they can affect normal spindle formation and induce cells to tend to stagnate in the S phase and increase the apoptotic rate. One possible mechanism to achieve this effect would involve the capacity of extracellular polysaccharides to affect spindle-related precise regulation and DNA synthesis during the cell cycle [3]. Similarly, antineoplastic polysaccharides extracted from mushrooms were shown to enhance the immune response *in vivo* and *in vitro* and act as a modifier of biological reactions [1].

Recent studies have revealed the promising clinical role of extracellular polysaccharides from multiple plants [4] [5], including *Brasenia schreberi* [6] [7]. *B. schreberi*, also known as water shield, horseshoe, lake vegetable, water anemone, sunflower and lotus leaf or cauliflower, is a perennial floating-leaf aquatic plant belonging to the family *Nymphaeaceae* [8] [9]. The main component of the viscous mucilage on the surface of *B. schreberi* is a viscous polysaccharide [10] with exceptionally high nutritional value and protection role [11]. As an indicator plant of the environment in wetlands, *B. schreberi* growth demands a high-quality water [12]. However, in recent years, habitat fragmentation and loss and human disturbance have led to a higher organic content in the original and wild water environment, causing a deterioration in the wild-living condition of *B. schreberi* [12]. Moreover, *B. schreberi* has been recently listed as a critically endangered species in several countries of East Asia [13]. In this context, seeking an alternative method to produce *B. schreberi* mucilage polysaccharide is of top priority.

Given that plants and their symbiotic endophytes can produce the same or similar functionally active ingredients, endophytes may be an important source of new active natural products [14]. Endophytes are microorganisms that survive in healthy plant tissues during part or all of their life cycles without causing obvious infection symptoms in their host. For example, previous results showed that *Erwinia* sp. SS2, an endosymbiotic bacterium found in Chinese yam, showed the ability to produce extracellular polysaccharides [15]. Further, a pre-

vious study showed that there are many gum-producing bacteria in the viscose polysaccharide of *B. schreberi* [16]. To comprehensively utilize *B. schreberi* and seek alternative bacteria that can produce mucilage polysaccharide similar to *B. schreberi*, this study aimed to isolate potential symbiotic bacteria from *B. schreberi* and study their polysaccharide-producing characteristics.

2. Materials and Methods

2.1. Experimental Materials

Stems and leaves of *B. schreberi* were obtained from Fubaoshan Nature Reserve in Lichuan, Hubei Province. These were rinsed with 30% hydrogen peroxide and then with distilled water for further use.

2.2. Isolation of Endophytic Bacteria from *B. schreberi*

Stems, leaves and roots of *B. Schreberi* were sterilized with 0.1% mercury for 10 min and rinsed with distilled water several times in a sterile environment. Thereafter, these were inoculated on LB solid medium and cultured in the dark. Three kinds of well-growing colonies were selected from the medium and transferred to the new LB medium plate. The colonies were purified and numbered as No. 1, No. 2 and No. 3. In addition, the flourishing bacteria were preserved in glycerol suspension for subsequent experiments.

2.3. Identification of Endophytic Bacteria in *B. schreberi*

The 16S rDNA sequence of bacteria (27F/1492R primer) or ITS sequence of fungi (ITS1 and ITS4 primer) were amplified based on standard procedure of bacteria/fungi identification, which is exerted by. The amplified products were sequenced by the Sanger method. The sequencing results were compared and analyzed on NCBI website (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Cases of homology greater than 97% and highest homology were used to identify the candidate species of endophytic bacteria.

16S rDNA sequence amplification primer sequence:

Primer 1 27F: 5-AGAGTTTGATCCTGGCTCAG-3'

Primer 2 1492R: 5'-TACGGCTACCTTGTACGACTT-3'

The size of amplified fragments is approximately 1400 - 1700 bp

ITS sequence amplification primer sequence:

Primer 1 ITS1: 5'-TCCGTAGGTGAACCTGCGG-3'

Primer 2 ITS4: 5'-TCCTCCGCTTATTGATATGC-3'

The size of amplified fragments is approximately 400 - 700 bp

2.4. Purification and Extraction of Polysaccharides from the Endosymbiont

Strains were selected from the plate of LB medium and transferred to the purified strains. The strains were inoculated into the liquid LB medium. The fermentation liquid was centrifuged for 5 min at 200 rpm and incubated at 37°C overnight for 2 days. The supernatant was obtained after centrifugation at 12,000

rpm for 5 min. Next, the supernatant and the organic solution were mixed with chloroform-n-butanol solution (chloroform: n-butanol = 4:1) at a 3:1 ratio for extraction in a centrifugal tube. The supernatant was oscillated for 20 - 30 min and centrifuged at 8000 rpm for 10 min. Precipitated protein between the two phases could not be discarded; thus, it continued to be removed five times according to the above operation. Then, the supernatant was filtered twice with a 0.45- μ m micron membrane. After filtration, it was heated and concentrated to 1/2 - 2/3 of its original volume at 70°C. Thereafter, it was added with a 4-fold volume of 95% ethanol. Finally, the supernatant was frozen overnight in a refrigerator and centrifuged at 12,000 rpm for 10 min. The precipitate was considered the endophytic bacterial polysaccharide.

2.5. Extraction and Separation of Crude Polysaccharide from *B. schreberi*

Tender sprouts of *B. schreberi* were extracted in NaOH (0.1 mol/L); the ratio of material to liquid was 1:2 (w/v) and the pH was adjusted to 7.0 after 1.5 h of alkali extraction at 25°C. The sprouts were removed, the residues were separated by centrifugation and the supernatant was concentrated at 60°C for 1 h using a magnetic stirrer. Thereafter, the Sevage method was used to treat the concentrated crude extract repeatedly to remove the free protein and concentrate again to approximately 2/3 of the original volume. A 4-fold volume of absolute ethanol was added to precipitate overnight; thereafter, the mixture was centrifuged for 10 min at 4000 rpm to obtain the precipitate. Next, a small amount of water was used to dissolve the precipitate, which was then concentrated and added with a 4-fold volume of ethanol to precipitate overnight. This operation was repeated three times before final drying to obtain the *B. schreberi* polysaccharide (BSP).

2.6. Determination of Sugar Content in the *B. schreberi* Polysaccharide Extract

Polysaccharide content of BSP was determined by the phenol-sulfuric acid method and D-glucose as standard. First, D-glucose was used to prepare the standard curve solution (0.1 mg/ml). Second, the reference solutions were placed into the test tubes (0.2, 0.4, 0.6, 0.8 and 1.0 ml) and the phenol solution was added to stabilize them. Then, concentrated sulfuric acid was quickly added for shaking. The tubes were allowed to stand for 10 min until cooled to room temperature and then the absorbance was measured at 490 nm. Double distilled water (DDW) was used as the blank control. The results were analyzed by linear regression analysis. Thereafter, the absorbance level of the sample was measured to determine the content of glucuronic acid.

2.7. Structural Analysis of Polysaccharides from *B. schreberi* and Comparison with Polysaccharides from Endophytes

The chemical composition of polysaccharide samples was determined by high performance liquid chromatography (HPLC) using an Agilent 1260 Infinity II LC System equipped with a Shodex OH-pak SB-804 HQ column (8 mm \times 300

mm). To further compare the glycosidic bonds of the polysaccharides between symbiotic bacteria and *B. schreberi*, the structure of polysaccharides was further analyzed by Fourier transform infrared spectroscopy (IR) (IRTracer-100).

2.8. Determination of Antioxidant Activity of Polysaccharides

In this experiment, the hydroxyl radical kit provided by Nanjing Jiancheng Bio-engineering Institute was used to determine polysaccharide activity. The polysaccharide sample and reagent were mixed evenly and reacted at 37°C for 1 min, after which the color reagent was added immediately to stop the reaction. After mixing evenly, the polysaccharide sample was placed at room temperature for 20 min. The spectrophotometer was zeroed with distilled water and the absorbance of each tube was measured at 550 nm. The experimental results showed the scavenging of hydroxyl radicals and some antioxidant activities of the polysaccharides obtained.

3. Results

3.1. There are Well-Developed Airways and Glandular Cells in the Stems and Leaves of *B. schreberi*

The stomata were distributed on the upper epidermis of the leaves (**Figure 1(A)**). The lower epidermis of the leaves was in contact with water, so there were more stomata on the upper epidermis for photosynthesis and gas exchange. Mucilage was abundant on the dorsal surface of the stem and the leaf and there were many black stripes on the lower epidermis of the leaf. Study showed these structures were glandular cells. In addition, red dots and black stripes were observed, which might be symbiotic bacteria (**Figure 1(B)**). There was a layer of pectin on the surface of the stem of fresh water shield. Under this pectin layer, there were glandular cells with a hollow airway in the middle to allow gas exchange (**Figure 1(C)**). After incubation in the laboratory for a period of time, the glandular cells disappeared and there was no pectin on the surface of the stem (**Figure 1(D)**). Therefore, pectin secretion may be related to glandular cells.

3.2. Isolation of Endophytes

The three colonies grew well in the LB medium plate. Among them, the diameter of colony No. 1 was approximately 1 - 2 mm, its shape was regular, round, smooth and tidy edged and was wet and milky yellow, with a transparent surface (**Figure 2(A)**); the diameter of colony No. 2 was approximately 4 - 6 mm, the colony bulged, the edge was not smooth, the hypha was visible and the color was opaque white (**Figure 2(B)**); the diameter of colony No. 3 was 5 mm and the colony was round and milky white (**Figure 2(C)**).

3.3. Symbiosis of Bacteria and Fungi with *B. schreberi*

According to the standard sequencing method of bacteria and fungi, the sequence of symbiotic bacteria *B. schreberi* was as follows:

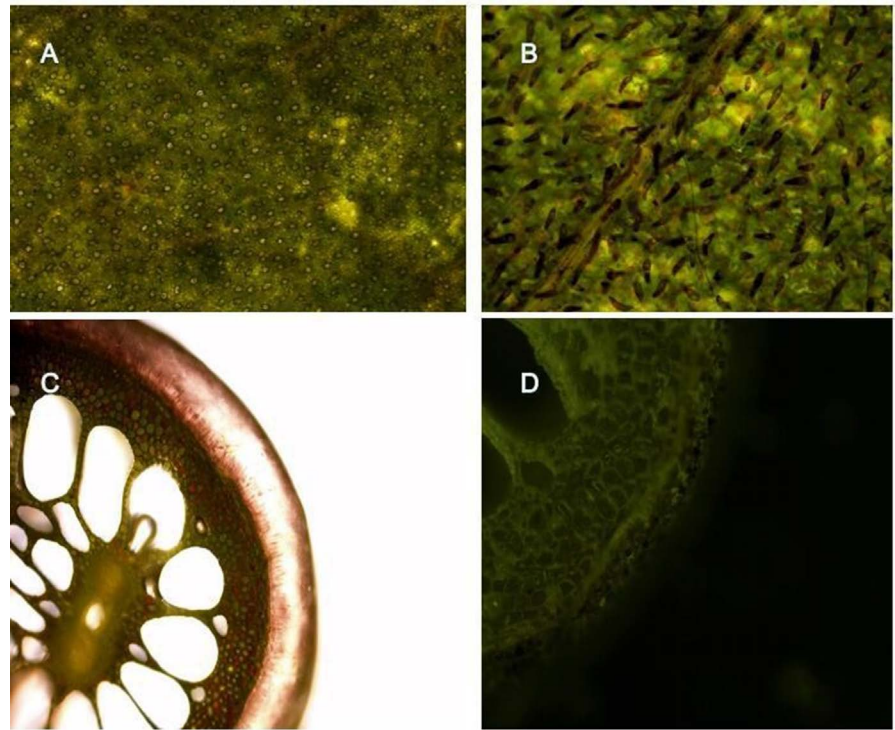


Figure 1. Transverse section of the stem and the leaf of *Brasenia schreberi*. (A) An upper epidermis of the leaf; (B) Lower epidermis of the leaf; (C) Transverse section of a fresh stem; (D) Transverse section of a stem grown in culture.

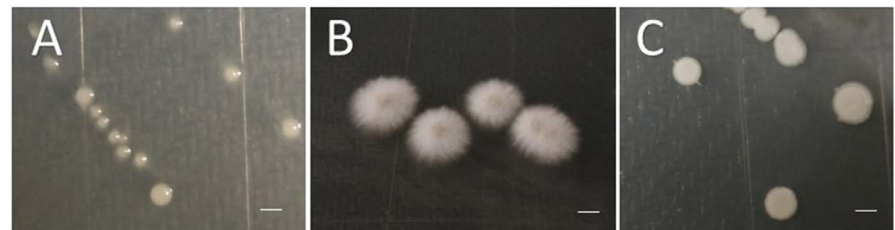


Figure 2. Morphology of the colonies of the three endophytic bacteria from *Brasenia schreberi*. (A) colony No. 1; (B) colony No. 2; (C) colony No. 3 (Bars = 2 mm).

No. 1:

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GCATGGCAGCGCTAGAGTTGCTCCTGGAGCGGAAGGCCCTTCGGGG
TACTCGAGCGGCCGAACGGGTGAGTAACACGTGAGTAACCTGCCCCAGG
CTTTGGGATAACCCTCGGAAACGGGGGCTAATACCGAATATGACCTCC
GGACGCATGTTTGGTGGTGAAAGTTTTTCGGCCTGGGATGGGCTCGC
GGCCTATCAGCTTGTGGTGGGGTGATGGCCTACCAAGGCGACGACGG
GTAGCCGGCCTGAGAGGGCGACCGGCCCACTGGGACTGAGACACGG
CCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGC
GGAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGT
TGTAACCTCTTTCAGCAGGGACGAAGCGTAAGTGACGGTACCTGCAG
AAGAAGCGCCGGCCAACTACGTGCCAGCAGCCGCGGTAAGACGTAGG
GCGCGAGCGTTGTCCGATTTATTGGGCGTAAAGAGCTCGTAGGCCGGC
TTGTGCGGTCGACCGTGAAAACCTTGGGGCTCAACCCCAAGCCTGCGGT
```

CGATACGGGCAGGCTAGAGTTCGGTAGGGGAGACTGGAATTCCTGGTG
 TAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGC
 GGGTCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGC
 GAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGTTGGGCGCT
 AGGTGTGGGGGGCCTCTCCGGTTCCTGTGCCGACGTAACGCATTA
 GCGCCCCGCTGGGGAGTACGGCCGCAAGGCTAAAACCTCAAAGGAAT
 TGACGGGGGCCCCGACAAAGCGGCGGAGCATGCGGATTAATTCGATGC
 AACGCGAAGAACCTTACCTGGGTTTGACATGGCCGCAAAACCTCCAGA
 GATGGGGGGTCTTTCGGGGGCGGTACACAGGTGGTGCATGGCTGTCGTC
 AGCTCGTGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
 TCGTTCGATGTTGCCAGCGCTTATGGCGGGACTCATCGAAGACTGC
 CGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCC
 CCTTATGTCCAGGGCTTACGCATGCTACAATGGCCGGTACAATGGGC
 TCGATACCGTGAGGTGGAGCGAATCCCAAAAAGCCGGTCTCAGTTCG
 GATCGGGGTCTGCAACTCGACCCCGTGAAGTCGGAGTCGCTAGTAATC
 GCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACAC
 CGCCCGTACGTCACGAAAGTCGTCAACGTAGGAAGTGCGGTGGCCCC
 ACCCTGGTGGAGGCAACGTTAGAGTAGAGCTATCCGCC

No. 2:

GTAGGTTGAACCTGCGGAGGGATCATTACTGAGTTCTACAAAAACT
 CCCAACCTTTGTGAACCTTACCGTCGTTGCCTCGGCGCCGAGCGGCG
 GCTACCCTGGAGAAGCTACCCGGGAGCCACCTACCCTGTAGGTGGCTA
 CCCTGGAGCTACCCTGTAGTAGTTTGCATTCTACGCTCCGCCGGCGGA
 CCTTCTACACTCTGTTTTGTATAGTGTATCTCTGAAACCTATAACGTAA
 TACGTTAAAACCTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAA
 GAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAATTCAGTGAA
 TCATCGAATCTTTGAACGCATATTGCGCCATTAGTATTCTAGTGGGCA
 TGCCTATTCGAGCGTCATTTCAACCCTTACGCCCTGTTGCGTAGTGTT
 GGGAACCTACAGGCCTGTAAAAGGACCTGTAGCTCCCTAAAGGTAGT
 GGCGGTGTTAGGTACACTCGTAGCGTAGTAACATCTTTTCTCGCTCCTG
 CAGTGTACCTAAGGCCTGCCGTGAAAAACCCCTATAACTTCTAGTGG
 TTGACTTCGGATTAGGTAGG

No. 3:

GAATGGCGGGCCGCTATAATGCTAGTCGAGCGAACAGATAAGGAGC
 TTGCTCCTTTGACGTTAGCGGCGGACGGGTGAGTAACACGTGGATAAC
 CTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGA
 TAACATATTGAACCGCATGGTTCAATAGTAAAAGGCGGCTTTGCTGTC
 ACTTATAGATGGATCCGCGCCGATTAGCTAGTTGGTAAGGTAACGGC
 TTACCAAGGCAACGATACGTAGCCGACCTGAGAGGGTATCGGCCACA
 CTGGAACCTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGG
 AATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTG
 ATGAAGGTCTTCGGATCGTAAAACCTCTGTTATCAGGGAAGAACAAATG
 TGTAAGTAACTGTGCACATCTTGACGGTACCTGATCAGAAAGCCACGG
 CTAACCTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTAT

CCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGA
 TGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAA
 CTTGAGTGCAGAAGAGGAAAGTGGAAATCCATGTGTAGCGGTGAAATG
 CGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTG
 TAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATA
 CCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTT
 TCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCTGGGGA
 GTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCAC
 AAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCCTTAC
 CAAATCTTGACATCCTTTGACCGCTCTAGAGATAGAGTCTTCCCCTTCG
 GGGGACAAAGTGACAGGTGGTGCATGGTTGTCTCAGCTCGTGTCTGTG
 AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTG
 CCATCATTAAGTTGGGCACTCTAAGTTGACTGCCGGTGACAAACCGGA
 GGAAGGTGGGGATGACGTCAAATCATCATGCCCTTATGATTTGGGCT
 ACACACGTGCTACAATGGACAATACAAAGGGCAGCTAAACCGCGAGG
 TCAAGCAAATCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAAC
 TCGACTACATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTA
 CGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGA
 GAGTTTGTAACACCCGAAGCCGGTGGAGTAACCATTTATGGAGCTAGC
 CGTCGAGTGGAGAGCTCAGTCT

The results of sequencing were compared and analyzed via browsing the NCBI website. The species with over 97% homology and highest homology were finally identified as endosymbiotic species. The results showed that two of the symbiotic bacteria isolated were bacteria (colonies No. 1 and No. 3), while the other was a fungus (colony No. 2) (**Table 1**). Endophytes were identified as *Micromonospora* sp.YG-1, Xylariaceae sp. strain UT-X and *Psychrobacter pulmonis* strain T-15.

3.4. Extracts from the Fermentation Broth of Symbiotic Bacteria Found in *B. schreberi* Contained Secreted Polysaccharides

Polysaccharide content in the fermentation broth of endophytic bacteria from *B. schreberi* was determined and compared. After polysaccharide was hydrolyzed by sulfuric acid, the content of secreted sugar in the bacterial solution was determined by DNS (3-amino-5-nitro salicylic acid) method. With D-glucuronic acid as the reference standard, the standard curve was drawn by the absorbance of different concentrations of standard glucose solution reacted with DNS reagent and sulfuric acid. The standard curve was $y = 0.0368x - 0.0436$ ($R^2 = 0.9807$). The content of polysaccharides was determined according to this standard curve (**Table 2**). The results showed that the polysaccharide contents in the fermented extracts were as follows: 0.30 ± 0.01 mg/ml in *B. schreberi* samples, 0.07 ± 0.001 mg/ml in *Micromonospora* sp.YG-1, 0.07 ± 0.002 mg/ml in *Xylariaceae* sp. strain UT-X and 0.06 ± 0.001 mg/ml in *Psychrobacter pulmonis* strain T-15 (**Table 2**).

Table 1. Species identification of three endophytic bacteria.

Code	Homology	Species
No. 1	99% (1354/137)	<i>Micromonospora</i> sp.YG-1
No. 2	98% (563/575)	Xylariaceae sp. strain UT-X
No. 3	99% (1440/144)	<i>Psychrobacter pulmonis</i> strain T-15

Table 2. Determination of polysaccharides from different species.

Extracts from fermentation/polysaccharide	Polysaccharide content (mg/ml)
<i>Brasenia schreberi</i> polysaccharide (BSP)	
<i>Micromonospora</i> sp.YG-1	0.30 ± 0.010
Xylariaceae sp. strain UT-X	0.07 ± 0.001
<i>Psychrobacter pulmonis</i> strain T-15	0.07 ± 0.002
	0.06 ± 0.001

3.5. Organic extracts of Endophytic Bacteria from *B. schreberi* Have Similar Peaks as Those of Polysaccharides from *B. schreberi* by HPLC

We used HPLC to compare polysaccharide components detected in the bacterial species identified. HPLC spectra showed that the polysaccharide extracts from *Micromonospora* sp.YG-1 had a peak at 19 min and a sub-peak at 21 min (**Figure 3(A)**). Polysaccharide extracts from the other two endophytic bacteria, *Xylariaceae* sp. strain UT-X and *Psychrobacter pulmonis* strain T-15, also showed similar peaks (**Figure 3(B)**, **Figure 3(C)**). Interestingly, the sub-peak at the 21st min was similar to the peak signal of the polysaccharides extracted from *B. schreberi* (**Figure 3(D)**), which suggests that symbiotic bacteria in *B. schreberi* can produce polysaccharides similar to *B. schreberi* polysaccharides, besides producing their species-specific polysaccharides.

3.6. Polysaccharides Secreted by Three Endophytic Bacteria in *B. schreberi* Have Infrared Absorption Peaks Similar to Those of *B. schreberi*

To further determine whether polysaccharides from endophytic bacteria and those from *B. schreberi* contain the same chemical functional groups, we performed FTIR (Fourier Transform infrared spectrum) analysis. Infrared spectrum analysis showed that the three symbiotic bacteria had similar infrared absorption peaks. Representative polysaccharides from *Psychrobacter pulmonis* strain T-15 showed nine characteristic absorption peaks (**Figure 4**). From the infrared spectra, it can be seen that the peak at 3392.79 cm⁻¹ is the stretching vibration absorption peak of -OH, while that at 2924.09 cm⁻¹ is the stretching vibration absorption peak of C-H; these two are peaks that are characteristic of polysaccharides and similar to those of *B. schreberi* polysaccharides (**Table 3**). Peaks at 2854.65 cm⁻¹, 1558.00 cm⁻¹ and 1458.18 cm⁻¹ are the stretching vibration

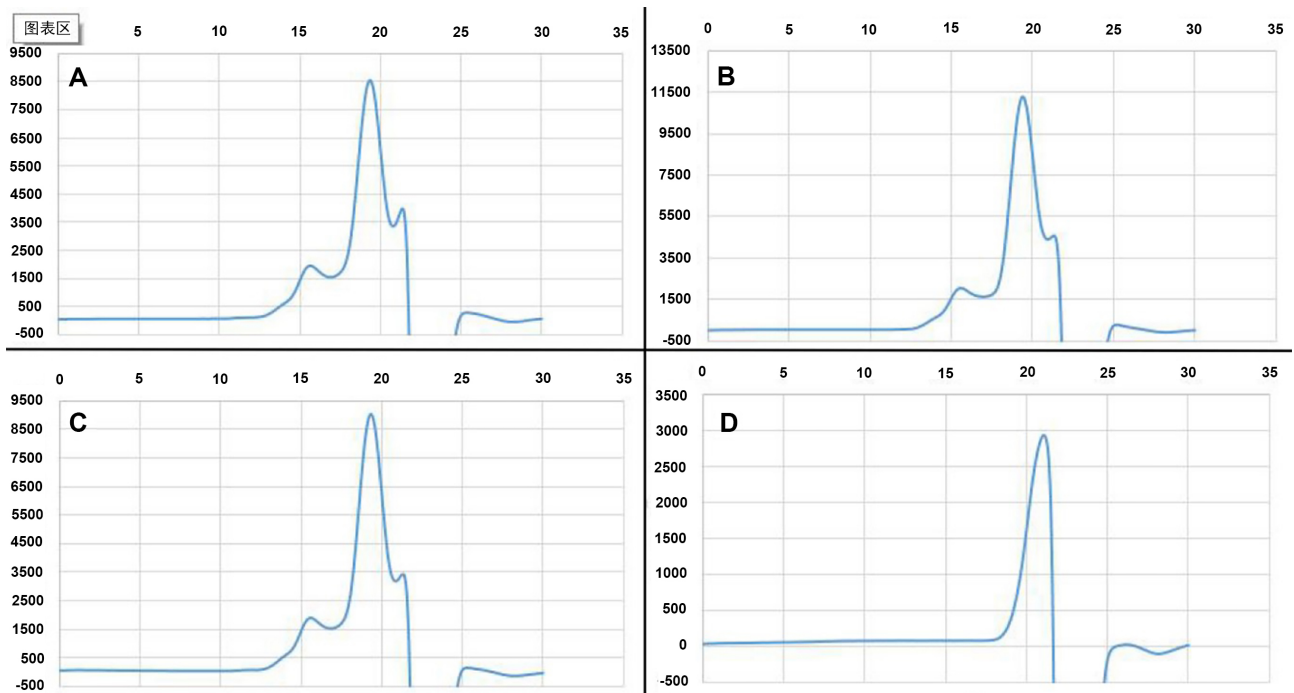


Figure 3. HPLC analysis of polysaccharides and secreted metabolites from *Brasenia schreberi* and its endophytic bacteria. (A) *Micromonospora sp. YG-1*; (B) *Xylariaceae sp. strain UT-X*; (C) *Psychrobacter pulmonis* strain T-15; (D) *Brasenia Schreberi*.

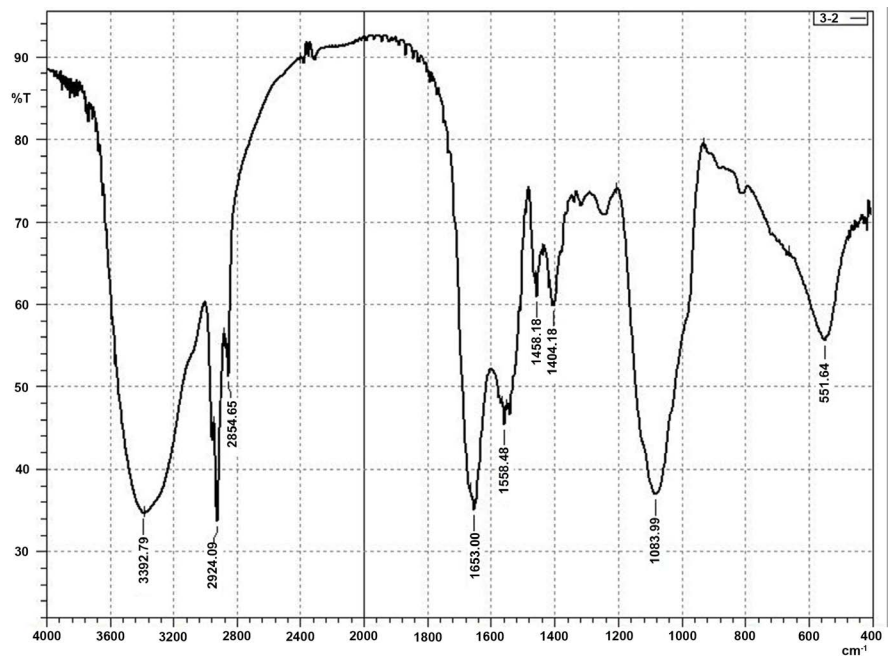


Figure 4. Infrared spectrum (IR) analysis of polysaccharides from *Psychrobacter pulmonis* strain T-15.

absorption peaks of C-H on the benzene ring, which are characteristic spectrum peaks of polysaccharides that are specific of symbiotic bacteria (Table 3); the peak at 1653.00 cm^{-1} is the carbon-oxygen double bond (C=O) absorption peak; the peaks at 1404.18 cm^{-1} and 551.64 cm^{-1} are all C-H stretching vibration

Table 3. Characteristic peak analysis of polysaccharides from different species.

Band (cm ⁻¹) From	<i>Micromonospora</i> <i>sp.</i>	Xylariaceae sp. strain UT-X	<i>Psychrobacter</i> <i>pulmonis</i> strain T-15	<i>Brasenia</i> <i>schreberi</i>	Characterization of chemical bonds
1	3385.07	3360.00	3392.79	3393	O-H
2	2926.01	2924.09	2924.09	2936	C-H
3	2854.65	2854.65	2854.65	-	CH2 from benzene ring
4	1653.00	1651.07	1653.00	1719 - 1617	C=O
5	1558.48	1556.55	1558.48	-	CH2 from benzene ring
6	1458.18	1456.26	1458.18	-	CH2 from benzene ring
7	1406.11	1404.18	1404.18	-	C-H
8	1083.99	1083.99	1083.99	1069	C-O
9	-	-	-	1420 - 1249	C-H
10	549.71	551.64	551.64	550 - 429	C-H

absorption peaks. Notably, the absorption peak at 1083.99 cm⁻¹ is associated with the C-O-H bond, which is the characteristic peak of beta-glucan (Table 3).

In addition, the polysaccharide produced by the symbiotic bacteria had the same absorption peaks as those of *B. schreberi* at 3392.79 cm⁻¹ and 2924.09 cm⁻¹ (Table 3), as well as unique characteristic peaks (2854.65 cm⁻¹, 1558.00 cm⁻¹ and 1458.18 cm⁻¹). Further, polysaccharides from symbiotic bacteria and from *B. schreberi* showed the same characteristic peaks of beta-glucan at 1083.99 cm⁻¹ (Table 3).

3.7. Polysaccharides from Different Species Show Significant Inhibitory Effects on the Production of Hydroxyl Radicals

The importance of polysaccharides lies in their physiological activities. The hydroxyl radical (OH) is a type of free radical with strong oxidation ability. Its chemical nature is very active and its oxidation rate of various organic and inorganic substances is very high. It is the main factor causing lipid peroxidation, nucleic acid and protein breakdown and polysaccharide decomposition in tissues. It is related to aging, cancer, radiation damage and cell phagocytosis. To determine the biological activity of polysaccharides from symbiotic bacteria present in *B. schreberi*, the antioxidant activity of their polysaccharides was evaluated by analyzing the hydroxyl radical production system. The results showed that the inhibitory effect of polysaccharides from symbiotic bacteria on hydroxyl radical production was concentration-dependent and maximum at 1.0 mg/ml (Table 4). The free radical scavenging effect of polysaccharides from symbiotic bacteria on hydroxyl radicals was significantly higher than that of polysaccharides from *B. Schreberi*.

Table 4. Inhibitory effect of polysaccharides from different species on hydroxyl radical production.

Source of polysaccharide	Inhibition efficiency (%)		
	0.5 (mg/ml)	1.0 (mg/ml)	2.0 (mg/ml)
Polysaccharide from <i>Brasenia schreberi</i>	19.46	27.82	45.39
<i>Micromonospora</i> sp. YG-1	92.7	93.87	89.27
Xylariaceae sp. strain UT-X	93.29	92.47	92.01
<i>Psychrobacter pulmonis</i> strain T-15	92.47	92.94	92.48

4. Discussion

B. schreberi is an economically important aquatic plant that requires high-quality water [12] [13]. It is becoming increasingly pertinent to pursue an alternative way for the production of polysaccharides from water shield due to the continuous deterioration of its natural environment [12]. A distinctive anatomical feature of water shield is the distribution of stomata mainly on the adaxial leaf surface and the abundance of glandular cells on the abaxial leaf surface (Figure 1). In the transverse section of the stem, there is a thick layer of pectin on the surface and many glandular cells [17]. It is preliminarily inferred that pectin is the product of glandular cell secretion and that the species' characteristic polysaccharide may be secreted by glandular cells or by endosymbiotic bacteria. In the present study, three species of bacteria were successfully isolated from sterilized stem and leaf tissues of *B. schreberi* and the polysaccharides they produce were studied. Three kinds of symbiotic bacteria were isolated and identified, in addition to one fungus (Figure 2, Table 1), reflecting the diversity of symbionts hosted by *B. schreberi*.

To determine whether the fermentation culture of symbiotic bacteria from *B. schreberi* contained polysaccharides, the polysaccharide content of the extract medium was determined by the DNS (3-amino-5-nitro salicylic acid) method and analyzed by HPLC (Table 2, Figure 3). The results confirmed that symbiotic bacteria in *B. schreberi* produce polysaccharides that show the same peaks on HPLC spectrum (Figure 3). IR showed that the polysaccharides from symbiotic bacteria and those from *B. schreberi* had similar IR characteristic peaks (Figure 4, Table 3). This is similar to the IR spectra of polysaccharides from the seaweeds, *Asparagus* [18] and *Ganoderma lucidum* [19]. This finding indicates that plant polysaccharides have similar or identical chemical functional groups. The function of a polysaccharide is reflected by its activity. The detection of hydroxyl free radicals indicated that polysaccharides from symbiotic bacteria present in *B. schreberi* remarkably inhibited the production of hydroxyl free radicals in a concentration-dependent manner (Table 4). Other biological activities of polysaccharides need to be further investigated using various cell types.

5. Conclusions

To circumvent the increasing deterioration of original living environment faced by *B. schreberi* in polysaccharide producing, seeking an alternative method to

produce *B. schreberi* mucilage polysaccharide is of top priority. In this investigation, three endophytic bacteria were isolated and purified from *B. schreberi*, namely *Micromonospora* sp. YG-1, Xylariaceae sp. strain UT-X and *Psychrobacter pulmonis* strain T-15. These endosymbiotic bacteria have the ability to produce polysaccharides with the similar retention time in HPLP analysis and FTIR spectrum that effectively scavenge reactive oxygen species. Our study provides a potential basis and feasible scheme for the comprehensive utilization of symbiotic bacteria in *B. schreberi* to produce effective natural polysaccharides.

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Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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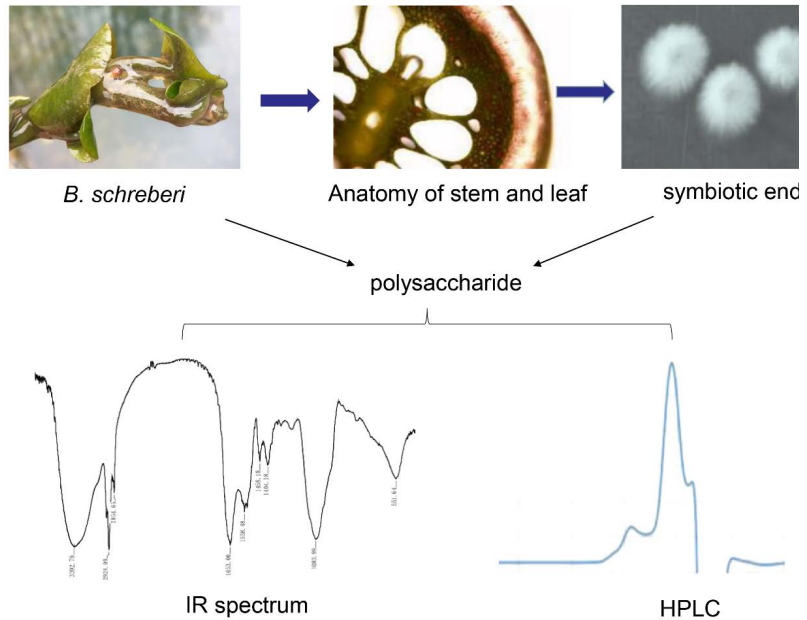
Abbreviations Used

BSP: *Brasenia schreberi* polysaccharide

FTIR: Fourier Transform infrared spectrum analysis

HPLC: High performance liquid chromatography

Abstract Graph



Highlights

The main component of viscous mucilage on the surface of *B. schreberi* is a viscous polysaccharide with exceptionally high nutritional value.

Three species of symbiotic endophytes of *B. schreberi* can produce the same or similar functional active ingredients of polysaccharide with the counterpart of *B. schreberi*.

The endosymbiotic bacteria have the promising application to produce polysaccharides and to circumvent the increasing deterioration of original living environment faced by *B. schreberi* in polysaccharide producing.